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Metabolic control of Schwann cell
myelination and demyelination

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DOCTORAL THESIS

**METABOLIC CONTROL OF
SCHWANN CELL MYELINATION
AND DEMYELINATION**

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DECLARATION OF CONFLICTS OF INTEREST

In accordance with ethical and transparency standards in research, I hereby declare that there are no conflicts of interest related to the present thesis entitled: “Metabolic control of Schwann cell myelination and demyelination”.

I conducted this research independently and objectively and have not received funding, support, or incentives that could influence the results or the interpretation of the data presented. I further declare that I have no personal, professional, or financial relationships that could compromise the impartiality of my work.

Any changes to this situation will be promptly communicated to my thesis committee.

Santiago de Compostela, December 2025

Alejandro Sánchez Rueda

A mi familia

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ABSTRACT

Schwann cells are specialized glial cells of the peripheral nervous system that elaborate myelin sheaths along large diameter axons to potentiate fast conduction of action potentials and provide vital trophic and metabolic support to axons. Defects in the formation of myelin sheaths or breakdown of myelin by disturbance to Schwann cells or the nerve environment, whether due to genetic or acquired disease, toxicity or microbial infections can result in severely debilitating, and even fatal, neurological disorders, such as Charcot-Marie-Tooth disease. For many of these diseases, there is essentially no cure. Conversely, after traumatic injury to nerves, Schwann cells undergo a process of adaptative reprogramming to convert to repair Schwann cells that can support axonal regeneration, forming the cornerstone of nerve repair. However, functional recovery in humans is often compromised, because Schwann cells lose their repair capacity in adverse situations, such as ageing and chronic denervation. All these situations represent a significant and unmet clinical need.

Recent studies have shown that Schwann cells undergo metabolic reprogramming to execute the bioenergetic and biosynthetic demands of differentiation and repair during development and after nerve injury respectively. However, the molecular switch that drives this metabolic reprogramming still remains unclear. It is well known that mitochondria are highly plastic organelles that can match their function according to their cell state-dependent metabolic needs. Metabolic reprogramming of mitochondria is coupled to changes in mitochondrial mass and shape, and these responses are complex and involve a combination of diverse molecular pathways that modulate mitochondrial biogenesis, metabolism, and dynamics. Mitochondria can undergo finely tuned, dynamic changes to their shape to control their function, including fusion, which represents the process of merging two or more mitochondria to generate a larger elongated organelle, or remodelling of internal structures such as cristae, which are dynamic infolds of the inner membrane.

In this study, we have delineated the functional and molecular role of mitochondrial fusion and cristae remodelling by targeting specific key genes (*Mfn1*, *Mfn2* and *Opa1*) using a variety of genetic models. In summary, we demonstrate that mitochondrial plasticity plays an essential role in the formation of peripheral myelin during development but only has a minimal role in driving Schwann cell responses to injury. This study thus provides key insights in the molecular mechanisms that drive the metabolic adaption of Schwann cells in developing and regenerating nerves.

RESUMO

O sistema nervioso constitúe unha rede de comunicación altamente organizada que integra as distintas rexións do organismo, permitindo a coordinación de funcións motoras, sensoriais e cognitivas. O desempeño eficaz destas tarefas require que a propagación do impulso nervioso sexa rápida e precisa. Con este fin, os vertebrados desenvolveron un mecanismo especializado: a formación de vaina de mielina arredor dos axóns. A mielina incrementa a resistencia e diminúe a capacitancia da membrana axonal, o que optimiza a velocidade de condución do potencial de acción e garante unha transmisión neuronal eficiente.

O sistema nervioso divídese en sistema nervioso central (SNC), constituído polo cerebro e a medula espiñal, e sistema nervioso periférico (SNP), composto polos nervios que conectan o SNC co resto do organismo. No SNP, a mielinización está a cargo das células de Schwann (CS), que xeran unha vaina de mielina arredor de axóns individuais, formando unha capa illante que permite a propagación rápida e eficiente do impulso nervioso ao longo de fibras de gran lonxitude. A formación de mielina esixe unha intensa síntese de lípidos e proteínas, o que require a integración precisa de múltiples sinais que activan programas transcrpcionais e traducionais específicos nas células de Schwann. Este proceso resulta esencial, dado que a mielinización implica unha expansión masiva da membrana plasmática, a cal se enrola concéntricamente arredor do axón. Ademais do seu papel como illante eléctrico, as células de Schwann desempeñan funcións tróficas e metabólicas fundamentais, aportando soporte aos axóns e asegurando así a súa integridade funcional e supervivencia. Por iso, a perda de mielina en caso de enfermidade ou lesión pode conducir a un abano de complicacións neurolóxicas, entre as que se inclúen déficits cognitivos e motores.

As CS derivan das células da crista neural (NCC) e atravesan diferentes estadios de diferenciación ata a súa maduración como células de Schwann mielinizantes (mCS) ou non mielinizantes (nmCS ou CS de Remak). Nos ratos, arredor de E12–13, as NCC diferéncianse en precursores de células de Schwann (SCP). Os SCP asóciase cos axóns en crecemento e rodéannos con prolongacións, proporcionándolles apoio metabólico esencial. Á súa vez, os SCP requiren da expresión axonal de neuregulina-1 (NRG1) tipo III para a súa supervivencia. Neste punto do desenvolvemento, axóns de todos os tamaños atópanse agrupados en fascículos ou feixes formados polas SCP, constituíndo o que se coñece como “familias”. Uns días despois, os SCP diferenciaranse en células de Schwann inmaturas (iCS), cuxa supervivencia xa non depende dos axóns. As iCS están conectadas coa lámina basal e son autosuficientes.

Xusto antes do nacemento, as iCS diferéncianse en células de Schwann promielinizantes. Nun proceso coñecido como clasificación radial (*radial sorting*), as células de Schwann escollen aqueles axóns cun diámetro maior de 1 μm e, mediante un evento de mitose e segregación, separan ese axón da familia. Unha vez se establece esta relación 1:1 entre CS e axón, fálase de célula de Schwann promielinizante. As células de Schwann destes axóns diferenciaranse en células de Schwann mielinizantes e mielinizarán o seu axón, mentres que o resto de axóns de menor diámetro permanecerán xuntos na familia, serán envoltos polo citoplasma da célula de Schwann non mielinizante e formarán os fascículos de Remak.

En función do destino final destes axóns, aparecen diferentes modos de propagación do potencial de acción: condución saltatoria (axóns mielinizados) ou propagación continua (axóns en fascículos de Remak). En consecuencia, cada fibra desempeñará funcións fisiolóxicas distintas. Os axóns mielinizados inclúen fibras de gran calibre (13–20 μm), de condución rápida, correspondentes a fibras tipo A esquelético-motoras e propioceptoras, así como fibras de calibre intermedio (6–12 μm), de condución rápida, correspondentes a mecanorreceptores tipo B e fibras eferentes autonómicas preganglionares. Pola contra, os axóns envoltos por células de Schwann de tipo Remak son de pequeno

diámetro (habitualmente $< 1 \mu\text{m}$) e corresponden a fibras tipo C de condución lenta, con velocidades de 0,5 a 4 m/s. Estas fibras inclúen axóns eferentes autonómicos postganglionares, así como aferencias sensoriais cutáneas que transmiten sinais de dor e temperatura.

As células de Schwann presentan un fenotipo moi plástico e son capaces de adaptarse a funcións moi diferentes. Isto é particularmente evidente en caso de lesión nerviosa. O SNP posúe unha gran capacidade rexenerativa grazas ás células de Schwann, xa que, tras a lesión, sofren un proceso de reprogramación que as transforma en células de Schwann de reparación. Esta transición inclúe a desactivación dos xenes relacionados coa mielina, a activación do programa de reparación e un aumento da proliferación celular. As células de Schwann de reparación son fundamentais para a resposta rexenerativa: participan activamente na dexeneración axonal e eliminan restos de mielina e axonais mediante autofaxia (mielinofaxia, mediada unicamente polas células de Schwann mielinizantes) e fagocitose. Así mesmo, modulan o microambiente rexenerativo recrutando células inmunes, regulando a barreira hemato-nerviosa e secretando factores neurotróficos que favorecen a supervivencia e rexeneración neuronal. Ademais, as células de Schwann de reparación forman as bandas de Büngner, que proporcionan orientación estrutural e molecular para o recrecemento axonal a través da zona lesionada e da porción do nervio distal. A recuperación funcional acádase finalmente mediante a rediferenciación das células de Schwann e a remielinización dos axóns rexenerados.

Todas estas transicións fenotípicas requiren unha remodelación masiva do metabolismo das células de Schwann. Nos nervios en desenvolvemento, as células de Schwann proliferativas experimentan unha reprogramación metabólica do metabolismo glucolítico ao oxidativo durante a diferenciación e a mielinización, nun proceso regulado por LKB1. Durante as fases sucesivas da desmielinización, reprogramación e remielinización tras unha lesión nerviosa, as células de Schwann recapitulán a transición observada durante o desenvolvemento. Xusto despois da lesión, as células de Schwann responden cunha activación glicolítica inmediata (dentro das primeiras 72 h) tras a lesión nerviosa aguda, un período que coincide co inicio da dexeneración Walleriana; porén, a evolución posterior (7–28 días tras a lesión por compresión) asóciase cunha activación metabólica intensa das células de Schwann de reparación, que inclúe un cambio cara a metabolismo oxidativo e un marcado incremento da respiración mitocondrial.

Mecanicamente, a adaptabilidade metabólica das mitocondrias está estreitamente ligada á súa plasticidade estrutural. A morfoloxía e a organización espacial das mitocondrias están reguladas pola actividade coordinada da maquinaria responsable da división, da fusión e da motilidade. En células nai embrionarias pluripotentes, con metabolismo glicolítico, as mitocondrias preséntanse fragmentadas e incompletas na organización das súas cristas internas. A medida que progresa a diferenciación das células nai, as mitocondrias experimentan un remodelado significativo: pasan a empregar a fosforilación oxidativa para a produción de enerxía, adquiren unha morfoloxía máis alongada e forman redes interconectadas con cristas ben desenvolvidas. Estes cambios estruturais están regulados, en parte, polo equilibrio entre a fisión e a fusión mitocondrial. Na diferenciación neuronal, un profundo remodelado mitocondrial asóciase co incremento na abundancia de orgánulos, con modificacións na súa distribución espacial e na súa función bioenerxética. Este remodelado está estritamente controlado polos reguladores da fusión MFN2 e OPA1, que facilitan a transición metabólica da glicólise á fosforilación oxidativa (OXPHOS), un paso esencial para a maduración neuronal.

Dada a natureza endosimbionte das mitocondrias, o proceso de fusión mitocondrial está dividido en dous pasos. Primeiro debe fusionarse a membrana mitocondrial externa, nun proceso regulado pola mitofusina 1 (MFN1) e a mitofusina 2 (MFN2). Estas proteínas posúen, en mamíferos, arredor dun 80% de homoloxía. Aínda que poidan compartir moitas das súas funcións, existen actividades específicas para cada mitofusina. Por exemplo, MFN1 é moito máis eficiente na fusión mitocondrial mentres que

MFN2 é indispensable para a comunicación con outros orgánulos. Unha vez fusionada a membrana externa, debe producirse a fusión da membrana interna. Este paso está gobernado pola proteína da atrofia óptica 1 (OPA1). Alén do seu rol na fusión mitocondrial, OPA1 desempeña funcións relacionadas co mantemento das cristas mitocondriais, a estabilidade do ADN mitocondrial e a eficiencia de OXPHOS.

A dinámica mitocondrial revelou-se como un factor importante no desenvolvemento do SNP, xa que mutacións en xenes relacionados con ela provocan diferentes tipos de alteracións neurolóxicas. Entre elas destacan a enfermidade de Charcot-Marie-Tooth (CMT) e a Atrofia Óptica Dominante (DOA). Mutacións en *MFN2* provocan CMT tipo 2A, unha forma neuronal da enfermidade que provoca a dexeneración axonal. Mutacións en *GDAP1*, xene que codifica unha proteína da membrana mitocondrial externa relacionada coa dinámica mitocondrial, causan CMT tipo 4A que presenta unha desmielinización progresiva nalgúns casos. Pola súa banda, a DOA é causada por mutacións en *OPA1* e certas mutacións producen neuropatía periférica e desmielinización.

En conxunto, estas achegas subliñan un papel central da fusión mitocondrial, tanto en células gliais como axonais, no mantemento da integridade funcional da unidade axón-mielina. Non obstante, o rol da fusión mitocondrial aínda non foi caracterizado nas células de Schwann. Por iso, o noso estudo baseouse na hipótese de que a fusión mitocondrial, a través dunha estreita coordinación entre os seus tres principais actores (MFN1, MFN2 e OPA1), regula a reprogramación metabólica necesaria para a correcta diferenciación das células de Schwann tanto en desenvolvemento como en lesión.

Para estudar o impacto da fusión mitocondrial no desenvolvemento, primeiro caracterizamos a expresión de MFN1, MFN2 e OPA1 nunha curva temporal dende o nacemento dos ratos ata a maduración das vaíñas de mielina (aos 2–3 meses de idade). Observamos que os niveis máximos das tres proteínas se acadaban xusto no momento de máxima expresión de xenes relacionados con OXPHOS, correlacionando a fusión mitocondrial coa activación do metabolismo oxidativo.

A pesar de que as tres proteínas coincidían no seu pico de expresión, MFN1 seguía un patrón diferente ao de MFN2 e OPA1. Mentres que MFN1 estaba expresada dende o inicio e despois de P15 os seus niveis diminuían, mentres que MFN2 e OPA1 ían aumentando progresivamente a súa expresión ata P15. Isto indica que as células de Schwann expresan as mitofusinas de maneira diferencial ao longo do desenvolvemento, sendo os niveis de MFN1 máis altos nos estadios máis temperáns e os de MFN2 nos máis diferenciados.

Usando imaxes de microscopía electrónica en seccións transversais de nervio ciático detectamos que a dinámica mitocondrial dos axóns e das células de Schwann era oposta. Ao longo do desenvolvemento, o número de mitocondrias axonais reducía, mentres que nas células de Schwann aumentaba.

Unha vez comprobado que a expresión dos compoñentes da fusión mitocondrial estaba altamente regulada durante o desenvolvemento das células de Schwann e se correlacionaba co cambio metabólico, decidimos establecer modelos xenéticos condicionais que nos permitisen analizar en profundidade os efectos da fusión mitocondrial especificamente en células de Schwann. Para iso, cruzamos ratos que tiñan as secuencias dos xenes *Mfn1*, *Mfn2* e *Opa1* flanqueadas por sitios *flox* (floxados) con ratos que tiñan a recombinasa Cre baixo o control do promotor de *Mpz*. Estes ratos expresarían a recombinasa a partir de E13,5 e producirían a recombinación daqueles xenes floxados. Dado que as mitofusinas comparten unha alta homoloxía, xeramos catro tipos de mutantes distintos: *Mfn1* cKO (knock-out condicional de *Mfn1*), *Mfn2* cKO, *Mfn1/2* dKO (dobre KO condicional de *Mfn1* e *Mfn2*) e *Opa1* cKO.

Para a caracterización destes modelos empregamos unha batería de probas para testar as funcións sensoriomotoras, rexistros electromiográficos, imaxes de microscopia electrónica, análises moleculares (*immunoblot* e *qPCR*) e ensaios de respirometría *Seahorse*.

Centrándonos nos mutantes de mitofusinas, todas estas análises arroxoaron un espectro fenotípico que ía dende o desenvolvemento absolutamente normal da mielina ata unha profunda desmielinización. Os mutantes de *Mfn2* non presentaron ningún defecto en ningunha das análises realizadas, o cal encaixa co cadro clínico de CMT2A onde se produce unha dexeneración axonal pero a mielina permanece intacta. As células de Schwann son perfectamente capaces de funcionar en ausencia de MFN2, o que nos leva a pensar que MFN1 pode suplir as funcións requiridas para MFN2. No caso dos ratos *Mfn1* cKO, tanto as análises *in vivo* como as imaxes de microscopía electrónica (EM) revelaron un desenvolvemento normal da mielina. Porén, as análises moleculares demostraron que, para manter unha mielina en condicións axeitadas, estes ratos activan un programa compensatorio mediante a indución da síntese de ácidos graxos, a activación da sinalización de estrés metabólico e da resposta integrada ao estrés (ISR), un aumento da bióxénese mitocondrial e mecanismos de protección mitocondrial.

A eliminación xenética de *Mfn1* provocaba máis problemas ás células de Schwann ca a de *Mfn2*, pero, a pesar diso, MFN2 era capaz de cubrir suficientemente a ausencia de MFN1 como para que os ratos tivesen un desenvolvemento normal. Esta situación cambiou radicalmente no dobre mutante das mitofusinas, *Mfn1/2* dKO. Estes ratos sufriron unha redución na súa esperanza de vida, xa que non sobrevivían alén de 3 meses. As probas funcionais sensoriomotoras demostraron que os ratos sufrían unha desmielinización drástica a partir dos 30 días e a condutividade eléctrica estaba comprometida xa a esa idade. Os recontos feitos nas imaxes de EM confirmaron unha desmielinización drástica desde os 30 días, o que levaría os ratos a unha perda das funcións fisiolóxicas básicas que causaría a súa morte arredor dos 90 días. Unha diferenza notable entre os 30 e os 60 días atopámola na análise morfolóxica das mitocondrias en EM. Aos 30 días, cando os *Mfn1/2* dKO aínda manteñen certa integridade da mielina, os axóns presentaban un incremento no número de mitocondrias comparado cos grupos control, mentres que as células de Schwann tiñan menos mitocondrias. Porén, esta compensación mitocondrial axonal perdíase aos 60 días, cando a desmielinización era xa evidente.

Os *immunoblots* dos nervios ciáticos dos *Mfn1/2* dKO indicaban que as células de Schwann tamén iniciaran unha resposta de emerxencia, mais, no caso da dobre ausencia das mitofusinas, non era suficiente para evitar a perda da integridade da mielina. Nesta situación, a resposta incluía unha supresión da síntese de ácidos graxos para redirixir o metabolismo cara á produción de enerxía, activación da ISR, indución dun programa de lesión específico das células de Schwann regulado por c-Jun e aumento da bióxénese mitocondrial a través de TFAM.

O modelo de dobre KO de *Mfn1* e *Mfn2* permitiunos explorar que acontecía cando só quedaba un dos alelos activos (dos catro posibles), ben un alelo de *Mfn1* (MFN1^{HT}MFN2^{KO}) ou un alelo de *Mfn2* (MFN1^{KO}MFN2^{HT}). Estes mutantes non presentaron redución da esperanza de vida respecto dos controis, pero si comezaron a amosar signos de neuropatía periférica en momentos diferentes. Nos resultados do *Rotarod*, os ratos MFN1^{KO}MFN2^{HT} comezaron a amosar unha caída no tempo de carreira arredor de P90, mentres que os MFN1^{HT}MFN2^{KO} non presentaron este declive ata P180. Nunha situación semellante atopámonos cos rexistros electromiográficos: os MFN1^{KO}MFN2^{HT} presentaban máis alteracións e a idades máis temperás ca os MFN1^{HT}MFN2^{KO}. Estes datos concordan cos atopados en *Mfn1* cKO e *Mfn2* cKO: aínda que as mitofusinas poidan compensarse mutuamente ata certo punto, as funcións de MFN1 son máis importantes nas células de Schwann ca as de MFN2. As análises de EM permitíronnos entender o que sucedía a nivel estrutural. En primeiro lugar, a mielina dos axóns de maior diámetro vese comprometida, xa que a súa estrutura máis extensa resulta máis difícil de manter.

As células de Schwann detectan este estrés mitocondrial e inician un proceso de desmielinización para adoptar un fenotipo non mielinizante, en forma de feixe de Remak. En fases iniciais poden observarse numerosos feixes de Remak, como se detecta en MFN1^{HT}MFN2^{KO}, pero posteriormente estes agrúpanse en feixes de Remak aumentados, como ocorre en MFN1^{KO}MFN2^{HT}.

Cos mutantes de *Opa1* atopamos un escenario no que, no canto de sufrir unha desmielinización abrupta, os defectos na mielina preséntanse desde o principio. Tanto nas probas funcionais, nos rexistros electromiográficos como nas análises de imaxe de EM, os ratos *Opa1* cKO presentan claros signos de desmielinización e neuropatía periférica en P30 ou incluso antes. Neste caso, a compensación mitocondrial axonal que atopabamos en P30 nos *Mfn1/2* dKO aparece a P120, unha idade moito máis tardía. Semella que ambos tipos de mutantes comparten certas características, pero a diferentes tempos. Por exemplo, as análises moleculares dos nervios ciáticos dos *Opa1* cKO tamén revelaron unha resposta de emerxencia similar á dos dobres mutantes de mitofusina, pero máis pronunciada, que comprendía o cese da síntese de mielina e un cambio cara a un estado de conservación enerxética, aumento da bióxénese mitocondrial e intento de sobrevivir mediante programas de estrés mediados pola ISR e c-Jun. Para comprender as consecuencias funcionais da inhibición de *Opa1*, realizamos ensaios de consumo de osíxeno *Seahorse*. O resultado foi que, cando se engade cAMP ás células de Schwann *in vitro*, o consumo de osíxeno aumenta notabelmente, reflectindo o cambio a metabolismo oxidativo descrito *in vivo*. Pola contra, nos mutantes de *Opa1* isto non sucedía: permanecían respirando a nivel basal, semellante ao que manteñen as células de Schwann inmaturoas en proliferación. Este resultado indica que a fusión mitocondrial, mediada por OPA1, é necesaria para que as células de Schwann realicen a reprogramación metabólica requirida durante a diferenciación. As células de Schwann que carecen de OPA1 permanecen nun estado non diferenciado no que non poden madurar nin formar correctamente as vaíñas de mielina.

Unha vez caracterizado o papel da fusión mitocondrial en desenvolvemento, centrámonos en estudar o contexto da lesión nerviosa. Igual que fixemos ao principio, primeiro caracterizamos a expresión de MFN1, MFN2 e OPA1 en diferentes modelos de lesión. Con este obxectivo, estudamos a resposta aguda tanto en ratos novos como en envellecidos, a resposta a lesión crónica e a resposta en rexeneración nerviosa. En todas as ocasións observamos un aumento inmediato dos niveis de proteína de MFN1 tras a lesión, mentres que os niveis de MFN2 e OPA1, máis elevados en nervios non lesionados, sufriron un lixeiro descenso progresivo tras o dano. Ademais, a expresión de MFN1 permanece elevada na lesión crónica pero non durante a rexeneración nerviosa. Este patrón de expresión en lesión, combinado cos altos niveis de MFN1 nos estadios máis inmaturos das células de Schwann, coincide co de proteínas expresadas en programas específicos de reparación en células de Schwann, como c-Jun. Polo tanto, poderíamos incluír MFN1 no conxunto de proteínas relacionadas con este estadio máis indiferenciado/reparador.

Para caracterizar o papel da fusión mitocondrial no contexto da lesión nerviosa e a súa reparación, foi necesario establecer dúas novas liñas de ratos transxénicos. Os mutantes condicionais constitutivos *Mfn1/2* dKO e *Opa1* cKO non podían empregarse para realizar lesións nos nervios, xa que presentaban de base defectos na mielina. En vez de usar a liña *Mpz-Cre*, cruzamos os ratos cos xenos *Mfn1/2* e *Opa1* floxados con ratos *PlpCre-ERT2*. Esta liña só expresa a recombinase Cre naquelas células que expresen *Plp* (oligodendrocitos e células de Schwann) tras a inxección de tamoxifeno. Polo tanto, a recombinación só a inducimos unha vez a vaíña de mielina estaba formada, producindo os ratos *Mfn1/2* idKO (dobre KO condicional inducible) e *Opa1* icKO (KO condicional inducible). Esta recombinación non produciu ningunha alteración na estrutura da mielina, polo que a fusión mitocondrial non é requirida para o mantemento da vaíña de mielina. Unha vez inducida a recombinación, procedeuse a practicar as cirurxías necesarias para os diferentes modelos de lesión. Os ratos *Mfn1* cKO e *Mfn2* cKO foron utilizados co mesmo propósito xa que non presentaban ningunha

alteración de base na mielina.

Aos 7 días de lesión por corte podemos observar un aumento na velocidade de eliminación de mielina nos ratos *Mfn2* cKO, caracterizado por unha diminución dos niveis de MPZ e MBP. Así aconteceu tamén nos ratos *Mfn1/2* idKO e *Opa1* icKO, aínda que só se detectaron niveis reducidos de MPZ. En canto aos mutantes de *Mfn1*, non atopamos ningunha diferenza con respecto aos controis.

Por último, analizamos o impacto que podían ter os mutantes *Mfn1/2* idKO e *Opa1* icKO na remielinización, xa que foran os que maior impacto tiveran na mielinización. Porén, non se atoparon diferenzas en ningún dos parámetros analizados ata 40 días despois da lesión, cando se considera que a remielinización é completa. Este aspecto da lesión nerviosa aínda require máis investigación, e é posible que novas análises reflectan outros resultados.

RESUMEN

El sistema nervioso constituye una red de comunicación altamente organizada que integra las distintas regiones del organismo, permitiendo la coordinación de funciones motoras, sensoriales y cognitivas. El desempeño eficaz de estas tareas requiere que la propagación del impulso nervioso sea rápida y precisa. Con este fin, los vertebrados han desarrollado un mecanismo especializado: la formación de vainas de mielina alrededor de los axones. La mielina incrementa la resistencia y disminuye la capacitancia de la membrana axonal, lo que optimiza la velocidad de conducción del potencial de acción y garantiza una transmisión neuronal eficiente.

El sistema nervioso se divide en sistema nervioso central (SNC), constituido por el cerebro y la médula espinal, y sistema nervioso periférico (SNP), compuesto por los nervios que conectan el SNC con el resto del organismo. En el SNP, la mielinización está a cargo de las células de Schwann (SC), que generan una vaina de mielina alrededor de axones individuales, formando una capa aislante que permite la propagación rápida y eficiente del impulso nervioso a lo largo de fibras de gran longitud. La formación de la mielina exige una intensa síntesis de lípidos y proteínas, lo cual requiere la integración precisa de múltiples señales que activan programas transcripcionales y de traducción específicos en las células de Schwann. Este proceso resulta esencial, dado que la mielinización implica una expansión masiva de la membrana plasmática, la cual se enrolla concéntricamente alrededor del axón. Además de su papel como aislante eléctrico, las células de Schwann desempeñan funciones tróficas y metabólicas fundamentales, proporcionando soporte a los axones y asegurando así su integridad funcional y supervivencia.

Por ello, la pérdida de la mielina en caso de enfermedad o lesión puede llevar a un abanico de complicaciones neurológicas, entre las que se incluyen déficits cognitivos y motores.

Las SC derivan de las células de la cresta neural (NCCs) y sufren diferentes estadios de diferenciación hasta su maduración como células de Schwann mielinizantes (mSC) o no mielinizantes (nmSC o Remak SC). En ratones, alrededor del E12-13, las NCCs se diferencian en precursores de las células de Schwann (SCPs). Las SCPs se asocian con los axones en crecimiento y los rodean con conos, aportándoles apoyo metabólico esencial. A su vez, las SCPs requieren de la expresión axonal de la neuregulina 1 (NRG1) tipo III para su supervivencia. En este punto, axones de todos los tamaños se encuentran agrupados en fascículos o haces formados por las SCPs, formando lo que se conoce como familias. Unos días después, los SCPs se diferenciarán en células de Schwann inmaduras (iSC), cuya supervivencia ya no depende de los axones. Las iSC están conectadas con la lámina basal y son autosuficientes.

Justo antes del nacimiento las iSC se diferencian en células de Schwann pro-mielinizantes. En un proceso conocido como clasificación radial (*radial sorting*), las células de Schwann escogen a aquellos axones con un diámetro mayor de una micra y a través de un evento de mitosis, separan ese axón de la familia. Una vez se establece esta relación 1:1 entre SC y axón, es cuando se le denomina célula de Schwann pro-mielinizante. Las células de Schwann de estos axones se diferenciarán en células de Schwann mielinizantes y mielinizarán a su axón, mientras que el resto de axones de menor diámetro permanecerán juntos en la familia, serán envueltos por el citoplasma de la célula de Schwann no mielinizante y formarán los fascículos de Remak.

En función del destino final de estos axones, aparecerán diferentes modos de propagación del potencial de acción: conducción saltatoria (axones mielinizados) o propagación continua (axones en fascículos de Remak). En consecuencia, cada fibra desempeñará funciones fisiológicas distintas. Los axones mielinizados incluyen fibras de gran calibre (13–20 μm), de conducción rápida, correspondientes a fibras tipo A esquelético-motoras y propioceptoras, así como fibras de calibre intermedio (6–12 μm), de conducción rápida, correspondientes a mecanorreceptores tipo B y fibras

eferentes autonómicas preganglionares. Por otro lado, los axones envueltos por células de Schwann de tipo Remak son de pequeño diámetro (habitualmente menores de 1 μm) y corresponden a fibras tipo C de conducción lenta, con velocidades de conducción entre 0,5 y 4 m/s. Estas fibras incluyen axones eferentes autonómicos postganglionares, así como aferentes sensoriales cutáneos que transmiten señales de dolor y temperatura.

Las células de Schwann presentan un fenotipo muy plástico y son capaces de adaptarse a funciones muy diferentes. Esto es particularmente evidente en caso de lesión nerviosa. El SNP posee una gran capacidad regenerativa gracias a las células de Schwann, ya que, tras la lesión, sufren un proceso de reprogramación que las transforma en células de Schwann reparadoras. Esta transición incluye la desactivación de los genes relacionados con la mielina, la activación del programa de reparación y un aumento de proliferación celular. Las células de Schwann de reparación son fundamentales para la respuesta regenerativa: participan activamente en la degeneración axonal y eliminan restos de mielina y axonales mediante autofagia (mielinofagia, mediada únicamente por las células de Schwann mielinizantes) y fagocitosis. Asimismo, modulan el microambiente regenerativo reclutando células inmunitarias, regulando la barrera hemato-nerviosa y secretando factores neurotróficos que favorecen la supervivencia y regeneración neuronal. Además, las células de Schwann de reparación forman las bandas de Büngner, que proporcionan orientación estructural y molecular para el recrecimiento axonal a través de la zona lesionada y del muñón nervioso distal. La recuperación funcional se alcanza finalmente mediante la rediferenciación de las células de Schwann y la remielinización de los axones regenerados.

Todas estas transiciones fenotípicas requieren de una remodelación masiva del metabolismo de las células de Schwann. En los nervios en desarrollo, las células de Schwann proliferativas experimentan una reprogramación metabólica de un metabolismo glucolítico a uno oxidativo durante la diferenciación y la mielinización, en un proceso regulado por LKB1. Durante las fases sucesivas de desmielinización, reprogramación y remielinización tras una lesión nerviosa, las células de Schwann recapitulan la transición observada durante el desarrollo. Justo después de la lesión, las células de Schwann responden con una activación glucolítica inmediata (dentro de las 72 h) tras la lesión nerviosa aguda, un periodo que coincide con el inicio de la degeneración Walleriana; sin embargo, la evolución posterior (7–28 días tras la lesión por compresión) se asocia con una activación metabólica intensa de las células de Schwann de reparación, que incluye un cambio hacia un metabolismo oxidativo y un marcado incremento en la respiración mitocondrial.

Mecánicamente, la adaptabilidad metabólica de las mitocondrias está estrechamente ligada a su plasticidad estructural. La morfología y la organización espacial de las mitocondrias están reguladas por la actividad coordinada de la maquinaria responsable de la división, la fusión y la motilidad. En células madre embrionarias pluripotentes con metabolismo glucolítico, las mitocondrias se presentan fragmentadas e incompletas en la organización de sus crestas internas. A medida que progresa la diferenciación de las células madre, las mitocondrias experimentan un remodelado significativo: pasan a utilizar la fosforilación oxidativa para la producción de energía, adquieren una morfología más elongada y forman redes interconectadas con crestas bien desarrolladas. Estos cambios estructurales están regulados, en parte, por el equilibrio entre la fisión y la fusión mitocondrial. En la diferenciación neuronal, un profundo remodelado mitocondrial se asocia con el incremento en la abundancia de orgánulos, modificaciones en su distribución espacial y en su función bioenergética. Este remodelado está estrictamente controlado por los reguladores de la fusión MFN2 y OPA1, que facilitan la transición metabólica de la glucólisis a la fosforilación oxidativa (OXPHOS), un paso esencial para la maduración neuronal.

Dada la naturaleza endosimbionte de las mitocondrias, el proceso de fusión mitocondrial está dividido

en dos pasos. Primero se debe fusionar la membrana mitocondrial externa, en un proceso regulado por la mitofusina 1 (MFN1) y mitofusina 2 (MFN2). Estas proteínas poseen, en mamíferos, alrededor de un 80% de homología. Aunque puedan compartir muchas de sus funciones, hay actividades específicas de cada mitofusina. Por ejemplo, MFN1 es mucho más eficiente en la fusión mitocondrial mientras que MFN2 es indispensable para la comunicación con otros orgánulos.

Una vez fusionada la membrana externa, debe producirse la fusión de la membrana interna. Este paso está gobernado por la proteína de la atrofia óptica 1 (OPA1). Más allá de su rol en la fusión mitocondrial, OPA1 desempeña funciones relacionadas con el mantenimiento de las cretas mitocondriales, la estabilidad del ADN mitocondrial y la eficiencia de la OXPHOS.

La dinámica mitocondrial se ha revelado como un factor importante en el desarrollo del SNP, ya que mutaciones en genes relacionados con ella provocan diferentes tipos de alteraciones neurológicas. Entre ellas destacan la enfermedad de Charcot-Marie-Tooth (CMT) y la atrofia óptica dominante (DOA).

Mutaciones en *MFN2* provocan CMT tipo 2A, una forma neuronal de la enfermedad que provoca la degeneración axonal. Mutaciones en *GDAP1*, gen que codifica para una proteína de la membrana mitocondrial externa relacionada con la dinámica mitocondrial, causan CMT tipo 4A que presenta una desmielinización progresiva en algunos casos. Por otro lado, la DOA es causada por mutaciones en *OPA1* y ciertas mutaciones producen neuropatía periférica y desmielinización.

En conjunto, estos hallazgos subrayan el papel central de la fusión mitocondrial, tanto en células gliales como axonales, en el mantenimiento de la integridad funcional de la unidad axón-mielina. No obstante, el rol de la fusión mitocondrial aún no ha sido caracterizado en las células de Schwann. Por ello, nuestro estudio se basó en la hipótesis de que la fusión mitocondrial, a través de una estrecha coordinación entre sus tres principales actores (MFN1, MFN2 y OPA1), regula la reprogramación metabólica necesaria para la correcta diferenciación de las células de Schwann tanto en desarrollo como en lesión.

Para estudiar el impacto de la fusión mitocondrial en el desarrollo primero caracterizamos la expresión de MFN1, MFN2 y OPA1 en una curva temporal desde el nacimiento de los ratones hasta la maduración de las vainas de mielina (a los 2-3 meses de edad). Lo que observamos es que los niveles máximos de las tres proteínas se alcanzaban justo en el momento de máxima expresión de genes relacionados con la OXPHOS, correlacionando la fusión mitocondrial con la activación del metabolismo oxidativo.

A pesar de que las tres proteínas coincidían en su pico de expresión, MFN1 seguía un patrón diferente al de MFN2 y OPA1. Mientras que MFN1 estaba expresado desde el principio y después de P15 sus niveles disminuían, MFN2 y OPA1 iban aumentando progresivamente su expresión hasta P15. Esto indica que las células de Schwann expresan las mitofusinas diferencialmente a lo largo del desarrollo, siendo los niveles de MFN1 más altos en los estadios más tempranos y MFN2 en los estadios más diferenciados.

Usando imágenes de microscopía electrónica en secciones transversales de nervio ciático detectamos que la dinámica mitocondrial de los axones y las células de Schwann era opuesta. A lo largo del desarrollo el número de mitocondrias axonales reducía, mientras que en células de Schwann aumentaba.

Una vez comprobado que la expresión de los componentes de la fusión mitocondrial estaba altamente regulada durante el desarrollo de las células de Schwann y correlacionaban con el cambio metabólico, decidimos establecer modelos genéticos condicionales que nos permitiese analizar en profundidad los

efectos de la fusión mitocondrial específicamente en células de Schwann. Para ello, cruzamos ratones que tenían las secuencias de los genes de *Mfn1*, *Mfn2* y *Opa1* flanqueadas con secuencias *flox* (floxeados) con ratones que tenían la recombinasa Cre bajo el control del promotor de *Mpz*. Estos ratones expresarían la recombinasa a partir del E13.5 y producirían la recombinación de aquellos genes floxeados. Dado que las mitofusinas comparten una alta homología generamos cuatro tipos de mutantes distintos: *Mfn1* cKO (*Knock-Out* condicional de *Mfn1*), *Mfn2* cKO, *Mfn1/2* dKO (doble KO condicional de *Mfn1* y *Mfn2*) y *Opa1* cKO.

Para la caracterización de estos modelos utilizamos una batería de pruebas para testear las funciones sensoriomotoras, registros electromiográficos, imágenes de microscopía electrónica, análisis moleculares (Western blot y qPCRs) y ensayos de respirometría *Seahorse*.

Centrándonos en los mutantes de mitofusinas, todos estos análisis arrojaron un espectro fenotípico que iba desde el desarrollo absolutamente normal de la mielina hasta una profunda desmielinización. Los mutantes de *Mfn2* no presentaron ningún defecto en ninguno de los análisis realizados, lo cual encaja con el cuadro clínico del CMT2A donde se produce una degeneración axonal pero la mielina permanece intacta. Las células de Schwann son perfectamente capaces de funcionar en ausencia de *Mfn2*, lo que nos lleva a pensar que MFN1 puede suplir las funciones requeridas para MFN2. En el caso de los ratones *Mfn1* cKO tanto los análisis *in vivo* como las imágenes de EM revelaron un desarrollo normal de la mielina. Sin embargo, los análisis moleculares demostraron que, para mantener una mielina en condiciones adecuadas, estos ratones habían activado un programa compensatorio a través de la inducción de la síntesis de ácidos grasos, activación de la señalización de estrés metabólico y de la respuesta integrada a estrés, biogénesis mitocondrial aumentada y mecanismos de protección mitocondrial.

La delección genética de *Mfn1* provocaba más problemas a las células de Schwann que la de *Mfn2*, pero a pesar de ello MFN2 era capaz de cubrir suficientemente la ausencia de MFN1 como para que los ratones tuvieran un desarrollo normal. Esta situación cambió radicalmente en el doble mutante de las mitofusinas, *Mfn1/2* dKO. Estos ratones sufrieron una reducción en su esperanza de vida, ya que no sobrevivían más allá de los 3 meses. Las pruebas funcionales sensoriomotoras demostraron que los ratones sufrían una desmielinización drástica a partir de los 30 días y la conductividad eléctrica estaba impedida ya a esta edad. Los contajes hechos en las imágenes de EM confirmaron una desmielinización drástica a partir de los 30 días, lo que llevaría a los ratones a una pérdida de las funciones fisiológicas básicas que causaría su muerte alrededor de los 90 días. Una diferencia notable entre los 30 y los 60 días la encontramos en el análisis morfométrico de las mitocondrias en las imágenes de EM. A los 30 días, cuando los ratones *Mfn1/2* dKO aún mantienen cierta integridad de la mielina, los axones presentaban un incremento en el número de mitocondrias comparado con los grupos control, mientras que las células de Schwann tenían menos mitocondrias. Sin embargo, esta compensación mitocondrial axonal se perdía a los 60 días, cuando la desmielinización era ya evidente.

Los *immunoblots* de los nervios ciáticos de los ratones *Mfn1/2* dKO indicaban que las células de Schwann también habían iniciado una respuesta de emergencia, pero, en el caso de la doble ausencia de las mitofusinas no era suficiente para evitar la pérdida de la integridad de la mielina. En esta situación, la respuesta disparada incluía una supresión de la síntesis de ácidos grasos para redirigir el metabolismo hacia la producción de energía, activación de la respuesta integrada al estrés, inducción de un programa de lesión específico de las células de Schwann regulado por c-Jun, y aumento de la biogénesis mitocondrial a través de TFAM.

El modelo de doble KO de *Mfn1* y *Mfn2* nos permitió explorar que ocurría cuando solo quedase uno de los alelos activos (de los cuatro posibles) ya fuese un alelo de *Mfn1* (MFN1^{HT}MFN2^{KO}) o un alelo de

Mfn2 (MFN1^{KO}MFN2^{HT}). Estos mutantes no presentaron una reducción en su esperanza de vida comparado con los controles, pero sí que empezaron a mostrar signos de una neuropatía periférica en diferentes momentos. En los resultados del Rotarod, los ratones MFN1^{KO}MFN2^{HT} empezaron a mostrar una caída en el tiempo que se mantenían corriendo alrededor de P90, mientras que los ratones MFN1^{HT}MFN2^{KO} no presentaron este declive hasta los P180. En una situación parecida nos encontramos con los registros electromiográficos: los ratones MFN1^{KO}MFN2^{HT} presentaban más alteraciones y a edades más tempranas que los MFN1^{HT}MFN2^{KO}. Estos datos concuerdan con aquellos encontrados en los *Mfn1* cKO y *Mfn2* cKO, aunque las mitofusinas puedan compensarse mutuamente hasta cierto punto, las funciones de MFN1 son más importantes en las células de Schwann que las de MFN2. Los análisis de EM nos permitieron entender exactamente que ocurría a nivel estructural. En primer lugar, la mielina de los axones de mayor diámetro se ve comprometida, ya que su estructura más extensa resulta más difícil de mantener. Las células de Schwann detectan este estrés mitocondrial e inician un proceso de desmielinización para adoptar un fenotipo no mielinizante, en forma de haz de Remak. En fases iniciales pueden observarse numerosos haces de Remak, como se detecta en MFN1^{HT}MFN2^{KO}, pero posteriormente estos se agrupan en haces de Remak aumentados, como ocurre en MFN1^{KO}MFN2^{HT}.

Con los mutantes de *Opa1* nos encontramos en un escenario donde en vez de sufrir una desmielinización abrupta, los defectos en la mielina se nos presentan desde el principio. Tanto en las pruebas funcionales, en los registros electromiográficos como en los análisis de imagen de EM los ratones *Opa1* cKO presentan claros signos de desmielinización y neuropatía periférica a P30 o incluso antes. En este caso, la compensación mitocondrial axonal que encontrábamos a P30 en los ratones *Mfn1/2* dKO, aparece a P120, una edad mucho más tardía. Parece ser que ambos tipos de mutantes comparten ciertas características, pero a diferentes tiempos. Por ejemplo, los análisis moleculares de los nervios ciáticos de los ratones *Opa1* cKO también revelaron una respuesta de emergencia similar a los dobles mutantes de mitofusina pero más pronunciada que comprendía el cese de la síntesis de mielina y un cambio hacia un estado de conservación energética, aumento la biogénesis mitocondrial e intento de sobrevivir mediante programas de estrés mediados por la ISR y c-Jun. Para comprender las consecuencias funcionales de la inhibición de *Opa1*, realizamos ensayos de consumo de oxígeno *Seahorse*. El resultado fue que cuando se añade cAMP a las células de Schwann *in vitro*, el consumo de oxígeno aumenta enormemente reflejando el cambio a metabolismo oxidativo descrito *in vivo*. Por el contrario, los mutantes de *Opa1* esto no sucedía, permanecían respirando a nivel basal similar al que mantienen las células de Schwann inmaduras en proliferación. Este resultado nos indica que la fusión mitocondrial, mediada por OPA1, es necesaria para que las células de Schwann realicen la reprogramación metabólica necesaria durante la diferenciación. Las células de Schwann que carecen de OPA1 permanecen en un estado no-diferenciado en el que no pueden madurar y formar correctamente las vainas de mielina.

Una vez caracterizado el papel de la fusión mitocondrial en desarrollo nos centramos en estudiar el contexto de la lesión nerviosa. Al igual que habíamos hecho al principio, primero caracterizamos la expresión de MFN1, MFN2 y OPA1 en diferentes modelos de lesión. Para este objetivo, estudiamos la respuesta aguda tanto en ratones jóvenes como en ratones envejecidos, la respuesta a lesión crónica y la respuesta en regeneración nerviosa. En todas las ocasiones observamos un aumento inmediato de los niveles de proteína de MFN1 tras la lesión, mientras que los niveles de MFN2 y OPA1 que estaban más elevados en nervios no lesionados sufrieron un ligero descenso progresivo tras la lesión. Es más, la expresión de MFN1 permaneció elevada en la lesión crónica pero no durante la regeneración nerviosa. Este patrón de expresión en lesión, combinado con los altos niveles de MFN1 en los estadios más inmaduros de las células de Schwann coincide con el de aquellas proteínas expresadas en los programas específicos de reparación en células de Schwann como c-Jun. Por lo que podríamos incluir a MFN1 en el conjunto de proteínas relacionadas con este estadio más indiferenciado/reparador.

Para poder caracterizar el papel de la fusión mitocondrial en el contexto de la lesión nerviosa y su reparación necesitamos el establecimiento de dos nuevas líneas de ratones transgénicos. Los mutantes condicionales constitutivos de *Mfn1/2* dKO y *Opa1* cKO no podían ser utilizados para realizar lesiones sobre los nervios ya que presentaban de base defectos en la mielina. En vez de usar la línea *MpzCre*, cruzamos los ratones con los genes *Mfn1/2* y *Opa1* floxeados con ratones *PlpCre-ERT2*. Esta línea solo expresa la recombinasa Cre en aquellas células que expresen Plp (oligodendrocitos y células de Schwann) tras la inyección de tamoxifeno. Por lo que la recombinación solo la indujimos una vez la vaina de mielina estaba formada produciendo los ratones *Mfn1/2* idKO (doble KO condicional inducible) y *Opa1* icKO (KO condicional inducible). Esta recombinación no produjo ninguna alteración en la estructura de la mielina, por lo que la fusión mitocondrial no es requerida para el mantenimiento de la vaina de mielina. Una vez inducida la recombinación se procedió a practicar las cirugías necesarias para los diferentes modelos de lesión. Los ratones *Mfn1* cKO y *Mfn2* cKO fueron utilizados con este mismo propósito ya que no presentaban ninguna alteración de base en la mielina.

A los 7 días de lesión por corte pudimos observar un aumento en la velocidad de eliminación de la mielina en los ratones *Mfn2* cKO caracterizado por una disminución de los niveles de MPZ y MBP. Así ocurrió también con los ratones *Mfn1/2* idKO y *Opa1* icKO, aunque solo se detectaron niveles reducidos de MPZ. En cuanto a los mutante de *Mfn1* no encontramos ninguna diferencia con respecto a los controles.

Por último, analizamos el impacto que podían tener los mutantes *Mfn1/2* idKO y *Opa1* icKO en la remielinización ya que habían sido los que mayor impacto en la mielinización. Sin embargo, no se encontraron diferencias en ninguno de los parámetros analizados hasta 40 días después de la lesión, cuando se considera que la remielinización es completo. Este aspecto de la lesión nerviosa aun requiere más investigación y es posible que nuevos análisis reflejen otros resultados.

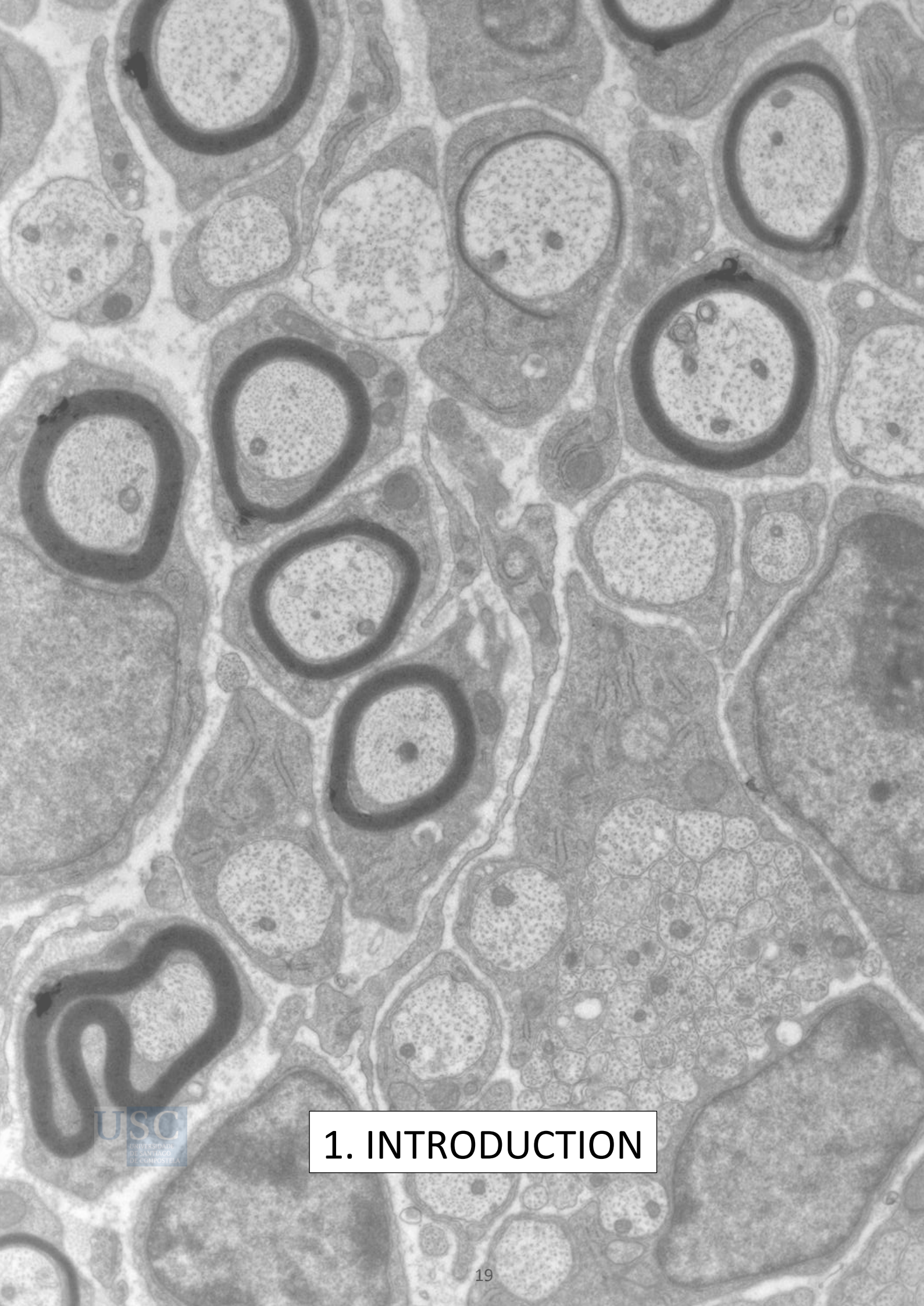
ABBREVIATIONS

ABBREVIATION	DEFINITION
ACC	Acetyl-CoA Carboxylase
Acetyl-CoA	Acetyl Coenzyme A
Actb	Actin b
ADP	Adenosine Diphosphate
AgRP	Agouti-Related Peptide
AMP	Adenosine Tphosphate
AMPK	AMP-activated Protein Kinase
AP-1	Activating Protein 1
ATP	Adenosine Triphosphate
ATP5A	ATP synthase F1 subunit alpha
BAT	Brown Adipose Tissue
BNB	Blood-Nerve Barrier
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3.
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
c-Jun	Jun Proto-Oncogene
cKO	Conditional Knock Out
CL	Cardiolipin
CMAP	Compound Muscle Action Potential
CMT	Charcot–Marie–Tooth disease
c-Myc	Myc Onco gene
CNS	Central Nervous System
COX10	Cytochrome C Oxidase assembly factor heme A:farnesyltransferase COX10
CV	Coefficient of Variation
<i>Cytb</i>	Cytochrome b
dKO	Conditional Double Knock Out
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic Acid
DOA	Dominant optic atrophy
DPI	Days Post-Injury
E12	Embryonic day 12
EDTA	Ethylenediaminetetraacetic Acid
EGR2	Early Growth Response 2
eIF2 α	Eukaryotic Initiation Factor 2 alpha subunit
EM	Electron Microscopy

ER	Endoplasmic Reticulum
<i>ErbB2</i>	Erythroblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-Regulated Kinase
ERMIN	ER Mitofusin 2
ERMIT	ER Mitofusin 2 Tether
ES	Embryonic Stem cells
FBS	Fetal Bovine Serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FSK	Forskolin
FUNDC1	FUN14 Domain-Containing Protein 1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GDAP1	Ganglioside Induced Differentiation Associated Protein 1
GLUT1	Glucose Transporter 1
GSEA	Gene Set Enrichment Analysis
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HCl	Hydrochloric acid
HIF1 α	Hypoxia-Inducible Factor 1 alpha
HKI	Hexokinase I
HKII	Hexokinase II
HR1	Heptad Repeat Domain 1
HR2	Heptad Repeat Domain 2
<i>Hsp60</i>	Heat Shock Protein 60
icKO	Inducible Conditional Knock Out
idKO	Inducible Conditional Double Knock Out
IMM	Intern Mitochondrial Membrane
ISR	Integrated Stress Response
JNK	c-Jun N-terminal Kinase
KO	Knock-Out
KOMP	Knock-Out Mouse Project
LC3	Microtubule-associated protein light chain 3
LDHA	Lactate Dehydrogenase A
LDHB	Lactate Dehydrogenase B
LKB1	Liver Kinase B1
LONP1	Lon Peptidase 1, Mitochondrial
L-OPA1	Long isoform of OPA1
MAMs	Mitochondria-Associated Membranes
MBP	Myelin Basic Protein
MCT1	Monocarboxylate Transporter 1

MCT2	Monocarboxylate Transporter 2
MCT4	Monocarboxylate Transporter 4
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MMRRC	Mutant Mouse Regional Resource Centers
MPZ	Myelin Protein Zero
mRNA	Messenger RNA
<i>Mrpl10</i>	Mitochondrial ribosomal protein L10
MSC	Mouse Schwann Cell
MTCO1	Mitochondrially encoded cytochrome c oxidase I
mtDNA	Mitochondrial DNA
mTORC1	Mechanistic Target of Rapamycin Complex 1
NaCl	Sodium Chloride
NAD ⁺	Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NAMPT	Nicotinamide phosphoribosyltransferase
NaOH	Sodium hydroxide
NB	Newborn
NCC	Neural Crest Cell
NCV	Nerve Conduction Velocity
ND1	NADH dehydrogenase subunit 1
ND4	NADH dehydrogenase subunit 4
NDUFS1	NADH:Ubiquinone Oxidoreductase Core Subunit S1
NF	Neurofilament
NRG1	Neuregulin 1
OCR	Oxygen Consumption Rate
OL	Oligodendrocyte
OMA1	Overlapping with the M-AAA protease 1 homolog
OMM	Outer Mitochondrial Membrane
OPA1	Optic Atrophy 1
OXPHOS	Oxidative Phosphorylation
P17	Post-natal day 17
pACC	Phospho Acetyl-CoA Carboxylase
pAMPK	Phospho AMP-activated Protein Kinase
peIF2 α	Phospho Eukaryotic Initiation Factor 2 alpha subunit
PERK	PKR-like ER Kinase
PFKFB3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 Enzyme
PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

Pgk1	Phosphoglycerate kinase 1
PHB1	Prohibitin 1
PHB2	Prohibitin 2
PINK1	PTEN-induced putative kinase 1
PLP	Proteolipid protein
PMP22	Peripheral Myelin Protein 22
PNS	Peripheral Nervous System
POMC	Pro-opiomelanocortin
Ppia	Peptidyl-prolyl cis-trans isomerase A
PR	Proline-Rich Domain
RHEB	Ras homolog enriched in brain
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
<i>Rpl13a</i>	Ribosomal protein L13a
<i>Rps26</i>	Ribosomal protein S26
RSC	Rat Schwann Cell
RT-qPCR	Real Time-quantitative Polymerase Chain Reaction
SC	Schwann cell
SCKO	Schwann Cell Knock Out
SCP	Schwann Cell Precursor
SDHA	Succinate dehydrogenase complex subunit A
SDS	Sodium dodecyl sulfate
SEM	Standard Error of the Mean
S-OPA1	Short isoform of OPA1
Tbp	TATA-box binding protein
TBS	Tris-buffered saline solution
TCA	Tricarboxylic Acid Cycle
TFAM	Transcription Factor A, Mitochondrial
UPR	Unfolded Protein Response
UQCRC2	Ubiquinol-Cytochrome c Reductase Core Protein II
VDAC	Voltage-Dependent Anion Channel
WT	Wild-type
YME1L1/YME1L	Yeast Mitochondrial Escape 1-Like 1



1. INTRODUCTION

1.1 PERIPHERAL NERVOUS SYSTEM

The peripheral nervous system is an intricate network of nerves and ganglia responsible for facilitating communication between the different regions of the body and supporting vital functions such as movement, sensory perception, and cognitive processes. It serves both as the primary control centre for physiological operations and as a key interface with the external environment. Efficient performance of these roles relies on the rapid transmission of nerve impulses. In certain organisms, this is accomplished by increasing the diameter of axons; a strategy effective primarily in invertebrates with relatively few nerve fibres, exemplified by the giant axon of the squid (Stassart et al., 2018).

The myelin sheath is a vertebrate innovation that allowed the emergence of large and complex nervous systems by enabling rapid and efficient nerve conduction. Phylogenetic evidence suggests that myelin first appeared in early gnathostomes, arising from a common glial progenitor that later diverged into the Schwann cell and oligodendrocyte lineages (Gould et al., 2008; Zalc, 2016).

These glial cells wrap the axons with a myelin sheath; a dense structure derived from their plasma membrane enriched in proteins and lipids that augments resistance and reduce axonal capacitance. This increases both speed and efficiency of electrical nerve impulse transmission as a result of saltatory nerve conduction (Nave, 2010; Saab & Nave, 2017). In myelinated axons, the regions wrapped in myelin are called internodes, while the uninsulated gaps between them are known as the nodes of Ranvier. These nodes serve as sites for the generation of electrical impulses. When a node undergoes depolarization, the membrane's electrical potential rises. If this change surpasses a critical threshold, it initiates depolarization in the neighbouring node. This process enables action potentials to "leap" from one node to the next, markedly increasing the speed of nerve impulse propagation (Bosch-Queralt et al., 2023). As a result, myelin sheaths facilitate the efficient transmission of electrical signals, ensuring synchronized responses and precise temporal control in neural communication.

Recent findings have revealed that myelin sheaths serve multiple functions beyond insulating axons, including providing metabolic support to axons and regulating neuronal activity and higher-order nervous system functions (Herbert & Monk, 2017; Jessen & Mirsky, 2019a, 2019b; McKenzie et al., 2014; Simons & Nave, 2016).

Consequently, the disruption or loss of myelin due to disease or injury can lead to a range of neurological impairments, including both cognitive and motor deficits (Gibson et al., 2018; Kamil et al., 2019). In the nervous system, myelin is produced by two distinct cell types: oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Oligodendrocytes and Schwann cell differ significantly in their structure, function, and developmental origins, reflecting their specialized roles within their respective systems (Cristobal & Lee, 2022).

1.2 SCHWANN CELL DEVELOPMENT

1.2.1 Neural crest cells

Schwann cells originate from neural crest cells (NCCs) and progress through different stages to finally transform into the mature myelinating or non-myelinating Schwann cells (Fig. 1A). NCCs are a group of multipotent cells that can differentiate to different cell lineages such as cardiac cells, skeletal and connective tissues of head, melanocytes and PNS glial cells. NCCs migrate ventrally during early embryonic development, and they are highly proliferative (reviewed extensively in Jessen & Mirsky, 2005, 2019a; Muppirala et al., 2021; Woodhoo & Sommer, 2008).

1.2.2 Schwann cell precursors

From their origin and throughout their lifespan, Schwann cells express the transcription factor Sox10 and remain closely associated with axons, establishing a relationship of mutual dependence. During the initial stages of PNS development, at around embryonic day 12-13 (E12-13) in mice, these NCCs rapidly differentiate into Schwann cell precursors (SCPs) (Jessen et al., 1994; Weston, 1963). SCPs associate with growing axons and surround their growth cones (Wanner et al., 2006), providing essential metabolic support. Notably, axonal integrity during this avascular developmental window depends on the trophic and energetic support from SCPs. In turn, the survival and proliferation of SCPs require axonal expression of type III Neuregulin 1 (NRG1), which signals via ErbB2/3 receptors on the SCP surface (Hancock et al., 2011; Meyer & Birchmeier, 1995). At this point, axons of all calibres are packed into bundles with SCPs. NRG1 and axonal interaction promotes SCP proliferation, inducing them to group axons into smaller bundles and forming a basal lamina. Schwann cells surrounding an axon bundle contained by the same basal lamina are known as Schwann cell families (Peters et al., 1991).

1.2.3 Immature Schwann cells

Subsequently, SCPs differentiate into immature Schwann cells (E15-17 to postnatal in mice) (Fig. 1A). Unlike SCPs, immature Schwann cells are connected to the basal lamina and have autocrine survival loops such that their survival no longer relies on direct axonal contact. Moreover, during this stage of PNS development, vascularization begins and connective tissue forms, with the first layers of the perineurium appearing (Jessen & Mirsky, 2005, 2019a).

1.2.4 Myelinating/non-myelinating Schwann cells

Just before birth, immature Schwann cells differentiate into promyelinating Schwann cells. In a process known as radial sorting, a Schwann cell select an axon with a diameter $\geq 1 \mu\text{m}$ and separate it from the family, a process involving mitosis. At this stage, these promyelinating Schwann cells, are in a relationship 1:1 with the axons. These axons will become myelinated

while the rest of the smaller diameter axons will remain together in the family, forming unmyelinated Remak bundles (Fig. 1B).

As promyelinating Schwann cells differentiate into myelinating Schwann cells, they upregulate *Egr2*, a key transcription factor that controls the myelination program by inducing the expression of myelin protein and lipid biosynthesis genes. Meanwhile, small-calibre axons that remain bundled after radial sorting become individually ensheathed within cytoplasmic pockets of a single Schwann cell, which differentiates into a mature Remak (non-myelinating) Schwann cell. Unlike the axon bundles seen during early development, Remak bundles are composed exclusively of small axons that are properly ensheathed by Schwann cells (Feltri et al., 2016) (Fig. 1A).

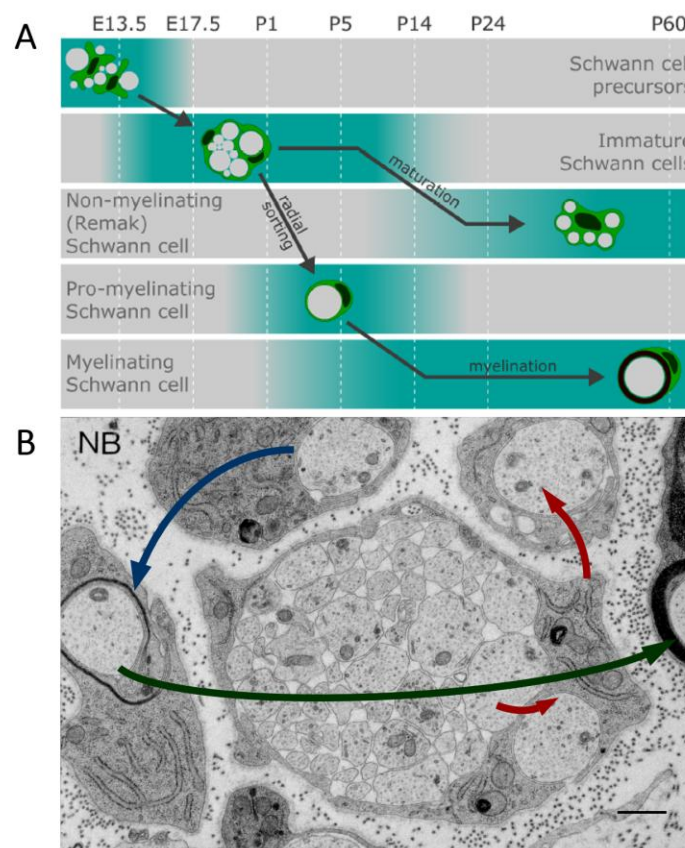


Figure 1. (A) Schematic representation of the temporal progression of Schwann cell (SC) development in peripheral nerves, aligned with key differentiation milestones. At E13.5, Schwann cell precursors are present in developing nerves. By E17.5, these precursors differentiate into immature Schwann cells, which proliferate and initiate radial sorting of axons—a process that continues through P1 and P5. During these stages, many Schwann cells establish a 1:1 relationship with large-calibre axons, becoming pro-myelinating Schwann cells. These cells undergo transcriptional reprogramming to initiate myelination, which progresses alongside radial and longitudinal expansion of the myelin sheath, particularly evident by P14 and P24. Small diameter axons remain associated with non-myelinating (Remak) SCs. By P60, the developmental myelination program concludes, and sciatic nerves are primarily composed of mature myelinating and Remak Schwann cells. Figure obtained from (Gerber et al., 2021). **(B) EM micrograph of a transversal section of a newborn sciatic nerve showing initial stages of the myelination process,** red arrows indicate radial sorting process; blue arrow reflect the initiation of myelination in the pro-myelinating stage and green arrow marks the full maturation of myelin sheath. Scale bar, 0.6 μ m. Figure adapted from (Jessen & Mirsky, 1999).

Depending on the differentiation pathway Schwann cells follow during development, the axons that become either myelinated or ensheathed in Remak bundles give rise to distinct modes of action potential propagation: saltatory conduction in myelinated fibers versus continuous conduction in non-myelinated fibers (Peters et al., 1991). Based on this, each fibre will perform distinct physiological roles. Myelinated axons comprise large-calibre (13 - 20 μm), rapidly conducting type A skeleton-motor and proprioceptor fibres and mid-calibre (6 - 12 μm), fast-conducting type B mechanoreceptors, and preganglionic autonomic efferent fibres. Axons ensheathed by Remak Schwann cells are small in diameter (typically less than 1 μm) and correspond to slowly conducting type C fibres, with conduction velocities ranging from 0.5 to 4 m/sec. These fibres include postganglionic autonomic efferent axons as well as sensory afferents from the skin that transmit pain and temperature signals (J. Salzer et al., 2024).

1.3 SCHWANN CELL MYELINATION

Myelin sheath formation entails a huge expansion of the Schwann cell plasma membrane. For example, it has been estimated that myelinating Schwann cells generate around 20 mm^2 of membrane for the largest axons (Kidd et al., 2013). This process occurs in a short lapse of time requiring an exquisite coordination of transcriptional and translational control, directing and inserting all myelin components to their membrane location. Additionally, stoichiometry of myelin components must be precise. The extensive membrane expansion required for myelination imposes considerable stress on Schwann cells, particularly within the endoplasmic reticulum (ER) (Scherer & Svaren, 2024).

1.3.1 Myelin structure

Myelin is a multilayered, lipid-rich membrane that wraps around axons to enable the rapid transmission of electrical signals (Fig. 2). It is a complex structure organized into different membrane domains, each of them with a singular set of proteins, and a communicating set of cytoplasmic compartments. This makes myelinating Schwann cells extremely polarized cells, both at a radial and longitudinal level. Radial polarity, which determines myelin thickness, is composed by an abaxonal membrane (faced to the exterior) and adaxonal membrane (faced to the axon). Longitudinal (axial) polarity is reflected in the structured organization of myelinating Schwann cells and axons into distinct domains: nodal, paranodal, juxtaparanodal, and internodal regions (Rasband & Peles, 2025).

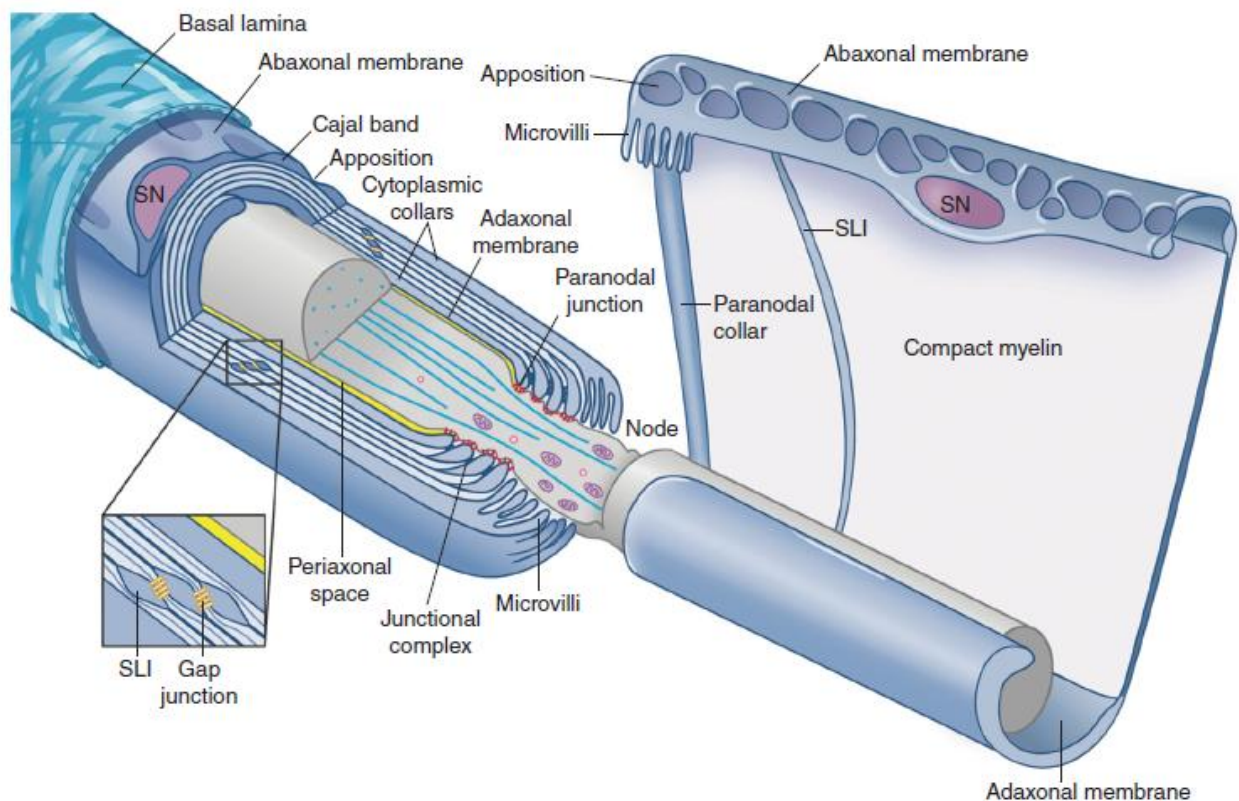


Figure 2. Schematic illustration of the structural organization of a myelinating Schwann cell ensheathing an axon. The Schwann cell is enclosed by a basal lamina, which interfaces with its abaxonal membrane. The abaxonal cytoplasmic domain, containing the Schwann cell nucleus, is organized into longitudinal Cajal bands interspersed with periodic appositions where the abaxonal membrane contacts the outermost myelin layer. The adaxonal membrane, facing the axon, is separated from it by a narrow periaxonal space. Compact myelin, which insulates the axon, is periodically interrupted by Schmidt–Lanterman incisures, cytoplasmic channels rich in autotypic junctions such as gap, tight, and adherent junctions. Similar junctional complexes are found in the paranodal loops, where the myelin sheath anchors near the node of Ranvier. Schwann cell microvilli extend toward the axonal surface at the node, where axonal diameter decreases, forming a specialized microenvironment for signal conduction. Figure obtained from (Salzer, 2015)

1.3.2 Myelin composition

The myelin sheath is a tightly packed, spiral structure composed of multiple membrane layers, interspersed with small cytoplasmic channels that support cellular communication and nutrient exchange (Fig. 3). These membrane layers are stabilized by specialized proteins, with myelin protein zero (MPZ) being the most abundant, alongside peripheral myelin protein 22 (PMP22) and myelin basic protein (MBP), all of which contribute to the structural compaction of the sheath. In addition, lipids such as cholesterol, phospholipids, galactolipids, and plasmalogens are essential components that confer membrane fluidity and structural integrity to the Schwann cell myelin (Salzer, 2015).

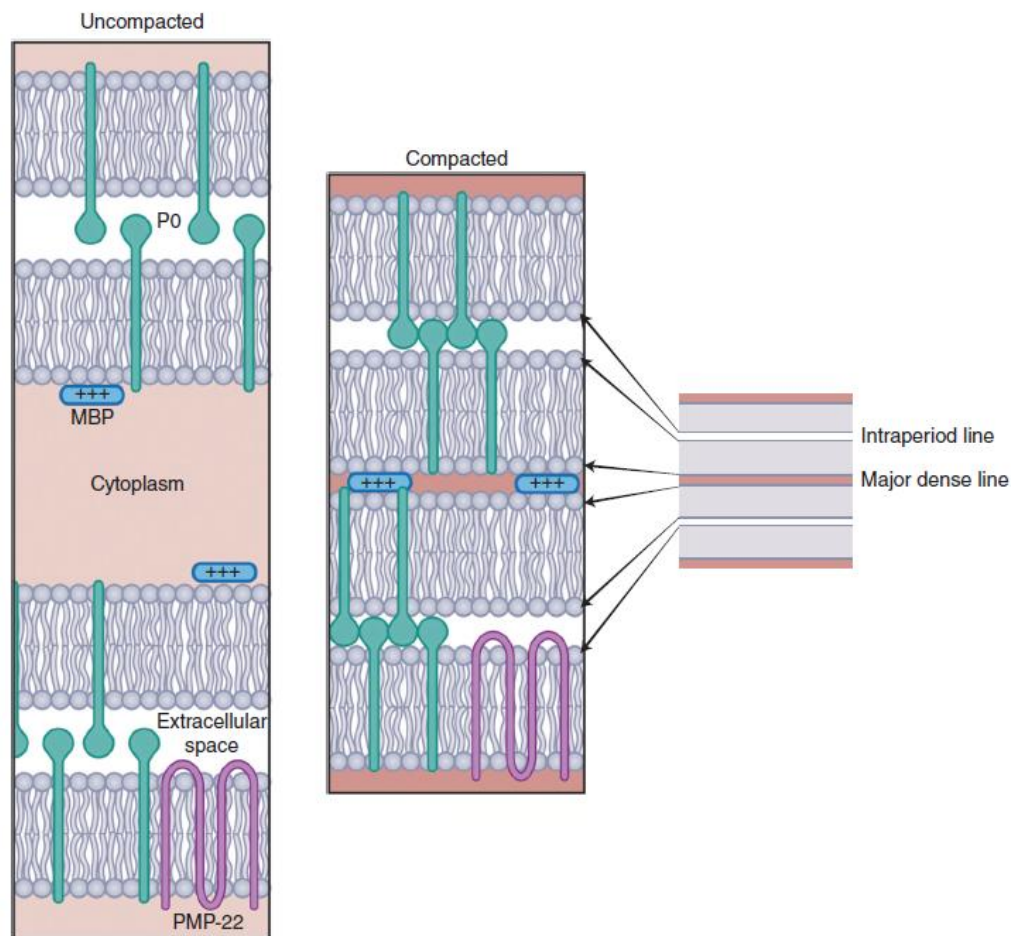


Figure 3. Schematic illustration of the structural maturation of the compact myelin sheath. Myelination begins with loose, non-compacted membrane, which wraps around the axon. As the myelinating program is activated, key myelin proteins (such as MPZ, MBP and PMP22) are upregulated. Myelin compaction involves both extracellular and cytoplasmic mechanisms: MPZ proteins form homophilic interactions across opposing extracellular membrane leaflets, while cytoplasmic compaction is driven by electrostatic binding of MBP to the inner membrane surface, supported by interactions with the cytoplasmic tail of MPZ. In electron microscopy pictures, compact myelin can be visualized as alternating structural features: major dense lines (MDLs), representing tightly apposed cytoplasmic leaflets, and intraperiod lines, corresponding to the aligned extracellular leaflets. Figure obtained from (Salzer, 2015). Copyright to Cold Spring Harbor Laboratory Press.

1.4 THE ROLE OF SCHWANN CELLS IN NERVE INJURY

In mammals, peripheral nerves have an intrinsic capacity to regenerate. Successful nerve repair relies on Schwann cells, which, because of their inherent plasticity, are able to transdifferentiate to repair Schwann cells, which organize and enable the repair response (Jessen et al., 2015).

Research on peripheral nerve injury has focused on two primary types: nerve transection (neurotmesis) and nerve crush (axonotmesis) (Seddon, 1942). In both cases, the damage initiates a well-characterized sequence known as Wallerian degeneration (Waller, 1851), which involves the rapid breakdown of axons distal to the injury site and the subsequent removal of axonal debris (Arthur-Farraj & Coleman, 2021).

The primary objective of nerve regeneration is to enable axons from surviving neurons to regrow toward their original targets. This regenerative response is highly effective after a crush injury, where the overall nerve architecture is largely preserved. In contrast, regeneration is significantly less precise following a transection injury, which disrupts not only the basal lamina tubes enclosing the Schwann cell/axon units but also the surrounding connective and perineurial tissues (Nguyen et al., 2002).

1.4.1 Schwann cell reprogramming

After injury, a dramatic cell reprogramming process takes place in which Schwann cells transdifferentiate into a specific progenitor-type phenotype called repair Schwann cells (Fig. 4). This reprogramming is activated by the ERK signalling pathway that induces this phenotypic switch. The Schwann cells downregulate their myelin program and conversely induce the repair program by activating a set of repair genes, under the overall influence of the c-Jun transcription factor and other secondary AP-1 transcription factors family members (such as *Atf3*, *c-Fos*, *Fos/2*) (Arthur-Farraj et al., 2012, 2017; Jessen & Arthur-Farraj, 2019; Jessen & Mirsky, 2019b; Stassart et al., 2013). These repair SCs perform several cell-autonomous and non-cell-autonomous functions in orchestrating nerve regeneration. Their key functions include: (1) regulating the disruption of the blood-nerve barrier (BNB) and the resulting infiltration of inflammatory cells, (2) clearing myelin and axonal debris through autophagy, and (3) promoting axonal regeneration. Notably, their ability to guide and support axons as they grow back toward their original targets is essential for effective peripheral nerve regeneration (Gomez-Sanchez et al., 2015; Jessen & Arthur-Farraj, 2019; Jessen & Mirsky, 2016; Stassart & Woodhoo, 2021; Stierli et al., 2019).

1.4.2 Success and failure of nerve function restoration

An important feature of the Schwann cell injury response is the creation of regeneration tracks. After injury, Schwann cell morphology changes radically: they take an elongated bipolar morphology, with repair Schwann cells forming an aligned column known as Büngner band inside of the basal lamina tubes that previously surrounded Schwann cell and axon. Schwann cells forming these Büngner bands provide vital substrate and guidance to the regenerating axons, so they can reach again nerve target areas. Schwann cell tracks offer a supportive substrate crucial for axonal regrowth across the injury site. However, their guidance relies on the orientation of polarized blood vessels, as neither SCs nor axons are capable of migrating through the three-dimensional wound matrix. Instead, SCs navigate by migrating along the surfaces of blood vessels (Cattin et al., 2015).

When nerve regeneration is impaired or delayed, the result is a state of chronic nerve injury. This constitutes a frequent and serious problem in clinical practice, since humans possess long nerves and their restoration after a nerve transection can be interrupted (Höke et al., 2006; Palispis & Gupta, 2017; Sulaiman & Gordon, 2013; Terenghi, 1995). Beyond the critical distance

to the distal stump, failure in maintaining repair Schwann cell phenotype is one of the limitations for successful regeneration (Arthur-Farraj et al., 2012; Boyd & Gordon, 2003; Eggers et al., 2010; Jessen & Mirsky, 2019b).

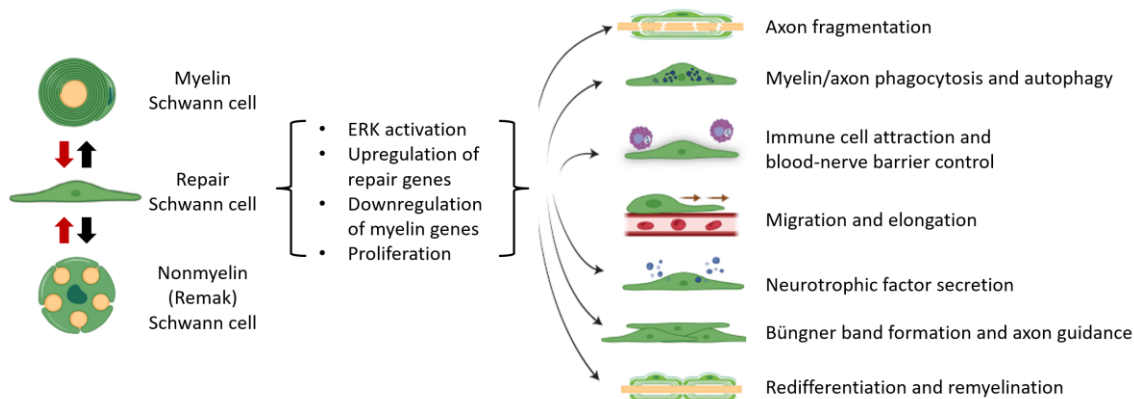


Figure 4. Schematic representation of the functions of repair Schwann cells. Following nerve injury, mature Schwann cells (myelinating or non-myelinating) undergo a rapid and efficient reprogramming process into repair Schwann cells, primarily driven by ERK signalling. This transition involves downregulation of myelin-associated genes, upregulation of repair-specific genes, and increased Schwann cell proliferation. Repair Schwann cells are central to the regenerative response: they actively participate in axonal degeneration, and clear myelin and axonal debris via autophagy and phagocytosis. They also modulate the regenerative microenvironment by recruiting immune cells, regulating the blood-nerve barrier, and secreting neurotrophic factors that support neuronal survival and regeneration. Furthermore, repair Schwann cells form Büngner bands, which provide structural and molecular guidance for axon regrowth across the lesion site and through the distal nerve stump. Functional recovery is ultimately achieved through SC redifferentiation and remyelination of regenerated axons. Figure adapted from: (Stassart et al., 2024). Copyright to Cold Spring Harbor Laboratory Press.

1.5 SCHWANN CELL METABOLISM

Myelination is a metabolically demanding process that requires massive biosynthesis of lipids and proteins in a short lapse of time. Some estimations calculate that the average Schwann cell total membrane area (plasma membrane and myelin) expands 2600-fold from day 1 to day 75 of rat development (Della-Flora Nunes et al., 2021). Moreover, axons of the PNS are either completely myelinated or fully surrounded by non-myelinating Schwann cells, which severely limits direct contact between the axons and the extracellular environment. There is emerging evidence that Schwann cells provide axons with essential metabolic and trophic support, a function that seems to be independent of myelination (Bosch-Queralt et al., 2023; Nave, 2010).

1.5.1 Mitochondrial function in Schwann cells

Mitochondria, pivotal organelles in cellular metabolism, are important regulators of myelin formation and maintenance in Schwann cells. In the last few years, several studies have demonstrated the consequence of the loss of function of mitochondrial proteins in Schwann cells *in vivo*. For example, conditional deletion of the respiratory chain component COX10 results in severe demyelination (malformed myelin) in the PNS (Fünfschilling et al., 2012),

whereas deletion of Prohibitin 2 (PHB2), a protein enriched in the inner mitochondrial membrane, in Schwann cells leads to a severe radial sorting defect in mice that is evident at P20 (Poitelon et al., 2015). This contrasts with the normal developmental or a mild developmental delay in myelination followed by a severe and progressive demyelinating peripheral neuropathy in Schwann cell-specific ablation of the mitochondrial transcription factor TFAM (Viader et al., 2011), the inner mitochondrial membrane protein Prohibitin1 (PHB1) (Della-Flora Nunes et al., 2021) or the mitochondrial network regulator GDAP1 (Niemann et al., 2014).

Viader and colleagues (2013) proposed that Schwann cell mitochondrial dysfunction caused by Schwann cell-specific *Tfam* ablation cause a maladaptive integrated stress response (ISR), leading to a deregulation in lipid metabolism and consequently inducing demyelination. However, Della-Flora Nunes and colleagues (2021) showed that treatment with an ISR inhibitor in *Phb1*-SCKO induced an exacerbated demyelination, discarding ISR induction as the demyelinating mediator and postulating it instead as a protective mechanism against demyelination.

1.5.2 The Schwann cell metabolic state

The metabolic state is critical for Schwann cell differentiation. When NAD⁺ homeostasis (which is fundamental for cellular metabolism since NAD⁺ serves as a central redox cofactor and signalling molecule) is deregulated, Schwann cell viability remains unchanged, but Schwann cell differentiation and myelination are impaired (Sasaki et al., 2018). In mice lacking NAMPT, a key enzyme involved in NAD⁺ metabolism, Schwann cells appear to undergo a dedifferentiation program driven by activation of the MEK/ERK/JUN signaling cascade, similar to that observed in *Phb1* Schwann cell mutants (Della-Flora Nunes et al., 2021). Schwann cells engage adaptive mechanisms to avoid cell death either via specific Schwann cell-stress response upregulating *c-Jun* or via general induction of ISR.

Key recent studies have shown that Schwann cell differentiation and myelination during development, and demyelination and remyelination following nerve injury, lead to a profound metabolic reprogramming in Schwann cells (Fig. 5).

In developing nerves, proliferating Schwann cells undergo metabolic reprogramming from glycolytic to oxidative metabolism during differentiation and myelination *in vivo*, with the metabolic regulator LKB1 playing an essential role in this metabolic shift. In the pre-myelinating sciatic nerve, axons are unmyelinated, and Schwann cells primarily rely on glycolysis to meet their bioenergetic demands, resulting in elevated lactate production relative to mitochondrial TCA cycle-derived acetyl-CoA. Activation of LKB1 enhances citrate synthase activity, promoting a metabolic shift from glycolysis toward oxidative phosphorylation. This transition enables Schwann cells to differentiate and initiate myelination. During this metabolic reprogramming, glucose is fully oxidized via the TCA cycle and oxidative phosphorylation, increasing acetyl-CoA

production. Acetyl-CoA then condenses with oxaloacetate to form citrate, which is exported to the cytoplasm to fuel *de novo* synthesis of myelin lipids (Pooya et al., 2014).

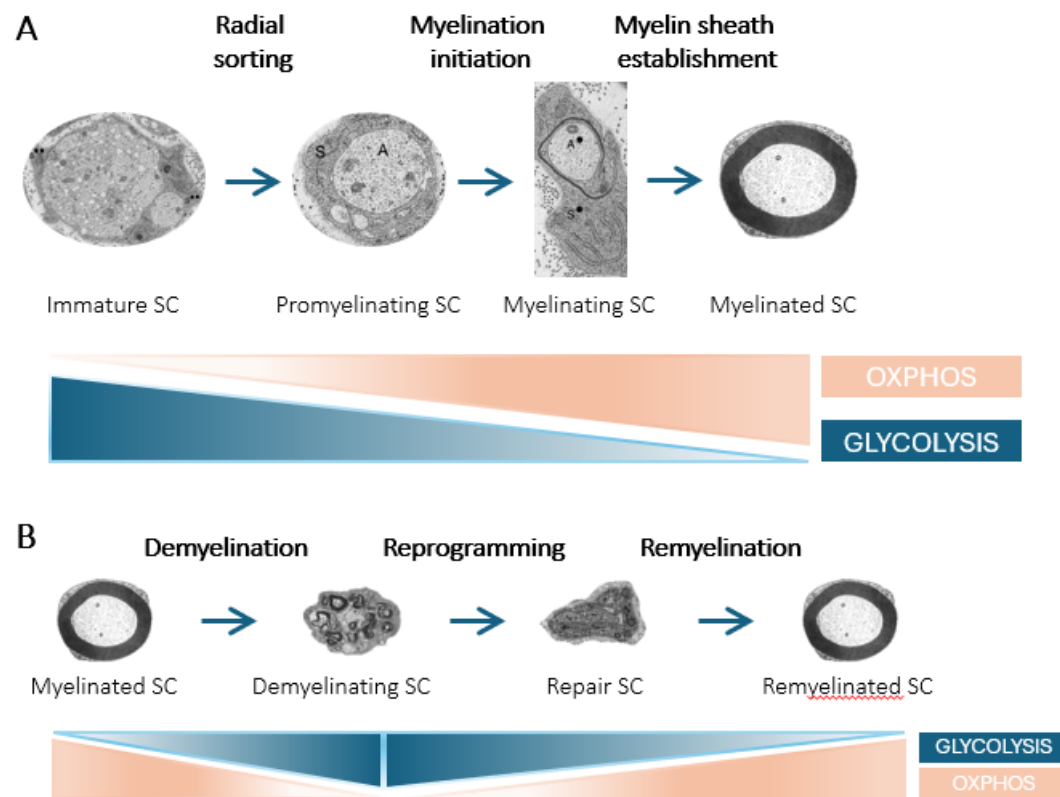


Figure 5. Schematic representation of the metabolic adaptations of Schwann cells. (A) In developing nerves, proliferating Schwann cells undergo metabolic reprogramming from glycolytic to oxidative metabolism during differentiation and myelination *in vivo*, a process governed by LKB1 (Pooya et al., 2014). (B) During the successive stages of demyelination, reprogramming and remyelination after nerve injury, Schwann cells undergo a profound remodelling of their metabolic programs. After injury, Schwann cells respond with glycolytic activation immediately (within 72 h) after acute nerve injury (Babetto et al., 2020), a period that covers the onset of Wallerian degeneration, but the subsequent time course of nerve de- and regeneration (7–28 days post crush) is associated with a profound metabolic activation of repair Schwann cells, including an oxidative switch and a marked increase in mitochondrial respiration (Sundaram et al., 2023). Figure created by the author. Schwann cell development: EM micrographs were taken from (Jessen & Mirsky, 1999).

As mentioned above, Schwann cells are able to transdifferentiate to repair Schwann cells after nerve injury. As part of the acute nerve cut response, Schwann cells undergo a rapid metabolic shift towards glycolysis in response to axonal energy depletion. This happens within 72h, a time frame that coincides with the initiation of Wallerian degeneration. This rewiring is driven by mTORC1 activation downstream of ErbB2 signaling, which induces HIF1 α and c-Myc to upregulate key glycolytic enzymes, including GLUT1, PFKFB3, and LDHA, enhancing pyruvate and lactate production from glucose. Concomitantly, monocarboxylate transporters (MCT1 and MCT4 in SCs; MCT2 in axons) are upregulated, facilitating the transfer of these metabolites to injured axons. While axonal mitochondria can directly utilize pyruvate for ATP synthesis, lactate must first be converted to pyruvate by LDHB (Babetto et al., 2020).

However, during the following process of nerve de- and regeneration (7 - 28 days post crush) Schwann cells undergo a profound metabolic activation characterized by an oxidative metabolic switch and a strong increase in mitochondrial respiration. The upstream regulatory control of this metabolic repair response relies on the leptin/leptin receptor signalling axis. Since the main source of circulating leptin in the body are adipocytes, it was shown that adipocytes respond to peripheral nerve injury with an increase in the mass of para-neural adipose tissue ipsilateral to the injury site., which is associated with augmented local leptin levels after nerve injury. This adipocyte-derived leptin response occurs in a non-cell-autonomous manner and contributes to nerve repair by enhancing Schwann cell function. Activation of leptin receptor signalling in repair Schwann cells involves the expression and translocation of receptors to the plasma membrane and results in glial metabolic modulation during peripheral nerve repair (Sundaram et al., 2023).

In summary, nerve degeneration and regeneration are defined by catabolic autophagy and anabolic remyelination in repair Schwann cells as early and late events, respectively, recapitulating the similar metabolic shift that occurs during development.

1.5.3 Schwann cell-axon metabolic coupling

Schwann cell metabolism not only affects their own differentiation but can also affect axonal health and survival. For example, disruption of mitochondrial pyruvate metabolism in Schwann cells via *Rheb* knockout impairs neuronal mitochondrial function, resulting in oxidative stress and axonal degeneration. This effect is mediated by elevated lactate production and release from Schwann cells (Jia et al., 2021). Although lactate-supported neuroenergetics may transiently benefit injured peripheral axons (Beirowski et al., 2014), sustained neuronal lactate metabolism induces oxidative damage, leading to energy deficits and progressive axonopathy.

Lactate transport is mediated by monocarboxylate transporters (MCTs), which are a family of proton-linked plasma membrane transporters that mediate the movement of monocarboxylates (such as lactate itself, pyruvate, and ketone bodies) across membranes. In the PNS, MCT1, MCT2 and MCT4 are expressed, and they are localized in regions where lactate is likely generated and/or traffics through defined cellular compartments towards its target. The direction of lactate movement appears to be governed primarily by substrate concentration gradients rather than by regulated expression of specific transporters (Domènech-Estévez et al., 2015). When *Mct1* is ablated, the lactate transport into Schwann cells is perturbed, which can impair lipogenesis, leading to elevated c-Jun, and resulting in myelination defects. Schwann cell-specific MCT1 ablation causes deficits in sensory (type A and B), but not in motor fibres (type C) at early stages (Jha et al., 2020).

However, MCT1, but not MCT4, expression in Schwann cells is required for the long-term maintenance of neuromuscular junction innervation and is linked to transcriptional alterations in lumbar motor neurons. These findings suggest that Schwann cell-mediated metabolic

support is essential for preserving motor neuron function. In the absence of this support, motor neurons appear to initiate compensatory mechanisms by upregulating genes involved in mitochondrial function, transcriptional regulation, and glycosylation—processes critical for the activity of transmembrane proteins, including neurotransmitter receptors and cell adhesion molecules (Bouçanova et al., 2021).

SC-axon metabolic coupling is also crucial for nerve regeneration since a delay in regeneration was observed in *Mct1* heterozygous null mice of motor axons as well as sensory and mixed axons (Morrison et al., 2015).

As described above, the Schwann cell-axon unit is not a homogeneous structure. Schwann cells associated with sensory or motor nerves have differential functions, distinct molecular phenotypes (Brushart et al., 2013) and different metabolic relationships. For example, in contrast to C fibres, which appear to directly uptake and metabolize fructose, A fibres rely on a Schwann cell-to-axon lactate shuttle to derive metabolic benefit from fructose (Rich & Brown, 2018).

1.6 MITOCHONDRIAL FUSION AND METABOLISM: A LINK BETWEEN SHAPE AND FUNCTION

Mitochondria are highly dynamic organelles capable of moving, fusing, dividing, and being degraded. Through continuous cycles of fission and fusion, they form flexible, interconnected networks that adapt their structure to meet the metabolic demands of the cell. Fusion plays a critical role in oxidative phosphorylation (OXPHOS), particularly through the regulation of mitochondrial DNA (mtDNA) levels (Mishra & Chan, 2016). Fission, in turn, is essential for generating new mitochondria during cell division, redistributing mitochondria within the cell, and isolating damaged organelles for removal. Fusion also promotes the exchange of contents between mitochondria, supporting mitochondrial function and quality control. Proper mitochondrial positioning is vital to meet localized energy requirements, making mitochondrial transport and mobility key for delivering these organelles to sites of high metabolic activity (El-Hattab et al., 2018; Suárez-Rivero et al., 2016).

1.6.1 Metabolic adaptation

Mechanistically, mitochondrial metabolic adaptability is closely tied to their structural plasticity (Friedman & Nunnari, 2014). Mitochondrial morphology and spatial organization are regulated by the coordinated activity of the machinery responsible for division, fusion, and motility. In glycolytic pluripotent embryonic stem cells, mitochondria appear fragmented and incomplete in their internal cristae structure. As stem cells differentiate, their mitochondria undergo significant remodelling: they transition to oxidative phosphorylation for energy production, adopt a more elongated morphology, and form interconnected networks with well-developed cristae. These structural changes are governed in part by the balance between mitochondrial fission and fusion (Amato et al., 2023; Suomalainen & Nunnari, 2024). In

neuronal differentiation, profound mitochondrial remodelling is associated with increase of organelle abundance, changes in spatial distribution and bioenergetic function. This remodelling is tightly controlled by the fusion regulators MFN2 and OPA1, which facilitate the metabolic transition from glycolysis to oxidative phosphorylation, an essential step for neuronal maturation. (Zanfardino et al., 2025).

Mitochondrial fusion is a coordinated two-step process involving the merging of the outer (OMM) and inner mitochondrial membranes (IMM), occurring nearly simultaneously. This process is mediated by three key proteins: mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy 1 (OPA1) (Fig. 6) (Chen et al., 2003; Wai & Langer, 2016).

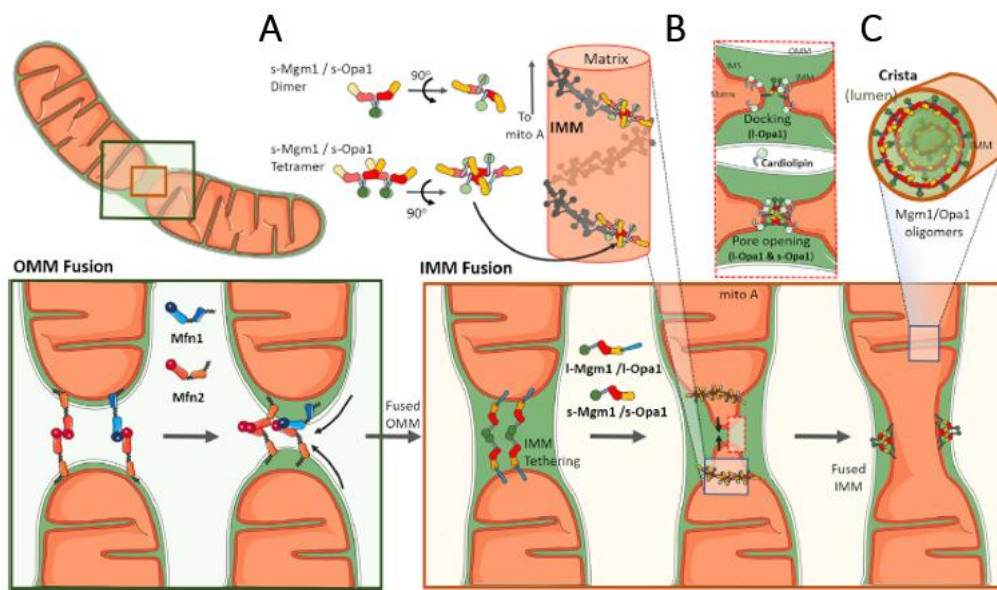


Figure 6. The fusion process of the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) pictured from left to right. (A) At the OMM, fusion is initiated by tethering and docking mediated by homotypic or heterotypic complexes of mitofusins MFN1 and MFN2, enabling membrane juxtaposition and subsequent fusion. Following OMM fusion, the IMM undergoes a separate but coordinated fusion process, initiated by long isoforms of OPA1 (L-OPA1). These mediate tethering and docking of the IMM, a process that requires interaction between L-OPA1 and cardiolipin on opposing membranes. Efficient IMM fusion is further promoted by heterotypic interactions between L-OPA1 and the short, proteolytically processed form of OPA1 (S-OPA1), in conjunction with GTP hydrolysis (B). This fusion process is associated with the assembly of Opa1 oligomers at the IMM, as described for both mammalian OPA1 and its yeast ortholog MGM1. As illustrated in (A), S-MGM1/OPA1 first form dimers, which then assemble into tetramers and grow into helical filaments that form a lattice on the IMM facing the IMS. Once IMM fusion is complete (bottom right), both L-OPA1 and S-OPA1 are redistributed across the fused membrane. In panel (C), an inset shows S-MGM1/OPA1 organized on the luminal side of cristae, forming helical structures. Figure obtained from (Quintana-Cabrera & Scorrano, 2023).

1.6.2 OMM fusion

Fusion of the OMM is regulated by MFN1 and MFN2, which possess C-terminal transmembrane domains that allow them to be anchored to the membrane. MFN1 and MFN2 are highly similar proteins in mammals (~80% similarity in humans), that are constituted by 737 and 757 amino acids, respectively. They are composed of a large, cytosolic, N-terminal GTPase domain, sequentially followed by a spacer, a first coiled-coil heptad-repeat (HR1)

domain, a spacer, two very close domains (TM) crossing the OMM, a spacer and a second, C-terminal heptad-repeat domain (HR2). Notably, between the HR1 and the TM domains, only MFN2 contains a proline-rich (PR) domain, which is thought to be responsible for specific protein–protein interactions. HR2 domains are relevant for the initial tethering between adjacent mitochondria, while the GTPase domain seems to be determinant for fusion completion (Filadi et al., 2018).

Interestingly, differential GTPase activity has been described for MFN1 and MFN2, and this could be one of the reasons for the different function they perform in mitochondrial fusion. MFN1 tethers mitochondrial membranes, depending on GTP hydrolysis, more efficiently than MFN2, which corresponds to changes in the oligomeric states of the MFN-containing complex. Purified MFN1 exhibited around eight-fold higher GTPase activity than MFN2 (Ishihara et al., 2004).

1.6.2 IMM fusion

IMM fusion is mediated by OPA1, a dynamin-like GTPase anchored to the IMM by an N-terminal transmembrane domain, with its functional domains facing the intermembrane space (Chen, 2010). OPA1 exists in long (L-OPA1) and short (S-OPA1) forms, generated through alternative splicing and proteolytic cleavage by the intermembrane enzymes OMA1 and YME1L1 (Ishihara et al., 2006). OPA1 is proteolytically processed at three distinct sites (S1, S2, and S3) resulting in long (L-OPA1) and short (S-OPA1) isoforms that regulate mitochondrial fusion. The S1 site is cleaved by the stress-activated protease OMA1 in response to mitochondrial depolarization, ATP depletion, and other stressors (Baricault et al., 2007; Merkwirth et al., 2008). In contrast, S2 and S3 are constitutively cleaved by the ATP-dependent metalloprotease YME1L, whose activity is upregulated under conditions of OXPHOS stimulation (Mishra et al., 2014). This processing typically maintains a balance between L-OPA1 and S-OPA1 (Gripic et al., 2007; Ishihara et al., 2006; Song et al., 2007; Wang et al., 2021), which is critical for mitochondrial morphology.

Disruption of this balance, through OMA1 activation or YME1L inactivation (which indirectly activates OMA1), leads to excessive accumulation of S-OPA1 and mitochondrial fragmentation (Anand et al., 2014; Wai et al., 2015). Although earlier studies proposed that L-OPA1 alone could support mitochondrial fusion under stress (Anand et al., 2014; Tondera et al., 2009), recent evidence indicates that an appropriate ratio of S-OPA1 to L-OPA1 is essential. Fusion efficiency is optimal at near-equimolar levels of the two isoforms, whereas an elevated S-OPA1:L-OPA1 ratio (e.g., $\geq 2:1$) compromises mitochondrial fusion (Ge et al., 2020).

To function properly, OPA1 requires mitofusin 1 to regulate mitochondrial fusion. In cells deficient in MFN1, OPA1 is unable to induce mitochondrial fusion. This impairment is restored by reintroduction of MFN1 but not MFN2, identifying outer membrane MFN1 as a crucial functional partner of OPA1. Furthermore, MFN1 cannot induce mitochondrial elongation if

Opa1 is ablated. Thus, OPA1 and MFN1 are presented as functionally depending on each other (Cipolat et al., 2004).

OPA1 is required on only one IMM of the two opposing mitochondria to drive fusion (Song et al., 2009). Indeed, OPA1 can bind in trans to the lipid cardiolipin (CL) in the IMM of the opposing mitochondria (Ban et al., 2017, 2018). In this minimal model, Opa1 and CL heterotypically interact and require Opa1-mediated GTP hydrolysis to mediate fusion but not tethering (Quintana-Cabrera & Scorrano, 2023).

1.7 MITOFUSINS BEYOND MITOCHONDRIAL FUSION

Both MFN1 and MFN2 are indispensable for embryonic development in mice (Chen et al., 2003). Deletion of either *Mfn1* or *Mfn2* results in mid-gestation lethality, whereas heterozygous animals are viable and fertile. Notably, *Mfn2* knockout embryos display defective placental formation, a phenotype not observed in *Mfn1*-KO mice. Combined deletion of *Mfn1* and *Mfn2* leads to even earlier embryonic lethality.

Conditional post-placentation ablation of *Mfn1* or *Mfn2* revealed distinct outcomes: *Mfn1* loss is compatible with postnatal survival, while *Mfn2* deficiency causes severe cerebellar defects, leading to motor impairments and death before postnatal day 17 (P17). Moreover, in conditional *Mfn1* KO mice, haploinsufficiency of *Mfn2* is lethal, underscoring both redundancy and functional divergence between the two mitofusins, which appear to be context- and stage-dependent (Chen et al., 2007).

One clear example of this partial overlapping function but clearly distinct roles played is shown when *Mfn1*-KO and *Mfn2*-KO mouse embryonic fibroblasts (MEFs) are cultured (Fig. 7). *Mfn1*-KO MEFs present a severe mitochondrial fragmentation, with mitochondria forming small uniform spheres. On the other hand, mitochondria in *Mfn2*-KO cells are spheric or oval in form with a great size range. Overexpression of MFN1 in *Mfn2*-KO cells, or MFN2 in *Mfn1*-KO cells, partially rescues mitochondrial fusion, indicating a degree of functional redundancy. However, overexpression of either MFN1 or MFN2 in wild-type cells induces mitochondrial aggregation and perinuclear collapse, suggesting that balanced expression levels are critical for normal mitochondrial dynamics (Eura et al., 2003; Filadi et al., 2015; Rojo et al., 2002).

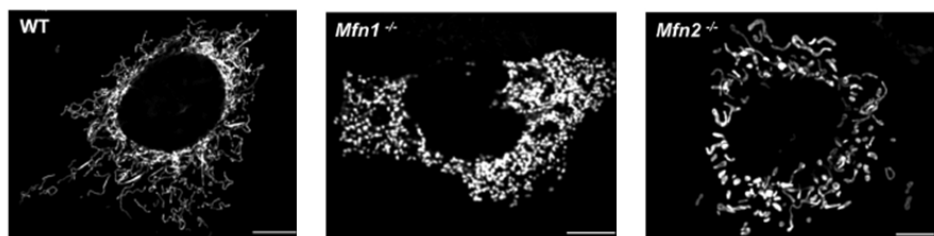


Figure 7. Representative confocal microscopy images of WT, *Mfn1*-KO and in *Mfn2*-KO MEF cells, expressing a mitochondrial matrix-targeted RFP. Scale bar: 5 μ m. The fragmented mitochondrial network in mitofusins ablated cells displays a different phenotype depending on the specific *Mfn*. *Mfn1*-KO induces small, uniformed spheres and *Mfn2*-KO, more enlarged structures of different size. Figure obtained from (Filadi et al., 2018).

1.7.1 Mitofusins expression pattern

Although MFN1 and MFN2 are ubiquitously expressed, their expression levels vary across tissues. They are present at similar levels in liver, kidney, and adrenal glands; however, MFN1 predominates in testis and heart, while MFN2 is more abundantly expressed in the brain, where MFN1 is only marginally present. This differential expression may underlie the tissue-specific phenotypes observed upon individual gene ablation, particularly the cerebellar vulnerability to MFN2 loss (Eura et al., 2003).

In response to external stimuli, both mitofusins participate in the transduction of metabolic signals across multiple organs to support systemic energy homeostasis (Schrepfer & Scorrano, 2016). Environmental and physiological challenges—such as food intake, temperature changes, stress, or physical activity—trigger adaptive metabolic responses in brown adipose tissue (BAT), brain, heart, and skeletal muscle, regulating feeding behaviour, body weight, contractile performance, oxidative stress responses, and insulin sensitivity:

(A) Central regulation via hypothalamic neurons: Adiposity signals (e.g. leptin and insulin) differentially affect two key neuronal populations in the hypothalamus: orexigenic AgRP and anorexigenic POMC neurons. In AgRP neurons, overnutrition induces MFN1/2-dependent mitochondrial elongation, which promotes increased food intake and fat accumulation (Chen et al., 2004). In contrast, overfeeding suppresses MFN2 expression in POMC neurons, leading to reduced mitochondria–ER contact sites (mitochondrial-associated membranes, MAMs). This disruption impairs POMC neuronal activity, resulting in decreased energy expenditure, leptin resistance, and obesity (Schneeberger et al., 2013). Additionally, POMC neurons are dependent on mitochondrial dynamics mediated by MFN1 for metabolic shifts. Lack of MFN1 in these cells alters central glucose sensing and a defective pancreatic insulin release. (Ramírez et al., 2017).

(B) Cardiac function: In the heart, MFN1 and MFN2 are essential for maintaining cardiomyocyte homeostasis. While their presence supports normal cellular function, their involvement in pro-apoptotic responses have also been noted under stress conditions such as calcium overload and ROS generation (X. Guo et al., 2007; Papanicolaou et al., 2011). Notably, simultaneous deletion of *Mfn1* and *Mfn2* leads to heart failure, highlighting their protective roles (Chen et al., 2011).

(C) Skeletal muscle metabolism: In skeletal muscle, *Mfn2* expression is upregulated in response to temperature changes, adrenergic stimulation and elevated energy demand, in a PGC-1 α –dependent manner (Cartoni et al., 2005; Soriano et al., 2006). Increased MFN2 enhances mitochondrial OXPHOS gene transcription, boosts mitochondrial respiration, and attenuates ROS production, ER stress, and JNK pathway activation (Jheng et al., 2012; R. Liu et al., 2014; Sebastián et al., 2012). These effects contribute to the preservation of insulin sensitivity. Accordingly, MFN2 deficiency is associated with metabolic disorders such as obesity

and type 2 diabetes (Diez-Juan et al., 2015; Misko et al., 2012; Patti & Corvera, 2010; Petersen et al., 2004; Schneeberger et al., 2013).

1.7.2 ER-mitochondria contacts

Beyond its well-established role in mitochondrial fusion, MFN2 has been implicated as a key regulator of ER-mitochondria juxtaposition. Unlike MFN1, a distinct pool of MFN2 localizes to ER membranes, particularly at MAMs, specialized ER subdomains closely localized in the OMM (Giorgi et al., 2015). At these sites, MFN2 is proposed to mediate ER–mitochondria tethering, potentially through homo- or heterotypic interactions with MFN2 or MFN1 on the OMM, reflecting its established role in mitochondrial tethering and fusion (De Brito & Scorrano, 2008).

The molecular mechanism by which MFN2 mediates ER–mitochondria tethering has long remained unclear. Unlike other known tethering complexes or protein pairs (Bhatt et al., 2008; Hara et al., 2002; St-Pierre et al., 2003), MFN2 lack an identified ER-resident binding partner. A recent study identified two splice variants of *Mfn2*, termed ERMIT2 and ERMIN2. ERMIT2 is specifically enriched at the ER–mitochondria interface and functions as the ER counterpart of mitochondrial MFNs, facilitating organelle tethering and promoting efficient Ca^{2+} and phospholipid transfer. In contrast, ERMIN2 is not enriched at MAMs and instead plays a role in regulating ER morphology (Naón et al., 2023).

MFN2 depletion triggers endoplasmic reticulum stress and activates components of the unfolded protein response (UPR) localized to the ER membrane. Specifically, MFN2 has been proposed to function as an upstream regulator of the ER-resident kinase PERK, maintaining it in an inactive state under basal conditions. Upon activation, the UPR promotes ER expansion, increases the expression of molecular chaperones, and attenuates global protein synthesis. In parallel, mitochondrial Ca^{2+} uptake and ATP production are elevated to meet the elevated energetic demands associated with ER stress. However, it remains unclear whether MFN2 ablation enhances or suppresses ER stress–induced apoptosis (Sebastián et al., 2012).

1.7.3 Mitophagy

Proteins that regulate mitochondrial dynamics are intimately linked to mitochondrial quality control mechanisms, referred to as mitophagy. Two key mediators of this pathway are serine/threonine kinase PINK1 and the E3 ubiquitin ligase Parkin. Under conditions of mitochondrial depolarization, PINK1 selectively accumulates on the OMM, where it recruits and activates cytosolic parkin. PINK1-mediated phosphorylation of MFN2 enables it to act as a receptor for parkin, which then ubiquitinates MFN2 to mark dysfunctional mitochondria for removal (Chen & Dorn, 2013). Ubiquitination of surface mitochondrial proteins disrupts mitochondrial interactions with the cytoskeleton and recruits mitophagy-initiating factors. Parkin subsequently ubiquitinates OMM proteins, such as MFN1 and MFN2, targeting for proteasomal degradation. This degradation contributes to mitochondrial fragmentation, a

process that facilitates the clearance of damaged organelles (Gegg et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Narendra & Youle, 2011; Tanaka, 2010; Ziviani et al., 2010).

MFN2 has been also described to be involved in the transport of mitochondria along axons, participating in their attachment to microtubules through interaction with Miro and Milton, the two main motor proteins (Misko et al., 2010).

1.8 THE MULTIFACETED FUNCTIONS OF OPA1 IN MITOCHONDRIA

1.8.1 Cristae structure

Similar to MFNs, OPA1 has a critical role in different mitochondrial and non-mitochondrial processes. One of the most relevant functions is related with mitochondrial cristae organization. When *Opa1* is deleted or presents pathogenic mutations, severe disorganization of the mitochondrial ultrastructure can be observed (Olichon et al., 2003; Zanna et al., 2008). Cristae junctions need the oligomerization of precise ratios of soluble and membrane-bound forms of OPA1 to be kept tight (Frezza et al., 2006). The preservation of cristae architecture relies on a complex network of protein interactions, with OPA1 functioning at the IMM in association with MICOS complex subunits and OMM components such as Sam50. This central regulatory hub also engages with phospholipids, including phosphatidylethanolamine and cardiolipin, thereby modulating IMM curvature, fusion capacity, and overall membrane properties. (Milenkovic & Larsson, 2015).

1.8.2 mtDNA maintenance

OPA1 plays an essential function in maintaining mitochondrial genome stability, as its genetic ablation leads to significant mtDNA depletion (Chen et al., 2010). Moreover, specific *OPA1* mutations have been associated with mtDNA instability, characterized by the accumulation of multiple deletions (Amati-Bonneau et al., 2008; Hudson et al., 2008), and, less frequently, mtDNA depletion (Spiegel et al., 2016). Similar phenotypes have been observed following loss or mutation of mitofusins, supporting the notion that mitochondrial fusion—and the associated continuous mixing of matrix contents—is essential for preserving mtDNA integrity.

1.8.3 Mitochondrial respiration

Ablation of *Opa1* has been linked to a profound bioenergetic dysfunction, as shown by reduced oxygen consumption rate and ATP levels, absence of respiratory super-complexes organization and partial assembly of complex V (Del Dotto et al., 2017; H. Lee et al., 2017; Patten et al., 2014).

The mitochondrial respiratory chain is the primary source of reactive oxygen species (ROS) in the cell. Given the role of OPA1 in organizing respiratory chain super-complexes and maintaining cristae architecture, its dysfunction is expected to disrupt redox homeostasis and promote ROS overproduction. Consistent with this, elevated ROS levels have been observed in *Drosophila* models harbouring homozygous or heterozygous *Opa1* mutations (Shahrestani et

al., 2009; Yarosh et al., 2008), as well as in cardiomyocytes from *Opa1*^{+/-} mice (Chen et al., 2012).

1.8.4 Apoptosis

OPA1 has also been involved in apoptosis, as shown by *OPA1* silencing in HeLa cells, which induces fragmentation of the mitochondrial network and the dramatic disruption of cristae structure. These events were linked to the release of cytochrome c from the OMM triggering caspase-dependent apoptotic nuclear events (Olichon et al., 2003). Interestingly, the anti-apoptotic function of OPA1 was found to be independent of its role with mitochondrial fusion (Cipolat et al., 2004).

As mentioned above, OPA1 also plays a role in mitophagy (Rodger et al., 2018; Twig & Shirihai, 2011), by interacting with different proteins. These include PINK1 and Parkin, similar to mitofusins (Narendra & Youle, 2011) or other mitophagy receptors such as BNIP3 (Hanna et al., 2012) and FUNDC1 (Liu et al., 2012), which mediate interaction with LC3 and recruitment to the autophagy machinery.

Unexpectedly, OPA1 has also been implicated in an extramitochondrial role within adipocyte lipid droplets, where it functions as an A-kinase anchoring protein (AKAP) for perilipin, thereby facilitating lipolysis (Pidoux et al., 2011).

1.8.5 OPA1 isoforms

Given the wide range of mitochondrial functions linked to OPA1, it is reasonable to hypothesize that these activities may depend on the diversity of its isoforms. Through complex proteolytic processing, OPA1 generates a characteristic mixture of long (L-OPA1) and short (S-OPA1) forms in each cellular context. This processing is mediated by the proteases OMA1 and YME1L, which cleave L-OPA1 to produce S-OPA1. The role of OPA1 in maintaining mitochondrial physiology—encompassing cristae architecture, mtDNA stability, and OXPHOS efficiency—can be rescued by the expression of any OPA1 isoform when *Opa1* expression is impaired (Del Dotto et al., 2017). However, the restoration of a fully interconnected mitochondrial network requires the coordinated action of both L-OPA1 and S-OPA1. They proposed a model where S-OPA1 forms a passive structural scaffold that shapes cristae membranes, anchors mtDNA (Elachouri et al., 2011) and organizes the respiratory chain by directly interacting with its complexes (Zanna et al., 2008). In contrast, L-OPA1 is membrane-anchored and actively promotes mitochondrial fusion, likely through interactions with MFN1 and MFN2. The interplay between the structural support provided by S-OPA1 and the fusion activity of L-OPA1 is essential for sustaining long-term mitochondrial network connectivity (Fig. 8).

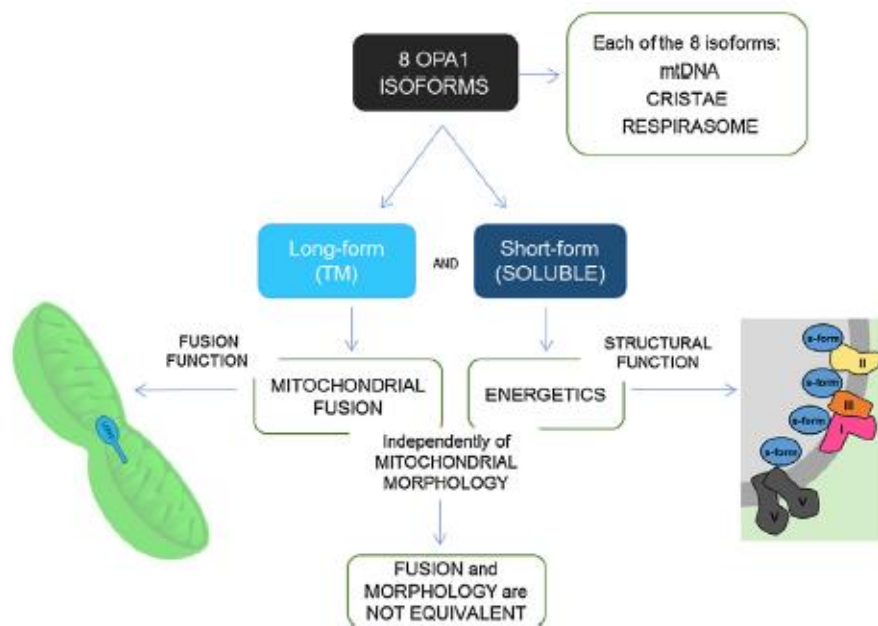


Figure 8. Each of the eight OPA1 isoforms contributes to mitochondrial DNA (mtDNA) maintenance, cristae architecture, and proper assembly of the respiratory chain supercomplexes (respirasomes). L-OPA1 alone is sufficient to mediate mitochondrial fusion, S-OPA1 independently supports mitochondrial energetics. These functions occur irrespective of overall mitochondrial morphology, indicating that mitochondrial fusion and morphology are mechanistically distinct processes and should be considered separately. Figure taken from (Del Dotto et al., 2018).

1.9 THE IMPACT OF MITOCHONDRIAL FUSION ON PERIPHERAL NEUROPATHIES

Disorders of mitochondrial dynamics are implicated in neurodegenerative, neoplastic, endocrine, and cardiovascular diseases. These include the PNS disorders Charcot–Marie–Tooth disease (CMT) and Dominant optic atrophy (DOA).

1.9.1 Charcot–Marie–Tooth

Charcot–Marie–Tooth disease is the most common inherited neurological disorder, affecting approximately 1 in 2,500 individuals (Suter & Scherer, 2003). It arises from mutations in genes encoding proteins essential for the structure and function of peripheral nerve axons or the myelin sheath. CMT is genetically heterogeneous and can follow autosomal dominant, autosomal recessive, or X-linked inheritance patterns (Fig. 9) (Pareyson & Marchesi, 2009). Based on the primary site of pathology, CMT is classified into two main types:

- CMT1 (demyelinating): affects Schwann cells, resulting in slow nerve conduction and myelin abnormalities, such as segmental demyelination and onion bulb formation.
- CMT2 (axonal): affects neurons, showing preserved nerve conduction velocity (NCV) but reduced compound muscle action potentials due to axonal degeneration.

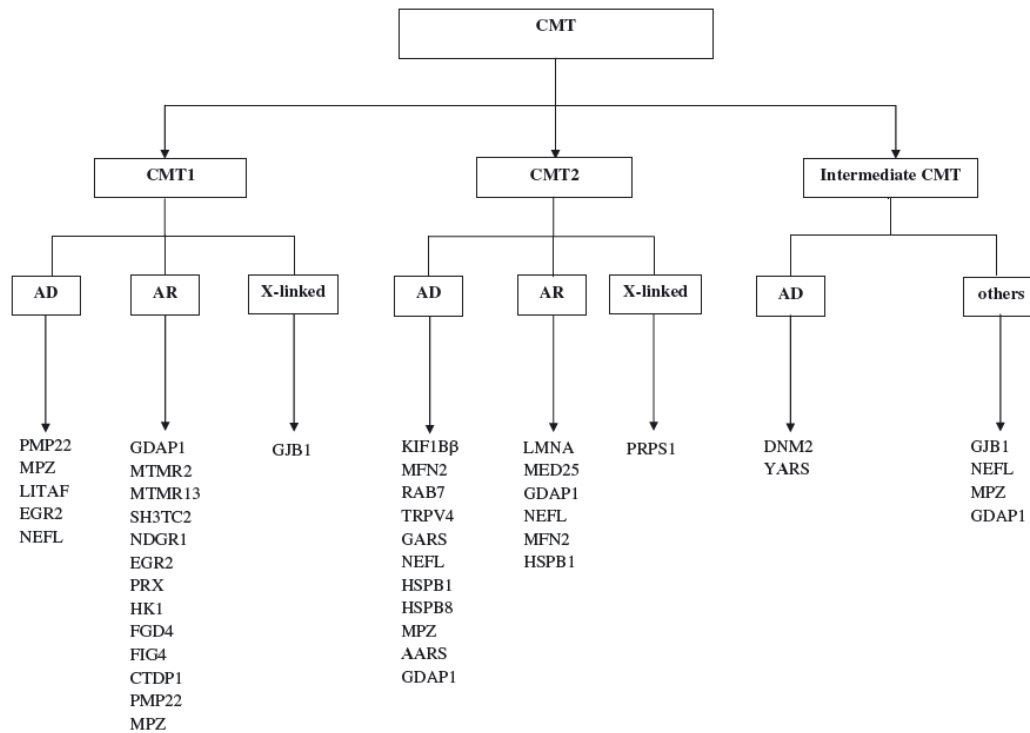


Figure 9. Genetic classification of Charcot-Marie-Tooth from (Reilly et al., 2011).

Intermediate CMT presents features of both demyelinating and axonal types. X-linked CMT (CMTX), typically caused by mutations in *GJB1* (encoding connexin 32), shows variable inheritance and presentation (Tazir et al., 2014). CMT3 (Dejerine–Sottas syndrome, DSS) is a severe, often sporadic, early-onset demyelinating neuropathy. CMT4 refers to autosomal recessive demyelinating forms (Reilly et al., 2011).

CMT2A, the most common axonal subtype, is frequently caused by autosomal dominant loss-of-function mutations in *MFN2*. While *MFN1* can rescue *MFN2* mutations via heterodimerization, variability in *MFN1* expression may explain phenotypic differences and offers a potential therapeutic target (Detmer & Chan, 2007; Longo & Massa, 2013).

Another relevant gene is *GDAP1*, which encodes a mitochondrial outer membrane protein involved in mitochondrial dynamics. Mutations in *GDAP1* cause CMT4A, originally identified in consanguineous Tunisian families (Ben Othmane et al., 1998), and have also been linked to autosomal recessive axonal CMT with vocal cord palsy (Cuesta et al., 2002; Sevilla et al., 2003). Depending on the mutation, *GDAP1*-related CMT can present with demyelinating, axonal, or intermediate phenotypes and often shows early-onset and severe progression. Recessive mutations reduce mitochondrial fission activity, while dominant mutations impair mitochondrial fusion (Niemann et al., 2005, 2009).

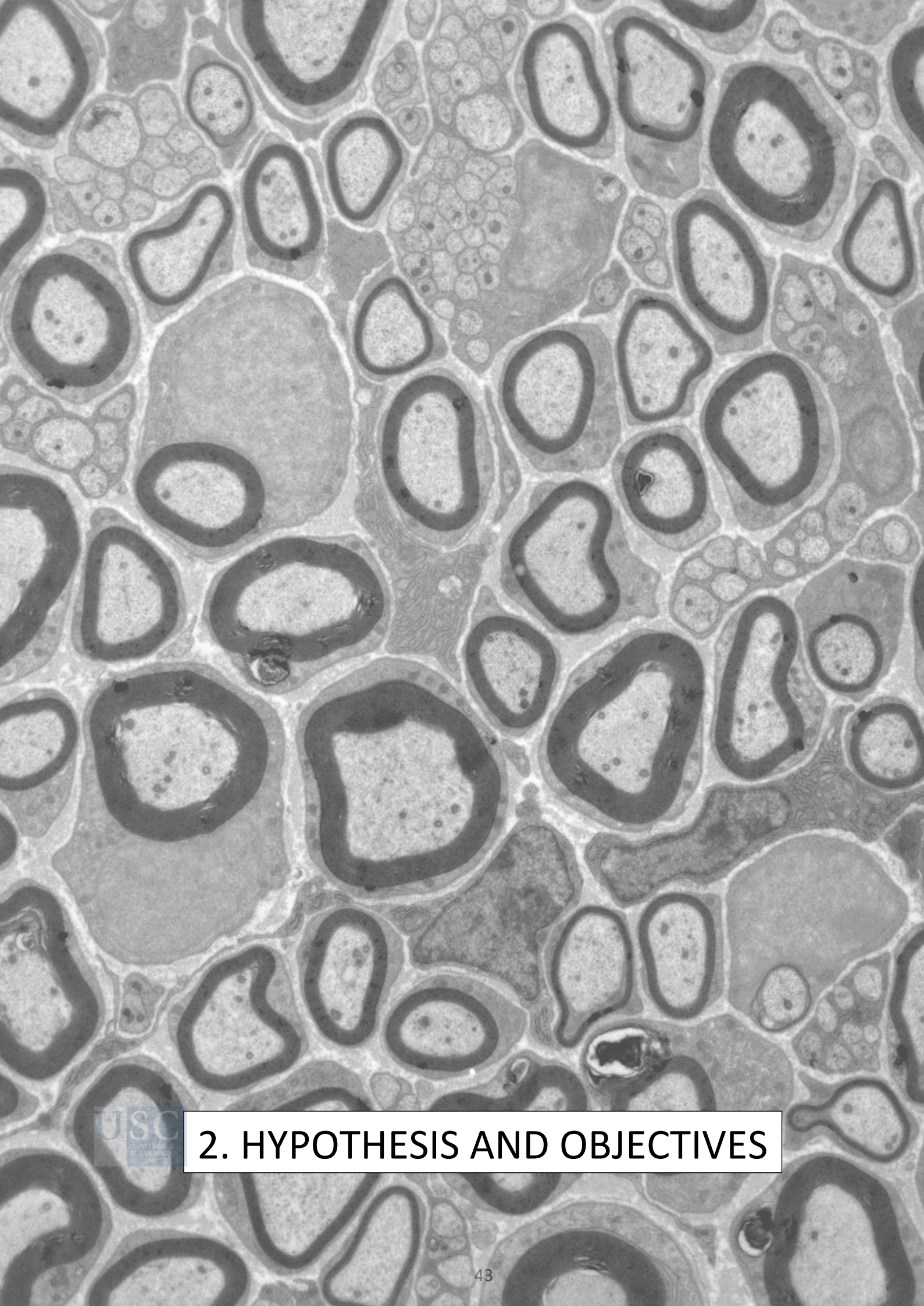
1.9.2 Dominant optic atrophy

Dominant optic atrophy is the most prevalent inherited optic neuropathy, with the majority of cases caused by heterozygous mutations in *OPA1* (Chun & Rizzo, 2017). As previously discussed, *OPA1* is crucial for mitochondrial inner membrane fusion and plays key roles in maintaining cristae architecture and organizing OXPHOS super-complexes. Numerous disease-causing *OPA1* mutations have been identified, and in many instances, the pathogenic mechanism involves haploinsufficiency.

Classical DOA typically presents with progressive, bilateral vision loss due to degeneration of retinal ganglion cells, leading to optic nerve pallor. However, certain *OPA1* mutations give rise to a more severe phenotype known as DOA-plus syndrome, which includes additional neurological features such as sensorineural hearing loss, myopathy, peripheral neuropathy, and demyelination (Amati-Bonneau et al., 2008; Bonneau et al., 2014; Hudson et al., 2008; Yu-Wai-Man et al., 2009). This peripheral demyelinating phenotype was confirmed by (Gonzalez et al., 2015) thanks to the generation of a mouse model of optic atrophy *OPA1*^{delTTAG} that mimicked the human disease (Sarzi et al., 2012). *Opa1* mutation induced not only optic nerve defects but also peripheral nerve demyelination. This demyelinating phenotype started 5 months after birth and worsened with aging.

While classical forms of CMT2A and DOA primarily affect distinct neuronal populations, motor and sensory neurons in CMT2A and retinal ganglion cells in DOA, there is increasing evidence of phenotypic overlap. Some *MFN2* mutations, traditionally linked to peripheral neuropathy in CMT2A, have also been associated with optic atrophy (Stuppia et al., 2015), blurring the clinical boundaries between the two disorders.

Collectively, these findings underscore the central role of mitochondria fusion, both in glial and axonal cells, in maintaining the functional integrity of the axon–myelin unit.



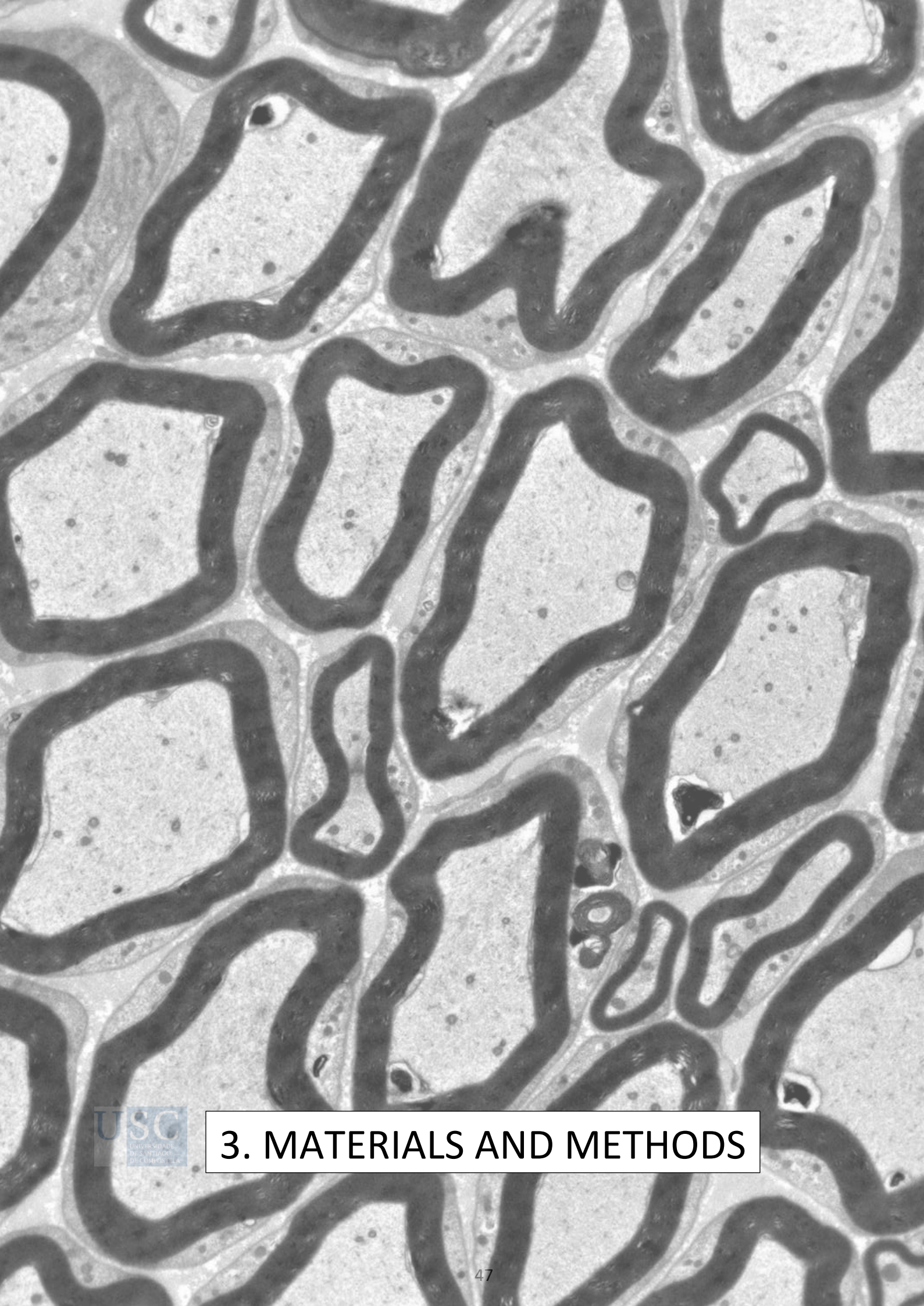
During myelin formation, Schwann cells undergo a metabolic shift from glycolysis to oxidative phosphorylation (Pooya et al., 2014). Following nerve injury, this metabolic switch recapitulates the developmental transition: Schwann cells initially revert to a glycolytic state to support the early repair program (Babetto et al., 2020), and subsequently shift back to oxidative metabolism to sustain long-term remyelination (Sundaram et al., 2023). During neuronal differentiation, a similar bioenergetic transition from glycolysis to oxidative phosphorylation is regulated by the mitochondrial fusion proteins MFN2 and OPA1 (Zanfardino et al., 2025). MFN1 is a homolog of MFN2 and although they share overlapping functions and can partially compensate for one another, distinct roles for each protein have also been described (Filadi et al., 2018).

Despite several studies showing a key role of mitochondrial metabolism in Schwann cell biology, the precise function of mitochondrial dynamics in this context has not yet been investigated.

Based on this, we hypothesize that mitochondrial fusion, that would require a tight coordination between its three main components (MFN1, MFN2 and OPA1), regulates this metabolic switch which is crucial for Schwann cell proper differentiation in both development and after injury.

To test this hypothesis, we aim to study the following specific aims:

- 1. The functional and molecular role of mitochondrial fusion in Schwann cell myelination during development.** In this objective, we aim to make an exhaustive characterization of nerve defects in genetic models, designed to study the contribution of mitochondrial fusion to Schwann cell differentiation using electrophysiological, behavioural, histological and biochemical analyses.
- 2. The functional role of mitochondrial plasticity in mature myelinating Schwann cells in adult nerves.** In this objective, we aim to examine the functional effects of genetically inhibiting mitochondrial fusion in mature Schwann cells in the maintenance of the myelin phenotype and axonal health.
- 3. The functional role of mitochondrial plasticity in peripheral nerve regeneration.** In this objective, we aim to investigate the functional effects of genetically inhibiting mitochondrial fusion in mature Schwann cells during Wallerian degeneration, nerve repair and regeneration in injury models.



3.1 ANIMALS

All the experiments performed in this project were done in agreement with the Rules of Laboratory Animal Care and International Law on Animal Experimentation. The procedures were approved by the Ethics Committee at the University of Santiago de Compostela and the Xunta de Galicia. The registration number of these procedures is: 15012/2023/003. These procedures were carried out in compliance with the legislative framework of Directive 2010/63/EU of the European Parliament and Council of 22 September 2010, transposed into Spanish law by Royal Decree 53/2013 of 1 February, which sets out the rules for the use and protection of animals used in experimentation and for other scientific purposes. Mice were bred at the Centro de Biomedicina Experimental (CEBEGA) – University of Santiago de Compostela animal facility.

Mice were maintained in groups of a maximum of five mice per cage, containing woodchip bedding, under standard housing conditions (20-22°C, 45±10 humidity, 12 hours day/night cycle). The cages were regularly cleaned, and bedding was changed every week to maintain hygienic conditions. Mice had *ad libitum* access to food and water, and the water was replenished daily. They were fed with a standard diet (2918 Teklad global 18% protein rodent maintenance diets, Envigo). To ensure animal welfare, all experiments and procedures were planned to reduce as much as possible the number of animals and minimize their suffering in compliance with the 3Rs principles (Replacement, Reduction and Refinement). Signs of distress or abnormal behavior were monitored daily, and animals showing signs of undue stress were humanely euthanized following approved protocols. All mice used had C57BL/6 genetic background.

To generate the conditional constitutive mice specifically in Schwann cells, mice homozygous for the floxed allele of *Mfn1* (H. Chen et al., 2007; Ramírez et al., 2017), *Mfn2* (H. Chen et al., 2007), *Mfn1* and *Mfn2* (combining both previous floxed mice) and *Opa1* (Rodríguez-Nuevo et al., 2018) were crossed with an MPZ-cre line, which expresses Cre recombinase specifically in Schwann cells from embryonic day E14.5 (Strain #017927, The Jackson Laboratory) (Feltri et al., 1999). To generate conditional tamoxifen-inducible *Mfn1/2* and *Opa1* knockout mice, *Mfn1/2* and *Opa1* floxed mice were crossed with Plp1-CreERT2 mice (MGI:2663093) (Leone et al., 2003), followed by tamoxifen administration. Floxed mice lines were provided by Prof. Antonio Zorzano (IRB Barcelona) (*Mfn1*, *Mfn2* and *Opa1* lines).

3.2 MICE GENOTYPING

Genotyping of mice pups was performed using PCR to confirm the presence of the floxed allele and the Cre transgene. DNA was extracted from ear biopsies collected from pups using 25 mM NaOH and 0.2 mM EDTA (pH 12) for 30 min at 95°C. Subsequently the solution was buffered with 40 mM tris-HCl (pH 5). 2X DreamTaq Green PCR Master Mix (ThermoFisher Scientific, K1081) was used for PCRs. PCR conditions and primers are detailed in the following tables:

Table 1. *MPZ-Cre/Mfn1/Opa1* Thermocycler conditions

Temperature	Time	Cycles
94 °C	3 minutes	x1
94 °C	30 seconds	x35
60 °C	30 seconds	
72 °C	1 minute	
72 °C	1 minute	x1

Table 2. *PLP-Cre* Thermocycler conditions

Temperature	Time	Cycles
94 °C	5 minutes	x1
94 °C	30 seconds	x35
54 °C	45 seconds	
72 °C	45 seconds	
72 °C	5 minutes	x1

Table 3. *Mfn2* Thermocycler conditions

Temperature	Time	Cycles
94 °C	3 minutes	x1
94 °C	30 seconds	x35
55 °C	30 seconds	
72 °C	1 minute	
72 °C	1 minute	x1

Table 4. Primers 5'-3' for *MPZ-Cre* genotyping

Geno_ <i>MPZ-Cre</i> _14203	CCACCACCTCTCCATTGCAC
Geno_ <i>MPZ-Cre</i> _oIMR7338	CTAGGCCACAGAATTGAAAGATCT
Geno_ <i>MPZ-Cre</i> _oIMR7339	GTAGGTGGAAATTCTAGCATCATCC
Geno_ <i>MPZ-Cre</i> _oIMR9377	ATGTTTAGCTGGCCCAAATG

Table 5. Primers 5'-3' for *PLP-Cre* genotyping

PLPCre_F	CACTCTGTGCTTGGTAACATGG
PLPCre_R	TCGGATCCGCCGCATAACC

Table 6. Primers 5'-3' for *Mfn1* genotyping

DC285	AGCAGTTGGTTGTGTGACCA
HC105	TTGGTAATCTTTAGCGGTGCTC

Table 7. Primers 5'-3' for *Mfn2* genotyping

Geno_MFN2_22496 (MFN2_P1)	TTTGGGAAGTAGGCAGTCTCCA
Geno_MFN2_22497 (MFN2_P2)	CAGGCAGCACTGAAAAGAGA

Table 8. Primers 5'-3' for *Opa1* genotyping

Opa1LoxP2_F	GGGTAGAATCAGAGACAGCC
OPa1LoxP2_R	TTCTGTGCACACTGGTGTC

3.3 TAMOXIFEN INJECTION

Tamoxifen (T5648, Sigma-Aldrich) was dissolved to a stock concentration of 20 mg/ml in a vehicle of ethanol and corn oil (C8267, Sigma-Aldrich). The mix was incubated for 2 hours at 65°C in agitation (700 rpm) protected from sunlight.

For adult mice (P60) treatment, 100 µl was administered by intraperitoneal injection once daily for five consecutive days (around 75mg/kg). Control mice (Plp-CreERT²) were treated identically.

3.4 SCIATIC NERVE SURGERY

Sciatic nerve surgeries were performed under deep surgical anesthesia (isoflurane; 5% induction, 2% maintenance). The hindlimbs and lower back were shaved and sterilized with iodine. An incision was made on the skin and the sciatic nerve exposed.

The anatomical target for the surgeries was the cross line between the sciatic nerve and the tendon below (Fig. 10). For nerve cut, curved scissors (Fine Science Tools, 91500-09) were used to cut at that anatomical point. In the case of chronic denervation, after nerve cut, the distal stump was stitched to the muscle 2cm away from the lesion point. For nerve crush the sciatic nerve was compressed once for 30 s using a pair of delicate forceps (Fine Science Instruments, catalog no. 11063-07) and again for 30 s at the same site but perpendicular to the initial crush.

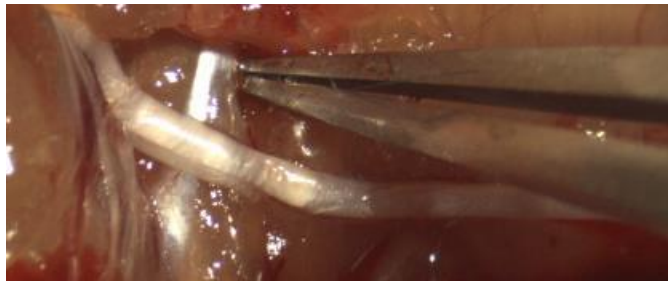


Figure 10. The tip of the forceps points to the tendon on which the crush or cut is performed. Picture taken during sciatic nerve surgery.

At defined time points following injury, mice were euthanized by cervical dislocation. The sciatic nerves, both injured (right hindlimb) and contralateral uninjured (left hindlimb), were

subsequently exposed and dissected. Animals were placed in the prone position, and the skin (Fine Science Tools, 14101-14), underlying muscle (Fine Science Tools, 16144-13), and sciatic nerves (Fine Science Tools, 91500-09) were sequentially incised. Dissection was performed proximally to the tendon in the case of crush injuries, and at the tibial branch near the knee in the case of cut lesions. Finally, the nerve was excised by grasping the distal end at the knee with forceps (Fine Science Tools, 91150-20).

For transmission electron microscopy, nerves were pre-fixed in situ for 15 min prior to dissection using a fixative solution containing 2% paraformaldehyde (Electron Microscopy Sciences, 15710) and 2.5% glutaraldehyde (Electron Microscopy Sciences, 16220) in 0.1 M cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, 12300).

For Western blot analyses, a 1-cm segment proximal to the tendon was collected from uninjured nerves and the first 8 mm of the distal stump were collected from injured nerves. Samples were stored at -80 °C until processing. For qPCR, the maximum possible length of uninjured nerves (from spinal column to foot) was collected and stored at -80 °C, whereas for injured nerves the first 8 mm of the distal stump were collected.

3.5 RAT PRIMARY SCHWANN CELL CULTURE

Primary rat Schwann cells (RSCs) were used for *in vitro* culture assays (Palomo Irigoyen et al., 2018). Sciatic nerves were obtained from P3 to P5 Wistar rats and digested in a solution containing 0.3% trypsin (Gibco, 27250-018), 0.6% type 2 collagenase (Worthington, LS004174) prepared in Hanks' balanced salt solution (Gibco, 14170112). Cells were cultured for 3 days in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) containing AraC (10^{-3} M) (Sigma-Aldrich, C6645-25MG) at 37°C, 5% CO₂. Then, Schwann cells were immunopanned to remove remaining fibroblasts and expanded in Expansion Medium [RSC Defined Medium (Table 9) supplemented with 0.5% FBS, 2 µM forskolin (EMD Millipore, 344270-10MG) and 10 µg/mL neuregulin 1 (R&D Systems, 396-HB-050)] in dishes coated with poly-D-lysine (Sigma-Aldrich, P6407-5MG) and laminin (Sigma-Aldrich, L2020-1MG). Only the first seven passages were used.

To induce differentiation, Schwann cells were cultured overnight in RSC Defined Medium and 0.5% FBS. The next day, they were switched to differentiation medium [RSC Defined Medium, 0.5% FBS and 1 mM dibutyryl cAMP (Sigma-Aldrich, D0627-1G)] for 48 hours to induce upregulation of myelin differentiation markers. The composition of each media is detailed in the following tables:

Table 9. RSC Defined Medium

Gibco™ DMEM/F-12, GlutaMAX™ supplemented	Base medium	Gibco 31331028
Antibiotic-Antimycotic (100X)	1X	Fisher Scientific 15-240-062
B27 supplement (50X)	1X	Thermo Scientific 17504044
SATO supplement (100X) (Table 10)	1X	Prepared Media
3,3',5-Triiodo-L-thyronine sodium salt	10.1 ng/ml	Sigma T6397
Hi Insulin solution (10 ⁻³ M)	10 ⁻⁶ M	Sigma I9278

Table 10. SATO supplement

Neurobasal medium	Base medium	Thermo Fischer, 21103-049
BSA	100 µg/mL	Sigma A4161
Transferrin	1 mg/mL	Sigma T1147
Putrescine	1600 µg/mL	Sigma P5780
Progesterone	60 ng/mL	Sigma P8783
Sodium selenite	40 ng/mL	Sigma S5261

3.6 MOUSE PRIMARY SCHWANN CELL CULTURE

Primary mouse Schwann cells (MSCs) were obtained from WT and *Opa1* cKO mice and used in the Seahorse assay to analyze oxygen consumption. The protocol was adapted from (Kim & Kim, 2018) and under guidance from Dr. Venkat Sundaram (University of Leipzig). Embryos at E13.5 were extracted from the embryonic sack and maintained on Leibovitz's L-15 medium (Fisher Scientific 15460554). The spinal cord was dissected and the dorsal root ganglia (DRGs) were extracted. DRGs were dissociated with 0.25% trypsin without EDTA (Thermo Fisher Scientific 15090046) and were plated in P35mm dishes with DRG Plating Media (Table 11). The heads of the embryos were stored for genotyping. All trypsin reaction were stopped with Trypsin Stop Media (Table 12).

The following day, the media was replaced with N2/NGF Serum Free Media (Table 13). Under the serum-free condition, fibroblast growth is suppressed, while axon-associated Schwann cell precursors continue to proliferate. At day 7, under a dissecting microscope, neuron-associated Schwann cells are visible as beaded structures along neurites.

The Schwann cell-neuron network was then detached from the fibroblasts below by mechanical stress. The cells were dissociated in trypsin (Thermo Fisher Scientific 15090046)

(0.25%)-collagenase type 1 (Worthington Biochemical LS004196) (0.1%) solution. The cells were cultured in poly-L-lysine (Sigma-Aldrich P1274) coated P60mm dishes with Proliferation Media. The next day, bipolar, spindle-shaped Schwann cells were visible with few neuronal cell bodies. The neuregulin and forskolin present in the Proliferation Media [MSC Defined Media (Table 14), 2.5 μ M Forskolin (EMD Millipore 344270-10MG), 10ng/ μ L NRG-1 (R&D Systems 396-HB-050)] stimulate Schwann cell proliferation, while suppressing growth of any contaminating fibroblasts under the serum-free condition. The neurons died off within 2 days in the absence of NGF in the medium. To induce myelinogenic conditions, MSCs were cultured for 48h in Differentiation Media [MSC Defined Media, 10ng/ μ L NRG-1 (R&D Systems 396-HB-050), 1 mM dibutyryl cAMP (Sigma-Aldrich, D0627-1G)]. The composition of each media is detailed in the following tables:

Table 11. DRG Plating Media

DMEM High Glucose	Base medium	VWRC392-0415
Antibiotic-Antimycotic (100X)	1X	Fisher Scientific 15240062
Glutamax	1X	35050-061 GIBCO
Heat inactivated Horse Serum	10%	Fisher Scientific 11510516
Recombinant Mouse beta-NGF Protein	50ng/ml	R&D Systems 1156-NG-100

Table 12. Trypsin Stop Media

DMEM High Glucose	Base medium	VWRC392-0415
Antibiotic-Antimycotic (100X)	1X	Fisher Scientific 15240062
Heat inactivated Horse Serum	10%	Fisher Scientific 11510516

Table 13. N2/NGF Serum Free Media

Gibco™ DMEM/F-12, GlutaMAX™ supplemented	Base medium	Gibco 31331028
Antibiotic-Antimycotic (100X)	1X	Fisher Scientific 15-240-062
Recombinant Mouse beta-NGF Protein	50ng/ml	R&D Systems 1156-NG-100
N2 supplement (100X)	1X	Gibco 17502048

Table 14. MSC Defined Media

Gibco™ DMEM/F-12, GlutaMAX™ supplemented	Base medium	Gibco 31331028
Antibiotic-Antimycotic (100X)	1X	Fisher Scientific 15-240-062
N2 supplement (100X)	1X	Gibco 17502048

3.7 ELECTRON MICROSCOPY (EM)

Proximal sciatic nerves (before they branch out to sural, peroneal, and tibial nerves) were fixed with EM fixing solution [2.5% glutaraldehyde (Electron Microscopy Sciences 16220), 2% paraformaldehyde (Electron Microscopy Sciences 15710) in 0.1M cacodylate (Electron Microscopy Sciences 12300) buffer] at 4°C overnight and then postfixed with 1% osmium tetroxide (Electron Microscopy Sciences 19150) at 4°C overnight. Following *en bloc* staining with 4% uranyl acetate (Electron Microscopy Sciences 22400) solution during 45 min at 4°C, the nerves were then dehydrated in graded alcohol, infiltrated with propylene oxide (Electron Microscopy Sciences 20411), and embedded in epoxy resin (Agar Scientific). Semithin sections (1 µm) and ultrathin (50 nm) sections were cut with a Leica ultramicrotome (Leica, Germany). Semithin sections were stained with 0.1% toluidine blue (Sigma-Aldrich T3260-5G) in ethanol for 15 s and visualized using a Nikon Optiphot-2 microscope. Ultrathin sections were stained with lead citrate and visualized under a transmission electron microscope FEI Tecnai G2 Spirit BioTwin (Thermo Fisher Scientific company, Oregon, USA). All images were acquired with a Xarosa digital camera (EMSIS GmbH, Münster, Germany) controlled by Radius software (version 2.1). Ultrathin sections and images were obtained by Mario Soriano (Centro de Investigación Príncipe Felipe, Valencia).

Different morphometric measurements were performed, including counts of myelinated axons, amyelinated axons (1:1) per nerve, proportion of Schwann cells in a 1:1 relationship with an axon that are myelinated or remain unmyelinated, total number of axons in a 1:1 relationship with a SC, demyelinating axons, Remak bundles, number of axons per Remak bundle, macrophages and total nerve area, in electron micrographs of ultrathin sections by manually counting all of the figures present in the sciatic nerve sections using the cell counter plugin from NIH ImageJ software (<http://rsb.info.nih.gov/ij/>). Five pictures of each experimental group (at least three biological replicates per group) were quantified, and the counts of the quantified area were extrapolated to the total sciatic nerve area.

For g-ratio analysis, the axons (without myelin) and fiber areas (including myelin) of randomly chosen fibers were measured, their diameters were estimated, and the g-ratio was calculated as axon diameter/fiber diameter. At least 100 nerve fibers per animal (n = 3 for each group) were analyzed.

For mitochondria morphometric analysis, EM images were taken with a magnification of 2900-fold. The number of SC mitochondria per mm² Schwann cell cytoplasm, the SC mitochondrial size in mm² per Schwann cell cytoplasm, the number of SC mitochondria per mm² axonal cytoplasm were quantified as well as the axonal mitochondrial size in mm² per axonal cytoplasm.

3.8 IMMUNOBLOTTING

For immunoblotting, sciatic nerves were dissected, and the epineurium was stripped before snap freezing and storage at -80°C. Nerves or rat Schwann cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [1.6 mM NaH₂PO₄, 8.4 mM NaHPO₄, 0.1% Triton X-100, 0.1 M NaCl, 0.1% SDS, 0.5% sodium azide, phosphatase (PhosSTOP EASYpack, Sigma-Aldrich 04906837001), and protease (Complete Mini Protease Inhibitor Cocktail, Sigma-Aldrich, no. 04693124001) inhibitors]. Cells were scraped directly from the plate, and sciatic nerves were processed in a 1.5ml Eppendorf tubes containing zirconium beads (Next Advance, ZrOB05) using Bullet Blender Homogenizer (Next Advance, BBX24-CE) for homogenizing. The homogenization protocol consisted of 2-3 cycles of 30 seconds, followed by cooling periods to prevent overheating. Samples were centrifuged at 13,000 rpm for 20 min at 4°C, and supernatants were quantified with the Pierce Micro BCA Assay Kit (Thermo Fisher Scientific, no. 23235).

Protein samples were separated by manually prepared SDS-polyacrylamide gel electrophoresis in 8 to 15% acrylamide gels using a Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad) or 4 to 12% NuPAGE Bis-Tris (Thermo Fisher Scientific, WG1403BOX,). Blue Star Protein marker from Nippon genetics was used as molecular weight marker. Proteins were transferred from gels into 0.2µm pore size nitrocellulose membranes (GE Healthcare, no. 10600001) using a Trans-Blot Cell system electroblotting or using a Bio-Rad Trans-blot SD Semi-dry Transfer Cell (Bio-Rad). The presence of total protein was detected by Ponceau S solution (Sigma-Aldrich, no. P7170) staining.

Non-specific binding was blocked by incubation of the membranes with 0.1% (v/v) Tween 20–tris-buffered saline (TBS), solution containing 5% (w/v) skimmed milk powder or 2% (w/v) BSA (Sigma-Aldrich, no. A3912) for 1 hour at room temperature, before addition of the primary antibody (Table 15). After washing the unbound primary antibody with 0.1% (v/v) Tween 20–TBS three times, membranes were incubated with the corresponding horseradish peroxidase (HRP)–linked secondary antibody (Table 15) for 1 hour at room temperature. Membranes were washed three times to remove the excess of secondary antibody and the Western Lightning Plus-ECL HRP substrate (PerkinElmer, no. NEL104001EA) was subsequently added.

Table 15. List of antibodies

Protein ID	Source	Catalogue number	RRID
MFN1	Abcam	ab126575	AB_11141234
MFN2	Abcam	ab124773	AB_10999860
OPA1	BD Bioscience	612606	AB_399888
MPZ	Aves Lab	PZ0877982	AB_2313561
MBP	Aves Lab	MBP88957981	AB_2313550
Neurofilament	Abcam	ab4680	AB_304560
pACC ^{S79}	Cell Signaling	3661	AB_330337
ACC	Cell Signaling	3662	AB_2219400
pAMPK α ^{T172}	Cell Signaling	2535	AB_331250
AMPK α	Cell Signaling	2532	AB_330331
peIF2 α ^{S51}	Cell Signaling	9721	AB_330951
eIF2 α	Cell Signaling	5324	AB_10692650
EGR2	Abcam	ab245228	AB_2934181
c-Jun	Cell Signaling	9165	AB_2130165
VDAC	Cell Signaling	4661	AB_10557420
β -Actin	Sigma-Aldrich	A5441	AB_476744
NDUFS1	Abcam	ab169540	AB_2687932
SDHA	Abcam	ab14715	AB_301433
UQCRC2	Abcam	ab14745	AB_2213640
MTCO1	Abcam	ab14705	AB_2084810
ATP5A	Abcam	ab14748	AB_301447
HKI	Cell Signaling	2024	AB_2116996
HKII	Cell Signaling	2867	AB_2232946
TFAM	Novus	NBP1-71648SS	AB_11025763
LONP1	Abcam	ab103809	AB_10858161
Vinculin	Sigma-Aldrich	V4505	AB_477617
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling	7074	AB_2099233
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling	7076	AB_330924
Horseradish Peroxidase Goat Anti-Chicken IgY Secondary Antibody	Aves Lab	H-1004	AB_2313517

The chemiluminescent signal from immunoreactive proteins was captured using an iBright™ Imaging System (model no. FL1500) or by exposition to Super RX Fuji Medical X-Ray films (Fujifilm, no. 06-SHRGH1824B), which were further developed in a Curix 60 processor (AGFA

Healthcare). Films were digitalized and transformed into 8-bit images. Bands were quantified by densitometric analysis using the open-source image processing program ImageJ (<http://rsbweb.nih.gov/ij>) and normalized to vinculin or β -actin housekeeping protein expression.

3.9 RNA ISOLATION AND RT-QPCR

Total RNA was isolated from Schwann cells or sciatic nerves with TRIzol reagent (Sigma-Aldrich, no. T9424-200ML) according to the manufacturer's instructions. Briefly, cells were lysed directly in Trizol and, after phase separation with chloroform, the aqueous phase was transferred to a new tube, and RNA was precipitated using isopropanol. Glycogen was added to correctly visualize the RNA pellet. Finally, the RNA was resuspended in RNase-free H₂O. Cells were scraped directly from the plate, and sciatic nerves were processed in a 1.5 ml Eppendorf tube containing zirconium beads (Next Advance, ZrOB05) using Bullet Blender Homogenizer (Next Advance, BBX24-CE) for homogenizing. The homogenization protocol consisted of 2-3 cycles of 30 seconds, followed by cooling periods to prevent overheating. RNA concentration was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

A minimum of 250 ng of total RNA and up to 1 μ g was reverse-transcribed in a 20 μ l of reaction containing deoxynucleotide triphosphates (Thermo Fisher Scientific, no. R0182), random hexamers (Thermo Fisher Scientific, no. 48190011), 0.1 M dithiothreitol (Invitrogen, no. Y00147), RNaseOUT (Thermo Fisher Scientific, no. 100000840), and 5 $\times$ Buffer and M-MLV Reverse Transcriptase (Thermo Fisher Scientific, no. 28025013) according to the manufacturer's instruction. The resulting cDNA was diluted 1/5 with 80 μ l of RNase free H₂O (Sigma-Aldrich). RT-qPCR was performed in a 5 μ l reaction mix containing 2.5 μ l of Applied Biosystems SYBR Select Master Mix (Thermo Fisher Scientific, no. 44-729- 19), 0.5 μ l of 10 μ M primers, and 2 μ l of cDNA. All reactions were performed in triplicates in a QS6 Real-Time PCR System (Applied Biosystems). Forty cycles with a melting temperature of 60°C and 30 s of each step were used. Specific primers were designed with Primer Blast database (www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Sigma-Aldrich (Table 16).

Quantification was performed using the $\Delta\Delta$ CT method and data was normalized using a workflow combining NormFinder, CV analysis along with visual representation of mRNA fold changes and one-way ANOVA for validating reference genes (11 genes collection: *Actb*, *Hsp60*, *Gapdh*, *Sdha*, *Tbp*, *Mrpl10*, *Pgk1*, *Rpl13a*, *Ppia*, *Rps26*, *Calnexin*, *Yy1* and *Mre11*) in longitudinal studies described in (Sundaram et al., 2019) that allowed us to choose the best combination of reference genes for each experiment.

Table 16. qPCR primers' list

Gene name	Sense	Sequence 5'-3'
<i>Mfn1</i>	Forward	GATAAAGTCCTCCCCAGCGG
	Reverse	TATCTCCATCGGTCCCCTCG
<i>Mfn2</i>	Forward	GGGAAGAGCACCGTGATCAAT
	Reverse	TTGGTGGTATGACCAATCCCAG
<i>Opa1</i>	Forward	CTGCAGGTCCCAAATTGGTT
	Reverse	TCCAGGTGAACCTAACAAGAGA
<i>Actb</i>	Forward	ATGGTGGGAATGGGTCAGAAG
	Reverse	TGCCATGTTCAATGGGGTAC
<i>Hsp60</i>	Forward	AGCACGCTGGTTTTGAACAG
	Reverse	TTCTCCAAACACTGCACCAC
<i>Gapdh</i>	Forward	GTGGACCTCATGGCCTACAT
	Reverse	TGTGAGGGAGATGCTCAGTG
<i>Sdha</i>	Forward	AGAAAGGCCAAATGCAGCTC
	Reverse	GTGAGAACAAGAAGGCATCAGC
<i>Tbp</i>	Forward	CAGCAGTTCAGTAGCTATGAGC
	Reverse	CTCTGCTCTAACTTTAGCACCTG
<i>Mrpl10</i>	Forward	AGCTGCGGAAACACAAGATC
	Reverse	AAAAGGGGTAGCAGGTTTCG
<i>Pgk1</i>	Forward	ACGGTGTTGCCAAAATGTCTG
	Reverse	TTGGAACAGCAGCCTTGATC
<i>Rpl13a</i>	Forward	TACGCTGTGAAGGCATCAAC
	Reverse	TTGGTATTCATCCGCTTCCG
<i>Ppia</i>	Forward	GGCAAATGCTGGACCAAAC
	Reverse	CATTCCTGGACCCAAAACG
<i>Rps26</i>	Forward	TGCCATCCATAGCAAGGTTG
	Reverse	AAACGGCCTCTTTACATGGG
<i>Calnexin</i>	Forward	CTTCCAGGGGATAAAGGACTTGT
	Reverse	ACATAGGCACCACCACATTCTA
<i>Yy1</i>	Forward	GCCCTCATAAAGGCTGCAC
	Reverse	CATTCTGCACAGACGTGGAC
<i>Mre11</i>	Forward	CTGGGGCCATGAACATGAGT
	Reverse	TCCGGGCTGAGACACATAGA

3.10 ELECTROMYOGRAPHIC MEASUREMENTS

For electromyographic measurements, the UltraPro S100 EMG/NCS/EP Neurodiagnostic System (Natus) was used following a protocol adapted from (Pollari et al., 2018). In brief, mice were anesthetized with isoflurane (5% induction, 2% maintenance), and positioned in prone position, and hindlimbs were extended with tape. Needle electrodes (27 gauge) were used to record hindlimb CMAP (Compound Muscle Action Potential) measurements. Stimulating electrodes were placed subcutaneously on both sides of the sciatic notch with a 2-cm distance,

approximately (anode = 1 and cathode = 2). The recording electrode was also placed subcutaneously aligned with the gastrocnemius muscle. The reference electrode was inserted subcutaneously next to the Achilles tendon in a 30° angle. The ground electrode was placed subcutaneously on the other side of the mouse. First supramaximal stimulation was searched by gradually increasing the stimulation, until a perfect defined curve was obtained (registered as “First response”). Afterward, stimulations were recorded at 10.2, 15.3, and 20.0 mA.

Electromyographic parameters were calculated at 20 mA as shown in (Fig. 11). “Latency” was the time-lapse between the stimulus and the depolarization. “Amplitude” was the voltage difference between the maximum depolarization point and the maximum repolarization point. “Duration” was the time the whole response lasted. “Nerve Conduction Velocity (NCV)” was the latency divided by the distance between cathode stimulation electrode and recording electrode.

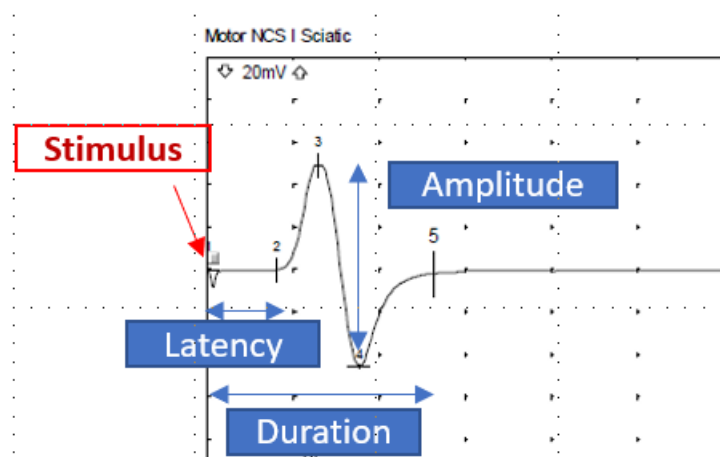


Figure 11. Example of how electromyographic measurements were calculated with Natus Elite – SynergyReader software. Latency is the time-lapse between mark 1 (stimulus) and mark 2 (response-starting point). Amplitude is the voltage difference between mark 3 (max. depolarization point) and mark 4 (max. repolarization point). Duration is the time-lapse between mark 1 (stimulus) and mark 5 (end of response).

3.11 MOTOR-SENSORY FUNCTIONAL TESTS:

3.11.1 Rotarod

Mice were initially trained on the rotating rod (UgoBasile, no. 47650) (5 rpm for 60 s) for a total of three trials separated by 10-min intertrial intervals. For the rotarod test, animals were tested in a session with three trials on an accelerating rotarod (4 to 40 rpm for 5 min) and the average time on the rod was reported.

3.11.2 Hargreaves plantar test

Thermal hyperalgesia was tested using a plantar test (Ugo Basile Plantar Test Apparatus, no. 7370;) as described previously (Hargreaves et al., 1988). Briefly, a radiant heat source beneath a glass floor was aimed at the plantar surface of the right hindpaw. Three measurements of hindpaw withdrawal latency were taken for each hindpaw in each test session. The hindpaw

was tested alternately with >5 min intervals between the consecutive tests. Three measurements of latency per side were averaged as the result of each test. A 20 s cutoff was set to prevent the tissue damage.

3.11.3 Hot Plate

The Hot Plate apparatus (UgoBasile, no. 35150) was set to a temperature of $55^{\circ} \pm 0.2^{\circ}\text{C}$. On the testing day, mice were placed on the surface of the hot plate and covered by a glass transparent cylinder, (25-cm high and 12-cm diameter). A 30-s cutoff time was assigned in this protocol. A remote foot-switch pad was used to control the start/stop/reset functions. The latency to response was recorded when the first hind paw lick or jump occurred.

3.11.4 Grip Strength

The protocol was adapted from Biomedical And Obesity Research Core (<https://cehs.unl.edu/borc/grip-strength-system/>). Forelimb measurement: Mice were lowered over the grid (UgoBasile, no. 47200) allowing only its forepaws to attach to the grid before any measurements are taken. They were gently pulled back by their tail ensuring that they grip the top portion of the grid keeping the torso horizontal. The maximal grip strength value displayed on the screen was recorded. Three forelimb grip strength measurements were obtained in total. Forelimb and hindlimb measurement: Mice were lowered over the grid keeping the torso parallel with the grid and allowing both their forepaws and hind paws to attach to the grid before any measurements are taken. Mice were gently pulled back by their tail ensuring that the torso remained parallel with the grid and the maximal grip strength value of the mouse that is displayed on the screen was recorded. Three forelimb/hindlimb grip strength measurements were obtained in total.

3.12 SEAHORSE MITOSTRESS ASSAYS

Mouse Schwann cells were cultured in Proliferation Media or Differentiation Media as described above for 48h. At the day of assay, cells were washed twice with the Seahorse XF DMEM media supplemented with 17.5mM Glucose, 0.5mM Pyruvate and 2mM Glutamine (adding NRG1+FSK in the case of the proliferating conditions and NRG1+cAMP in the case of differentiation conditions) and then incubated in this media for 1 hour at 37°C in a non-CO2 incubator before the Mitostress Assay. Just before the assay, all wells were washed and filled with fresh supplemented Seahorse media without any additional factors. The assay was designed to measure 3 cycles of basal respiration, 3 cycles after Oligomycin injection (Sigma O4876, 2μM final concentration), 3 cycles after FCCP injection (Sigma C2920, 2μM final concentration) and 3 Cycles after Rotenone + Antimycin A injection (Sigma R8875, Sigma A8674, 2μM final concentration of both compounds). Upon completion, total DNA was extracted from the cells with DNA extraction buffer (100mM Tris-HCl pH 8, 10mM EDTA, 200mM NaCl) followed by Proteinase K digestion (Thermo Scientific E00491, 1mg/mL) at 55°C for 1 hour. Proteinase K was heat inactivated at 95°C for 10 min and total DNA was then

measured using the Quant-iT PicoGreen dsDNA Assay (P7589, Thermo) according to the prescribed protocol. Oxygen Consumption Rates (OCRs) were then normalized to 1 μ g of total DNA for each well. OCR values were calculated using the Wave Desktop Software (version 2.6.3).

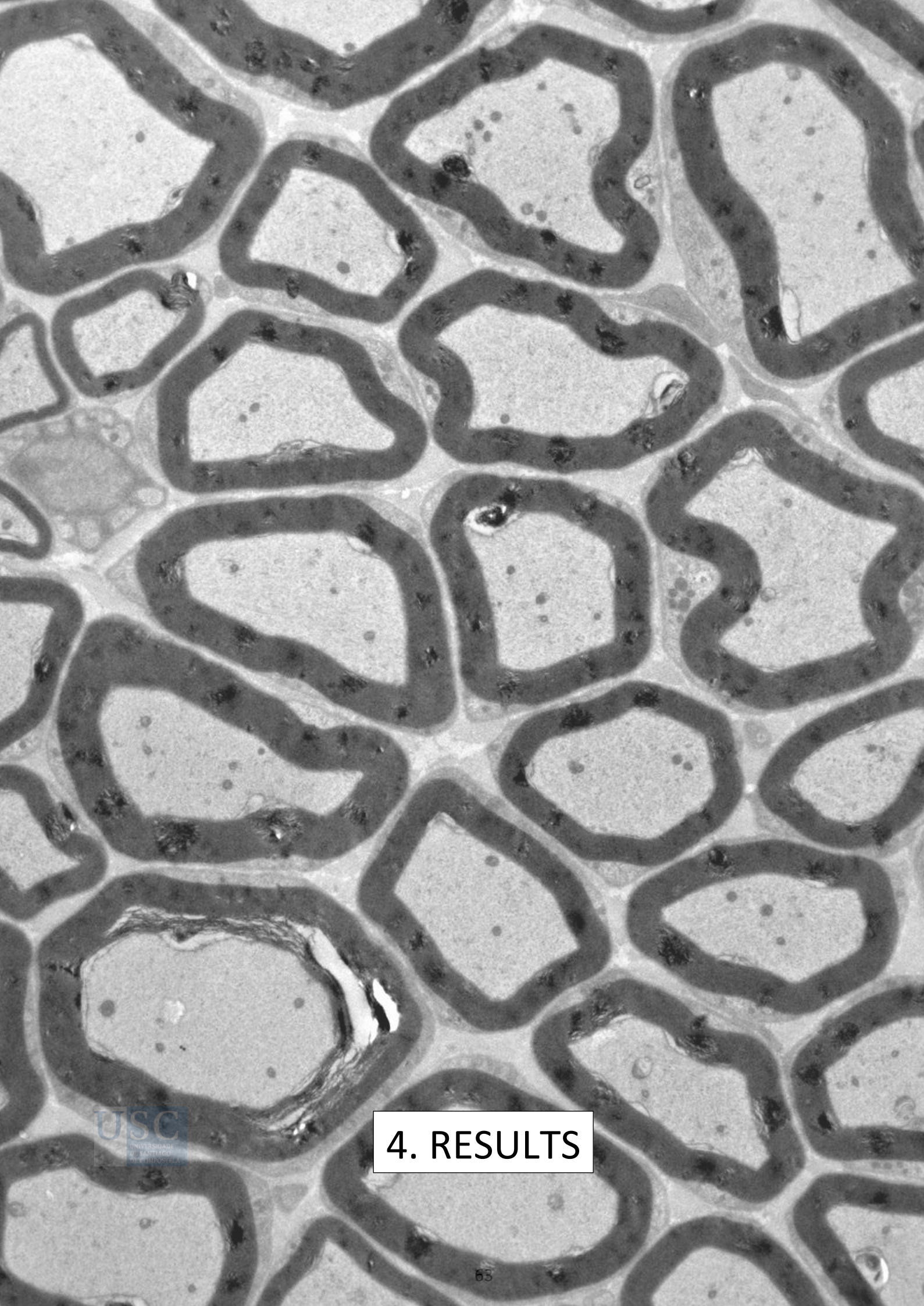
3.13 BIBLIOGRAPHIC RESOURCES AND AI-ASSISTED TECHNOLOGIES

PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) and Google Scholar (<https://scholar.google.es/>) were used as bibliographic resources for this thesis. “Consensus” AI search engine (<https://consensus.app/>) was used to complement the bibliographic screening. “Mendeley Reference Manager” Version 2.137.0 was employed to manage the bibliography.

During the preparation of this PhD thesis manuscript part of the text editing and language polishing was assisted using ChatGPT (GPT-5, OpenAI, 2025). The model was employed exclusively for linguistic improvements and not for generating scientific content or data interpretation. After using this tool, I thoroughly reviewed and edited the content as necessary and take full responsibility for the manuscript’s final content.

3.14 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 8 software. Data were represented as means $\pm$ SEM for each experimental group. Statistical significance was determined by parametric tests when sample normality was assumed. Student’s t test, one-way Anova, two-way Anova, nested t-test and nested Anova were applied in the corresponding analysis. A p-value < 0.05 was considered statistically significant. When p-value is not specified: (ns) non-significant, (*) < 0.05, (**) < 0.01, (***) < 0.001, (****) < 0.0001.



4.1 THE CORE COMPONENTS OF MITOCHONDRIAL FUSION ARE EXPRESSED DURING SCHWANN CELL DIFFERENTIATION

The role of mitochondrial fusion in the PNS has not been studied in detail. To explore its potential involvement in Schwann cell myelination, we analysed the expression levels of the mitochondrial fusion proteins MFN1, MFN2 and OPA1 as well as VDAC, an abundant and stable outer mitochondrial membrane protein, by immunoblotting in sciatic nerve lysates at various developmental stages that broadly correspond to key phases of the myelination process. As mentioned above, in developing nerves, immature Schwann cells envelop groups of axons communally, forming irregular axon/Schwann cell columns or families. Large diameter axons then become segregated from the immature Schwann cell families to form a 1:1 relationship with a Schwann cell, a process known as radial sorting, giving rise to pro-myelinating Schwann cells (J. Salzer et al., 2024). Thus, we collected sciatic nerves from newborn (NB) WT mice, enriched in immature Schwann cells, as well as from P7 and P15, which are characterized by active myelination, and from P30 and P90 nerves, which contain terminally differentiated Schwann cells (Fig. 12A). We found that levels of MFN1, MFN2 and OPA1 generally increased during Schwann cell myelination with a peak expression in actively myelinating Schwann cells. (Fig. 12B, C). At the mRNA level, we did not detect major changes, except a decrease in *Mfn1* levels in perinatal nerves, and an increase in *Mfn2* levels in P90 nerves (Fig. 12D). The data suggest that mitochondrial fusion appears to be most tightly regulated at the onset of the Schwann cell myelination program.

Next, we examined EM micrographs of sciatic nerve sections from P2 to P120 mice and analysed the number and size of axonal and Schwann cell mitochondria (Fig. 13A). We found an increase in SC mitochondrial number (Fig. 13B) and a decrease in axonal mitochondria number (Fig. 13D) with development, whereas there were no major changes in size (Fig 13C, E).

Finally, we examined expression levels of the mitochondrial fusion proteins in primary rat Schwann cells treated with dibutyl cAMP analogs [dibutyl (db) cAMP], which can potently up-regulate the myelin protein MPZ, as a surrogate *in vitro* model of Schwann cell differentiation (Palomo Irigoyen et al., 2018) (Fig. 14A). Under these myelinogenic conditions, we found a general increase in MFN1 and OPA1 protein levels with db cAMP treatment (Fig. 14B, C), coinciding with an increase in their mRNA levels (Fig. 14D). *Mfn2* mRNA levels were, on the other hand, unaltered by db cAMP treatment (Fig. 14D), whereas MFN2 protein levels were sharply decreased (Fig. 14B, C).

These data above show that key components involved in mitochondrial fusion are expressed and regulated during Schwann cell myelination.

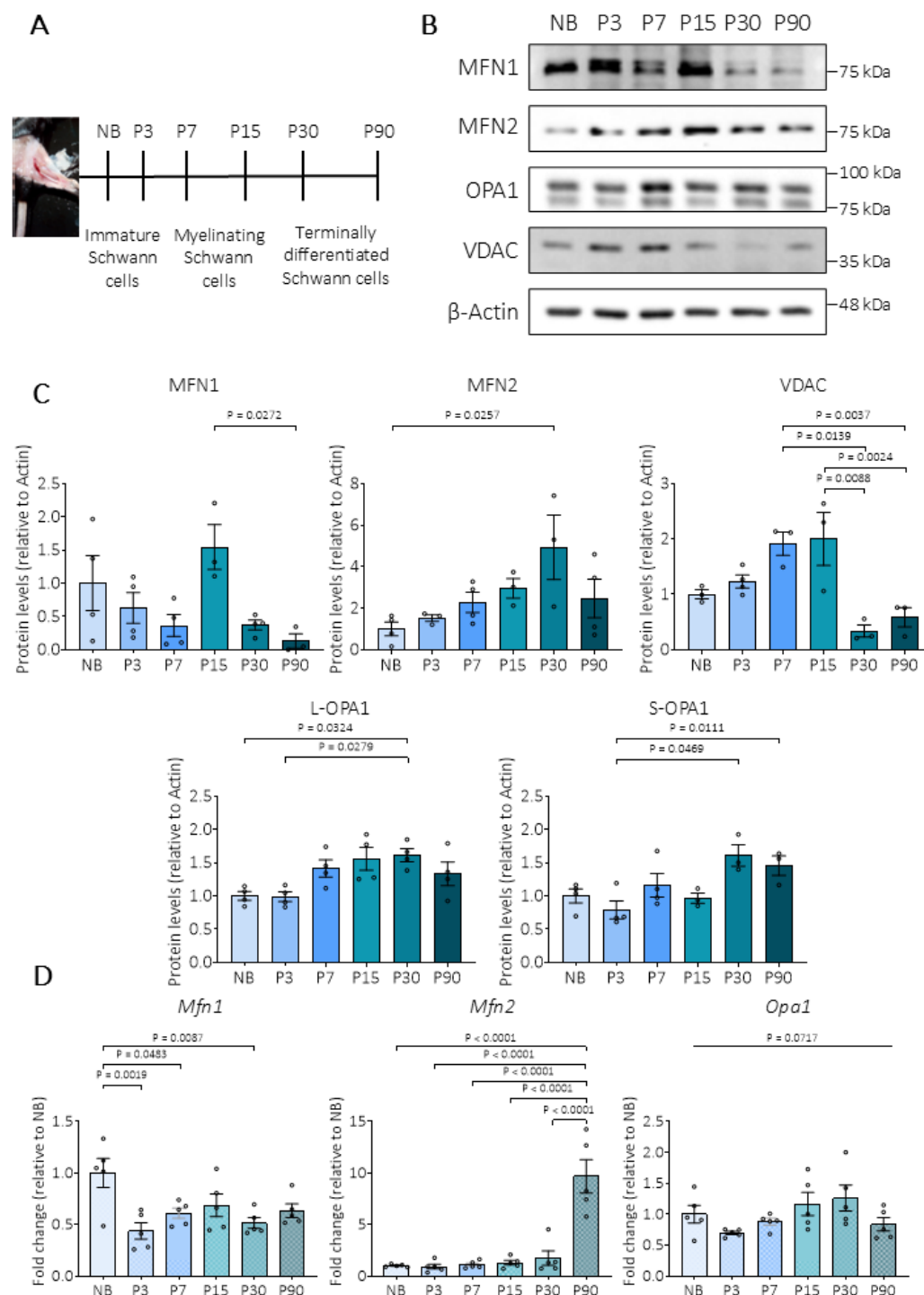


Figure 12. *In vivo* characterization of mitochondrial fusion during Schwann cell (SC) myelination. (A) Schematic representation of key developmental time points highlighting critical stages of SC myelination. (B) Immunoblot analysis of MFN1, MFN2, OPA1, VDAC and β -Actin expression in total sciatic nerve extracts at the indicated developmental ages. (C) Densitometric quantification of MFN1, MFN2, OPA1, and VDAC protein levels normalized to β -Actin (loading control). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (D) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in total sciatic nerve extracts at the indicated ages. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Mrpl10* and *Sdha* mRNA for each data point. Data are presented as means $\pm$ SEM, $n = 3$ to 4. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test.

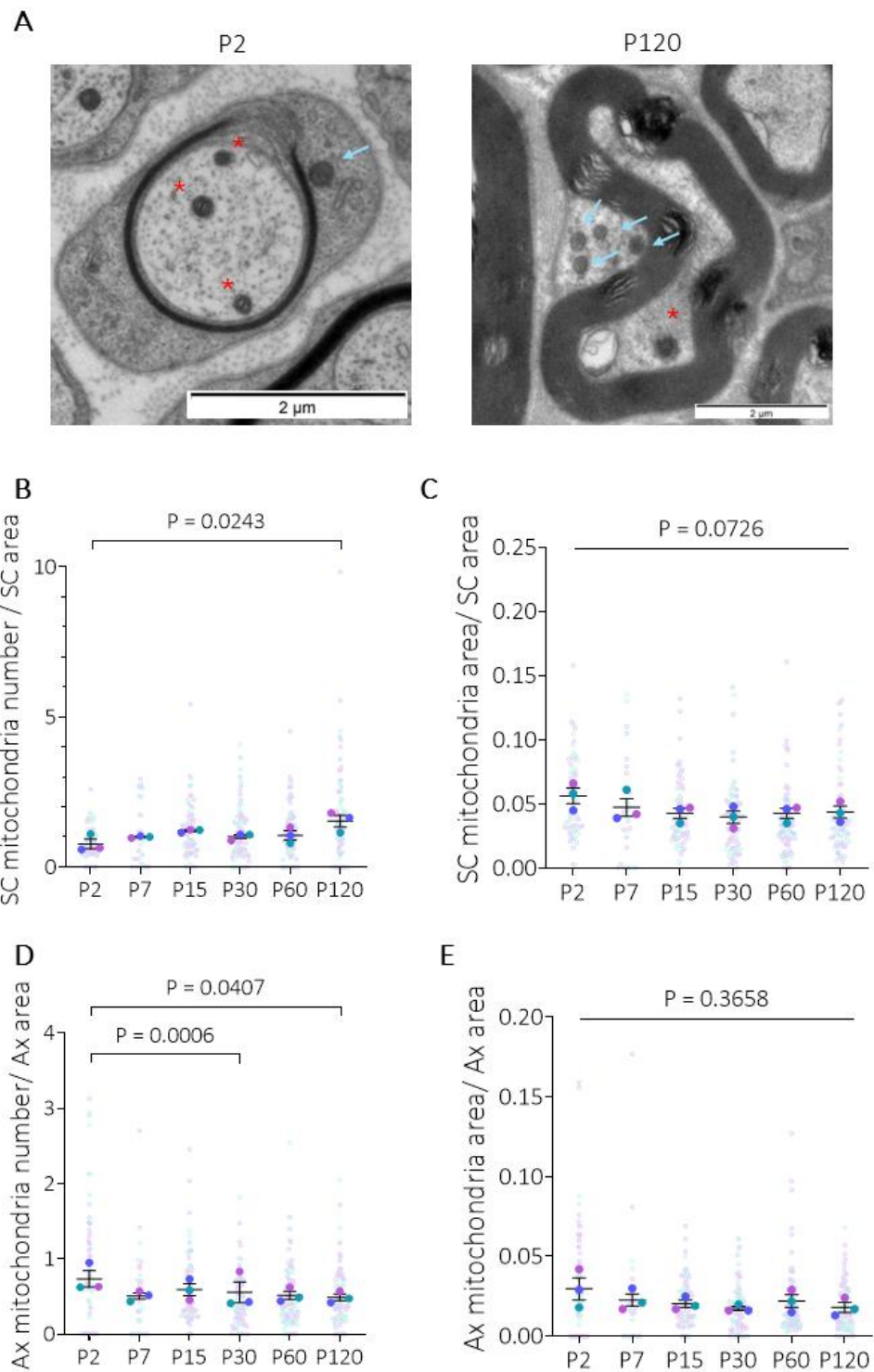


Figure 13. Ultrastructural characterization of Schwann cell and axonal mitochondria in peripheral nerves. (A) Representative electron micrographs of nerve cross sections at P2 and P120d. Blue arrows highlight Schwann cell mitochondria; red asterisks denote axonal mitochondria. Scale bar, 2 μ m. (B, C) Transmission electron microscopy (TEM) analysis of Schwann cell mitochondria in sciatic nerves at the indicated developmental stages. Morphometric assessment of (B) Schwann cell mitochondrial number, and (C) size (normalized to Schwann cell cytoplasmic area). (D, E) TEM analysis of axonal mitochondria in sciatic nerves at the indicated developmental stages. Morphometric assessment of (D) axonal mitochondrial number, and (E) size (normalized to axonal cytoplasmic area). Data are presented as super-plot combining biological replicates $\pm$ SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, $n = 3$ per group. Statistical significance was determined using nested one-way ANOVA with Tukey's post hoc test.

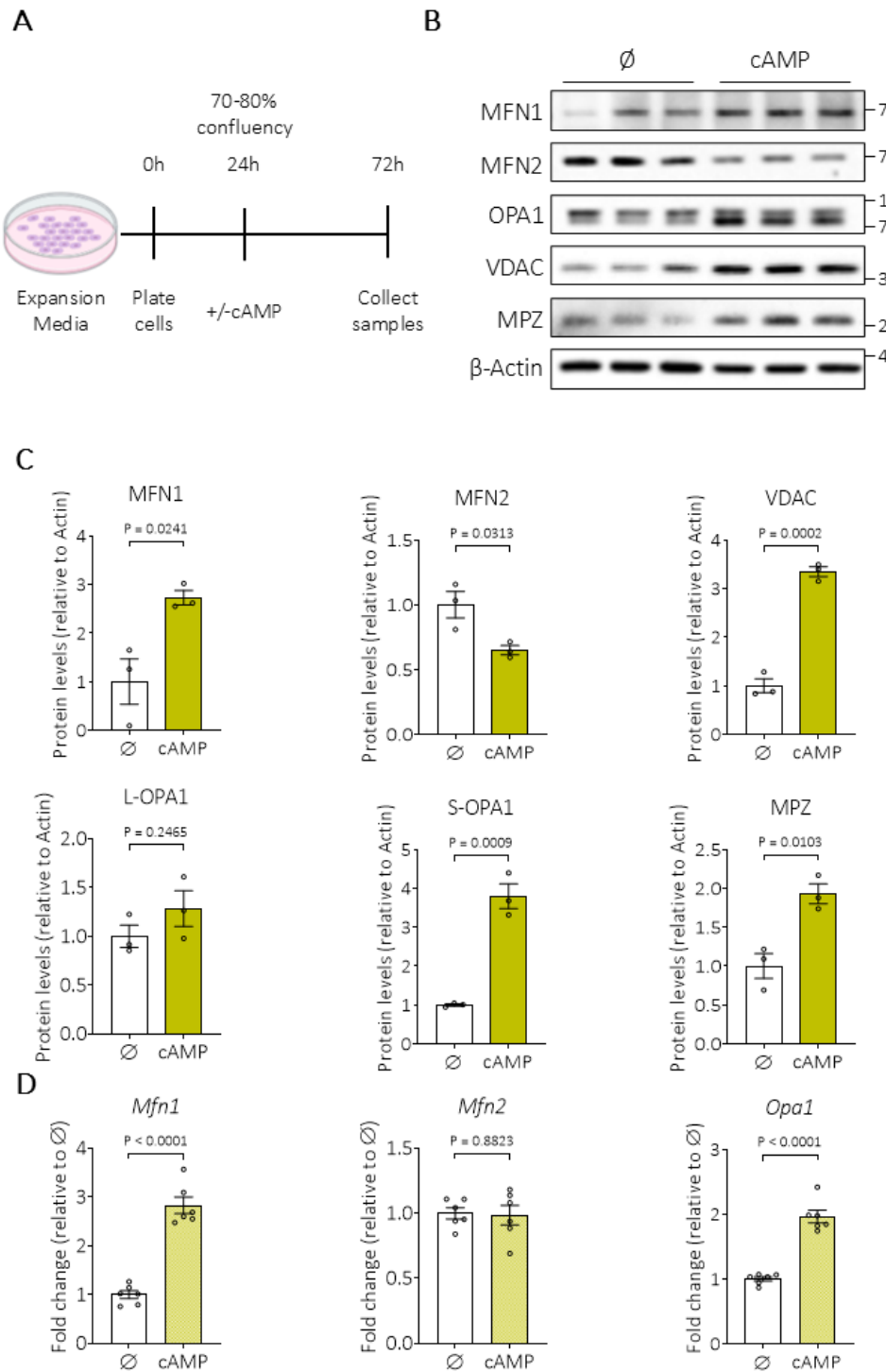


Figure 14. Characterization of mitochondrial fusion in Schwann cells *in vitro* under myelinogenic conditions. (A) Schematic representation experimental set-up. Primary rat Schwann cells (RSC) were cultured under basal (Ø) and myelinogenic conditions [dibutylcyclic adenosine 3',5'-monophosphate (db cAMP) treatment]. (B) Immunoblot analyses of MFN1, MFN2, OPA1, β-Actin, VDAC and MPZ expression in RSC lysates under basal (Ø) and myelinogenic conditions (cAMP). (C) Densitometric quantification of MFN1, MFN2, OPA1, VDAC and MPZ protein levels normalized to β-Actin (loading control). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (D) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in RSC lysates under basal (Ø) and myelinogenic conditions (cAMP). The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Yy1* and *Mre11* mRNA for each data point. Data are presented as means ± SEM. Two-tailed unpaired Student's *t* test.

4.2 ESTABLISHMENT OF CONSTITUTIVE, SCHWANN CELL–SPECIFIC KNOCKOUT MODELS TARGETING MITOCHONDRIAL FUSION.

To further investigate the role of mitochondrial fusion in Schwann cell development, we generated a series of conditional mice model lacking MFN1, MFN2 or OPA1 specifically in Schwann cells. For this, we crossed floxed mice for *Mfn1* (Chen et al., 2007; Ramírez et al., 2017), *Mfn2* (Chen et al., 2007), *Mfn1/2* (combining both previous floxed mice) and *Opa1* (Rodríguez-Nuevo et al., 2018) with the MPZ-Cre mice, which expresses Cre recombinase specifically in Schwann cells from embryonic day E13.5 (Feltri et al., 1999).

MPZ-Cre⁺; *Mfn1*^{fl/fl} mice were referred to as *Mfn1* conditional knockout (cKO) mice or MFN1^{KO}, and their littermates MPZ-Cre⁻; *Mfn1*^{fl/wt} or MPZ-Cre⁻; *Mfn1*^{fl/fl} mice were used as controls. Similar terminology was used for *Mfn2* and *Opa1* SC-KO models. For *Mfn1* and *Mfn2* double KO mice, MPZ-Cre⁺; *Mfn1*^{fl/fl}; *Mfn2*^{fl/fl} mice are referred to as *Mfn1/2* conditional double knockout (dKO) mice or MFN1^{KO}MFN2^{KO} and their littermates MPZ-Cre⁻; *Mfn1*^{fl/wt}; *Mfn2*^{fl/fl}, MPZ-Cre⁻ *Mfn1*^{fl/fl}; *Mfn2*^{fl/wt} or MPZ-Cre⁻; *Mfn1*^{fl/fl}; *Mfn2*^{fl/fl} mice were used as controls. In addition, we included mice retaining only one functional mitofusin allele to examine partial gene dosage effects: MPZ-Cre⁺; *Mfn1*^{fl/wt}; *Mfn2*^{fl/fl} and MPZ-Cre⁺; *Mfn1*^{fl/fl}; *Mfn2*^{fl/wt}, which we refer to as MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT} respectively.

RT-qPCR analyses confirmed the deletion of *Mfn1*, *Mfn2*, and *Opa1* in P15 mutant nerves, and we did not observe any compensatory expression between them (Fig. 15A, D and Fig. 16A, D). Immunoblot analyses of nerve lysates revealed different landscapes depending on the mutant line. *Mfn1* cKO mice showed a clear reduction in Mfn1 levels compared to their WT littermates, as well as compensatory increased levels of Mfn2 and Opa1 (both isoforms) (Fig. 15B, C). In contrast, *Mfn2* cKO mice exhibited reduced MFN2 protein levels but no changes in MFN1 nor OPA1 levels (Fig. 15E, F). Surprisingly, we did not see any decrease in protein levels of either MFN1 or MFN2 levels in *Mfn1/2* dKO nerves (Fig. 16B, C), despite the sharp decrease in *Mfn1* and *Mfn2* mRNA (Fig. 16A) and the decrease in protein levels observed in single knockouts (Fig. 15B, C, E and F). This could be due to compensatory increased levels in contaminating cell types present in sciatic nerve lysates, including fibroblasts or immune cells, and it would be important to confirm the decreased expression in purified Schwann cells. In *Opa1* cKO mice, OPA1 protein levels were diminished along with MFN1 levels, while MFN2 levels remained unchanged (Fig 16E, F).

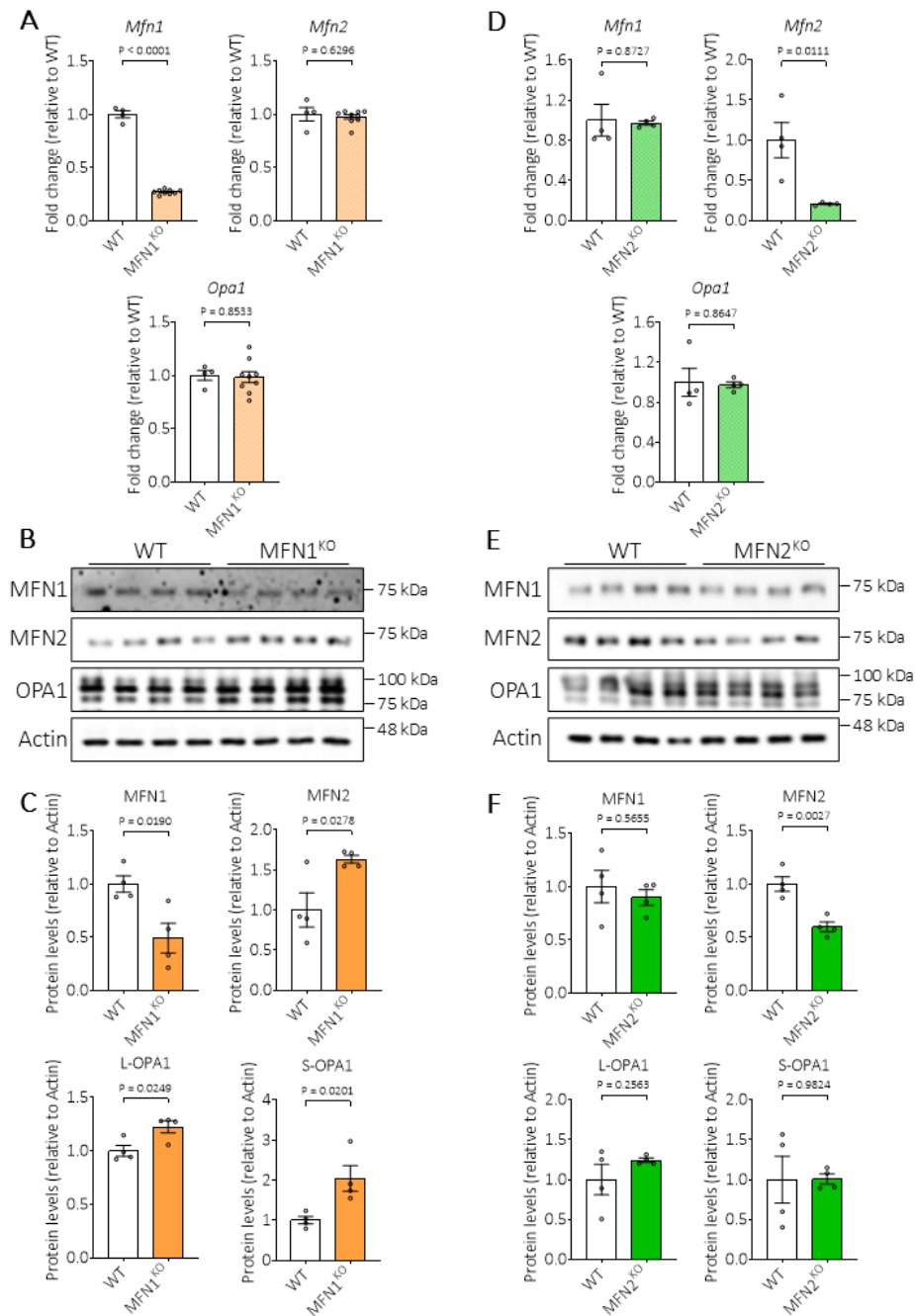


Figure 15. Generation of Schwann cell-specific *Mfn1* and *Mfn2* single knockout models. (A) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in sciatic nerve extracts from WT and *Mfn1* cKO mice. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Mrpl10* and *Gapdh* mRNA for each data point. (B) Immunoblot analyses of MFN1, MFN2, OPA1 and β -Actin expression in sciatic nerve extracts from WT and *Mfn1* cKO mice. (C) Densitometric quantification of MFN1, MFN2 and OPA1 protein levels normalized to β -Actin (loading control) from immunoblots in (B). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (D) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in sciatic nerve extracts from WT and *Mfn2* cKO mice. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Ppia* mRNA for each data point. (E) Immunoblot analyses of MFN1, MFN2, OPA1 and β -Actin expression in sciatic nerve extracts from WT and *Mfn2* cKO mice. (F) Densitometric quantification MFN1, MFN2 and OPA1 protein levels normalized to β -actin (loading control) from immunoblots in (E). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. Data are presented as means $\pm$ SEM, $n = 4$ to 9. Two-tailed unpaired Student's *t* test.

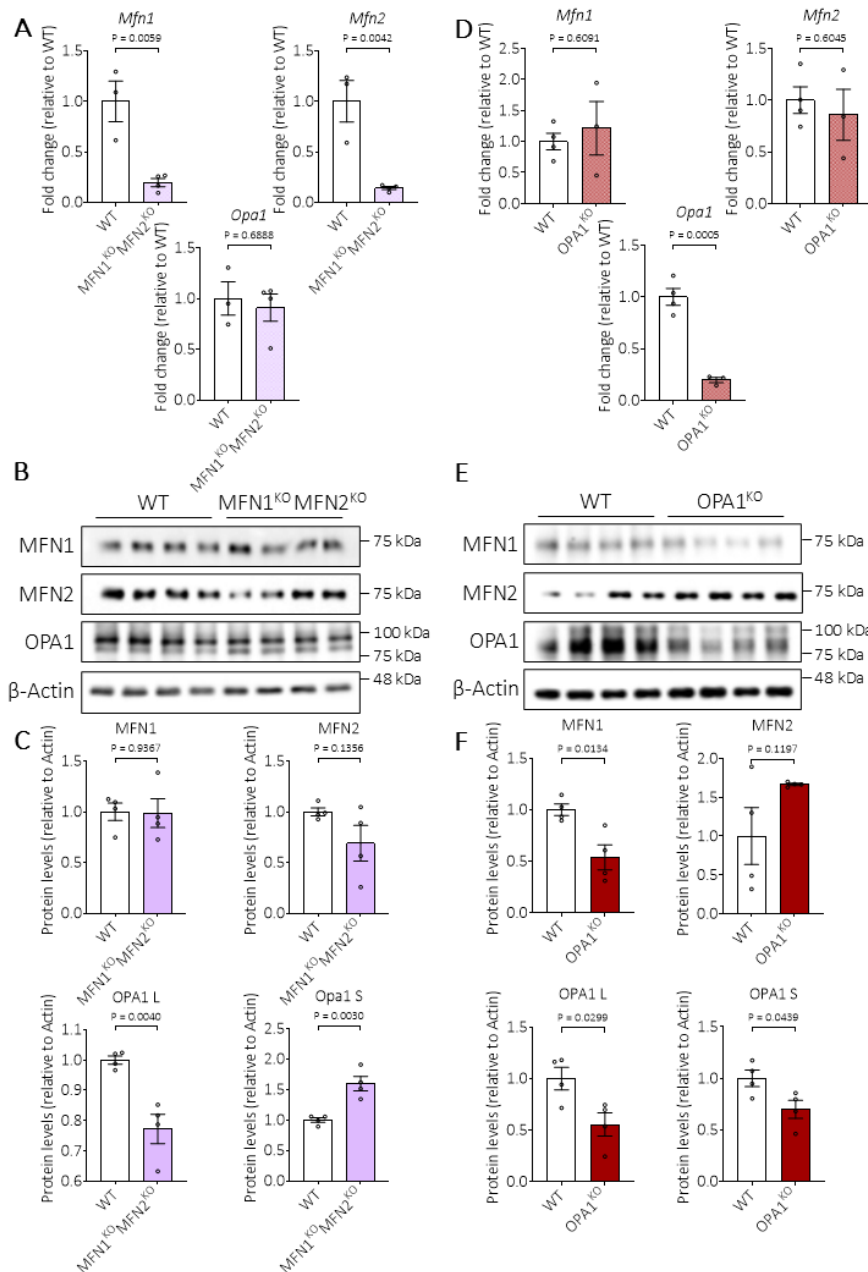


Figure 16. Generation of Schwann cell-specific *Mfn1/2* double KO and *Opa1* KO models. (A) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in sciatic nerve extracts from WT and *Mfn1/2* dKO mice. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Ppia* mRNA for each data point. (B) Immunoblot analyses of MFN1, MFN2, OPA1 and β-Actin expression in sciatic nerve extracts from WT and *Mfn1/2* dKO mice. (C) Densitometric quantification of MFN1, MFN2 and OPA1 protein levels normalized to β-Actin (loading control) from immunoblots in (B). L-OPA1 refers to the long and S-OPA1 to the short isoform. (D) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in sciatic nerve extracts from WT and *Opa1* cKO mice. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Ppia* mRNA for each data point. (E) Immunoblot analyses of MFN1, MFN2, OPA1 and β-Actin expression in sciatic nerve extracts from WT and *Opa1* cKO mice. (F) Densitometric quantification of MFN1, MFN2 and OPA1 protein levels normalized to β-Actin (loading control) from immunoblots in (E). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. Data are presented as means ± SEM, $n = 3$ to 4. Two-tailed unpaired Student's *t* test

4.3 GENETIC DELETION OF *MFN1* AND *MFN2* OR *OPA1* IN VIVO LEADS TO DEVELOPMENT OF PERIPHERAL NEUROPATHY

Mfn1 cKO and *Mfn2* cKO mice were born at the expected Mendelian ratio and were essentially indistinguishable from littermate controls with no major phenotypic alterations observed. We performed a battery of motor and sensory functional tests to examine any nerve defects (Ayuso-García et al., 2024), including latency to fall off the accelerating rotarod and grip strength of all four limbs or forelimbs for motor function, and nociceptive responses to thermal stress using the Hargreaves plantar test and hot-plate test for sensory function. We found that *Mfn1* cKO and *Mfn2* cKO mice were indistinguishable from their WT littermates in any of the functional tests. (Fig. 17 and Fig. 18, respectively). In addition, we did not find any differences in weight of these mice with age.

To evaluate nerve integrity, we performed electromyographic recordings at different ages and analysed the following parameters: latency to response, mean peak amplitudes of CMAPs, average durations of CMAPs, and nerve conduction velocities at 20 mA and first clear response when increasing mA (Ayuso-García et al., 2024). All these electromyographic parameters were almost identical in *Mfn1* cKO and *Mfn2* cKO mice, when compared to control mice (Fig. 19 and Fig. 20, respectively).

Similar to *Mfn1* and *Mfn2* single knockouts, *Mfn1/2* dKO mice were born at the expected Mendelian ratio and were essentially indistinguishable from littermate controls initially. However, *Mfn1/2* dKO mice died prematurely or were sacrificed for ethical reasons at around P90 (Fig. 21A). Starting from the second month, the mice began to develop severe tremor and unsteady gait. Upon tail suspension, they showed abnormal hindlimb claspings (Fig. 21B), weighed less than controls at P90 (Fig. 21C and Fig. 22A), and performed poorly in motor and sensory functional tests during development (Fig. 21D–H and Fig. 22B–F). Electrophysiological recordings showed a marked reduction in all analysed parameters (Fig. 23A–F and Fig. 24 A–E), clearly showing marked conduction blocks, accounting for the defect in motor function in these mice at P60.

In addition to the *Mfn1/2* double knockout mice, we also examined mice retaining a single mitofusin allele (*MFN1*^{HT}*MFN2*^{KO} or *MFN1*^{KO}*MFN2*^{HT}). These mice initially showed no significant alterations but started to show signs of peripheral neuropathy at later time-points. *MFN1*^{HT}*MFN2*^{KO} mice showed no obvious deficits in motor-sensory tests at early time-points (Fig. 21D–H and Fig. 22B–F), but at P120, electromyographic analyses revealed decreased amplitude and a marked reduction in nerve conduction velocity (Fig. 23D, F and Fig. 24C, E). In contrast, *MFN1*^{KO}*MFN2*^{HT} mice performed worse with deficits in motor function, as shown by the rotarod test and grip strength measurements, whereas no defects in sensory function were observed (Fig. 21D–H and Fig. 22B–F). Electrophysiological recordings also showed earlier and more profound deficits than *MFN1*^{HT}*MFN2*^{KO} mice in most parameters, including latency, amplitude, first response, duration and NCV (Fig. 23A–F and Fig. 24 A–E).

Opa1 cKO mice were born at the expected Mendelian ratio and were essentially indistinguishable from littermate controls initially. However, *Opa1* cKO mice died or were sacrificed for ethical reasons at around P180 (Fig. 25A). Starting from the second month, the mice began to develop severe tremor and unsteady gait. Upon tail suspension, they showed abnormal hindlimb clasp (Fig. 25B) and performed poorly in motor and sensory functional tests with development (Fig. 25C–H). Electromyographic recordings showed a marked reduction in all analysed parameters (Fig. 26A–F), clearly showing marked conduction blocks, accounting for the defect in motor function in these mice at P30.

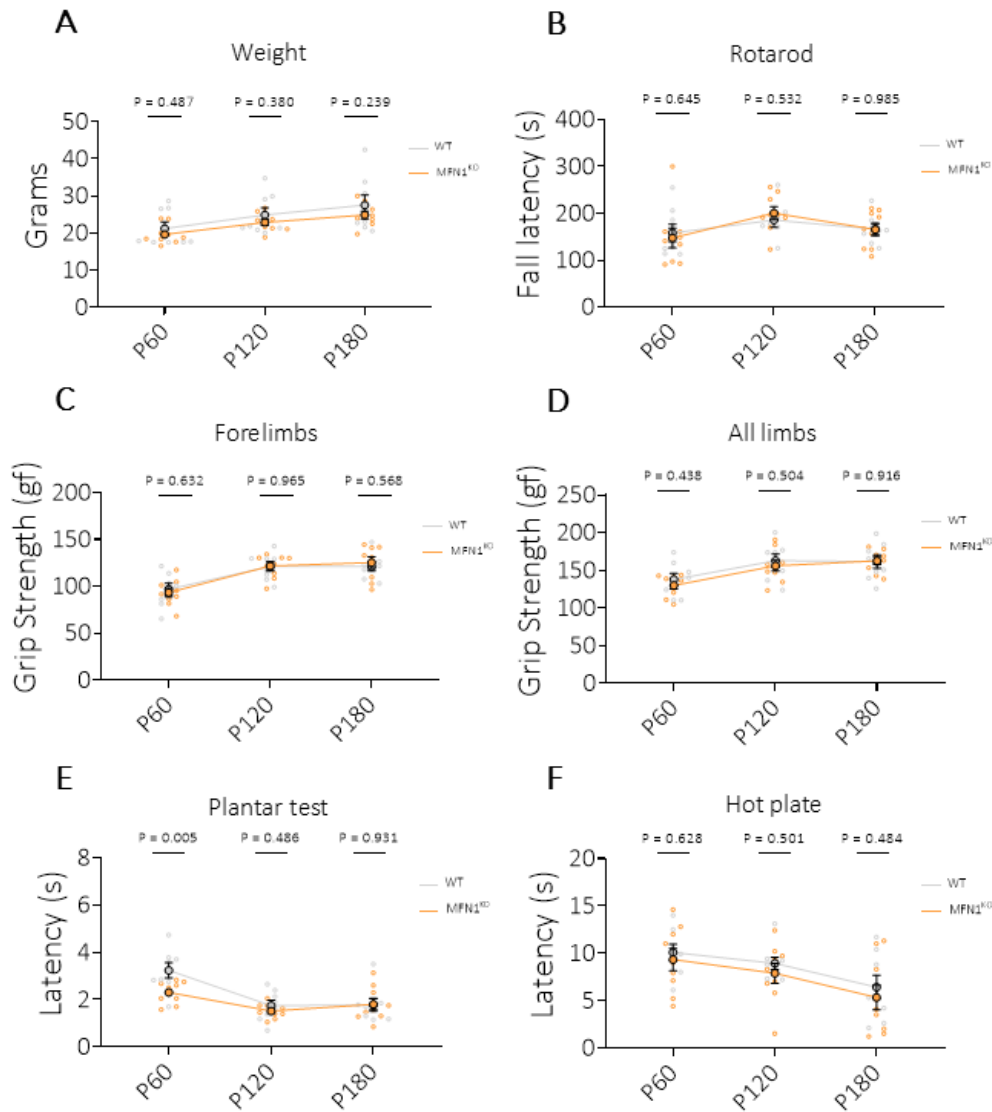


Figure 17. *Mfn1* mutant mice show no alterations in sensory-motor function. (A–E) *Mfn1* cKO mice exhibit no significant differences compared to control littermates in (A) weight, motor function, as shown by (B) latency to fall off the accelerating rotarod, (C, D) grip strength of forelimbs or all four limbs, and in sensory responses using (E) the plantar plate test and (G) hot plate test, at indicated ages. Data are presented as means $\pm$ SEM; $n = 7$ to 9. Two-tailed unpaired Student's *t* test.

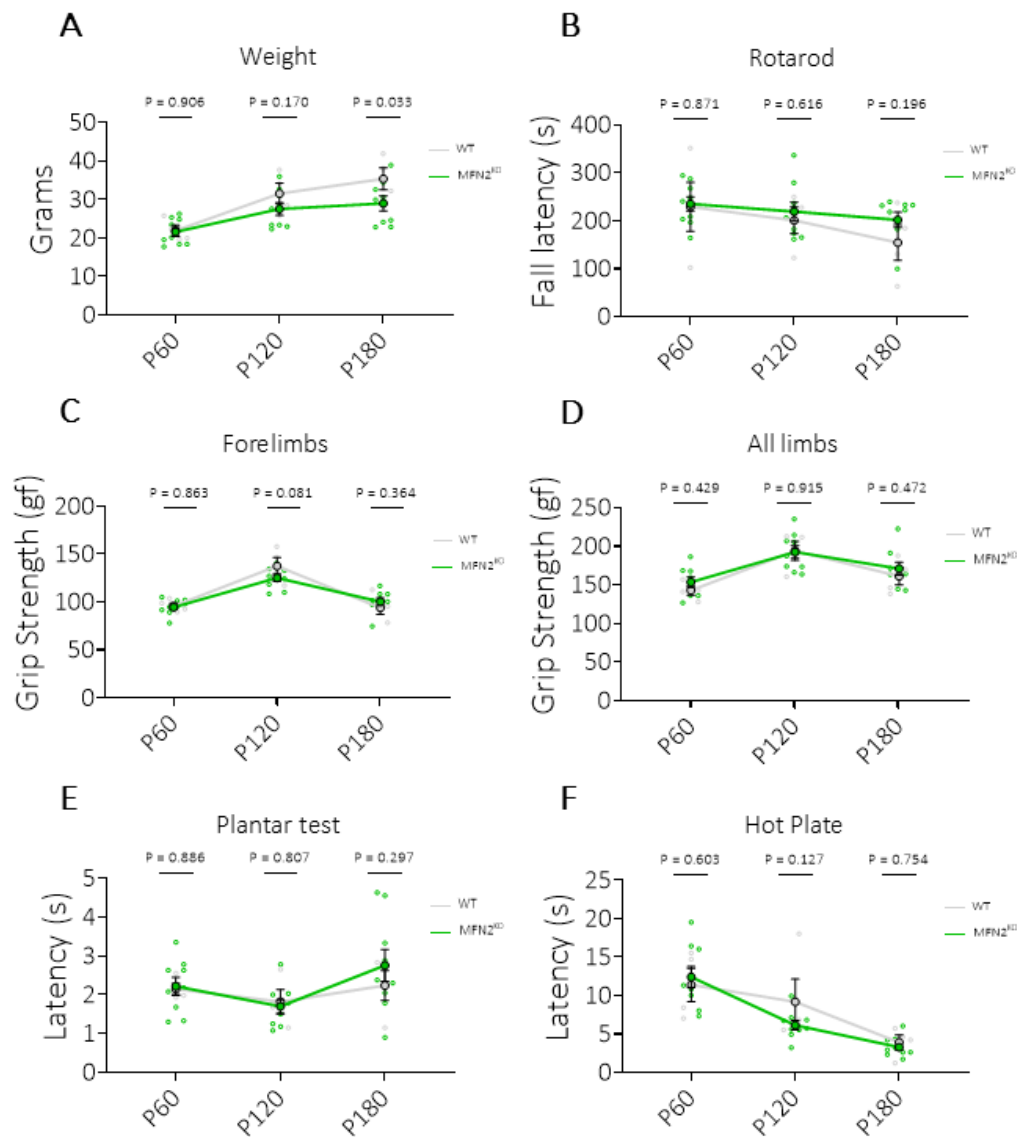


Figure 18. *Mfn2* mutant mice show no alterations in sensory-motor function. (A–E) *Mfn2* cKO mice exhibit no significant differences compared to control littermates in (A) weight, motor function, as shown by (B) latency to fall off the accelerating rotarod, (C, D) grip strength of forelimbs or all four limbs, and in sensory responses using (E) the plantar plate test and (G) hot plate test, at indicated ages. Data are presented as means $\pm$ SEM; $n = 4$ to 9. Two-tailed unpaired Student's *t* test.

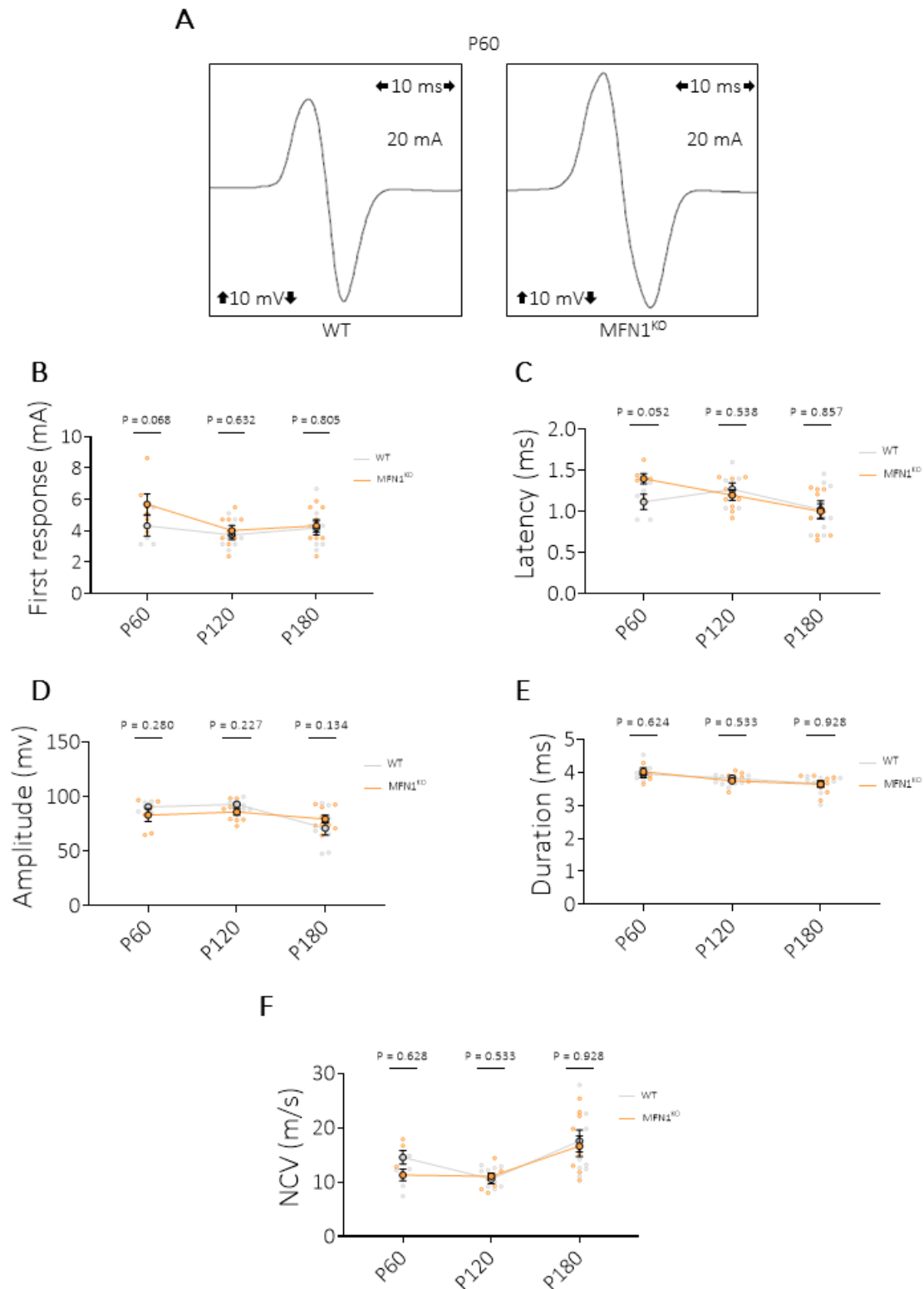


Figure 19. *Mfn1* mutant mice exhibit normal electrophysiological profiles. (A–F) *Mfn1* mutant mice display normal electromyography responses. (A) Electrophysiological recording of CMAPs from sciatic nerves of *Mfn1* cKO and control mice at P60. Representative traces are shown. Graphs show (B) first clear response when increasing mA, (C) latency to response, (D) mean peak amplitudes of CMAPs, (E) average durations of CMAPs, and (F) nerve conduction velocities at 20 mA in sciatic nerves of control, and *MFN1* cKO mice at indicated ages. Data are presented as means $\pm$ SEM; $n = 5$ to 9. Two-tailed unpaired Student's t test.

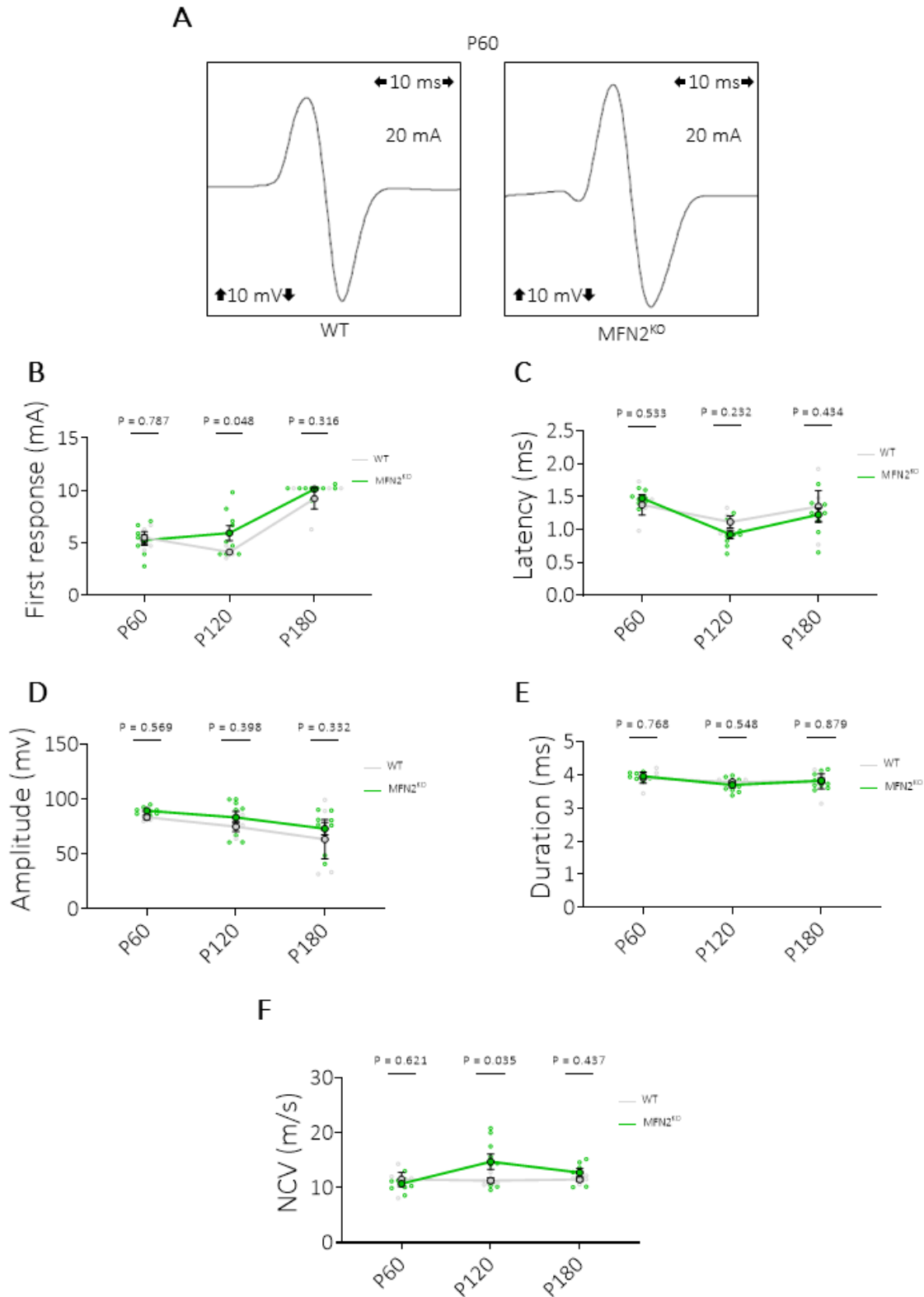


Figure 20. *Mfn2* mutant mice exhibit normal electrophysiological profiles. (A–F) *Mfn2* mutant mice display a normal electromyography response. (A) Electrophysiological recording of CMAPs from sciatic nerves of *Mfn2* cKO and control mice at P60. Representative traces are shown. Graphs show (B) first clear response when increasing mA, (C) latency to response, (D) mean peak amplitudes of CMAPs, (E) average durations of CMAPs, and (F) nerve conduction velocities at 20 mA in sciatic nerves of control, and *Mfn2* cKO mice at indicated ages. Data are presented as means $\pm$ SEM; $n = 4$ to 9. Two-tailed unpaired Student's t test.

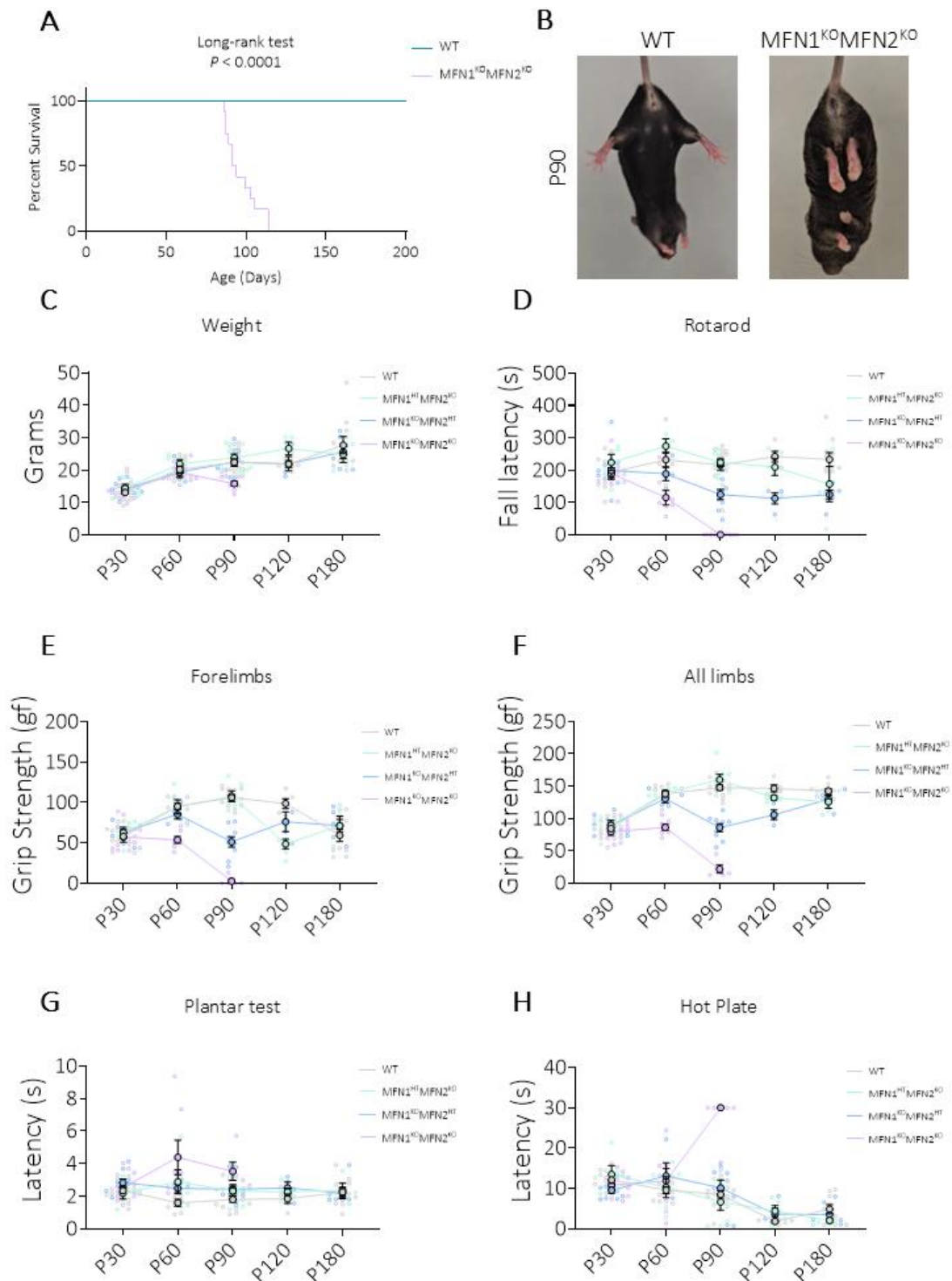


Figure 21. *Mfn1/2* double mutant mice (MFN1^{KO}MFN2^{KO}) show profound deficits in sensory-motor function. (A) Survival curves of WT and *Mfn1/2* dKO mice ($n = 12$ per group). P values are from a log-rank test between groups. (B) Tail suspension test showing abnormal hindlimb clasp in *Mfn1/2* dKO mice but not in WT littermate control at P90, a common sign of peripheral neuropathy in mice. (C) Weight curves of WT and *mutant* mice. (D–H) MFN1^{KO}MFN2^{KO} mice show poor responses compared to the rest of groups (WT, MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT}) starting at 60 days in motor function, as shown by (D) latency to fall off the accelerating rotarod and (E, F) grip strength of forelimbs or all four limbs, and in sensory responses using (G) the plantar plate test and (H) hot plate test. MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT} show no overall differences with control groups in any test except for modest effects on motor function. Tests were performed during development from P30–P180. Tables in (Fig. 22) report the statistical values for the corresponding comparisons between different mice groups. Data are presented as means $\pm$ SEM; $n = 5$ to 14. Two-way ANOVA with Sidak's multiple comparisons test.

A Weight				
P30	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.8410	0.7043	0.9791
	MFN1 ^{HT} MFN2 ^{KO}		>0.9999	0.9686
	MFN1 ^{KO} MFN2 ^{HT}			0.9541
	MFN1 ^{KO} MFN2 ^{KO}			
P60	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.7358	0.9831	0.9495
	MFN1 ^{HT} MFN2 ^{KO}		0.6890	0.5010
	MFN1 ^{KO} MFN2 ^{HT}			0.9999
	MFN1 ^{KO} MFN2 ^{KO}			
P90	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.8079	>0.9999	0.0002
	MFN1 ^{HT} MFN2 ^{KO}		0.8823	0.0001
	MFN1 ^{KO} MFN2 ^{HT}			0.0050
	MFN1 ^{KO} MFN2 ^{KO}			
P120	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.1522	0.9997	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.3055	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
P180	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.7115	0.8206	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.9606	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
B Rotarod				
P30	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.7043	0.9961	0.9979
	MFN1 ^{HT} MFN2 ^{KO}		0.8755	0.6862
	MFN1 ^{KO} MFN2 ^{HT}			0.9870
	MFN1 ^{KO} MFN2 ^{KO}			
P60	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.5609	0.5231	0.0124
	MFN1 ^{HT} MFN2 ^{KO}		0.1079	0.0041
	MFN1 ^{KO} MFN2 ^{HT}			0.1538
	MFN1 ^{KO} MFN2 ^{KO}			
P90	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9788	0.0028	<0.0001
	MFN1 ^{HT} MFN2 ^{KO}		0.0012	<0.0001
	MFN1 ^{KO} MFN2 ^{HT}			0.0002
	MFN1 ^{KO} MFN2 ^{KO}			
P120	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.5599	0.0025	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.0411	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
P180	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.4654	0.0021	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.8359	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
C Forelimbs				
P30	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9202	0.7720	0.9996
	MFN1 ^{HT} MFN2 ^{KO}		>0.9999	0.9301
	MFN1 ^{KO} MFN2 ^{HT}			0.8629
	MFN1 ^{KO} MFN2 ^{KO}			
P60	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		>0.9999	0.5495	<0.0001
	MFN1 ^{HT} MFN2 ^{KO}		0.8206	0.0235
	MFN1 ^{KO} MFN2 ^{HT}			0.0191
	MFN1 ^{KO} MFN2 ^{KO}			
P90	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9958	<0.0001	<0.0001
	MFN1 ^{HT} MFN2 ^{KO}		0.0003	<0.0001
	MFN1 ^{KO} MFN2 ^{HT}			0.0002
	MFN1 ^{KO} MFN2 ^{KO}			
P120	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.0007	0.3115	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.2071	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
P180	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.6959	0.5116	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.9997	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
D All limbs				
P30	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9318	0.8644	0.8755
	MFN1 ^{HT} MFN2 ^{KO}		>0.9999	0.7147
	MFN1 ^{KO} MFN2 ^{HT}			0.5373
	MFN1 ^{KO} MFN2 ^{KO}			
P60	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9929	0.7985	<0.0001
	MFN1 ^{HT} MFN2 ^{KO}		0.6475	<0.0001
	MFN1 ^{KO} MFN2 ^{HT}			0.0057
	MFN1 ^{KO} MFN2 ^{KO}			
P90	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.5854	<0.0001	<0.0001
	MFN1 ^{HT} MFN2 ^{KO}		<0.0001	<0.0001
	MFN1 ^{KO} MFN2 ^{HT}			<0.0001
	MFN1 ^{KO} MFN2 ^{KO}			
P120	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.1122	0.0115	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.0645	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
P180	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.3409	0.2003	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.9380	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
E Plantar test				
P30	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9718	0.3409	0.9354
	MFN1 ^{HT} MFN2 ^{KO}		0.4473	0.8561
	MFN1 ^{KO} MFN2 ^{HT}			0.8747
	MFN1 ^{KO} MFN2 ^{KO}			
P60	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.4182	0.1505	0.1350
	MFN1 ^{HT} MFN2 ^{KO}		0.9518	0.6596
	MFN1 ^{KO} MFN2 ^{HT}			0.3744
	MFN1 ^{KO} MFN2 ^{KO}			
P90	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.4827	0.2966	0.1081
	MFN1 ^{HT} MFN2 ^{KO}		0.9943	0.3100
	MFN1 ^{KO} MFN2 ^{HT}			0.3659
	MFN1 ^{KO} MFN2 ^{KO}			
P120	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.5046	0.3627	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.8254	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
P180	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9760	0.9888	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.9488	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
F Hot Plate				
P30	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9241	0.0732	0.7481
	MFN1 ^{HT} MFN2 ^{KO}		0.4094	0.7159
	MFN1 ^{KO} MFN2 ^{HT}			0.7166
	MFN1 ^{KO} MFN2 ^{KO}			
P60	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.9927	0.7293	0.8802
	MFN1 ^{HT} MFN2 ^{KO}		0.9018	0.9775
	MFN1 ^{KO} MFN2 ^{HT}			0.9922
	MFN1 ^{KO} MFN2 ^{KO}			
P90	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.8402	0.8751	<0.0001
	MFN1 ^{HT} MFN2 ^{KO}		0.6206	0.0001
	MFN1 ^{KO} MFN2 ^{HT}			<0.001
	MFN1 ^{KO} MFN2 ^{KO}			
P120	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.2403	0.5399	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.9208	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			
P180	WT	MFN1 ^{HT} MFN2 ^{KO}	MFN1 ^{KO} MFN2 ^{HT}	MFN1 ^{KO} MFN2 ^{KO}
		0.0947	0.7101	Deceased animals
	MFN1 ^{HT} MFN2 ^{KO}		0.5191	
	MFN1 ^{KO} MFN2 ^{HT}			
	MFN1 ^{KO} MFN2 ^{KO}			

Figure 22. Statistical values for the corresponding comparisons between different mice groups from (Fig. 21). (A–F) Tables showing the statistical values between control, MFN1^{HT}MFN2^{KO}, MFN1^{KO}MFN2^{HT}, and MFN1^{KO}MFN2^{KO} at indicated ages of (A) weight and motor-sensory functional tests (B) latency to fall off the accelerating rotarod and (C, D) grip strength of forelimbs or all four limbs, (E) the plantar plate test and (F) hot plate test.

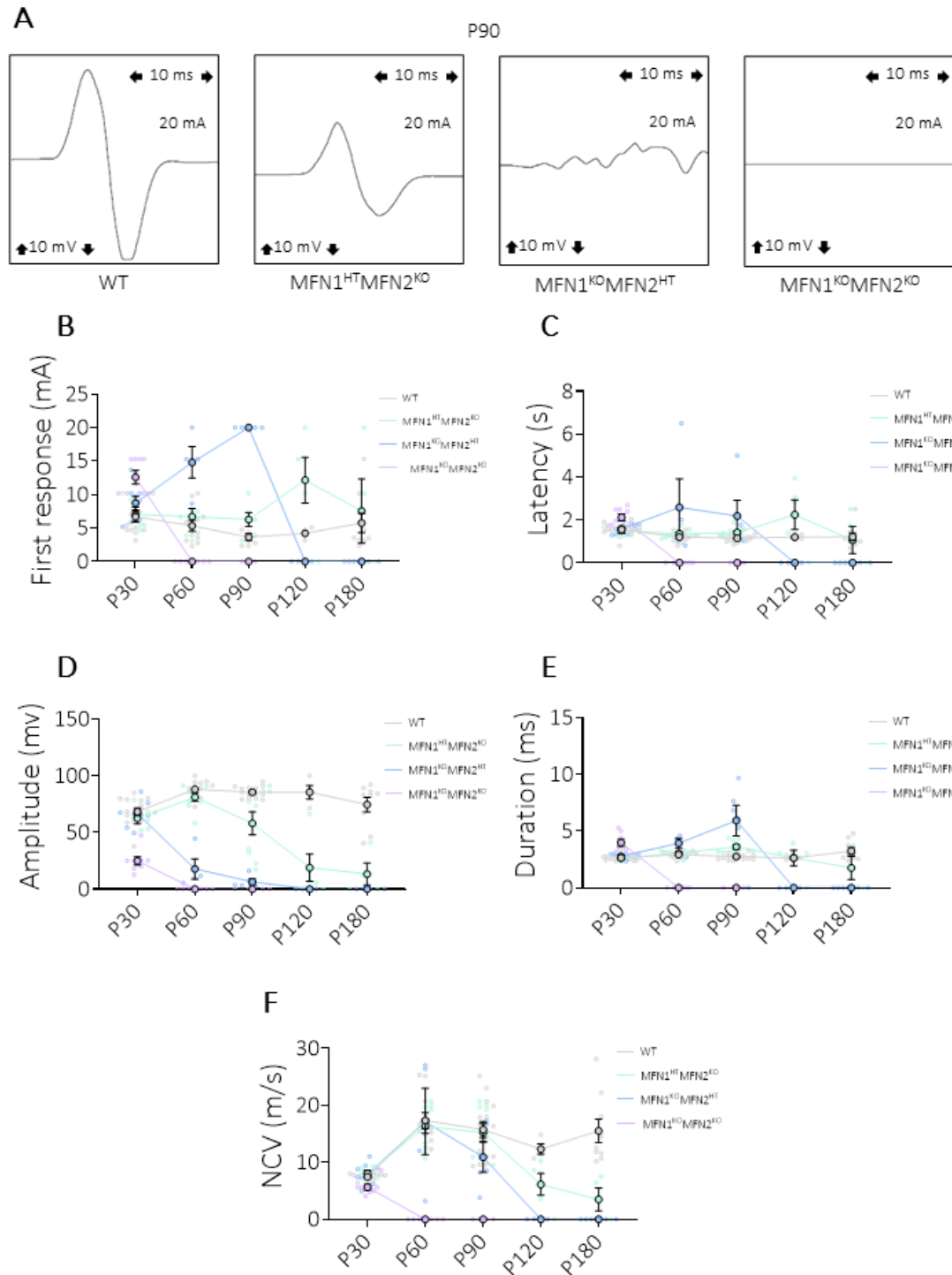


Figure 23. *Mfn1/2* double mutant mice (MFN1^{KO}MFN2^{KO}) display a marked reduction in conduction velocity. (A–F) *Mfn1/2* double mutant mice display a marked reduction in conduction velocity at early timepoints, whereas MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT} mice show a delayed onset of this reduction. (A) Electromyographic recording of CMAPs from sciatic nerves of MFN1^{KO}MFN2^{KO}, MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT} and control mice at P90. Representative traces are shown. Graphs show (B) first clear response when increasing mA, (C) latency to response, (D) mean peak amplitudes of CMAPs, (E) average durations of CMAPs, and (F) nerve conduction velocities at 20 mA in sciatic nerves of control, MFN1^{KO}MFN2^{KO}, MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT} mice at indicated ages. MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT} show a worsening electrical response when aging. Tables in (Fig. 24) report the statistical values for the corresponding comparisons between different mice groups. Data are presented as means $\pm$ SEM; $n = 5$ to 14. Two-way ANOVA with Sidak's multiple comparisons test.

A First response				
P30	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.9896	0.9913	0.0340
	MFN1 ^{+/+} MFN2 ^{+/+}		0.9864	0.3301
	MFN1 ^{+/+} MFN2 ^{+/+}			0.313
P60	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.2123	0.6508	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.7385	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}			0.3030
P90	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.1287	0.4863	0.0075
	MFN1 ^{+/+} MFN2 ^{+/+}		0.6894	0.0049
	MFN1 ^{+/+} MFN2 ^{+/+}			0.2198
P120	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.2076	0.0005	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0215	
	MFN1 ^{+/+} MFN2 ^{+/+}			
P180	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.9687	0.0003	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.3459	
	MFN1 ^{+/+} MFN2 ^{+/+}			
B Latency				
P30	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.7698	0.9882	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.8986	0.0012
	MFN1 ^{+/+} MFN2 ^{+/+}			<0.0001
P60	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.4316	0.0096	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0097	0.0012
	MFN1 ^{+/+} MFN2 ^{+/+}			0.3610
P90	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.0999	<0.0001	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0037	0.0030
	MFN1 ^{+/+} MFN2 ^{+/+}			0.1918
P120	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.0012	0.0016	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.2667	
	MFN1 ^{+/+} MFN2 ^{+/+}			
P180	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.0050	<0.0001	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.4330	
	MFN1 ^{+/+} MFN2 ^{+/+}			
C Amplitude				
P30	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.9998	0.5472	0.0132
	MFN1 ^{+/+} MFN2 ^{+/+}		0.6307	0.0125
	MFN1 ^{+/+} MFN2 ^{+/+}			0.00237
P60	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.8346	0.7586	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.3244	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}			0.0079
P90	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.0071	0.1983	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.4018	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}			0.0246
P120	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.8487	<0.0001	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0142	
	MFN1 ^{+/+} MFN2 ^{+/+}			
P180	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.4758	<0.0001	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.3319	
	MFN1 ^{+/+} MFN2 ^{+/+}			
D Duration				
P30	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.7160	0.8677	0.0749
	MFN1 ^{+/+} MFN2 ^{+/+}		0.9993	0.0753
	MFN1 ^{+/+} MFN2 ^{+/+}			0.0403
P60	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.9502	<0.9999	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.9969	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}			0.1659
P90	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.9946	0.4793	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}		0.5318	<0.0001
	MFN1 ^{+/+} MFN2 ^{+/+}			0.0470
P120	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.0474	0.0017	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0220	
	MFN1 ^{+/+} MFN2 ^{+/+}			
P180	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.0067	0.0002	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.3324	
	MFN1 ^{+/+} MFN2 ^{+/+}			
E NCV				
P30	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.9947	0.3969	0.0018
	MFN1 ^{+/+} MFN2 ^{+/+}		0.6739	0.0195
	MFN1 ^{+/+} MFN2 ^{+/+}			0.0745
P60	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.7800	0.0668	0.0003
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0998	0.0021
	MFN1 ^{+/+} MFN2 ^{+/+}			0.0239
P90	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.1909	<0.0001	0.0016
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0002	0.0067
	MFN1 ^{+/+} MFN2 ^{+/+}			<0.0001
P120	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.0663	0.0048	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.0150	
	MFN1 ^{+/+} MFN2 ^{+/+}			
P180	WT	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}	MFN1 ^{+/+} MFN2 ^{+/+}
		0.9327	0.0095	Deceased animals
	MFN1 ^{+/+} MFN2 ^{+/+}		0.3798	
	MFN1 ^{+/+} MFN2 ^{+/+}			

Figure 24. Statistical values for the corresponding comparisons between different mice groups from (Fig. 21). (A–F) Tables showing the statistical values between control, MFN1HTMFN2KO, MFN1KOMFN2HT, and MFN1KOMFN2KO at indicated ages of the electromyographic records: (A) first clear response when increasing mA, (B) latency to response, (C) mean peak amplitudes of CMAPs, (D) average durations of CMAPs, and (E) nerve conduction velocities at 20 mA in sciatic nerves.

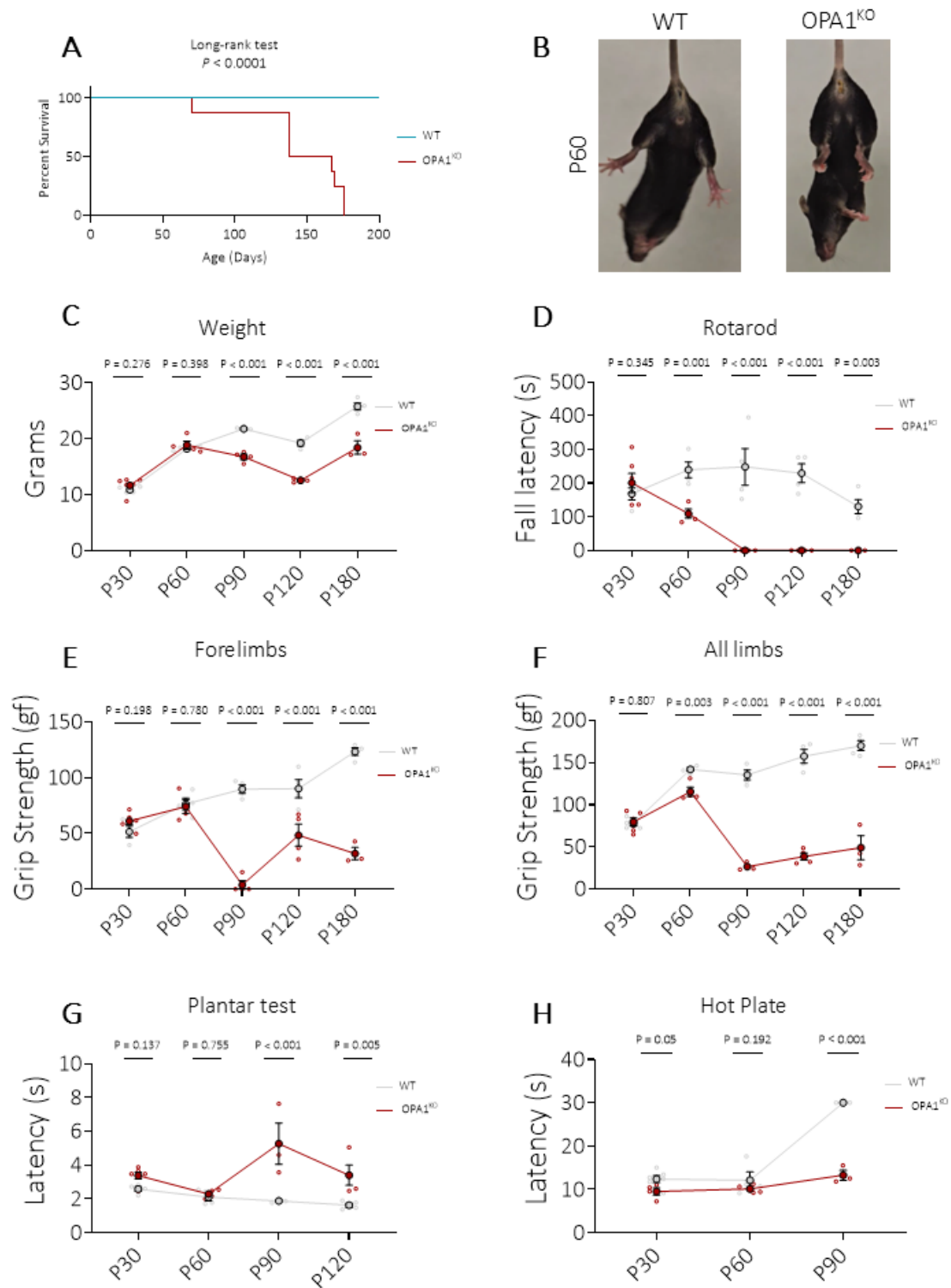


Figure 25. *Opa1* mutant mice show severe nerve deficits. (A) Survival curves of WT and *Opa1* cKO mice ($n = 8$ per group). P values are from a log-rank test between groups. (B) Tail suspension test showing abnormal hindlimb claspings in *Opa1* cKO mice but not in WT littermate control at P60, a common sign of peripheral neuropathy in mice. (C) Weight curves of WT and *Opa1* cKO mice. (D–H) *Opa1* cKO mice show poor responses compared to control mice in motor function, as shown by (D) latency to fall off the accelerating rotarod and (E, F) grip strength of forelimbs or all four limbs, and in sensory responses using (G) the plantar plate test and (H) hot plate test. Data are presented as means $\pm$ SEM; $n = 4$ to 6. Two-tailed unpaired Student's t test.

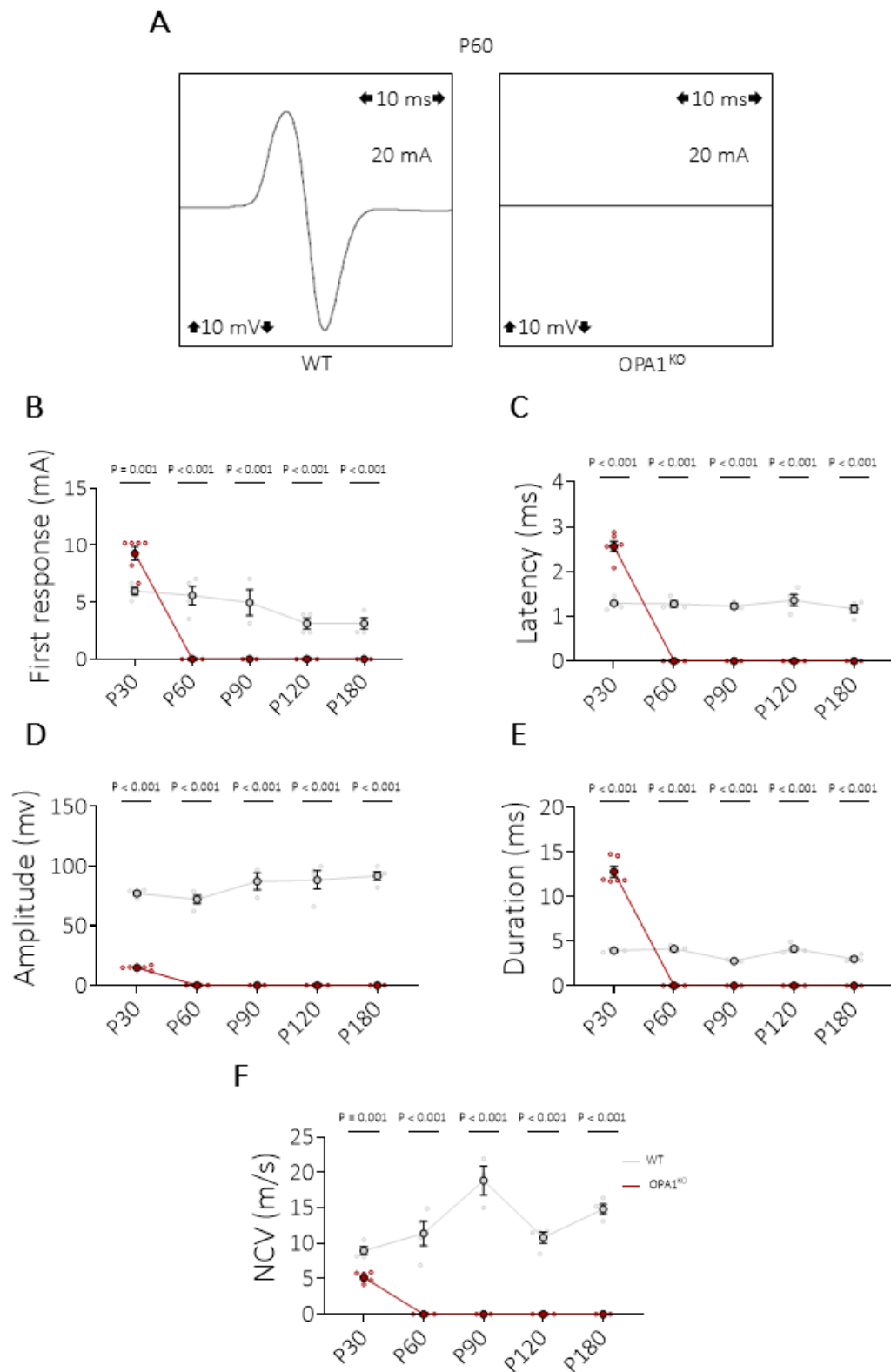


Figure 26. *Opa1* mutant mice display a marked reduction in conduction velocity. (A–F) *Opa1* mutant mice display a marked reduction in conduction velocity. (A) Electromyographic recording of CMAPs from sciatic nerves of *Opa1* cKO and control mice at P60. Representative traces are shown. Graphs show (B) first clear response when increasing mA, (C) latency to response, (D) mean peak amplitudes of CMAPs, (E) average durations of CMAPs, and (F) nerve conduction velocities at 20 mA in sciatic nerves of control, and *Opa1* cKO mice at indicated ages. Data are presented as means $\pm$ SEM; $n = 4$ to 6. Two-tailed unpaired Student's *t* test.

4.4 GENETIC DELETION OF *Mfn1* AND *Mfn2*, OR *Opa1* IN VIVO LEADS TO DEFECTIVE SCHWANN CELL MYELINATION

Our results above on motor function and nerve conduction velocity in the mutant mice suggested major defects in myelination in these mice. To confirm this, we performed ultrastructural examination of sciatic nerves from *Mfn1* cKO, *Mfn2* cKO, *Mfn1/2* dKO and *Opa1* cKO at different timepoints by transmission electron microscopy (TEM) (Chen et al., 2024; Feltri et al., 2016).

We did not find any overt differences when comparing *Mfn1* cKO or *Mfn2* cKO mice with control littermates, in any of the ultrastructural parameters analysed, including number of myelinated axons, g-ratio or nerve area (Fig. 27). For *Mfn1/2* dKO mice, on the other hand, overt defects in myelination could be observed at later time-points of development (Fig. 28A). Initially, myelin differentiation was normal till about P30 but at later time-points (as from P60), a marked reduction of myelinated axons was observed (Fig. 28B), which was accompanied by an increase in the number of amyelinated axons (Fig. 28C, D). Furthermore, we found that the total number of axons in a 1:1 relationship with a Schwann cell was clearly reduced as from P60 (Fig. 28E), indicating a marked axonal loss. We also found that there was a striking increase in number of demyelinating profiles (Fig. 28F) as well as a decrease in total nerve area (Fig. 28G).

Next, we analyzed myelination in sciatic nerves from MFN1^{HT}MFN2^{KO} or MFN1^{KO}MFN2^{HT} mice at P90 and at P180. No noticeable differences were seen between control and mutant nerves at P90 whereas a clear demyelinating phenotype was observed in mutant nerves at P180 (Fig. 29A). G-ratio measurements showed that myelin sheaths in mutant SCs at P180 were thinner compared to WT Schwann cells (Fig. 29B, C), an effect, which was more obvious for larger diameter axons (Fig. 29D). On the other hand, we did not observe major differences in total number of myelinated axons in mutant nerves at either P90 or P180, although we did observe a subtle increase in number of amyelinated axons at P180 (Fig. 30A, B and C). We found an increase in nerve area from MFN1^{HT}MFN2^{KO} mice only (Fig. 30D), and these mice were also characterized by a small increase in number of macrophages (Fig. 30E) as well as demyelinating profiles at P180 (Fig. 30F).

We also examined the Remak bundles in these mutant mice and found there were more Remak bundles in MFN1^{HT}MFN2^{KO} sciatic nerves, whereas for MFN1^{KO}MFN2^{HT} nerves, we observed more axons per Remak bundle instead (Fig. 31A–D). Altogether our data show that there is an absence of apparent myelination defects in *Mfn1* or *Mfn2* single-mutants and that one allele of either *Mfn* gene is sufficient to compensate for deficiency in the other gene at least during the initial stages of myelination. This suggests that MFN1 and MFN2 function redundantly during SC development.

In contrast to *Mfn1/2* dKO mice, we found that *Opa1* cKO mice were characterized by myelination defects already at the initial stages of myelination. (Fig. 32A). There were fewer myelinated axons in mutant nerves already at P30 (Fig. 32B) and elevated amyelinated axons at P15 (Fig. 32C, D). *Opa1* mutant nerves also had a reduced number of total axons in a relationship 1:1 with SC (Fig. 32E), an elevated number of demyelinating axons (Fig. 32F) and smaller total nerve area compared with control mice (Fig. 32G). Altogether, these data suggest that OPA1 is critical for the formation as well as maturation/maintenance of myelin sheath, whereas MFN1 and MFN2 play a more prominent role in the maturation/maintenance of myelin sheath without affecting their initial formation.

4.5 OPA1, AND MFN1 AND MFN2 REGULATE SCHWANN CELL MITOCHONDRIA HEALTH AND SIZE

Next, we examined the mitochondrial response in mutant Schwann cells from *Mfn1/2* and *Opa1* mice. For this, we performed morphometric analysis of Schwann cell and axonal mitochondria by electron microscopy of sciatic nerve cross sections from WT and mutant nerves at different time-points (Fig. 33A and 34A, respectively) (Sundaram et al., 2023).

Strikingly, we found a clear reduction in number and size of Schwann cell mitochondria in *Mfn1/2* dKO mice (Fig. 33B, C). Conversely, we found an increase in number and size of axonal cell mitochondria in these nerves, at least initially (P30), and effect that was, however, not maintained at later stages (P60).

In *Opa1* cKO mice, on the other hand, we found a clear tendency of a decrease in Schwann cell mitochondrial numbers at all ages till P120, although in this case, no alterations in mitochondrial size were observed (Fig. 34B). We also found a tendency of an increase in axonal mitochondrial numbers at later stages, but without affecting size (Fig. 34D).

These data suggest that OPA1 as well as MFN1 and MFN2 are necessary for regulating Schwann cell mitochondrial health, and that deficient Schwann cell mitochondria induce a compensatory effect in axons in the mutant mice to maintain energy balance. In addition, our data also suggest that MFN1 and MFN2 are important for regulating mitochondrial size, possibly by affecting mitochondrial fusion.

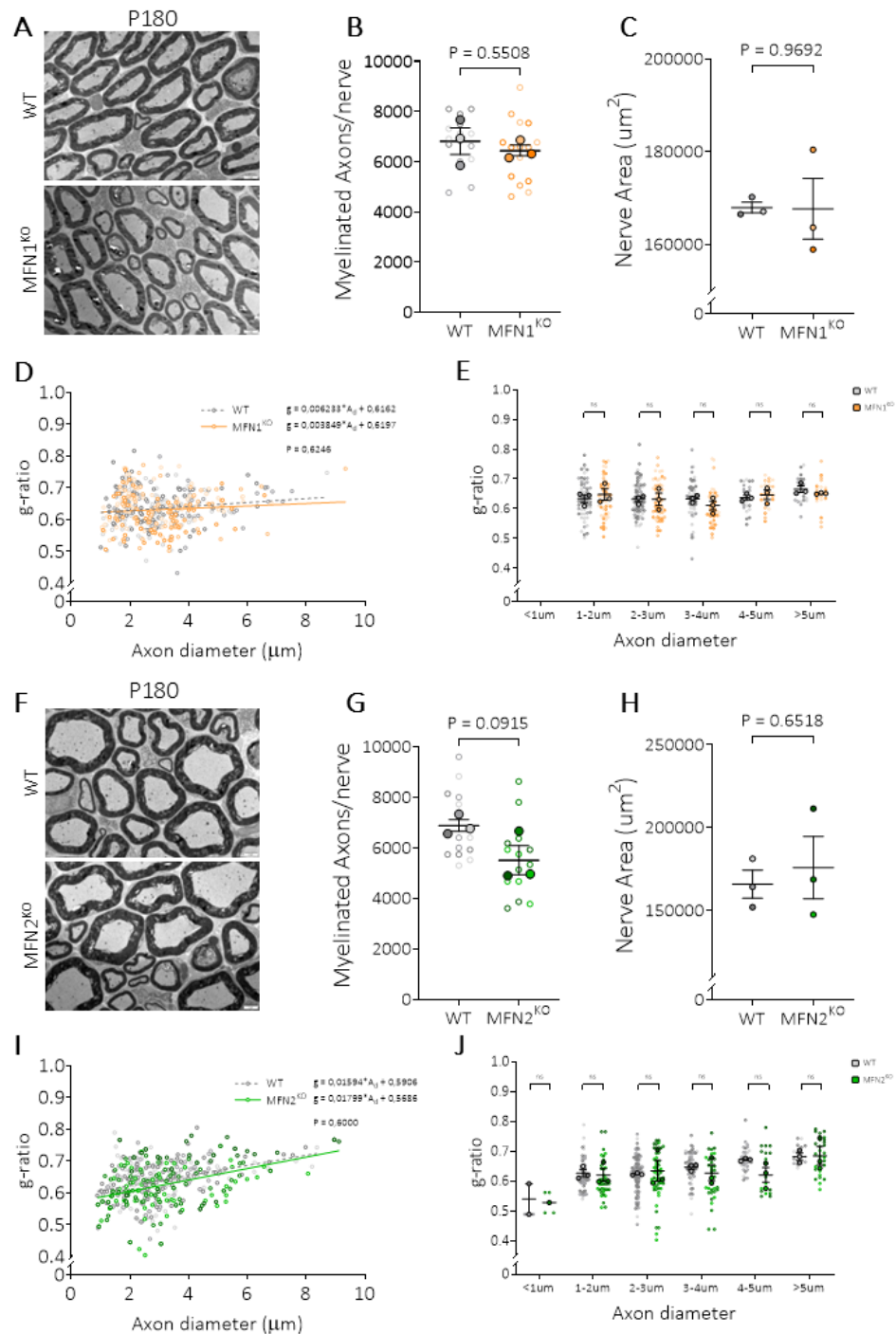


Figure 27. *Mfn1* cKO and *Mfn2* cKO mice do not show defects in Schwann cell myelination. (A) Representative EM pictures showing ultrastructure of control and *Mfn1* cKO sciatic nerves, at P180. Scale bar, 2 μm. (B–E) Graphs show quantification of (B) myelinated axons, (C) total nerve area, and (D, E) myelin sheath thickness assessed by g-ratio plotted against axon diameter in control and *Mfn1* cKO mice at P180. (F) Representative EM pictures showing ultrastructure of control and *Mfn2* cKO sciatic nerves, at P180. (G–J) Graphs show quantification of (G) myelinated axons (H) total nerve area, and (I, J) myelin sheath thickness assessed by g-ratio plotted against axon diameter in control and *Mfn2* cKO mice at P180. In (C, H) data are presented as means ± SEM; n = 3 per group. Two-tailed unpaired Student's *t* test. In (B), (E), (G) and (J) data are presented as super-plot combining biological replicates ± SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, n = 3 per group. Statistical significance was determined using nested t-test. In (D, I), g-ratio values were analysed by linear regression against axon diameter; significance of differences in slopes between control and mutant groups was assessed using a two-tailed unpaired Student's *t* test.

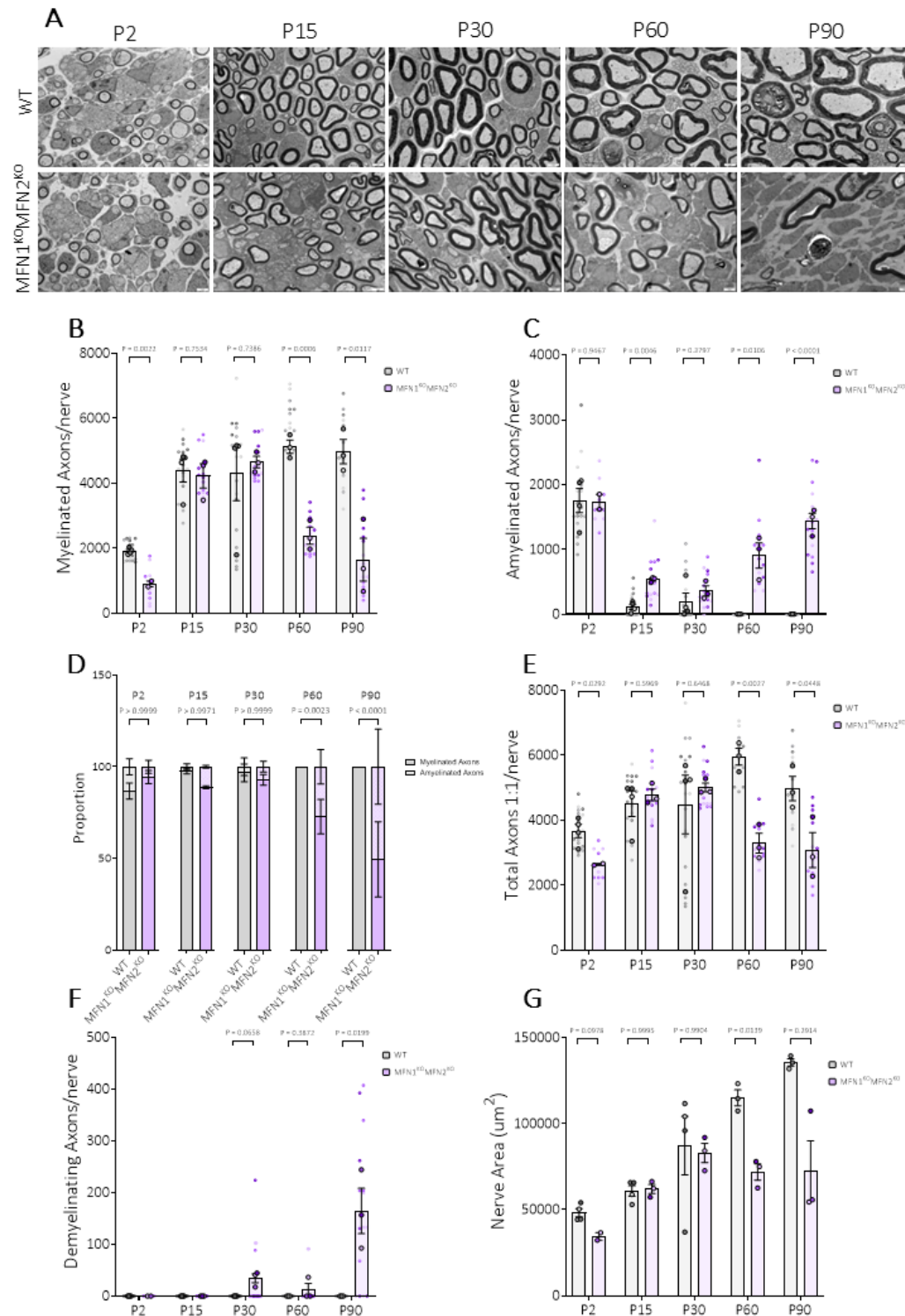


Figure 28. Simultaneous deletion of *Mfn1* and *Mfn2* lead to severe defects in myelination. (A) Representative EM pictures showing ultrastructure of control and *Mfn1/2* dKO sciatic nerves, at indicated ages. Scale bar, 2 μm. (B–G) Graphs show quantification of (B) myelinated axons, (C) amyelinated axons (1:1) per nerve, (D) proportion of Schwann cells in a 1:1 relationship with an axon that are myelinated or remain unmyelinated, (E) total number of axons in a 1:1 relationship with a SC, (F) demyelinating axons and (G) total nerve area of control and *Mfn1/2* dKO mice at indicated ages. In (B, E, F) data are presented as super-plot combining biological replicates ± SEM (large circles) with their technical replicates (small circles). Each color codes for a specific animal, *n* = 3 per group. Statistical significance was determined using nested t-test. In (D) data are presented as means ± SEM; *n* = 2 to 4 to 6. Two-way ANOVA with Sidak's multiple comparisons test. In (G) data are presented as means ± SEM; *n* = 3 per group. Two-tailed unpaired Student's *t* test.

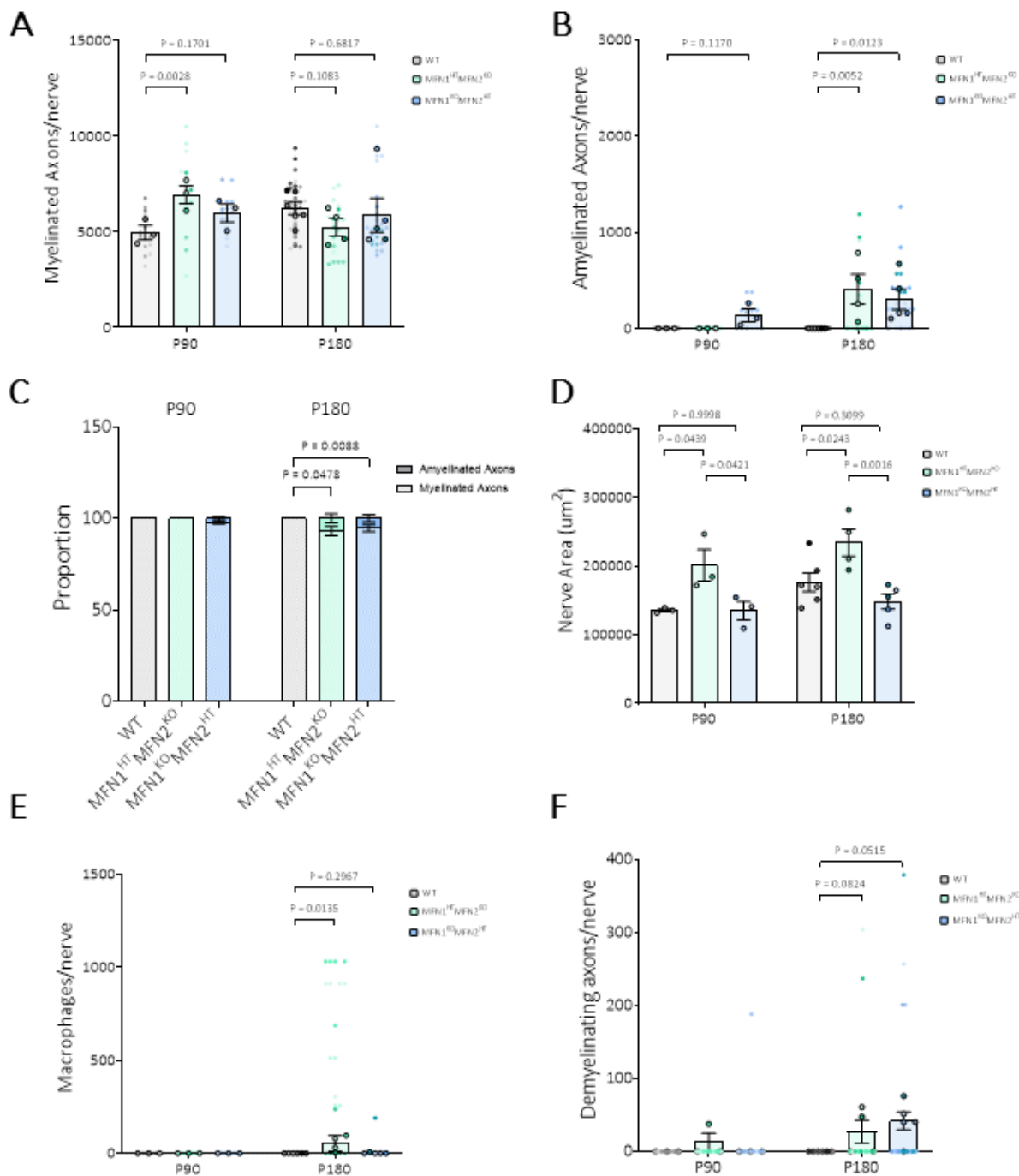


Figure 30. MFN1 and MFN2 function redundantly during SC development, general EM counts. (A–F) Graphs show quantification of (A) myelinated axons, (B) amyelinated axons (1:1) per nerve, (C) proportion of Schwann cells in a 1:1 relationship with an axon that are myelinated or remain unmyelinated, (D) total nerve area, (E) macrophages, (F) demyelinating axons at indicated ages. In (C, D) data are presented as means $\pm$ SEM; $n = 3$ to 6. Two-way ANOVA with Sidak's multiple comparisons test. In (A, B, D, E, F) data are presented as super-plot combining biological replicates $\pm$ SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, $n = 3$ to 6 per group. Statistical significance was determined using nested t-test.

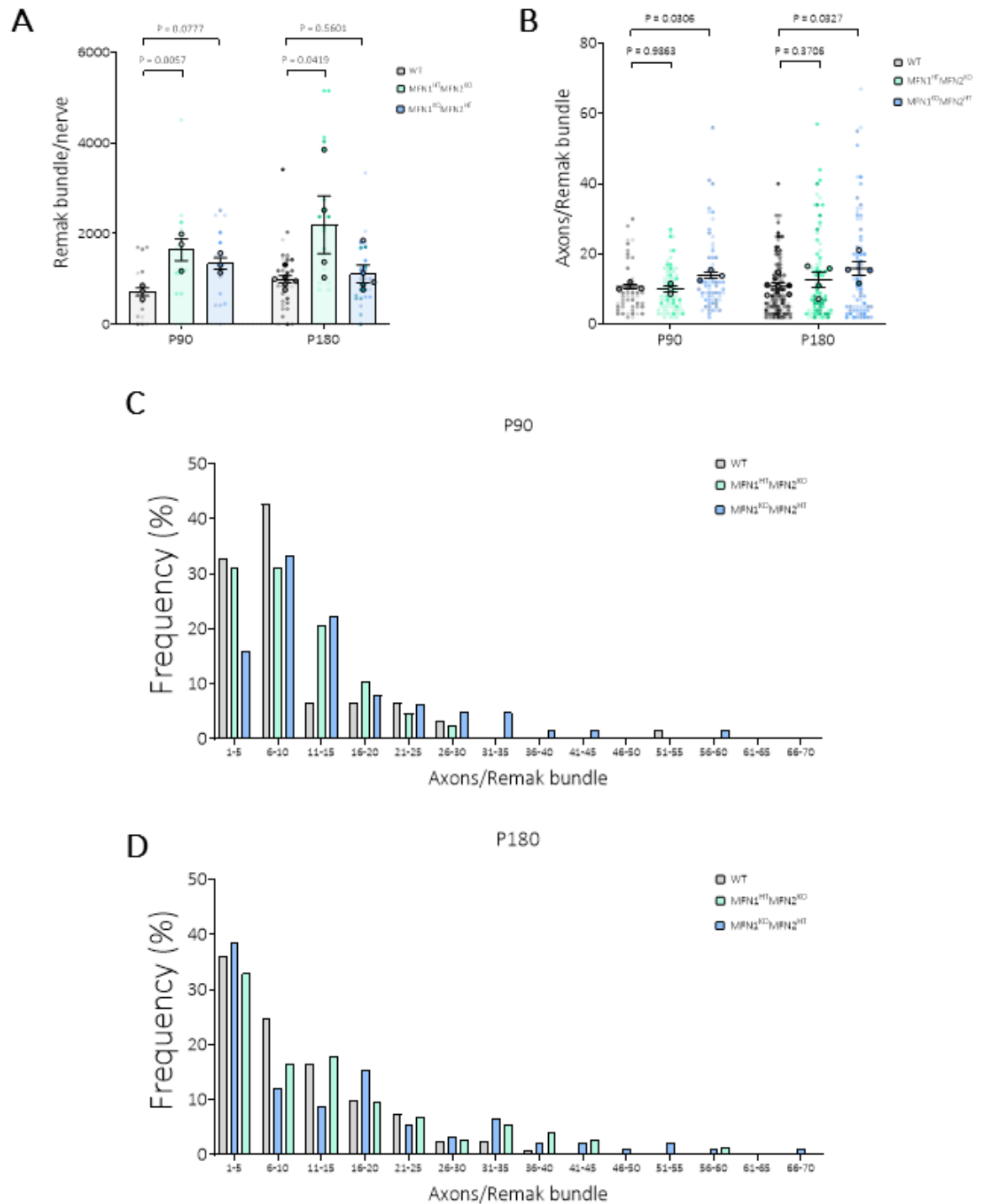


Figure 31. MFN1 and MFN2 function redundantly during SC development, Remak bundle counts. (A–D) Graphs show quantification of (A) number of Remak bundles, (B) number of axons per Remak bundle, (C, D) frequency (%) distribution profile of the number of axons per unmyelinated nerve bundle in control MFN1^{HT}MFN2^{KO} and MFN1^{KO}MFN2^{HT} mice at indicated ages. In (A, B) data are presented as super-plot combining biological replicates $\pm$ SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, $n = 3$ to 6 per group. Statistical significance was determined using nested t-test.

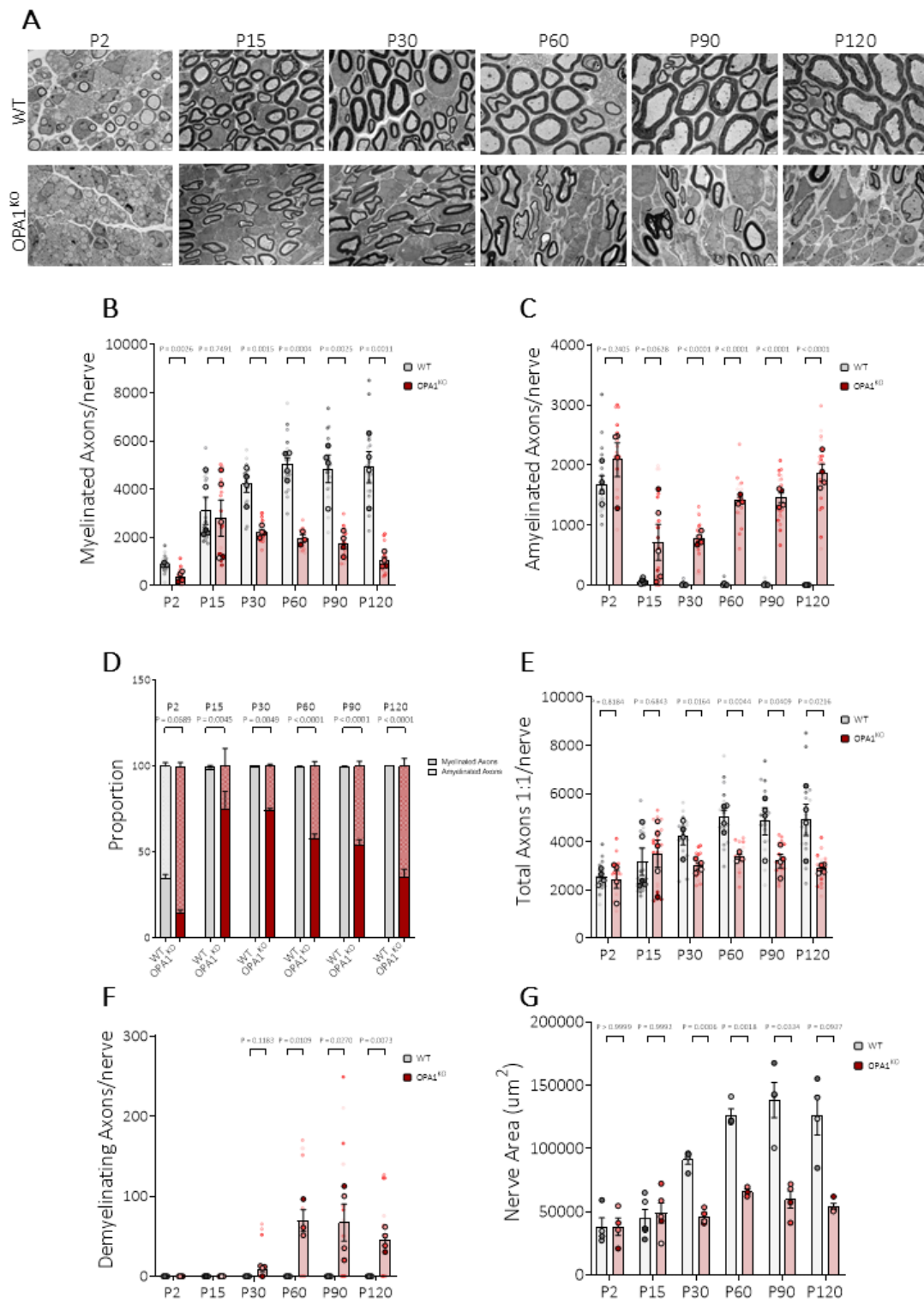


Figure 32. Genetic deletion of *Opa1* leads to severe defects in Schwann cell myelination. (A) Representative EM pictures showing ultrastructure of control, *Opa1* cKO sciatic nerves, at indicated ages. Scale bar, 2 μ m. (B–E) Graphs show quantification (B) myelinated axons, (C) amyelinated axons (1:1) per nerve, (D) proportion of Schwann cells in a 1:1 relationship with an axon that are myelinated or remain unmyelinated, (E) total number of axons in a 1:1 relationship with a SC, (F) demyelinating axons and (G) total nerve area of control and *Opa1* cKO mice at indicated ages. In (B, C, D) data are presented as super-plot combining biological replicates (large circles) with their technical replicates (small circles). Bars show biological replicates mean. Each colour codes for a specific animal, $n = 3$ per group. Statistical significance was determined using nested t-test. In (E) data are presented as means $\pm$ SEM; $n = 3$ per group. Two-tailed unpaired Student's *t* test.

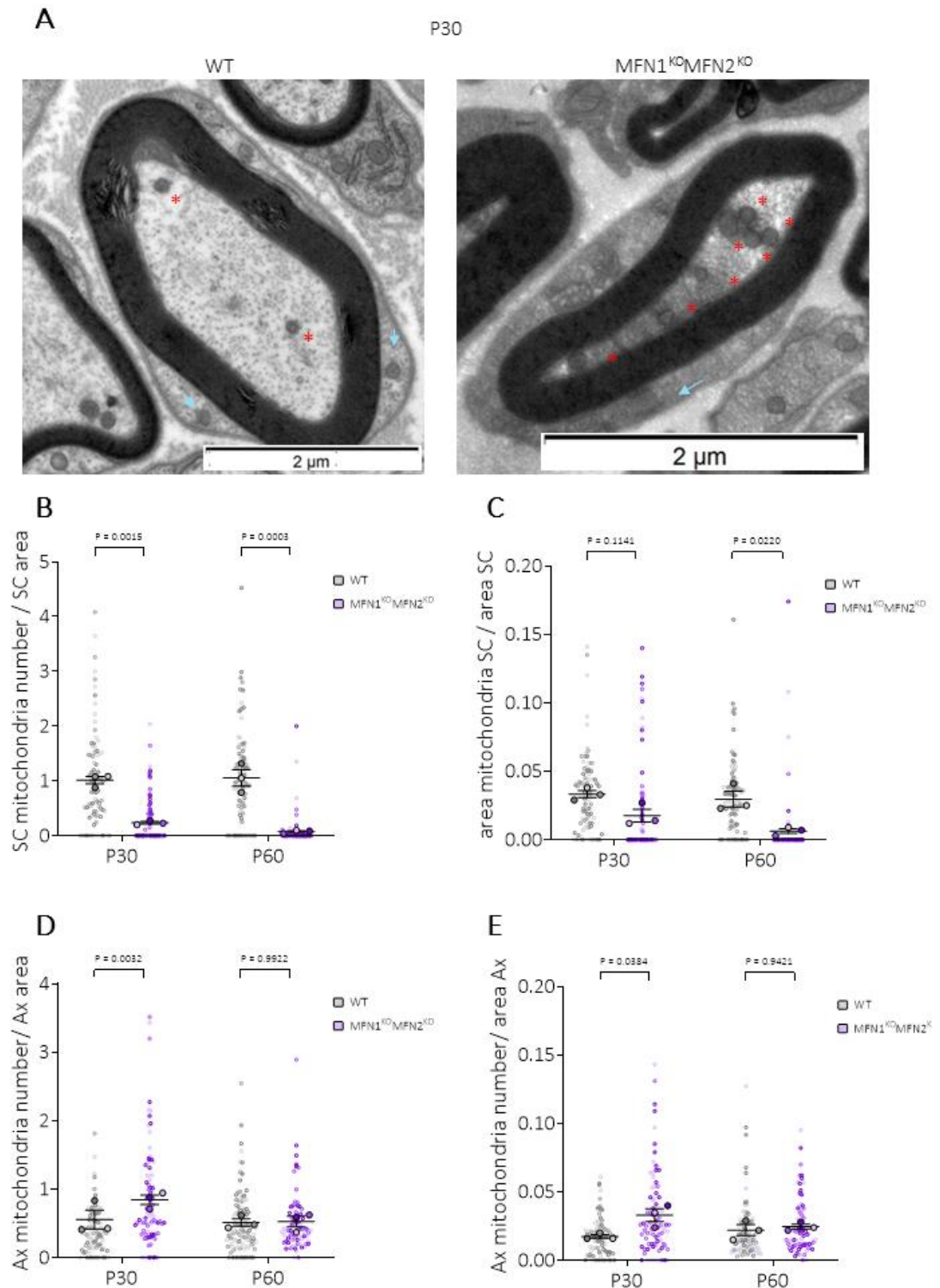


Figure 33. Genetic ablation of *Mfn1* and *Mfn2* alters mitochondrial number and size in both Schwann cells and axons of sciatic nerves. (A) Representative EM pictures of sciatic nerves from P30 control and *Mfn1/2* dKO. Blue arrows highlight SC mitochondria; red asterisks denote axonal mitochondria. Scale bar, 2 μ m. (B, C) Morphometric assessment of (B) Schwann cell mitochondrial number, and (C) size (normalized to Schwann cell cytoplasmic area) in control and *Mfn1/2* dKO mice. (D, E) Morphometric assessment of (D) axonal mitochondrial number, and (E) size (normalized to axonal cytoplasmic area) in control and *Mfn1/2* dKO mice. Data are presented as super-plot combining biological replicates $\pm$ SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, $n = 3$ per group. Statistical significance was determined using nested t-test.

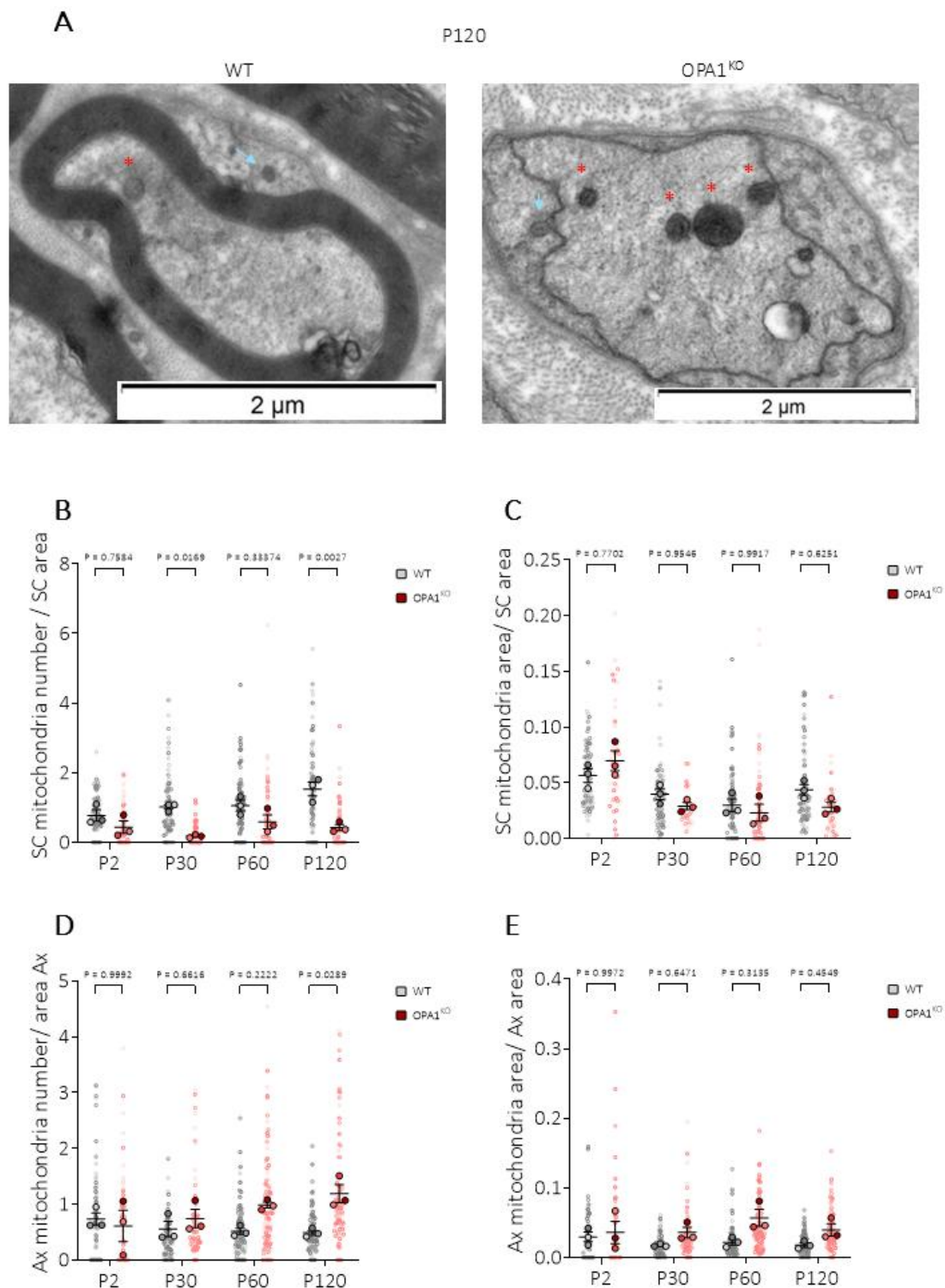


Figure 34. Genetic ablation of *Opa1* alters mitochondrial number and size in both Schwann cells and axons of sciatic nerves. (A) Representative EM pictures of sciatic nerves from P120 control and *Opa1* cKO. Blue arrows highlight Schwann cell mitochondria; red asterisks denote axonal mitochondria. Scale bar, 2 μm. (B, C) Morphometric assessment of (B) Schwann cell mitochondrial number, and (C) size (normalized to Schwann cell cytoplasmic area) in control and *Opa1* cKO mice. (D, E) Morphometric assessment of (D) axonal mitochondrial number, and (E) size (normalized to axonal cytoplasmic area) in control and *Opa1* cKO mice. Data are presented as super-plot combining biological replicates ± SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, $n = 3$ per group. Statistical significance was determined using nested t-test.

4.6 GENETIC ABLATION OF *MFN1* AND *MFN2*, AND *OPA1* TRIGGERS SCHWANN CELL STRESS SIGNALLING

Previous studies have shown that a disruption in Schwann cell metabolism or mitochondrial function can lead to activation of a number of pathways and stress signals, including the c-Jun pathways (Beirowski et al., 2014; Della-Flora Nunes et al., 2021; Poitelon et al., 2015; Pooya et al., 2014; Viader et al., 2011, 2013). We thus performed immunoblot analyses in P30 nerve lysates, and examined whether these pathways were similarly dysregulated in our mutant mice (Fig. 35 and 36). As expected, we found no differences in expression in levels of the structural myelin proteins MPZ and MBP in *Mfn1* cKO nor *Mfn2* cKO (Fig. 35A–D), whereas a strong downregulation was seen in *Mfn1/2* dKO and *Opa1* cKO mice, in line with our EM analyses above (Fig. 36A–D).

Surprisingly, we found some alterations in *Mfn1* cKO nerves, including a lower pACC^{S79}/ACC ratio, that could indicate more active ACC protein and consequently an increased fatty acid synthesis (C.-W. Kim et al., 2010). We also found higher pAMPK α ^{T172}/AMPK α and pelf2 α ^{S51}/elf2 α , indicating metabolic and general cellular stress respectively (Cantó & Auwerx, 2010; Pakos-Zebrucka et al., 2016). In addition, VDAC levels, which usually are used as mitochondrial biogenesis marker (Dubey et al., 2016), were also elevated in *Mfn1* cKO nerve compared with WT mice (Fig. 35).

In *Mfn1/2* dKO and *Opa1* cKO mice, on the other hand, we observed an increase in pACC^{S79}/ACC ratio, pointing to a metabolic shift from lipid synthesis toward energy production, a classic response to cellular stress, energy deficit, or increased AMP/ATP ratio (Fullerton et al., 2013; C.-W. Kim et al., 2010; Winder et al., 1997). In addition, the elevated levels of pelf2 α ^{S51}/elf2 α in *Mfn1/2* dKO nerves and the general elevated levels of both pelf2 α ^{S51} and elf2 α in *Opa1* cKO nerves indicate the triggering of integrated stress response (Pakos-Zebrucka et al., 2016). Similar to *Mfn1* cKO, we also found an increase in VDAC levels, and notably we also found an increase in c-Jun levels, which has previously been linked with demyelination in other mice models with Schwann cell-specific mitochondrial perturbations (Della-Flora Nunes et al., 2021) (Fig. 36).

NF is often used as axonal health state marker (Ayuso-García et al., 2024) and no differences were seen, coinciding with our EM data that shows that axonal loss started only after P60 in both *Mfn1/2* dKO and *Opa1* cKO mice. Levels of EGR2, a key transcriptional regulator of myelination (Decker et al., 2006; Topilko et al., 1994) were unaffected, pointing to the normal activation of the myelin transcriptional program (Fig. 36).

Collectively, these data suggest that ablation of *Mfn1/2* or *Opa1* lead to the activation of cellular and energetic stress response pathways that could help compensate mitochondrial disruptions to maintain myelin integrity.

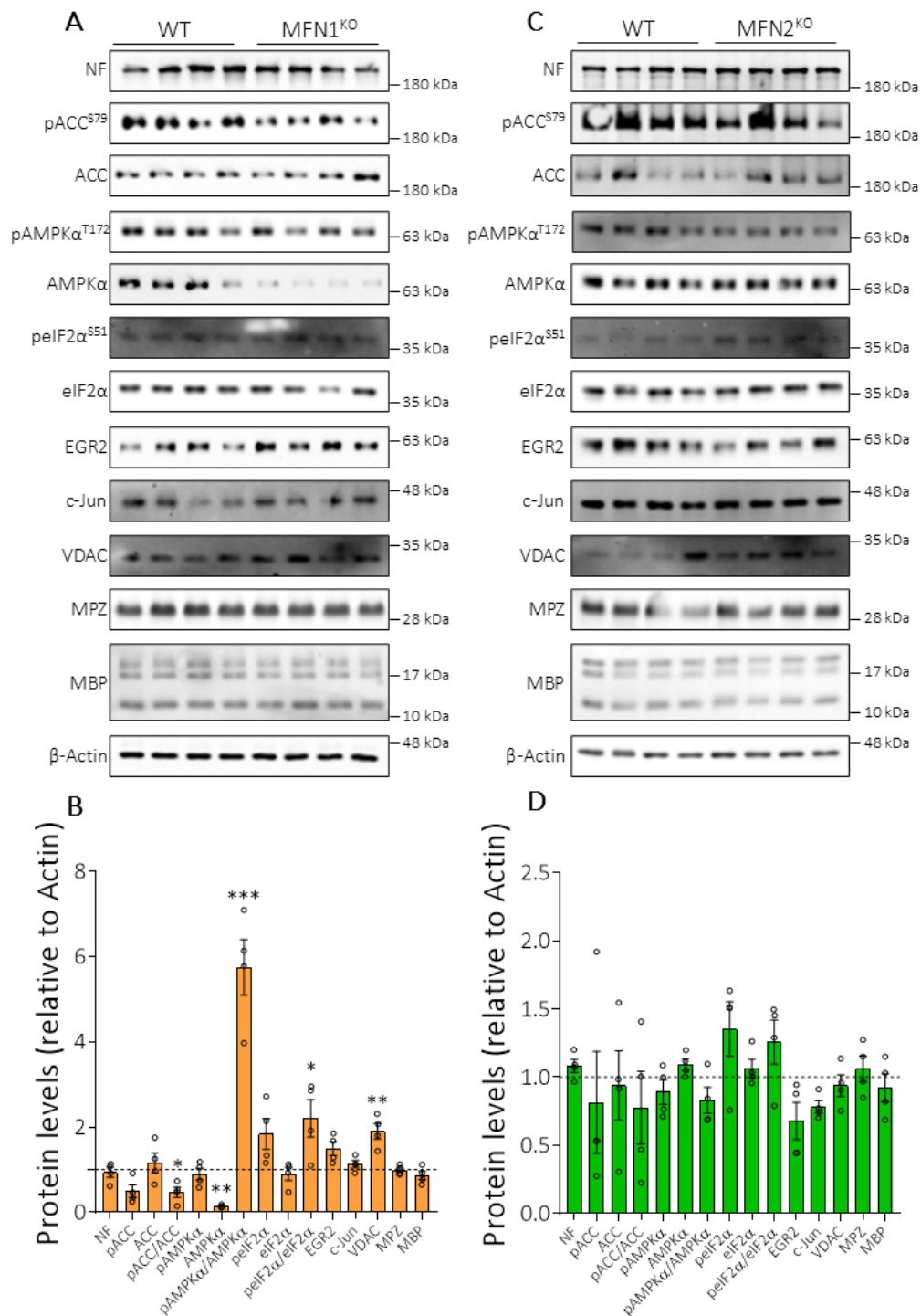


Fig. 35. Immunoblot analyses of myelin proteins and metabolic regulators in *Mfn1* cKO and *Mfn2* cKO mice. (A, B) (A) Immunoblot analyses and (B) densitometric quantification in sciatic nerve lysates from control and *Mfn1* cKO mice of an axonal marker (NF), structural proteins (MPZ, MBP), cellular energetics pathway component (ACC, pACC^{S79}, AMPK, pAMPKα^{T172}), integrated stress response (eIF2α, peIF2α^{S51}), negative myelin regulator (c-Jun), positive myelin regulator (EGR2), relative amount of mitochondria (VDAC), β-actin (loading control). (C, D) (C) Immunoblot analyses and (D) densitometric quantification in sciatic nerve lysates from control and *Mfn2* cKO mice of an axonal marker (NF), structural proteins (MPZ, MBP), cellular energetics pathway components (ACC, pACC^{S79}, AMPK, pAMPKα^{T172}), integrated stress response (eIF2α, peIF2α^{S51}), negative myelin regulator (c-Jun); positive myelin regulator (EGR2), relative amount of mitochondria (VDAC), β-actin (loading control). Data are presented as means ± SEM; *n* = 4 per group, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Two-tailed unpaired Student's *t* test.

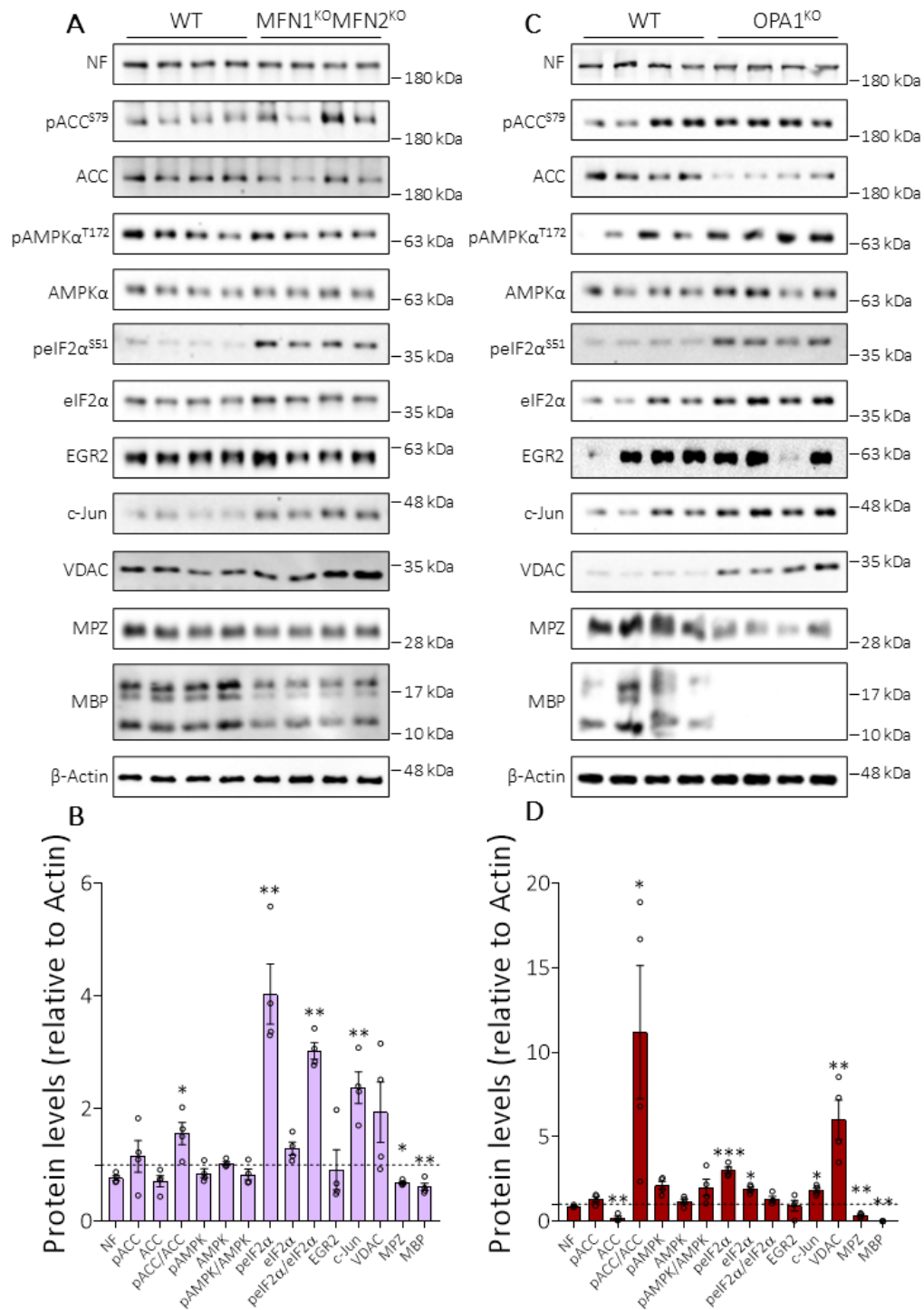


Fig 36. *Mfn1/2* or *Opa1* deletion leads to a downregulation of downregulates myelin structural proteins and trigger an integrated stress response. (A, B) (A) Immunoblot analyses and (B) densitometric quantification in sciatic nerve lysates from control and *Mfn1* cKO mice of an axonal marker (NF), structural proteins (MPZ, MBP), cellular energetics pathway component (ACC, pACC^{S79}, AMPK, pAMPK^{T172}), integrated stress response (eIF2^α, pEIF2^{S51}), negative myelin regulator (c-Jun) and positive myelin regulator (EGR2) relative amount of mitochondria (VDAC), β-actin (loading control). (C, D) (C) Immunoblot analyses and (D) densitometric quantification in sciatic nerve lysates from control and *Mfn2* cKO mice of an axonal marker (NF), structural proteins (MPZ, MBP), cellular energetics pathway components (ACC, pACC^{S79}, AMPK, pAMPK^{T172}), integrated stress response (eIF2^α, pEIF2^{S51}), negative myelin regulator (c-Jun), positive myelin regulator (EGR2) and relative amount of mitochondria (VDAC), β-actin (loading control). Data are presented as means ± SEM; *n* = 4 per group, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Two-tailed unpaired Student's *t* test.

4.7 OXPHOS COMPONENTS ARE ALTERED IN MITOCHONDRIAL MUTANTS

Our data above identify possible upstream regulators are activated in our mutant mice models. We next examined the functional consequences of the mutations on mitochondrial energy metabolism in Schwann cells by examining expression of components of mitochondrial oxidative phosphorylation (OXPHOS) and other metabolism-related proteins by immunoblot analysis (Bennett et al., 2022; Tang et al., 2020; Vercellino & Sazanov, 2022).

Similar to our data above, we found more alterations in *Mfn1* cKO nerve lysates compared to *Mfn2* cKO lysates. We found a reduction in UQCRC2 (complex III) and MTCO1 (complex IV) protein levels in *Mfn1* cKO nerves when compared to WT mice, along with a striking reduction in TFAM levels. HKII and LONP1, on the other hand, were upregulated in *Mfn1* mutant nerves (Fig. 37A, B), indicating that Schwann cells could be undergoing a metabolic shift from OXPHOS to glycolysis, and maintaining mitochondrial proteostasis respectively. *Mfn2* cKO mice, on the other hand, showed minor alterations including subtle reductions in protein levels of NDUFS1 (complex I), MTCO1 (complex IV) and HKII (glycolytic metabolism) (Fig. 37C, D), indicating that the lack of *Mfn2* does not induce any major metabolic adaptations. HKI, on the other hand, did not present any observable differences. HKI is broadly and consistently expressed across mammalian tissues, with its levels remaining largely stable under most physiological or stress conditions. In contrast, HKII is primarily expressed in muscle and heart, is frequently upregulated in various cancers, and its expression is modulated by hormonal and metabolic cues (D. Guo et al., 2023).

Mfn1/2 dKO mice showed more pronounced reductions in several OXPHOS components, including NDUFS1 (complex I), SDHA (complex II), UQCRC2 (complex III). We also observed a significant increase in TFAM levels (Fig. 38A, B), likely in an attempt to increase respiratory chain function to compensate for decreased OXPHOS capacity observed, given the central role TFAM plays in mitochondrial DNA (mtDNA) maintenance and expression (Kozhukhar & Alexeyev, 2023).

Opa1 cKO mice were also likely characterized by a partial impairment in OXPHOS capacity, since we found a strong upregulation of MTCO1 (complex IV) protein levels as well as a reduction in ATP5A (complex V). Similar to *Mfn1/2* dKO mice, TFAM levels are also upregulated (Fig. 38C, D).

In summary, our data show notable alterations in OXPHOS protein expression in the mutant mice, together with an attempt to compensate for this by upregulating critical regulators of mitochondrial DNA (mtDNA) maintenance and expression (Fig. 39).

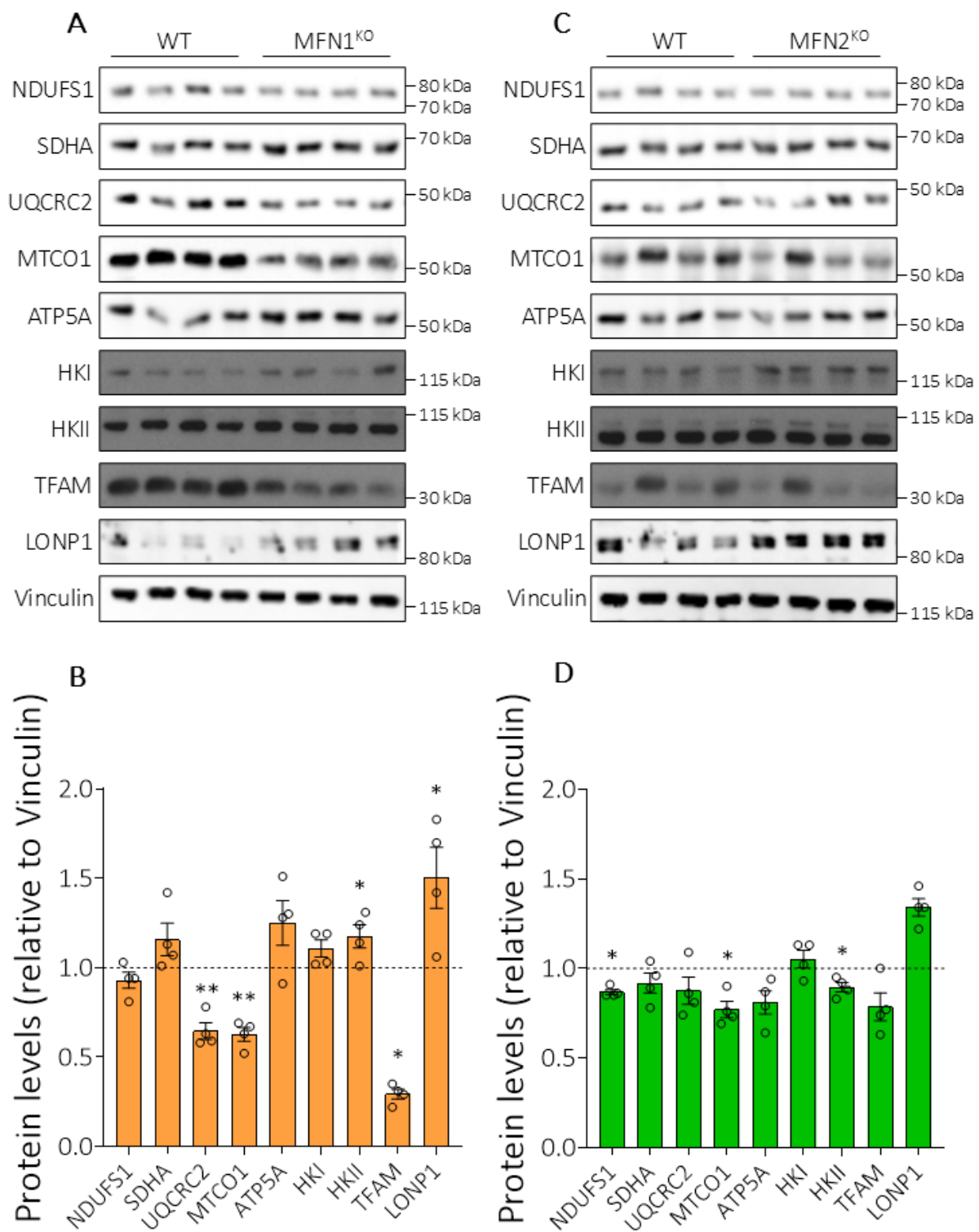


Fig. 37. *Mfn1* and *Mfn2* single mutants show mild alterations in mitochondrial protein expression. (A, B) (A) Immunoblot analyses and (B) densitometric quantification in sciatic nerve lysates from control and *Mfn1* cKO mice of OXPHOS components (NDUFS1, UQCRC2, MTCO1, ATP5A), glycolysis components (HKI, HKII), relative mitochondrial quantity (TFAM) and mitochondrial stress (LONP1), and vinculin (loading control). (C, D) (C) Immunoblot analyses and (D) densitometric quantification in sciatic nerve lysates from control and *Mfn2* cKO mice of OXPHOS components (NDUFS1, UQCRC2, MTCO1, ATP5A), glycolysis components (HKI, HKII), relative mitochondrial quantity (TFAM) and mitochondrial stress (LONP1), and vinculin (loading control). Data are presented as means $\pm$ SEM; $n = 4$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Two-tailed unpaired Student's t test.

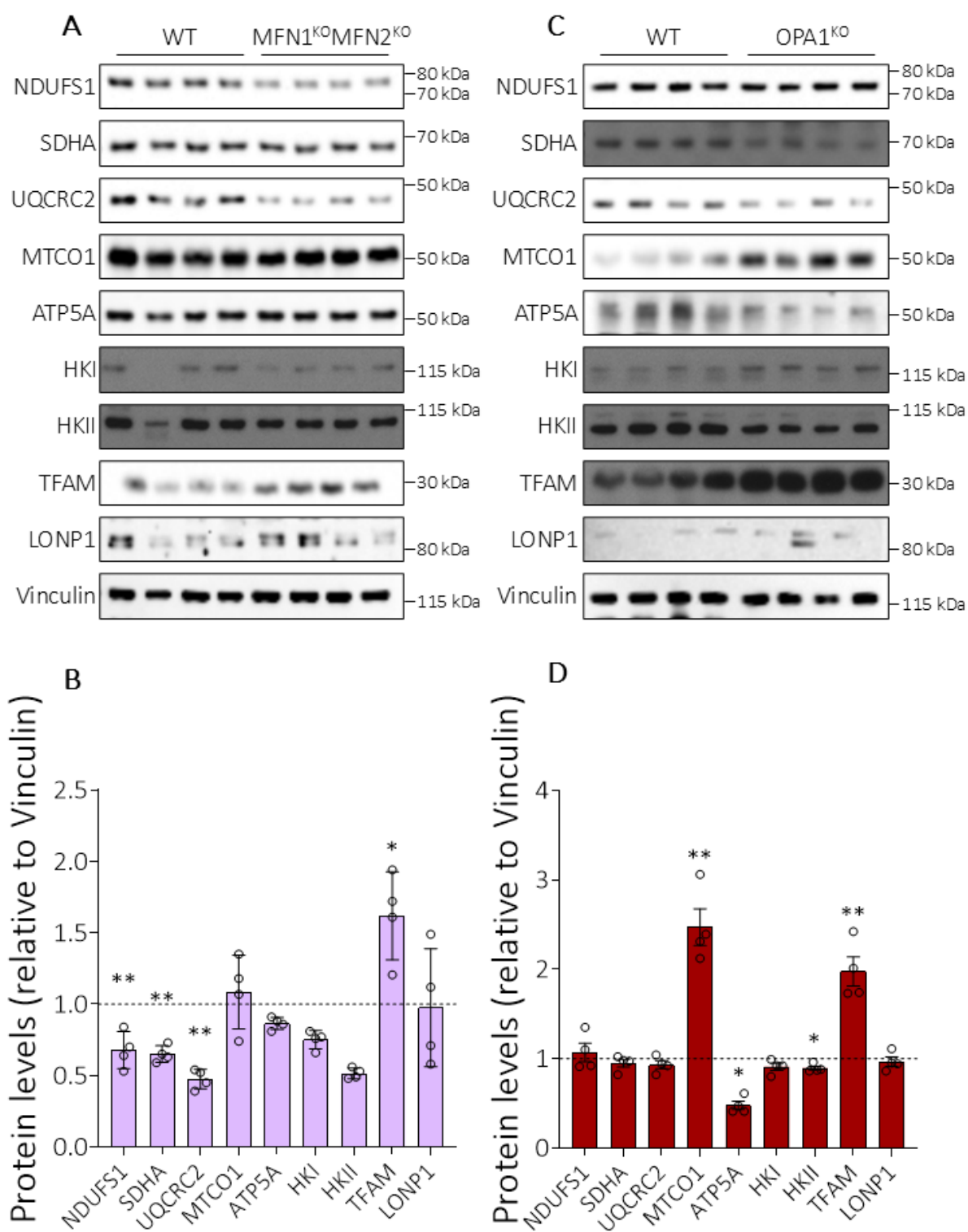


Fig. 38. *Mfn1/2* dKO and *Opa1* cKO mice show profound alterations in mitochondrial protein expression (A, B) (A) Immunoblot analyses and (B) densitometric quantification in sciatic nerve lysates from control and *Mfn1/2* dKO mice of OXPHOS components (NDUFS1, UQCRC2, MTCO1, ATP5A), glycolysis components (HKI, HKII), relative mitochondrial quantity (TFAM) and mitochondrial stress (LONP1), and vinculin (loading control). (C, D) (C) Immunoblot analyses and (D) densitometric quantification in sciatic nerve lysates from control and *Opa1* cKO mice of OXPHOS components (NDUFS1, UQCRC2, MTCO1, ATP5A), glycolysis components (HKI, HKII), relative mitochondrial quantity (TFAM) and mitochondrial stress (LONP1), and vinculin (loading control). Data are presented as means $\pm$ SEM; $n = 4$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Two-tailed unpaired Student's t test.

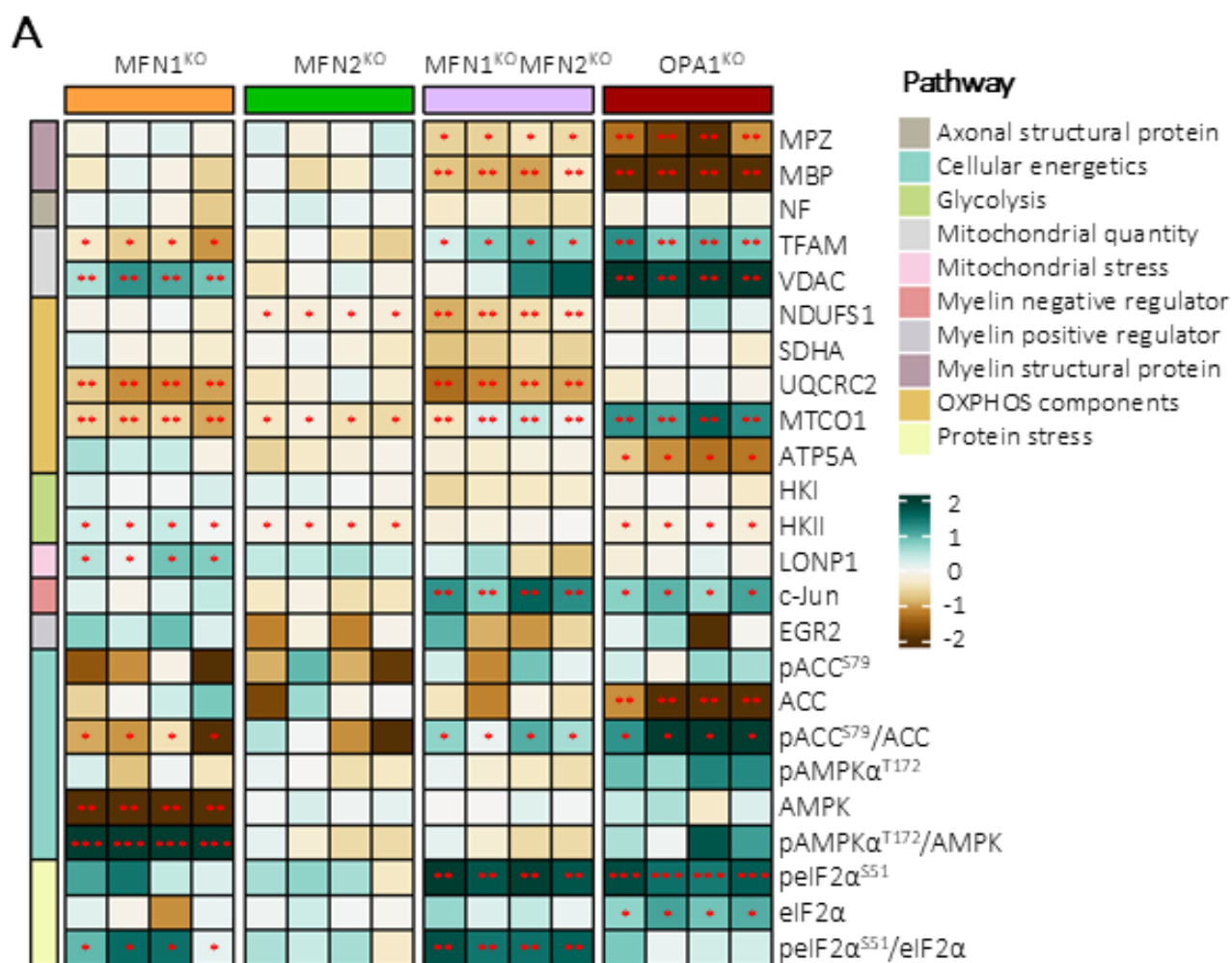


Fig. 39. Heatmap summarizing changes in expression of mitochondrial proteins and regulators (A) Heatmap showing changes in protein expression in sciatic nerve lysates from *Mfn1*, *Mfn2*, *Mfn1/2* and *Opa1* mutant mice, relative to their control littermates, classified in the following molecular categories: myelin proteins (MPZ, MBP), axonal structural protein (NF), cellular energetics pathway components (ACC, pACC^{S79}, AMPK, pAMPKα^{T172}), integrated stress response (eIF2α, peIF2α^{S51}), negative myelin regulator (c-Jun), positive myelin regulator (EGR2), relative mitochondrial quantity (VDAC, TFAM), OXPHOS components (NDUFS1, UQCRC2, MTCO1, ATP5A), glycolysis components (HKI, HKII) and mitochondrial stress (LONP1). Data are presented as fold change of mutant protein expression over control, $n = 4$ per group * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Two-tailed unpaired Student's t test.

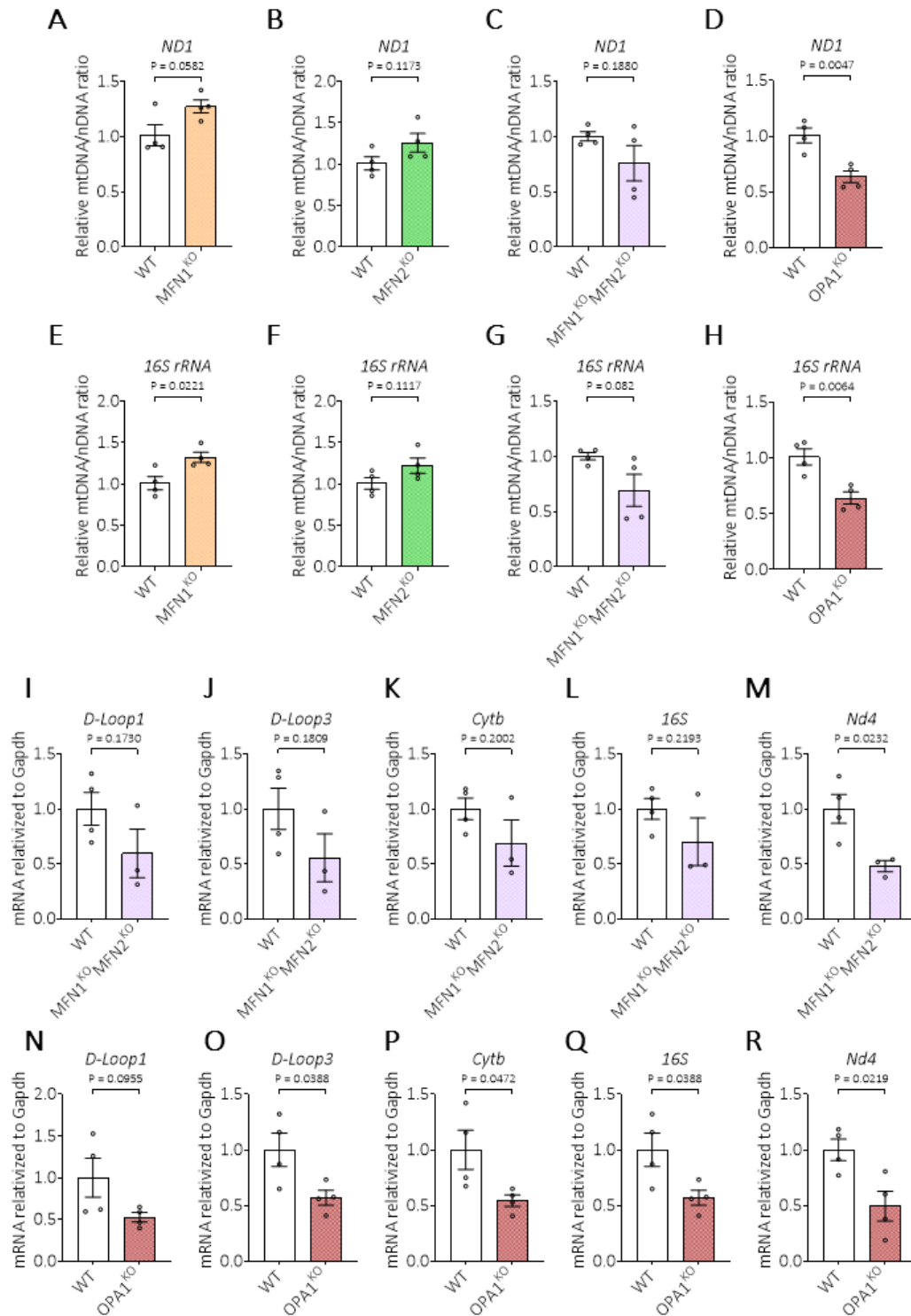
4.8 OPA1 DEFECTS DIMINISHES mtDNA CONTENT IN SCHWANN CELLS.

Opa1 deletion can induce mtDNA depletion that has been linked to mitochondria defects in skeletal muscle (Rodríguez-Nuevo et al., 2018). We thus examined relative mtDNA content in nerves from mutant mice by analyzing the expression of *ND1* and *16S* rRNA, two RNAs contained in the mitochondrial genome that we compared with *HKII*, the selected nuclear reference gene (Quiros et al., 2017)(Fig. 40A–H). In *Mfn1* cKO mice, we observed a strong tendency to increase mtDNA content since both *ND1* expression (Fig. 40A) and *16S* rRNA were increased (Fig. 40E), whereas *Mfn2* cKOs did not show any alterations compared with their control littermates (Fig. 40B, F). For *Mfn1/2* dKO mice, we found a tendency towards reduced expression of both *ND1* and *16S* rRNA (Fig. 40C, G), whereas we observed a marked reduction in both *ND1* and *16S* expression in *Opa1* cKO mice, as expected. (Fig 40D, H). To confirm these data, we analyzed a further 5 regions of the mitochondrial genome (*D-Loop1*, *D-Loop3*, *Cytb*, *16S* and *Nd4* using *Gapdh* as nuclear reference gene) in *Mfn1/2* dKO and *Opa1* cKO mice (Rodríguez-Nuevo et al., 2018). As above, we found a strong tendency in reduced expression in *Mfn1/2* dKO mice (Fig. 40I–M), whereas a significant decrease was observed in *Opa1* cKO mice (Fig. 40N–R).

Collectively, these data show that genetic ablation of *Opa1* and *Mfn1/2* in Schwann cells lead to profound depletion of mitochondrial DNA (mtDNA), which could account for the defective respiratory chain complexes observed, and an attempt to override this by upregulating TFAM levels.

4.9 MITOCHONDRIAL FUSION IMPAIRMENT BLOCKS METABOLIC SWITCH NEEDED FOR SCHWANN CELL DIFFERENTIATION

Previous studies have shown that proliferating Schwann cells undergo metabolic reprogramming from glycolytic to oxidative metabolism during differentiation and myelination *in vivo*, with the metabolic regulator LKB1 playing an essential role in this metabolic shift (Pooya et al., 2014). To examine whether these mitochondrial alterations could have functional consequences in mitochondrial respiration, we performed high-resolution respirometry measurements. For this, we conducted bioenergetic analysis of primary Schwann cells obtained from WT and mutant *Opa1* mice, cultured under proliferative or differentiation conditions by measuring oxygen consumption using the Seahorse XF analyzer. We found that basal oxygen consumption was potently increased in control Schwann cells during differentiation and this transition was blunted in *Opa1*-mutant Schwann cells. Maximal respiration during proliferation and differentiation was also blunted in mutant Schwann cells, suggesting reduced oxidative capacity of these cells (Fig. 41). These results confirm that Schwann cells undergo metabolic reprogramming during differentiation and that *Opa1* is required for this process. It would be essential to measure mitochondrial respiration in *Mfn1/2*-deficient Schwann cells in future experiments.



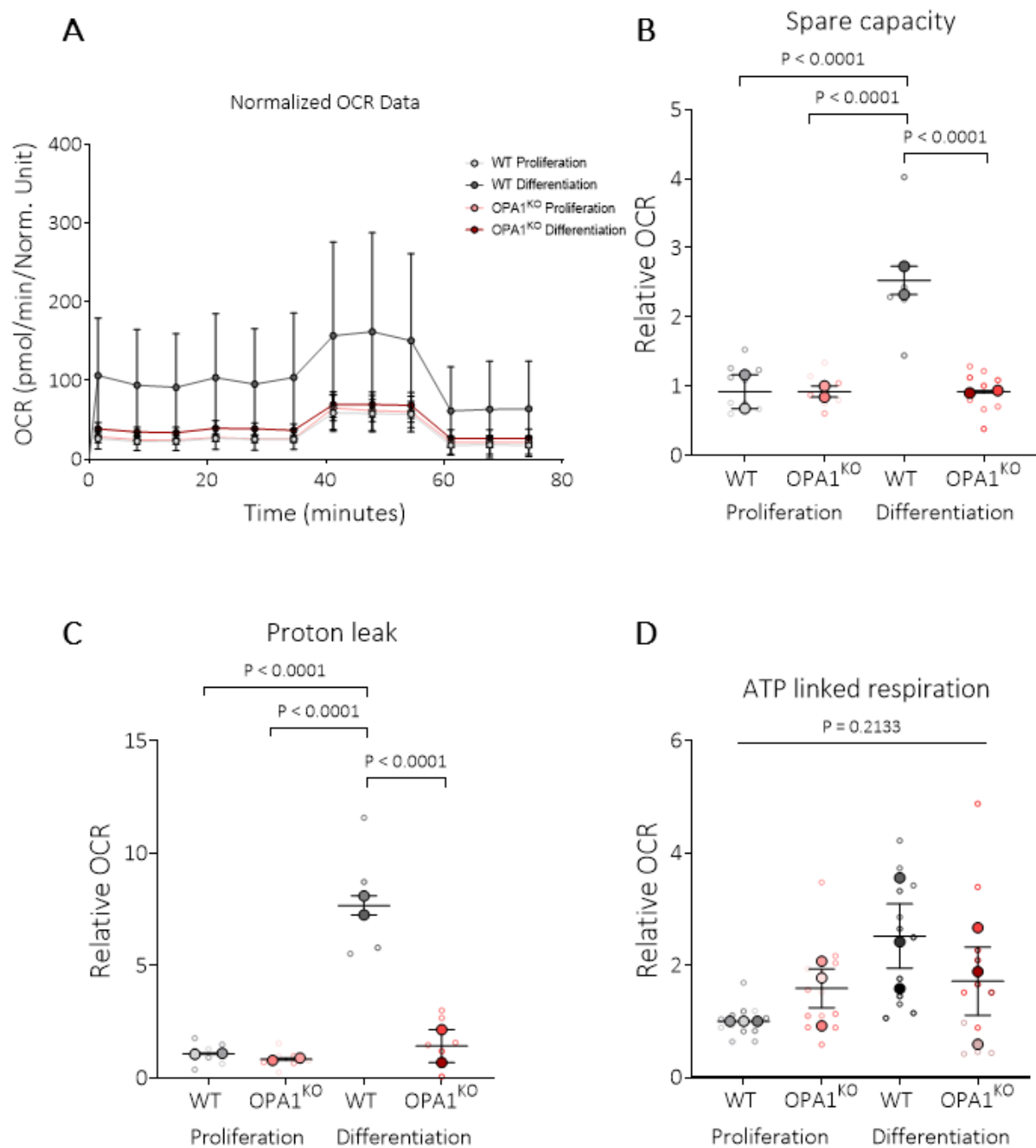


Fig. 41. Opa1 is required for metabolic reprogramming during SC differentiation. (A) Representative respirometric traces of the oxygen consumption rate (OCR) in control and *Opa1* cKO mouse Schwann cells cultured under proliferative or differentiation conditions (B–D) Graphs showing (B) spare capacity (FCCP - Baseline), (C) proton leak (Oligomycin - Rotenone/AA), and (D) ATP linked respiration (Baseline - Oligomycin). Data are presented as super-plot combining biological replicates $\pm$ SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, $n = 2-3$ per group. Statistical significance was determined using nested one-way ANOVA with Tukey's

4.10 MFN1, MFN2 AND OPA1 SHOW DYNAMIC CHANGES IN EXPRESSION IN INJURED NERVES

As mentioned above, peripheral nerves possess a remarkable capacity for regeneration. Following injury, mature Schwann cells undergo reprogramming to assume a repair phenotype that is specialized to facilitate nerve regeneration. These repair Schwann cells offer both metabolic and trophic support while guiding the regrowth of axons. Once axons re-establish connections with their targets, Schwann cells re-differentiate and restore myelin around the regenerated axons (Stassart & Woodhoo, 2021). Schwann cells respond with glycolytic activation immediately (within 72 h) after acute nerve injury, a period that covers the onset of Wallerian degeneration (Babetto et al., 2020), but the subsequent time course of nerve de- and regeneration (7-28 days post crush) is associated with a profound metabolic activation of repair Schwann cells, including an oxidative switch and a marked increase in mitochondrial respiration (Sundaram et al., 2023).

First, to examine whether mitochondrial dynamics could be involved in these processes, we analyzed mRNA and protein of MFN1, MFN2, OPA1 and VDAC at different time-points following an acute cut injury (Fig. 42A), as well as situations, in which nerve repair is compromised, as seen in aged cut nerves (Fig. 43A), or after chronic cut injury (Fig. 30A).

In all three situations, MFN1 protein levels exhibited the same trend: there was a progressive strong upregulation of MFN1 after nerve injury (Fig. 42B, C), which was also observed in aged nerves (Fig. 43B, C) or chronically denervated nerves (Fig. 44B, C). On the other hand, there was a significant reduction in both MFN2 and OPA1 protein after nerve injury (Fig. 42B, C), and in aged (Fig. 43B, C) and chronically denervated nerves (Fig. 44B, C). VDAC levels showed a general downregulation in these 3 models, suggesting a relative decrease in mitochondrial abundance in line with the mitochondrial proteins analyzed. *Mfn1* and *Mfn2* mRNA levels were, in general, relatively stable in these models (Fig. 42D, 43D and 44D), suggesting that post-transcriptional or translational mechanisms could account for the changes in protein levels. In contrast, we found a general downregulation of *Opa1* mRNA levels, which coincide with the reduced OPA1 protein levels observed (Fig. 43D, 44D).

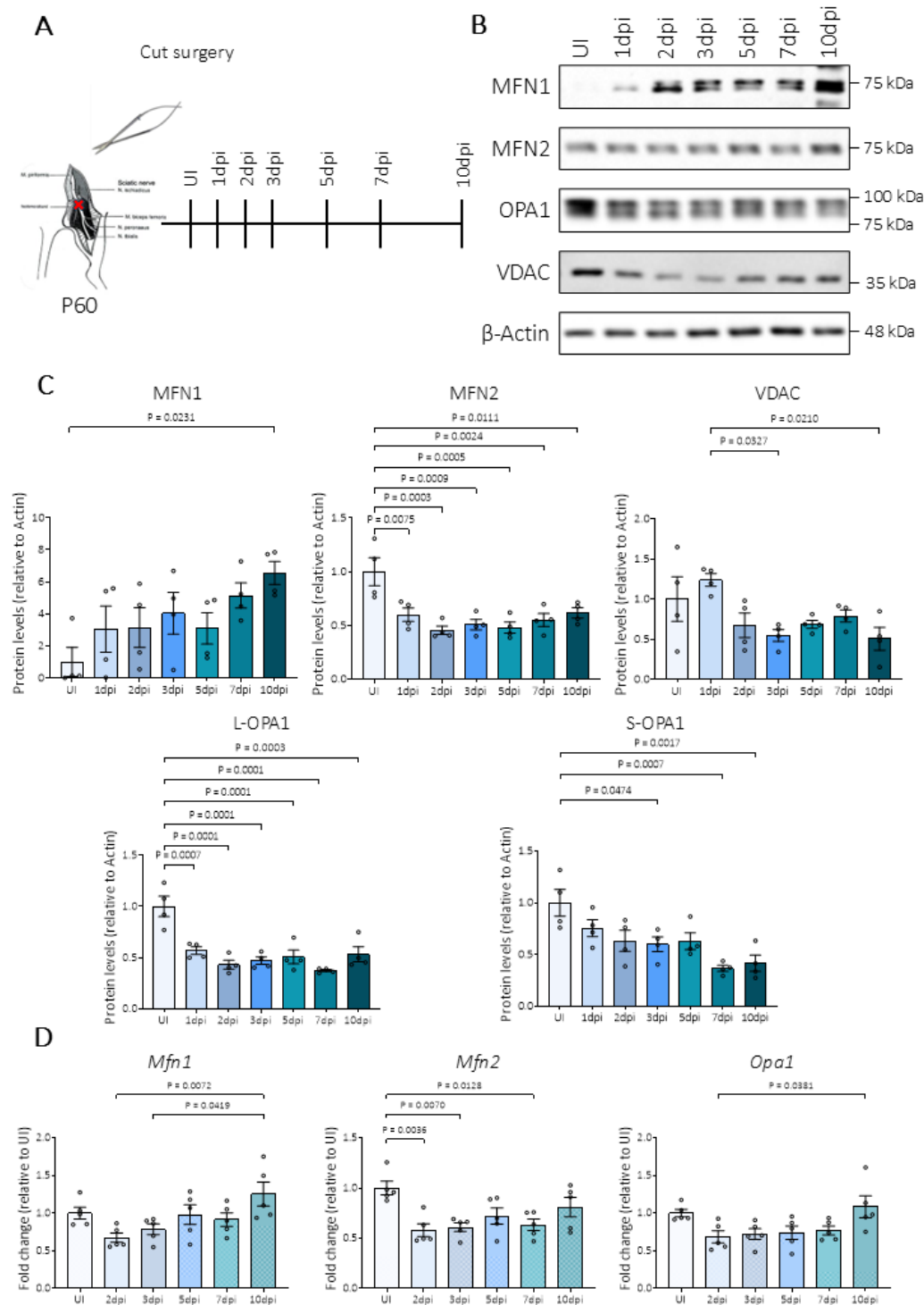


Figure 42. MFN1, MFN2 and OPA1 show dynamic changes in expression after nerve cut injury *in vivo* (A) Schematic representation of sample collection at different time-points after sciatic nerve cut surgery. (B) Immunoblot analysis of MFN1, MFN2, OPA1, VDAC and β-Actin expression in total sciatic nerve extracts at the indicated cut injury timepoints. (C) Densitometric quantification of MFN1, MFN2, OPA1 and VDAC protein levels normalized to β-Actin (loading control). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (D) RT-qPCR analysis of *Mfn1*, *Mfn2*, and *Opa1* mRNA expression in total sciatic nerve extracts at the indicated cut injury timepoints. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Mrpl10* and *Ppia* mRNA for each data point. Data are presented as means ± SEM, $n = 4$ to 5 per group. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test.

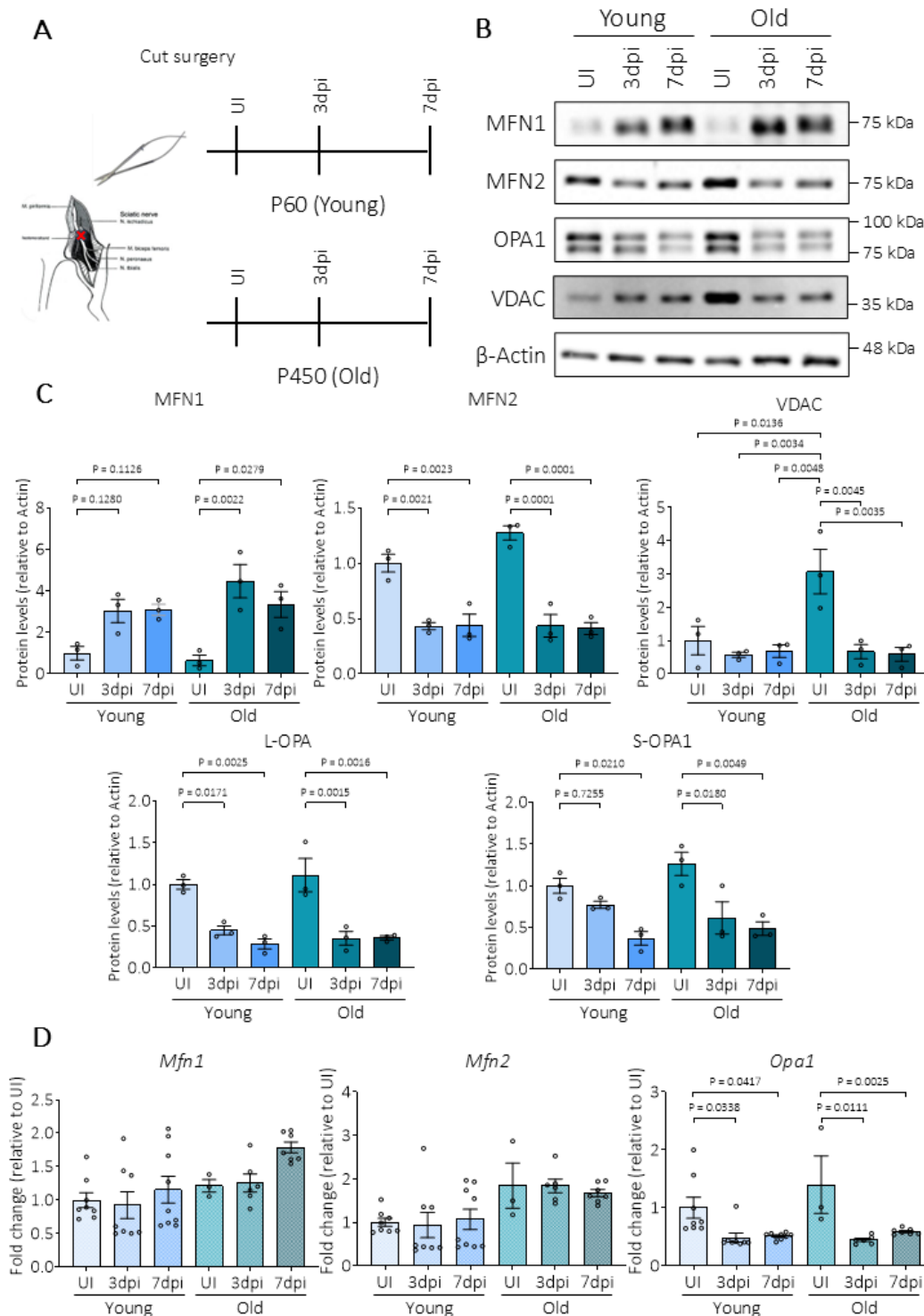
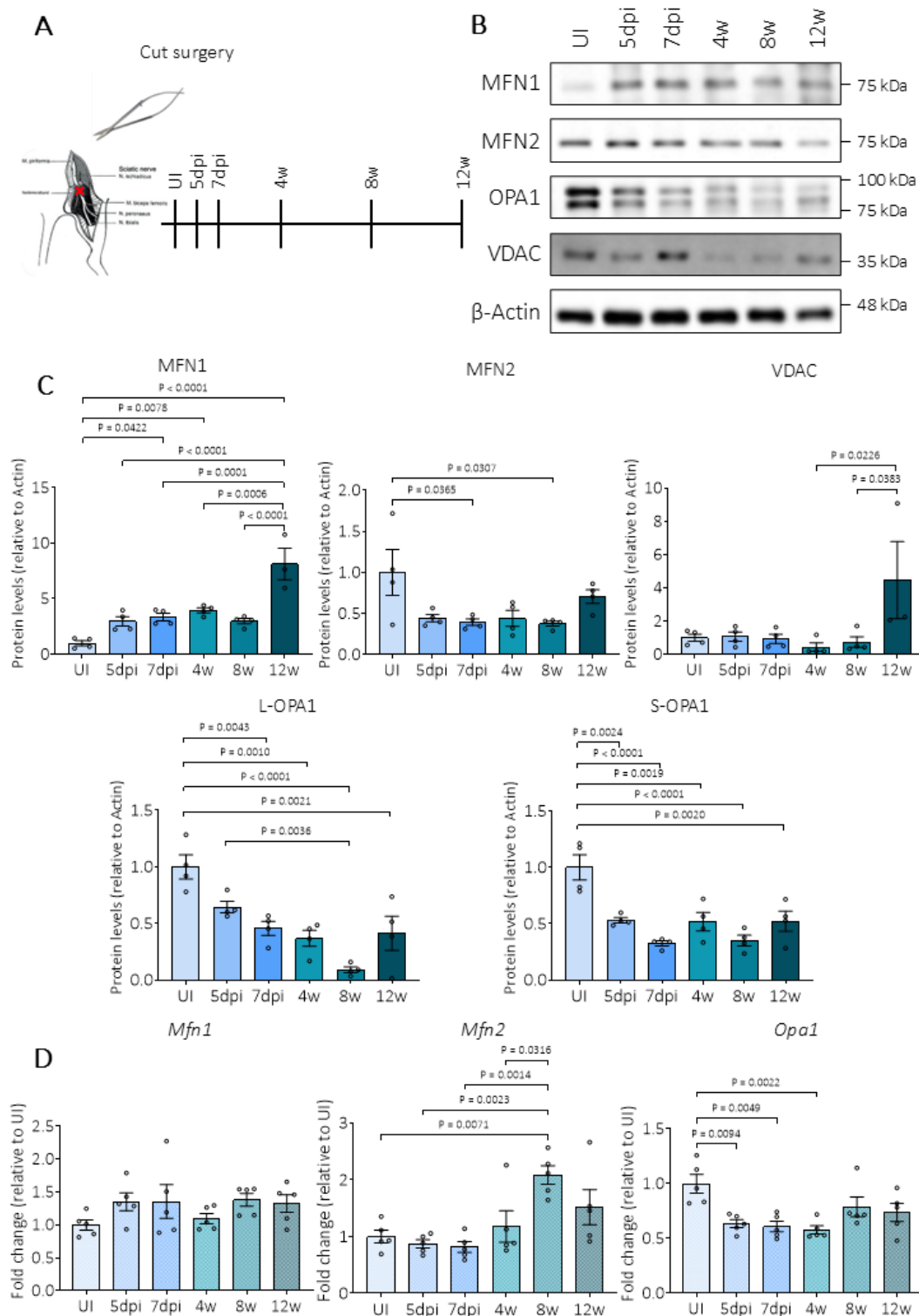


Figure 43. Dynamic changes in expression of MFN1, MFN2 and OPA1 in aged nerve cut injury. (A) Schematic representation of sample collection after sciatic nerve cut surgery in young (P60) and old (P450) mice. (B) Immunoblot analysis of MFN1, MFN2, OPA1, VDAC and β -Actin expression in total sciatic nerve extracts at the indicated cut injury timepoints in young and aged mice. (C) Densitometric quantification of MFN1, MFN2, OPA1, and VDAC protein levels normalized to β -Actin (loading control). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (D) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in total sciatic nerve extracts at the indicated cut injury timepoints in young and aged mice. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Calnexin* mRNA for each data point. Data are presented as means $\pm$ SEM, $n = 3$ to 9. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test.



4.11 GENERATION OF INDUCIBLE SCHWANN CELL-SPECIFIC KNOCKOUT MICE LINE

Our data above show that MFN1, MFN2 and OPA1 play key roles in peripheral nerve development. We next wanted to examine the functional role of these genes in adult nerves, specifically in the maintenance of the myelin phenotype, and in Schwann cell responses to injury. Thus, to bypass the developmental defects seen after ablation of these genes using the constitutive MPZ-cre line, we decided to generate tamoxifen-inducible knockout mice lines.

For this, we crossed floxed mice (as above) with a mouse line that expresses tamoxifen-inducible Cre recombinase driven by the PLP promoter (Plp-Cre^{ERT2}), which is expressed in mature Schwann cells and oligodendrocytes (Leone et al., 2003). P60 mutant mice for *Mfn1/2* (*Mfn1*^{ikO}*MFN2*^{ikO}) and *Opa1* (*Opa1*^{ikO}) were (which we refer to as idKO and icKO mice, respectively) were treated daily with tamoxifen solution (75 mg/kg) for 5 consecutive days by intraperitoneal injection, and the recombination levels determined 1 month later by WB and qPCR analysis of relevant genes.

In *Mfn1/2* idKO nerve lysates, we found a tendency, although not significant, reduction in both MFN1 and MFN2 protein levels (Fig. 45A, B) and a significant reduction in their mRNA levels (Fig. 45C). For *Opa1* icKO mice, immunoblot showed a significant decrease in S-OPA1 protein levels (Fig. 45D, E) that was accompanied by a significant reduction in *Opa1* mRNA levels (Fig. 45F).

4.12 MFN1/2 OR OPA1 ARE NOT REQUIRED FOR MYELIN MAINTENANCE

Similar to the formation of myelin sheaths, the maintenance of the myelination phenotype in adult nerves is also under strict transcriptional control. Altering this control leads to severe phenotypic defects. For example, the genetic inactivation of *Egr2* or *Sox 10* in mature myelinating Schwann cells, by using tamoxifen-inducible Cre mice lines (e.g. Plp Cre^{ERT2}), leads to severe demyelination (Bremer et al., 2011; Decker et al., 2006).

We thus examined the functional consequences of genetic ablation of mitochondria-related genes in mature Schwann cells on myelin homeostasis. Using immunoblot analyses, we did not find any alterations in myelin proteins MPZ and MBP in either model, at 1 month following tamoxifen injections in P60 mice, *Mfn1/2* idKO (Fig. 45A, B) nor *Opa1* icKO (Fig. 45D, E). Furthermore, we did not find any significant differences in the battery of sensory-motor functional tests and different parameters of electrophysiological recordings we performed of *Mfn1/2* idKO (Fig. 46A–U) or *Opa1* icKO (Fig. 47A–U). These data suggest that these genes are not required for maintenance of mature myelin sheaths.

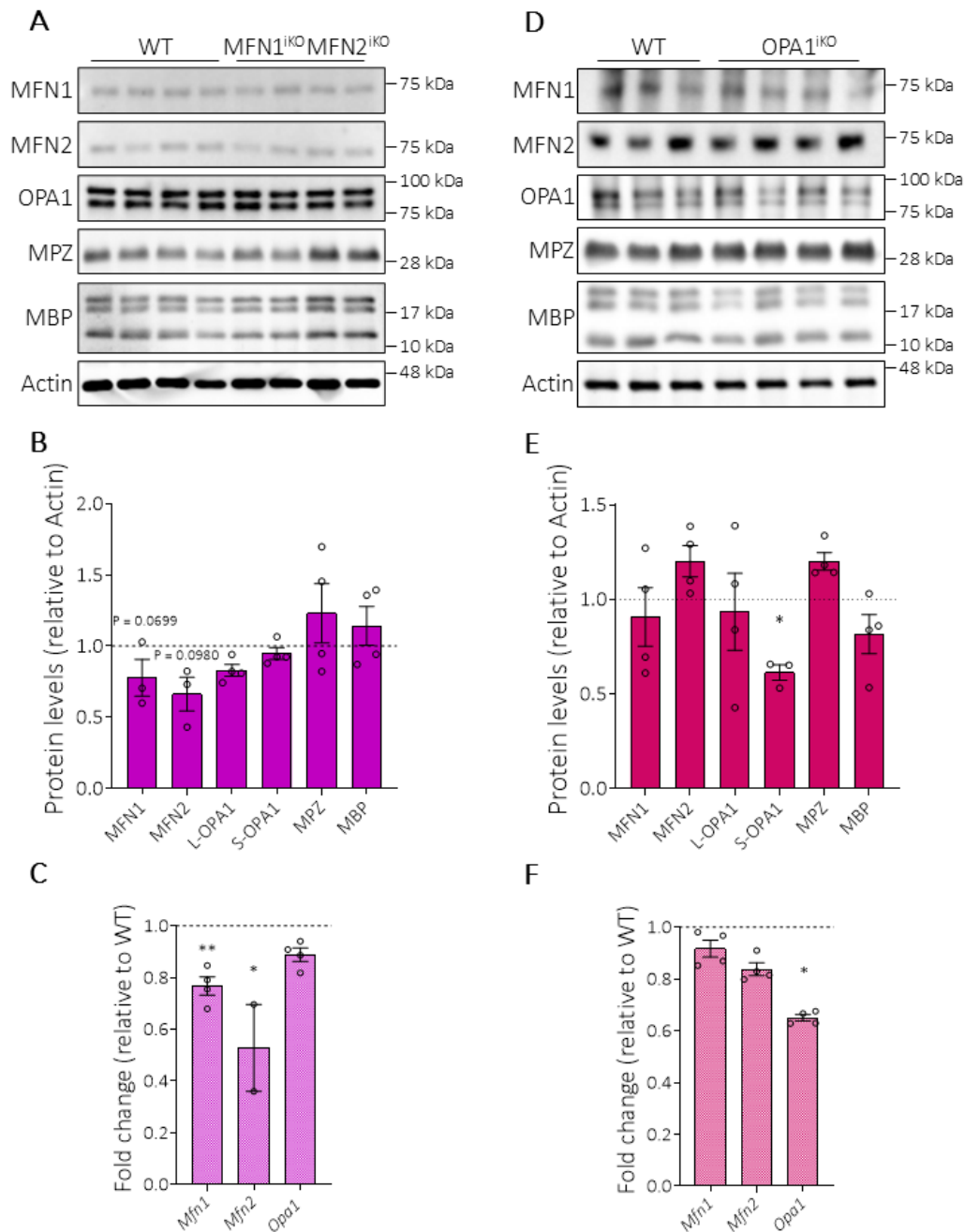


Figure 45. Generation of Schwann cell-specific *Mfn1/2* and *Opa1* inducible knockout models. (A) Immunoblot analyses of MFN1, MFN2, OPA1, MPZ, MBP and β -Actin in sciatic nerve extracts from adult WT and *Mfn1/2* idKO mice, 30 days after tamoxifen injection. (B) Densitometric quantification of MFN1, MFN2, OPA1, MPZ and MBP protein levels normalized to β -Actin (loading control). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (C) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in sciatic nerve extracts from WT and *Mfn1/2* idKO mice, 30 days after tamoxifen injection. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Calnexin* mRNA for each data point. (D) Immunoblot analyses of MFN1, MFN2, OPA1, MPZ, MBP and β -Actin in sciatic nerve extracts from WT and *Opa1* icKO mice, 30 days after tamoxifen injection. (E) Densitometric quantification of MFN1, MFN2, OPA1, MPZ, MBP protein levels normalized to β -actin (loading control). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (F) RT-qPCR analysis of *Mfn1*, *Mfn2*, and *Opa1* mRNA expression in sciatic nerve extracts from WT and *Opa1* icKO mice, 30 days after tamoxifen injection. Data are presented as means $\pm$ SEM, $n = 2$ to 4. Two-tailed unpaired Student's t test. The relative expression values of each mRNA were calculated using the comparative Ct method and were normalized using *Calnexin* mRNA for each data point.

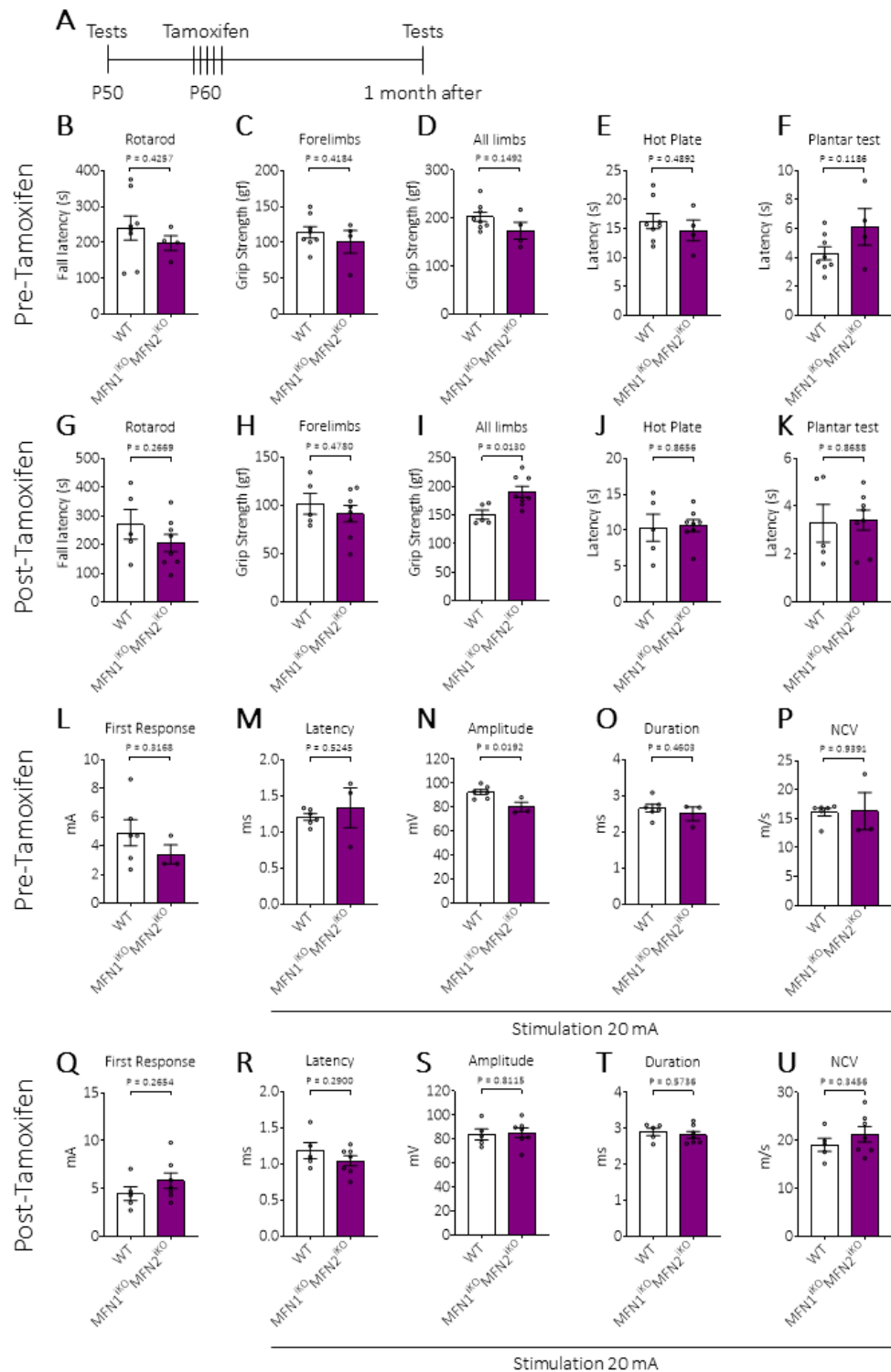


Figure 46. *Mfn1/2* are not required for myelin maintenance. (A) Schematic diagram depicting the strategy for recombination induction via tamoxifen injection. (B–K) *Mfn1/2* idKO exhibit no significant differences compared to control littermates (B–F) before, and (G–K) after tamoxifen injection in motor function, as shown by (B, G) latency to fall off the accelerating rotarod and grip strength of (C, H) forelimb or (D, I) all four limbs, and in sensory responses using (E, J) the plantar plate test and (F, K) hot plate test. (L–U) *Mfn1/2* idKO mice display normal electromyography responses (L–P) before and (Q–U) after tamoxifen injection. Graphs show (L, Q) first clear response when increasing mA, (M, R) latency to response, (N, S) mean peak amplitudes of CMAPs, (O, T) average durations of CMAPs and (P, U) nerve conduction velocity at 20 mA. Data are presented as means $\pm$ SEM, $n = 3$ to 7. Two-tailed unpaired Student's *t* test.

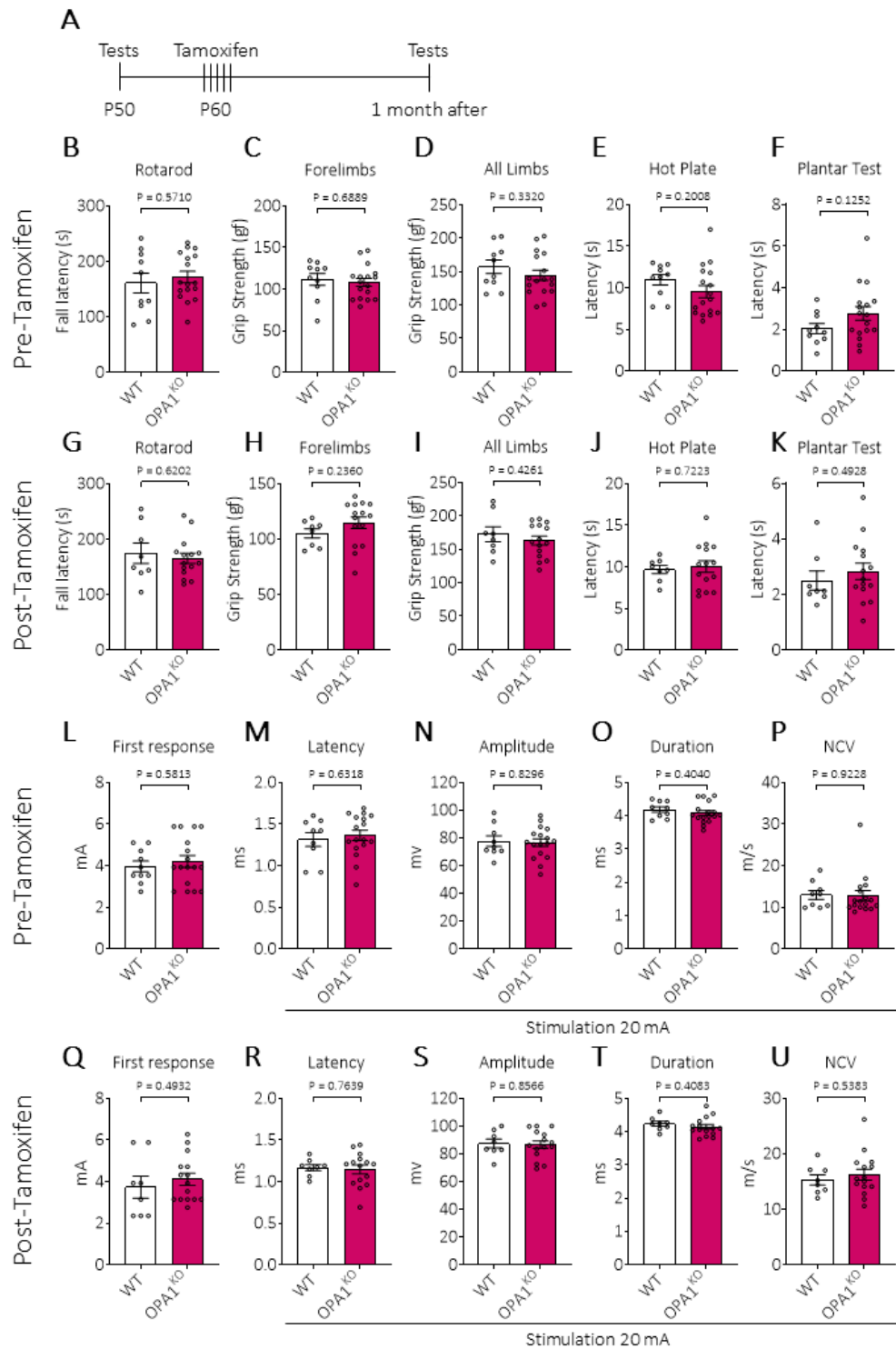


Figure 47. *Opa1* is not required for myelin maintenance. (A) Schematic diagram depicting the strategy for recombination induction via tamoxifen injection. (B–K) *Mfn1/2* idKO exhibit no significant differences compared to control littermates (B–F) before, and (G–K) after tamoxifen injection in motor function, as shown by (B, G) latency to fall off the accelerating rotarod and grip strength of (C, H) forelimb or (D, I) all four limbs, and in sensory responses using (E, J) the plantar plate test and (F, K) hot plate test. (L–U) *Mfn1/2* idKO mice display normal electromyography responses (L–P) before and (Q–U) after tamoxifen injection. Graphs show (L, Q) first clear response when increasing mA, (M, R) latency to response, (N, S) mean peak amplitudes of CMAPs, (O, T) average durations of CMAPs and (P, U) nerve conduction velocity at 20 mA. Data are presented as means ± SEM, *n* = 3 to 7. Two-tailed unpaired Student's *t* test.

4.13 IMPAIRED MITOCHONDRIAL FUSION LEADS TO FASTER MYELIN CLEARANCE AFTER INJURY

Our data above showing dynamic changes in expression of these genes suggest that they could be involved in nerve repair. One key requisite for successful nerve repair is that myelin debris present in the distal nerves following nerve injury must be cleared, and previous studies have shown that Schwann cells themselves can break down their myelin using autophagy (Gomez-Sanchez et al., 2015). We thus examined the functional role of MFN1, MFN2 and OPA1 in myelin clearance by performing nerve cut injury in our genetic models, including the *Mfn1/2* idKO and *Opa1* icKO mice, as well as the *Mfn1* cKO and *Mfn2* cKO constitutive models to examine the individual contribution of each gene in this process. We did not generate tamoxifen-inducible single knockout models for *Mfn1* or *Mfn2* since our previous data show no obvious defects in adult nerves at any level (Fig. 17, 18, 19, 20, 27 and 35).

Notably, using immunoblot analyses, we found a generally faster clearance of the myelin proteins MPZ or MBP after nerve cut injury in the *Mfn2* cKO, *Mfn1/2* idKO and *Opa1* icKO mice (Fig. 49B, D, E, G, F, H), whereas no differences were seen for *Mfn1* cKO mice (Fig. 49A, C). These data indicate that these mitochondria-related genes play a role in myelin breakdown.

4.14 MFN1, MFN2 AND OPA1 SHOW DYNAMIC CHANGES IN EXPRESSION IN REGENERATING NERVES

Next, we examined the role of these genes in nerve regeneration. As mentioned, following the initial responses to injury, such as myelin clearance, Schwann cells convert to repair Schwann cells and proliferate to establish columns of cells (Büngner Schwann cells) that aid regenerating axons to grow back to the targets, which are remyelinated by the Schwann cells to complete functional recovery (Stassart et al., 2024).

We first examined expression of the mitochondrial-related genes during the regenerative process using the extensively used nerve crush model (Ayuso-García et al., 2024). In brief, in this model, the sciatic nerve is exposed and compressed, and the mice are then allowed to recover (Fig. 35A). Nerves are then dissected out at different time-points, corresponding to the stages of the regenerative process. Thus, during the initial first few days (5-7), Schwann cells and macrophages contribute to myelin breakdown and Schwann cells proliferate to establish columns of cells (Büngner Schwann cells), and the regenerated axons are then remyelinated by the Schwann cells, with functional recovery complete within 60 days (Jessen & Mirsky, 2016).

Similar to the nerve cut injury model above (Fig. 42, 43 and 44), MFN1 protein levels were significantly upregulated in the crushed nerves (Fig. 50B, C), whilst mRNA levels remained relatively stable (Fig 50D). In contrast, MFN2 and OPA1 levels were relatively unaltered (Fig. 50B, C), whereas their mRNA levels were downregulated (Fig. 50D).

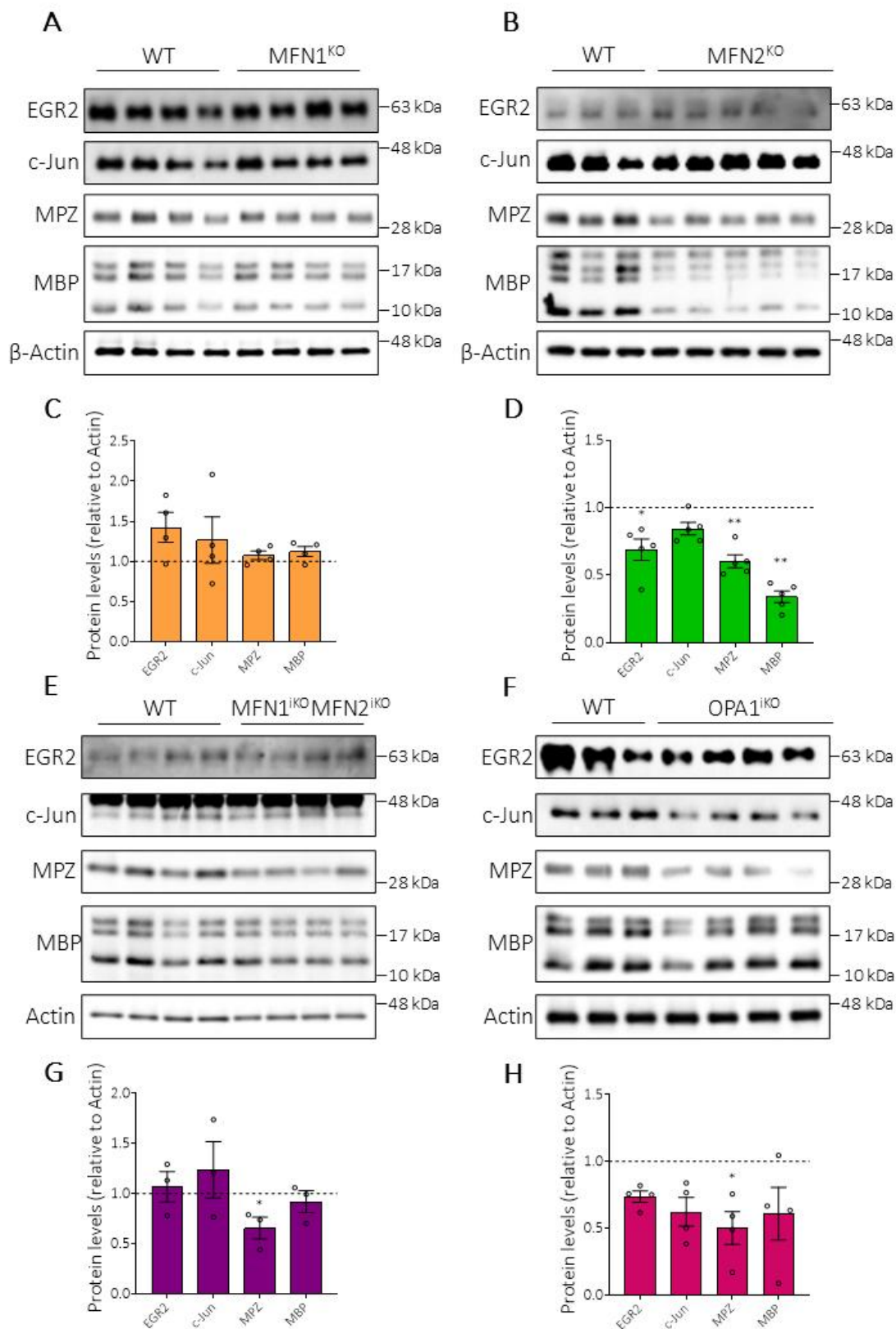


Figure 49. *Mfn2* cKOs, *Mfn1/2* idKOs and *Opa1* idKOs mice have accelerated myelin clearance. (A–H) (A, B, G, H) Immunoblot and (C, D, G, H) densitometric analysis of EGR2, c-Jun, MPZ, MBP and β-Actin (loading control) in sciatic nerve extracts from WT and (A, C) *Mfn1* cKO, (B, D) *Mfn2* cKO, (E, G) *Mfn1/2* idKO, (F, H) *Opa1* idKO mice, 7 days after injury. Data are presented as means ± SEM, $n = 3$ to 5. Two-tailed unpaired Student's t test.

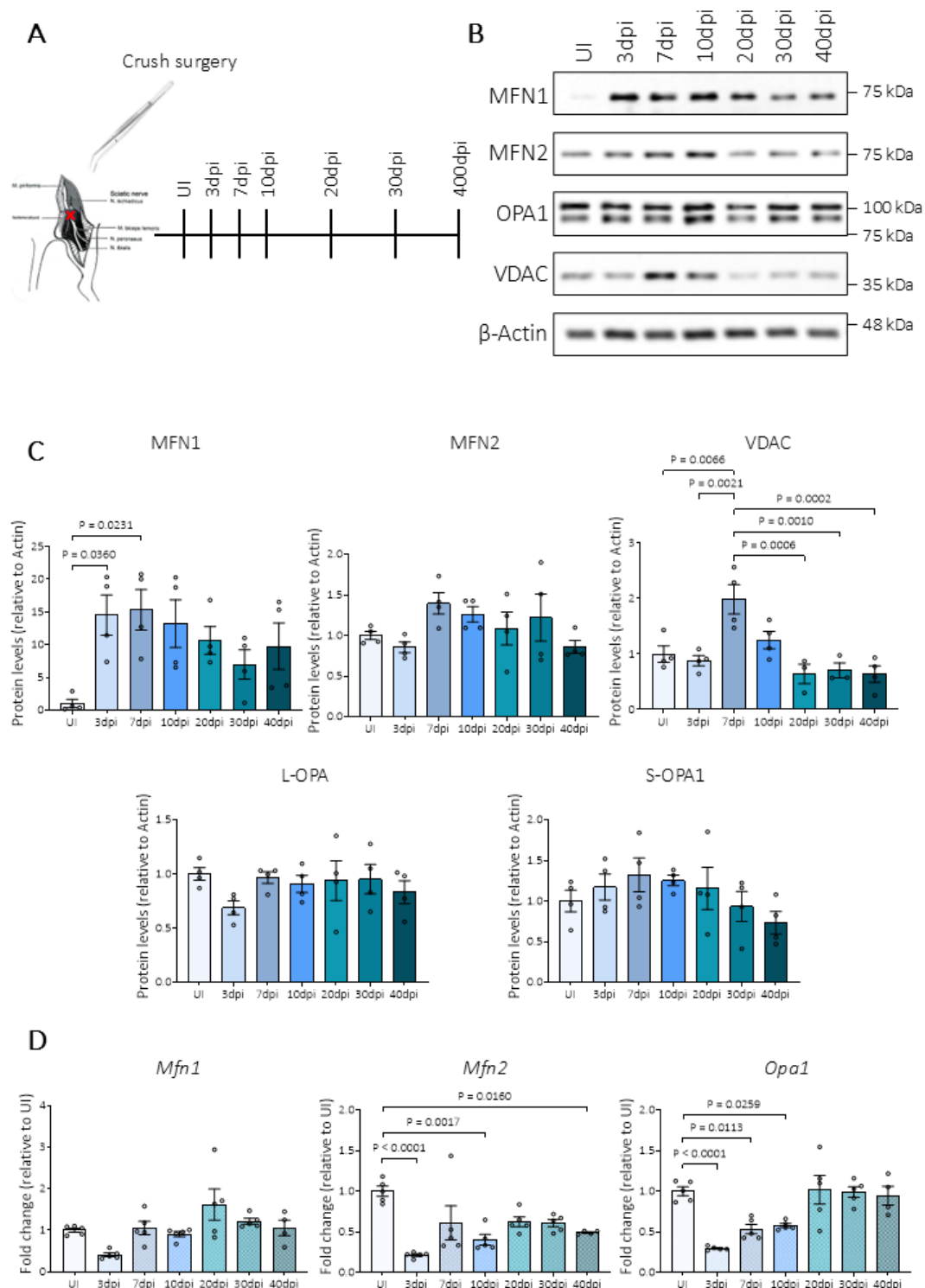


Figure 50. MFN1, MFN2 and OPA1 show dynamic changes in expression after nerve crush injury *in vivo*. (A) Schematic representation of sample collection after sciatic nerve crush surgery curve. (B) Immunoblot analysis of MFN1, MFN2, OPA1, VDAC and β-Actin expression in total sciatic nerve extracts at the indicated timepoints following crush injury. (C) Densitometric quantification of MFN1, MFN2, OPA1 and VDAC protein levels normalized to β-Actin (loading control). L-OPA1 refers to the long isoform and S-OPA1 to the short isoform. (D) RT-qPCR analysis of *Mfn1*, *Mfn2* and *Opa1* mRNA expression in total sciatic nerve extracts at the indicated timepoints following crush injury. Data are presented as means ± SEM, $n = 4$ to 5. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test.

4.15 MFN1/2 OR OPA1 ARE DISPENSABLE FOR NERVE REPAIR

To examine the functional role of these genes in nerve regeneration, we performed nerve crush injury in P90 *Mfn1/2* idKO and *Opa1* icKO mice, 30 days after tamoxifen injection, and examined functional recovery at different days post-injury (dpi) (Fig. 51 and Fig. 52, respectively).

We did not find any significant differences in sensory-motor functional tests at either 20dpi (Fig. 51B, C and Fig. 52B, C) and 40dpi (Fig. 51D, E and Fig. 52D, E) or in electrophysiological recordings at 40dpi (Fig. 51F–J and Fig. 52F–J) between control and mutant mice. Similarly, when we examined remyelination by EM, we did not find significant differences between control and *Mfn1/2* idKO (Fig. 53A–F) or *Opa1* icKO mice (Fig. 53G–L). These data suggest that these mitochondrial-related genes are not required for nerve regeneration.

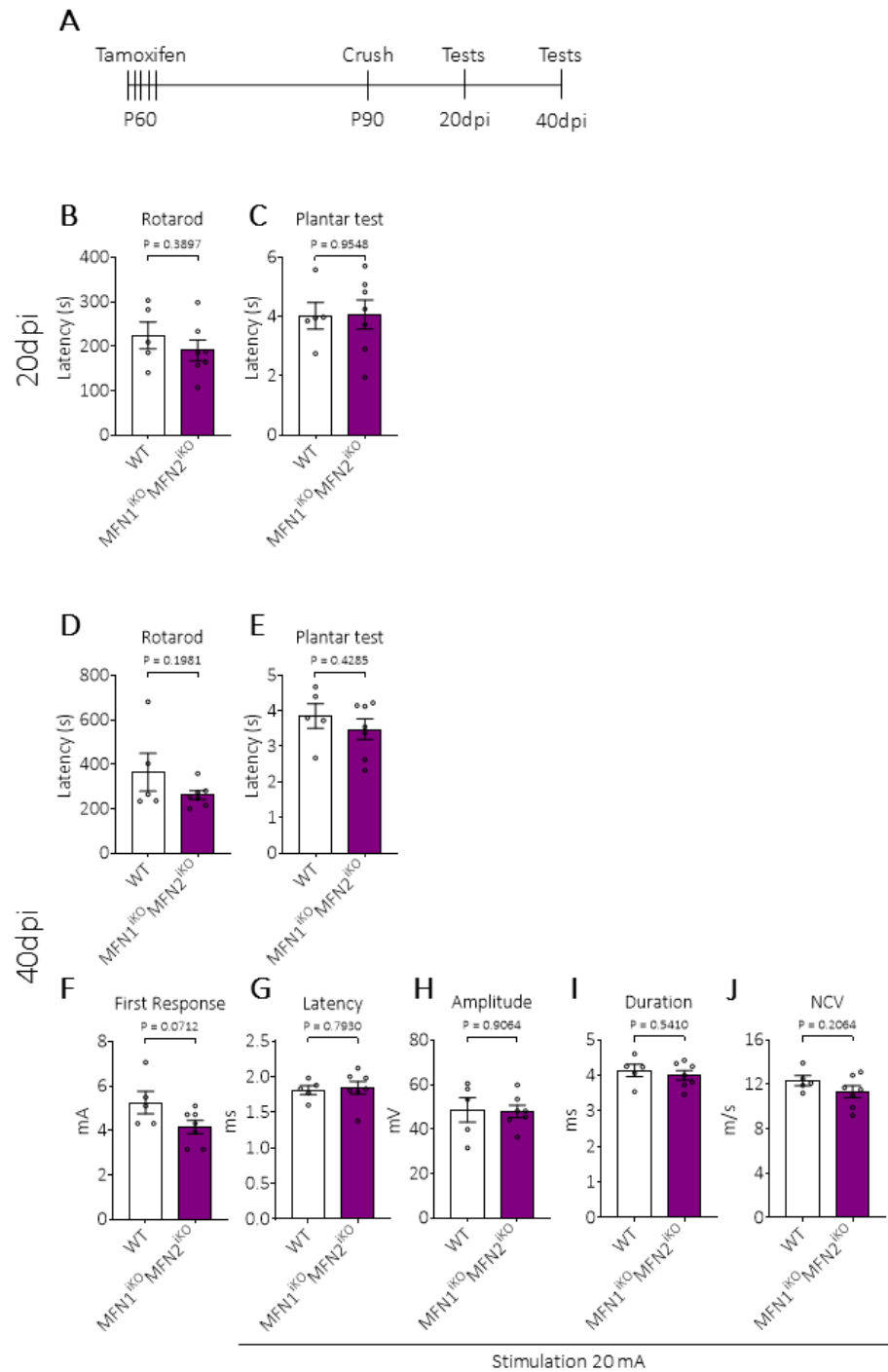


Figure 51. *Mfn1/2* idKO mice show normal functional recovery after nerve crush injury. (A) Schematic diagram depicting the strategy for nerve regeneration paradigm. P60 *Mfn1/2* idKO mice were treated with tamoxifen, followed by crush injury 40 days later, and were then sacrificed at P130. (B, C) *Mfn1/2* idKO mice exhibit no significant differences compared to control littermates when (B) latency to fall off the accelerating rotarod, and (C) plantar test at 20 days post crush were performed. (D–J) *Mfn1/2* idKO mice exhibit no significant differences compared to control littermates when (D) latency to fall off the accelerating rotarod, (E) plantar test, (F) first clear response when increasing mA, (G) latency to response, (H) mean peak amplitudes of CMAPs, (I) average durations of CMAPs, (J) nerve conduction velocities at 20 mA after 40 days post crush were performed. Data are presented as means $\pm$ SEM, $n = 5$ to 7. Two-tailed unpaired Student's t test.

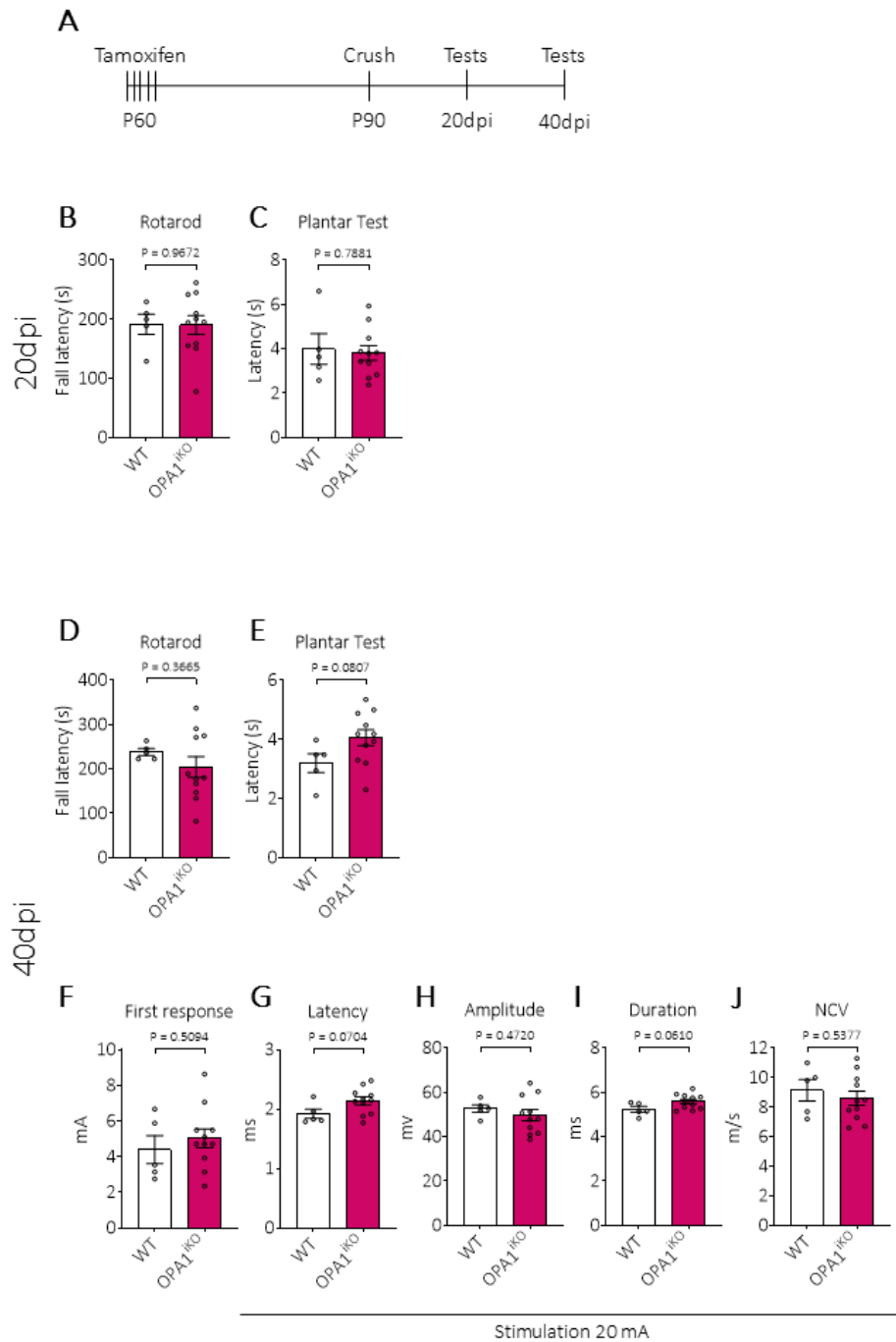


Figure 52. *Opa1* iKO mice show normal functional recovery after nerve crush injury. (A) Schematic diagram depicting the strategy for nerve regeneration paradigm. P60 *Opa1* iKO mice were treated with tamoxifen, followed by crush injury 40 days later, and were then sacrificed at P130. (B, C) *Opa1* iKO mice exhibit no significant differences compared to control littermates when (B) latency to fall off the accelerating rotarod and (C) plantar test at 20 days post crush were performed. (D–J) *Opa1* iKO mice exhibit no significant differences compared to control littermates when (D) latency to fall off the accelerating rotarod, (E) plantar test, (F) first clear response when increasing mA, (G) latency to response, (H) mean peak amplitudes of CMAPs, (I) average durations of CMAPs, (J) nerve conduction velocities at 20 mA after 40 days post crush were performed. Data are presented as means $\pm$ SEM, $n = 5$ to 10. Two-tailed unpaired Student's t test.

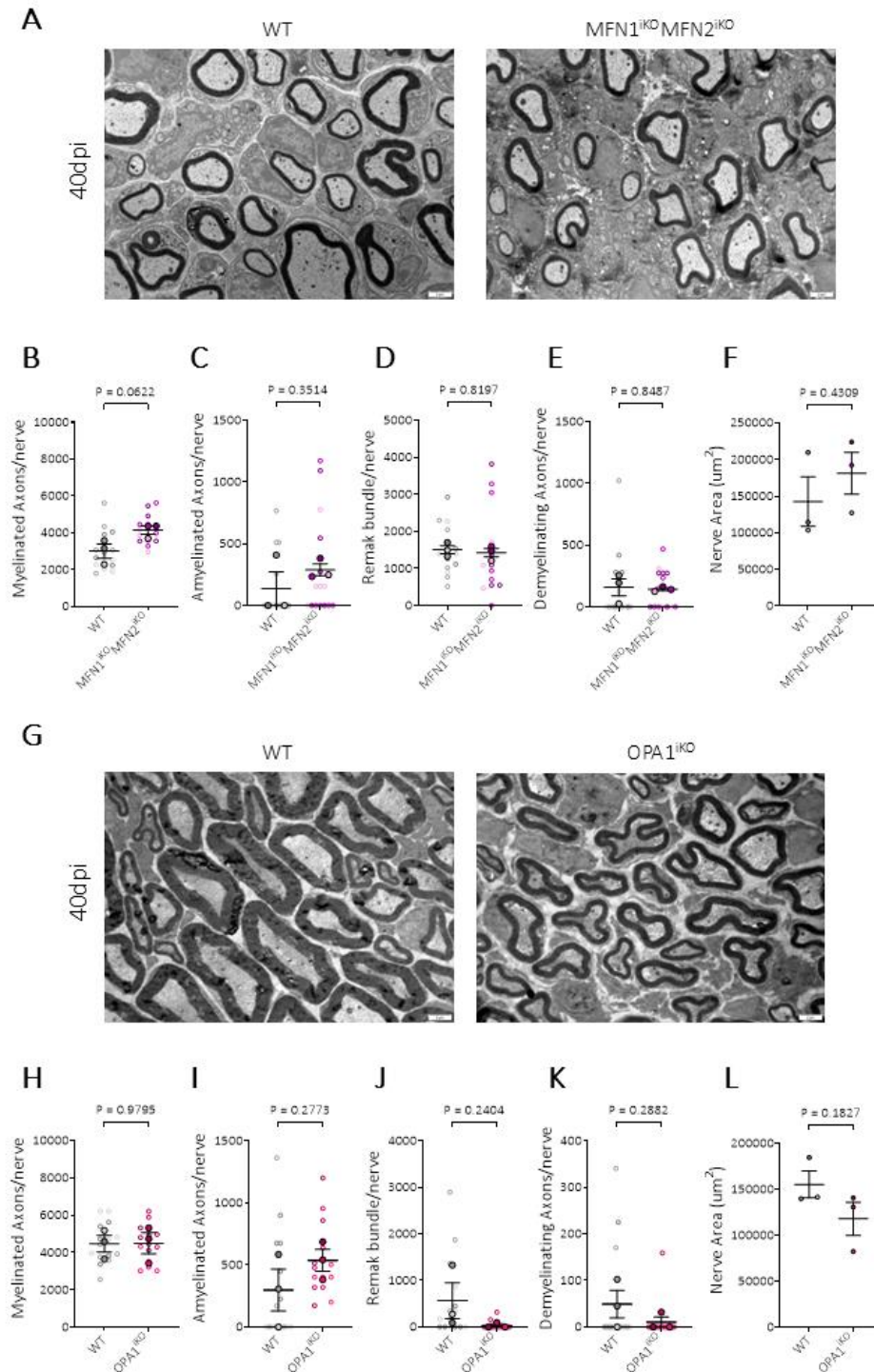
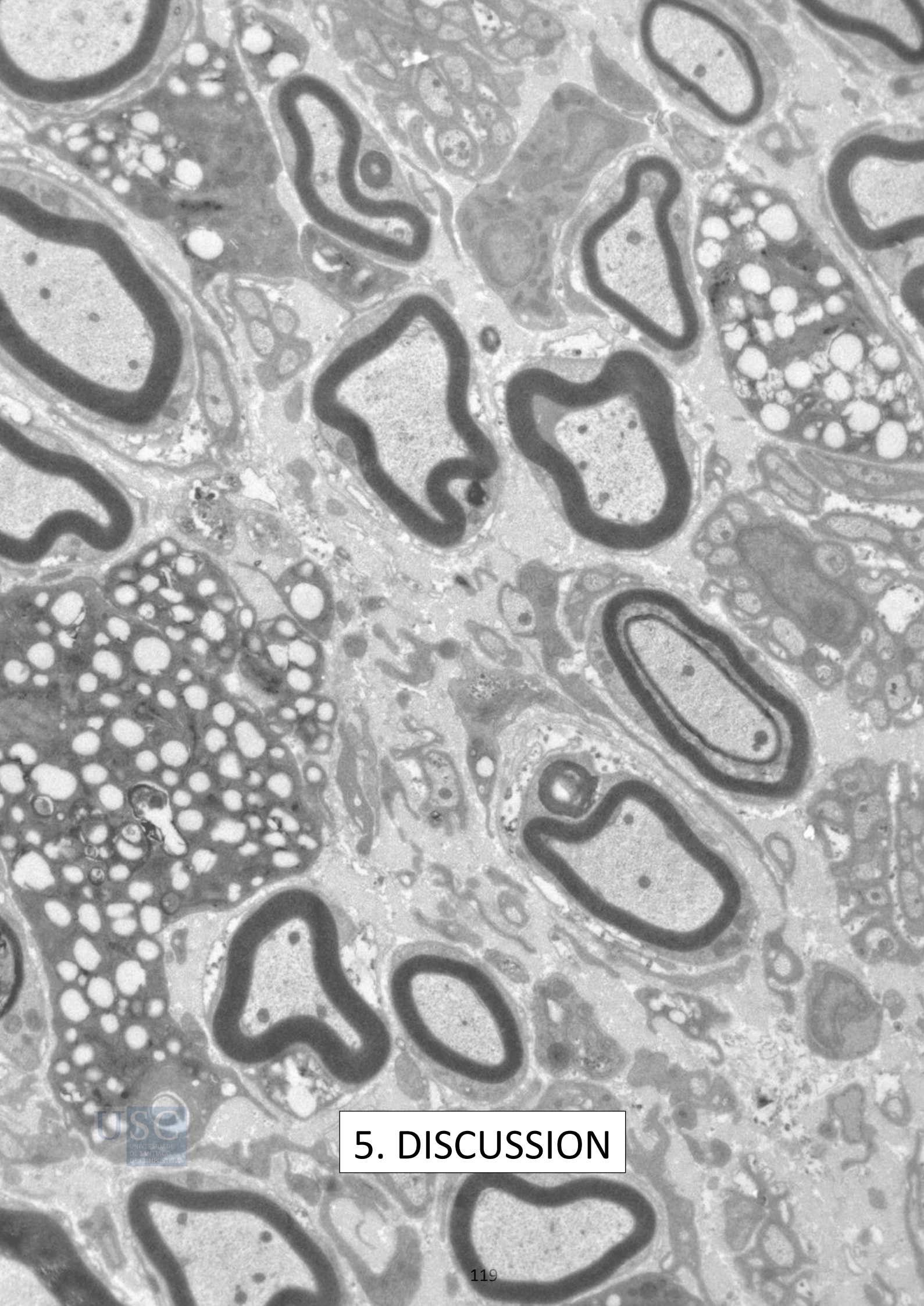


Figure 53. EM analyses show normal remyelination profiles *Mfn1/2* idKO and *Opa1* icKO after nerve crush injury. (A) Representative EM pictures showing ultrastructure of control and *Mfn1/2* idKO sciatic nerves, 40 days post-crush surgery. Scale bar, 2 μm. (B–F) Graphs show quantification of (B) myelinated axons, (C) amyelinated axons (1:1) per nerve, (D) Remak bundles, (E) demyelinating axons, (F) total nerve area in control and *Mfn1/2* idKO mice, 40 days post-crush surgery. (G) Representative EM pictures showing ultrastructure of control and *Opa1* icKO sciatic nerves, 40 days post-crush surgery. Scale bar, 2 μm. (H–L) Graphs show quantification of (H) myelinated axons, (I) amyelinated axons (1:1) per nerve, (J) Remak bundles, (K) demyelinating axons, (L) total nerve area in control and *Opa1* icKO mice, 40 days post-crush surgery. Nerve area data are presented as means ± SEM, *n* = 3 per group. Two-tailed unpaired Student's *t* test. Rest of data are presented as super-plot combining biological replicates ± SEM (large circles) with their technical replicates (small circles). Each colour codes for a specific animal, *n* = 3 per group. Statistical significance was determined using nested *t*-test.



5.1 METABOLIC ADAPTATION IN SCHWANN CELLS DURING DEVELOPMENT

Myelination is a metabolically demanding process, requiring massive biosynthesis of lipids and proteins in a short period of time. In peripheral nerves, the generation of myelinating Schwann cells is a complex, tightly regulated process that requires the orchestrated action of lineage-specific transcriptional and epigenetic regulators, signalling pathways, and interactions with extracellular matrix components (Salzer et al., 2024).

Mitochondria, which are pivotal organelles in cellular metabolism, are important regulators of myelin formation and maintenance in Schwann cells. In the last few years, several studies have explored the impact of the loss of function of mitochondrial proteins in SCs. For example, conditional deletion of the respiratory chain component COX10 results in severe dysmyelination (malformed myelin) in the PNS (Fünfschilling et al., 2012), whereas deletion of PHB2, a protein enriched in the inner mitochondrial membrane, in Schwann cells leads to a severe radial sorting defect in mice that is evident at P20 (Poitelon et al., 2015). This contrasts with the normal developmental or a mild developmental delay in myelination followed by a severe and progressive demyelinating peripheral neuropathy in Schwann cell-specific ablation of the mitochondrial transcription factor TFAM (Viader et al., 2011), the inner mitochondrial membrane protein PHB1 (Della-Flora Nunes et al., 2021) or the mitochondrial network regulator GDAP1 (Niemann et al., 2014).

There is also clear evidence of the importance of mitochondrial function in patients. For example, mutations in *MFN2* are associated with CMT2A, a neurological disorder characterized not only by peripheral neuropathy but also by complex symptoms with implications for the central nervous system (Stuppia et al., 2015). Other CMT subtypes have been linked to additional regulators of mitochondrial dynamics, such as *GDAP1*, whose mutations underlie CMT4A (Ben Othmane et al., 1998). Furthermore, specific *OPA1* mutations give rise to the more severe DOA-plus syndrome, which, beyond optic atrophy, includes neurological complications such as sensorineural hearing loss, myopathy, peripheral neuropathy, and demyelination (Yu-Wai-Man et al., 2009).

Key recent studies have shown that in developing nerves, proliferating Schwann cells undergo metabolic reprogramming from glycolytic to oxidative metabolism during differentiation and myelination *in vivo*, with the metabolic regulator LKB1 playing an essential role in this metabolic shift (Pooya et al., 2014). Although it is clear that Schwann cells undergo this metabolic reprogramming to execute the bioenergetic and biosynthetic demands of differentiation and repair, the molecular switch that is responsible for this remains unclear. In this study, we identify mitochondrial plasticity as essential for these processes.

It is well known that mitochondria are highly plastic organelles that can match their function according to their cell state-dependent metabolic needs. Metabolic reprogramming of

mitochondria is coupled to changes in mitochondrial mass and shape (Suomalainen & Nunnari, 2024). These responses are complex and involve a combination of diverse molecular pathways that modulate mitochondrial biogenesis, metabolism, and dynamics. Mitochondrial fusion, which is associated with increased ATP production, involves the merging of two or more mitochondria to generate a larger elongated organelle, a process controlled by a set of “mitochondria-shaping” proteins, including MFN1/2 and OPA1 (Quintana-Cabrera & Scorrano, 2023).

Here, we find that these key “mitochondria-shaping” proteins (MFN1, MFN2 and OPA1) are highly expressed in sciatic nerves at ages that correspond to active myelination, and that genetic ablation of these genes in Schwann cells *in vivo* lead to a severe neuropathy, associated with poor responses to motor and sensory functional tests (Fig. 10, 12), and a marked reduction in conduction velocity using electrophysiological measurements (Fig. 11, 13). Using EM and immunoblot analyses, we found that peripheral nerves in *Opa1* and *Mfn1/2* mutant mice lacked myelin and exhibited mitochondrial defects (Fig. 15, 17, 24). Furthermore, *Opa1*-deficient Schwann cells showed decreased respiratory rates in Seahorse analyses (Fig. 25). Altogether, these data strongly support that mitochondrial plasticity plays a key role in Schwann cells, at least in the formation of myelin sheaths during development.

At an ultrastructural level, we found that Schwann cell mitochondrial numbers increased with normal development, although we did not find any differences in size (Fig. 2). On the other hand, we did find a clear reduction both in number and size of Schwann cell mitochondria in *Mfn1/2* dKO mice (Fig. 18). In contrast, in *Opa1*-deficient mice, we found only a clear tendency of a decrease in Schwann cell mitochondrial numbers without affecting mitochondrial size (Fig. 19). This suggest that *Mfn1* and *2*, but not *Opa1*, could be playing a role in mitochondrial fusion during Schwann cell myelination. However, these measurements are derived from two-dimensional cross-sections, which do not capture the full spatial organization of mitochondrial networks. Recent advances emphasize the importance of 3D reconstructions to resolve the architecture of cellular structures (Meschkat et al., 2022; Steyer et al., 2023). Indeed, volume EM studies demonstrate that mitochondria often form elongated, interconnected tubules (Franco-Barranco et al., 2022; J. Liu et al., 2020). In agreement with this, findings from the Fledrich laboratory, presented at the Glia 2025 meeting, revealed that Schwann cell mitochondria extend as elongated structures along the plasma membrane surrounding axons. Thus, conventional 2D EM allows estimation of mitochondrial cross-sectional diameter but cannot reliably inform on network density. A comprehensive understanding of how mitochondrial architecture evolves during sciatic nerve development would therefore require 3D reconstruction approaches.

In addition to the severe myelination defects, we found that the defective Schwann cells in *Mfn1/2* dKO and *Opa1* cKO mice could not provide adequate support to maintain axonal integrity, as we found notable secondary axonal loss in the nerves, especially at later stages of

development. This is similar to another model of Schwann cell mitochondrial dysfunction, induced by genetic deletion of *Prohibitin 1* in Schwann cells, which also leads to a severe demyelinating peripheral neuropathy that is accompanied by secondary axonal loss (Della-Flora Nunes et al., 2021). Interestingly, we find that the mitochondrial loss in the *Mfn1/2* dKO and *Opa1* cKO Schwann cells is counterbalanced by a marked increase in axonal mitochondria. These data suggest that Schwann cell mitochondrial dysfunction compromises the ability of axons to meet their energy demands, triggering a compensatory increase in axonal mitochondrial abundance to attempt to meet these needs. This study thus adds to a growing body of evidence that indicate that Schwann cells could provide metabolic supply to their axonal counterparts, similar to the CNS, in which oligodendrocytes have the capacity to cater to their own and axonal energy needs (Fünfschilling et al., 2012; Y. Lee et al., 2012). Schwann cells and axons have a strict interdependent relationship, with each cell type providing essential support for the other. Schwann cell metabolic support to axons could be a cornerstone of this relationship, since Schwann cells may serve as a local source of energy and metabolites for axons, compensating for limitations imposed by the long distances from neuronal cell bodies and the restricted access to extracellular substrates resulting from Schwann cell ensheathment.

We also found that when Schwann cells sense this metabolic impairment caused by mitochondria dysfunction (via *Mfn1/2* or *Opa1* deletion) they trigger a specific stress response (Fig. 24). This is driven by activation of the integrated stress response, induction of a Schwann cell-specific injury program governed by c-Jun, and upregulation of mitochondrial biogenesis through TFAM. This stress response is similar to the one observed in other SC mitochondrial mutants. ISR was activated by upregulation of p-eIF2 α when TFAM is missing (Viader et al., 2013) or when *Phb1* is deleted (Della-Flora Nunes et al., 2021). Elevated levels of c-Jun have been also described in *Phb1* and *Nampt* Schwann cell mutants (Della-Flora Nunes et al., 2021; Sasaki et al., 2018). SC seem to enter in an emergency state that helps them to counteract mitochondria dysfunction, characterized primarily by activation of c-Jun and ISR. Furthermore, when Schwann cells enter in this emergency state, pACC^{S79}/ACC ratio is increased indicating a metabolic shift from lipid synthesis to energy production, a classic response to cellular stress, energy deficit, or increased AMP/ATP ratio (Fullerton et al., 2013; C.-W. Kim et al., 2010; Winder et al., 1997).

5.1.1 The role of Mitofusins in Schwann cell metabolic reprogramming

MFN1 and MFN2 are essential for embryonic survival, as global deletion of either protein results in lethality before embryonic day 12.5 in mice. Simultaneous ablation of both mitofusins leads to even earlier mortality and, in mouse embryonic fibroblasts, causes profound mitochondrial network fragmentation (Chen et al., 2003). Beyond embryogenesis, however, their roles become highly context-dependent and vary across tissues. In certain systems, such as sperm development, both mitofusins are dispensable (Miao et al., 2021). In

other tissues, lack of both mitofusins is critical provoking myocardial hypertrophy, heart tube contractile dysfunction, vascular smooth muscle cell proliferation, and skeletal muscle atrophy and impaired osteoclast precursor differentiation for example (Ballard et al., 2020; Chen et al., 2010; Eschenbacher et al., 2012; Guo et al., 2007; Sebastián et al., 2016). Tissue specificity is also evident in cases where one mitofusin predominates: MFN2 plays a dominant role in macrophages and POMC neurons (Bhatia et al., 2022; Schneeberger et al., 2013), whereas MFN1 is indispensable in oocytes (Carvalho et al., 2020). In pancreatic β -cells, their functions are sufficiently redundant to allow compensation by either mitofusin (Sidarala et al., 2022). Additionally, MFN1 has been implicated in modulating cellular activity without compromising survival, as observed in POMC neurons and cardiac cells (Papanicolaou et al., 2012; Ramírez et al., 2017).

Similar to pancreatic β -cells, our findings show that MFN 1 and 2 are functionally redundant in Schwann cells, since we find profound alterations in Schwann cells when both *Mfns* were deleted, whereas no apparent defects are seen with single deletion of either *Mfn1* or *Mfn2* (Fig. 6, 7, 8, 9 and 14). This is likely because both MFN1 and MFN2 localize at contact sites between adjacent mitochondria, where they can assemble into homotypic or heterotypic complexes to mediate mitochondrial fusion. Thus, loss of function of one of the MFN could lead to formation of homotypic complexes that can still mediate mitochondrial fusion. Nevertheless, we found striking differences when we compared our double mutant mice with one mitofusin copy present. Mice haploinsufficient for *Mfn1* (MFN1^{HT}MFN2^{KO}) maintained nerve integrity better than mice haploinsufficient for *Mfn2* (MFN1^{KO}MFN2^{HT}), which presented earlier signs of demyelination and axonal loss *in vivo* (Fig. 10, 11), as shown by earlier appearance of motor deficits in functional tests, and electrophysiological recordings.

These data suggest that MFN1 have a more profound functional role in Schwann cells than MFN2. In line with this, although we do not find any structural defects in myelination in *Mfn1* or *Mfn2* single cKO mice, we do find profound differences in activation of stress pathways. Immunoblot analyses clearly show activation of metabolic and integrated stress responses, increased mitochondrial biogenesis and protective mitochondrial mechanisms regulated by HKII and LONP1 in *Mfn1* cKO mice only, whereas *Mfn2* cKO mice are barely affected. Although MFNs share around an 80% of homology, MFN1 tethers mitochondrial membranes, depending on GTP hydrolysis, more efficiently than MFN2, which corresponds to changes in the oligomeric states of the MFN-containing complex. Furthermore, purified MFN1 has been shown to exhibit around eight-fold higher GTPase activity than MFN2 (Ishihara et al., 2004). Thus, this differential GTPase activity could likely be the reason for a more predominant role of MFN1 in Schwann cell metabolic reprogramming during myelination.

5.1.2 The role OPA1 in Schwann cell metabolic reprogramming

For *Opa1* mutants, although we found similar myelination defects as in the *Mfn1/2* dKO mice, peripheral neuropathy, as examined by our sensory-motor functional tests,

electrophysiological recordings and EM analyses, developed at an earlier onset (Fig. 12, 13 and 17). Immunoblots also confirmed a stronger demyelinating phenotype and a more marked stress response (Fig. 21, 23 and 24), accompanied with a striking decrease in mtDNA content (Fig. X). These data suggest that OPA1 has a potentially more pronounced role in Schwann cell myelination than MFN1/2.

Mitochondria also undergo remodelling of internal structures such as cristae, which are dynamic infolds of the inner membrane. Cristae are critical for efficient oxidative phosphorylation and contribute to the diffusion of proteins, metabolites, and ions including Ca²⁺ (Quintana-Cabrera & Scorrano, 2023; Suomalainen & Nunnari, 2024). Therefore, it is not surprising that changes in cristae structure influence mitochondrial bioenergetics and that cristae reshaping are observed according to the cellular energetic state. OPA1, in addition to its role in mitochondrial fusion, is a master regulator of cristae remodelling by participating in cristae junction formation and maintenance, which can improve oxidative phosphorylation through stabilization of respiratory chain supercomplexes (Fry et al., 2024; Gómez-Valadés et al., 2021). Thus, it is quite likely that *Opa1* deletion affects not only mitochondrial fusion but also cristae remodelling in Schwann cells, which could exaggerate the metabolic deficits leading to the more severe myelin defects in these mice.

To assess the functional consequences of mitochondrial failure caused by *Opa1* deletion, we examined mitochondrial respiration. Under proliferative conditions, no differences were detected between control and mutant Schwann cells. However, upon induction of myelination with cAMP, control cells displayed a robust increase in all oxygen consumption rate (OCR) parameters, whereas *Opa1*-deficient cells maintained respiration at baseline levels comparable to the proliferative state (Fig. 25). As previously established, proliferating Schwann cells undergo metabolic reprogramming from glycolytic to oxidative metabolism during differentiation (Pooya et al., 2014). Our results indicate that in the absence of OPA1, Schwann cells fail to undergo this metabolic switch to OXPHOS, with their metabolic status locked in this glycolytic state. As discussed above, this undifferentiated-like state is an emergency state that Schwann cells activate for their survival by prioritizing their energy production. This state is reminiscent of a repair SC and immature SC state, which are also characterized by upregulated c-Jun levels (Arthur-Farraj et al., 2012; Della-Flora Nunes et al., 2021; Stassart et al., 2024). Collectively, these findings highlight the essential role of mitochondrial fusion and/or cristae remodelling in enabling the metabolic reprogramming required for Schwann cell differentiation.

5.2 Metabolic adaptation in Schwann cells during nerve repair

After injury, Schwann cells initiate a specialized repair program, which consists of a partly overlapping sequence of phenotypic transformations. This includes myelin autophagy, secretion of cytokines that recruit macrophages to complete myelin clearance, induction of

trophic factor expression, and morphological changes such as elongation and branching to generate regeneration tracks known as Büngner bands. Through these mechanisms, repair Schwann cells actively clear myelin debris, promote neuronal survival, support axonal regrowth, and facilitate reinnervation of target tissues. These repair cells populate the distal stump of injured nerves, where they provide guidance to regenerating axons (Bosch-Queralt et al., 2023; Jessen & Mirsky, 2019; Stassart et al., 2024; Stassart & Woodhoo, 2021).

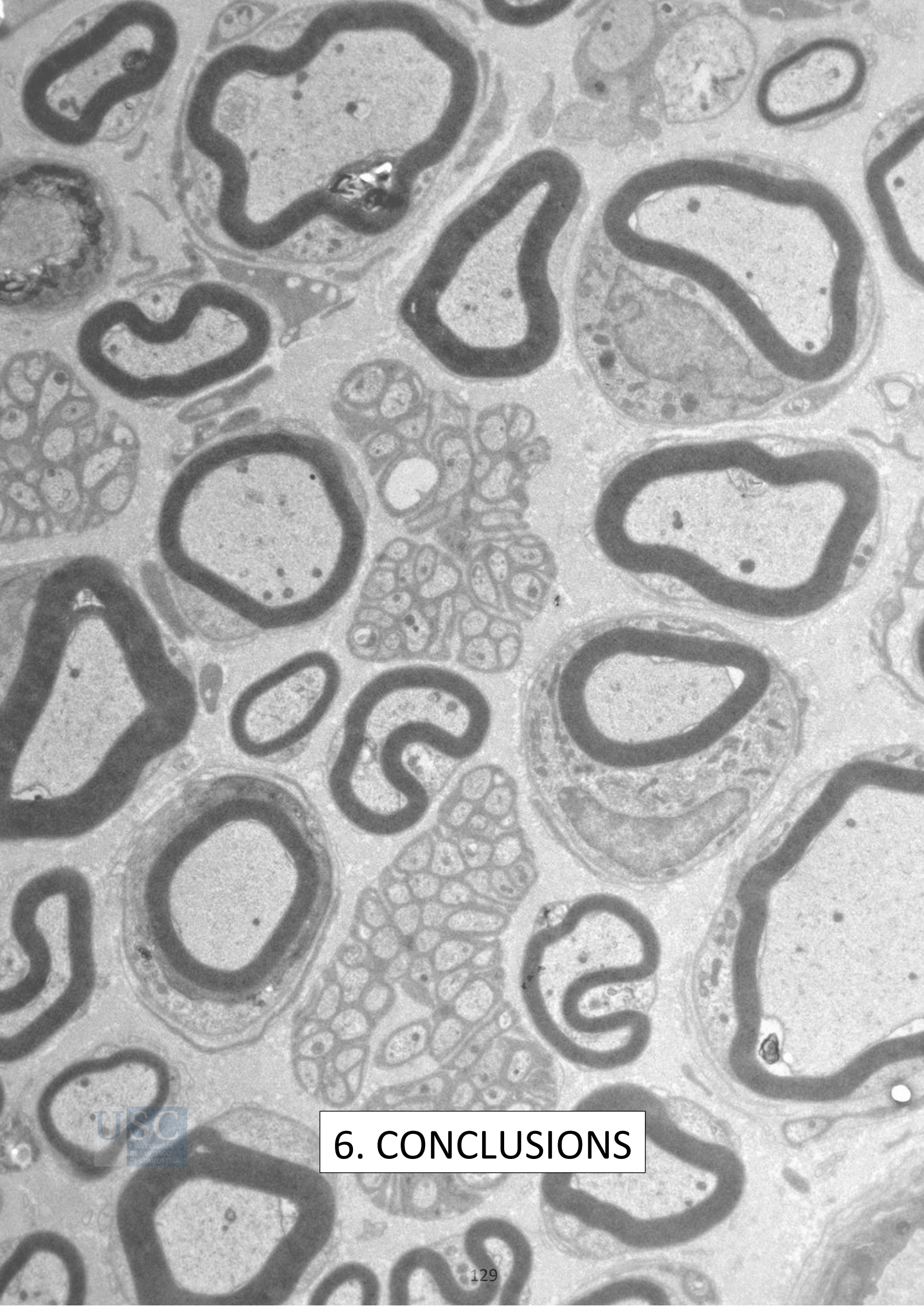
In injured nerves, Schwann cells respond with glycolytic activation immediately (within 72 h) after acute nerve injury, a period that covers the onset of Wallerian degeneration (Babetto et al., 2020), but the subsequent time course of nerve de- and regeneration (7-28 days post crush) is associated with a profound metabolic activation of repair Schwann cells, including an oxidative switch and a marked increase in mitochondrial respiration (Sundaram et al., 2023). In general, nerve de- and regeneration are characterized by catabolic autophagy and anabolic remyelination in repair Schwann cells as early and late events, respectively.

MFN1, MFN2, and OPA1 display distinct regulatory dynamics after nerve injury. MFN1 expression is strongly induced following the lesion and remains elevated only while the damage persists. In contrast, MFN2 and OPA1 are constitutively expressed in the mature myelinated state, but their levels decline after injury (Fig. 28-30). Taken together with the developmental expression profile, these findings suggest that MFN1 is associated with a less differentiated, repair-like state, whereas MFN2 and OPA1 are linked to Schwann cell maturation.

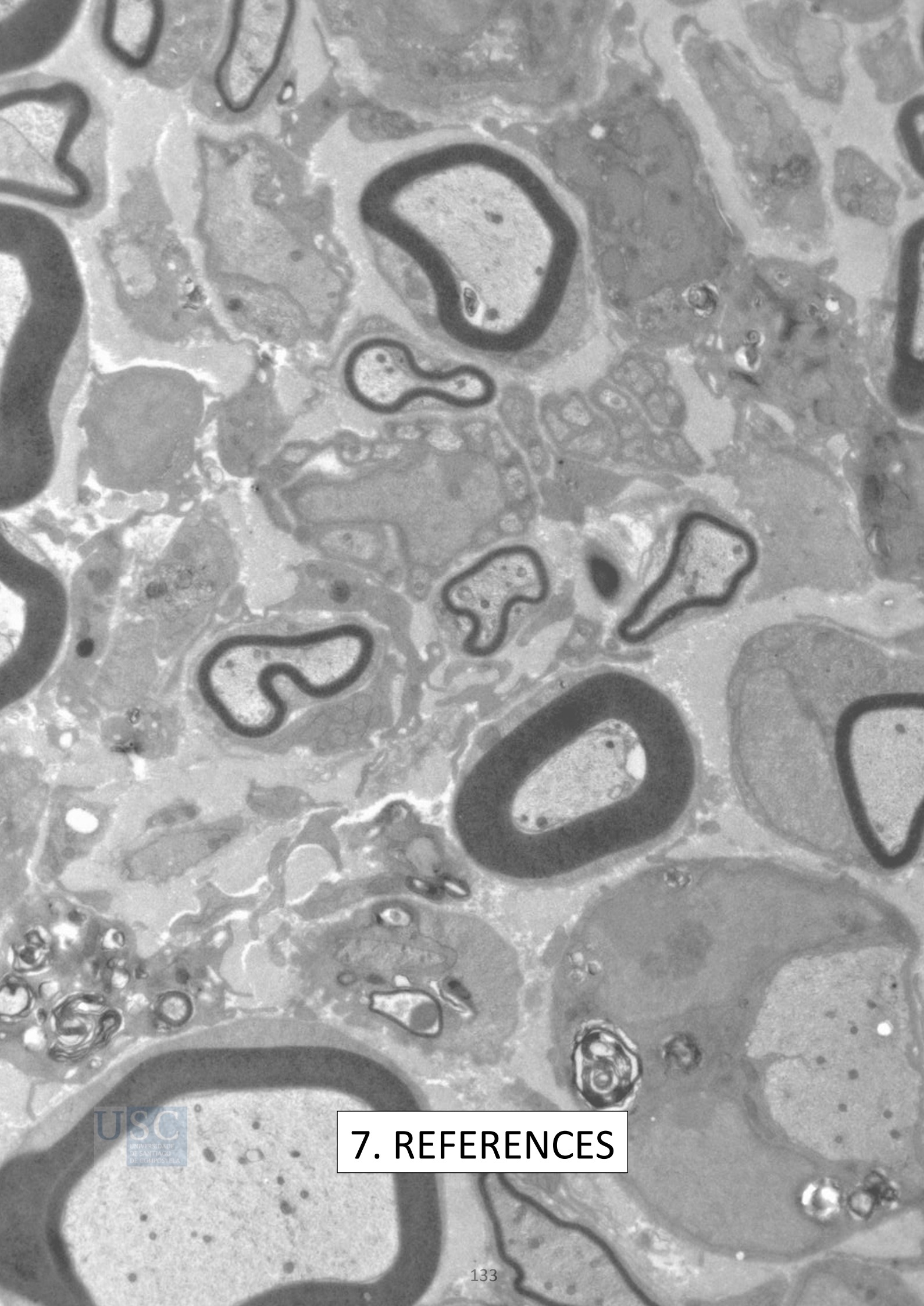
To investigate the role MFN1, MFN2, and OPA1 in nerve injury, we established the inducible models *Opa1* icKO and *Mfn1/2* idKO. Recombination was induced after 60 days, when myelin is fully matured, by tamoxifen injection. Our studies indicate that OPA1 and, MFN1 and MFN2 absence did not provoke any impact in myelin maintenance.

Myelin clearance in injured nerves occurs in two sequential phases: an initial phase in which Schwann cells initiate myelin breakdown, followed by a secondary phase largely mediated by infiltrating hematogenous macrophages through phagocytosis. Following nerve injury, Schwann cells trigger the degradation of myelin proteins and lipids primarily through autophagy, a conserved cellular process that enables the intracellular degradation of self-components (Gomez-Sanchez et al., 2015). By examining expression of the myelin proteins MPZ and MBP, we found that *Mfn2* cKO mice, as well as *Mfn1/2* idKO and *Opa1* icKO, but not *Mfn1* cKO mice, were characterized by a faster clearance of myelin, suggesting a hyperactivation of autophagy. We can only speculate that genetic ablation of these genes could lead to a faster switch to the glycolytic state after nerve injury described by Babetto and colleagues (2020), an effect, which would be consistent with our findings during developmental myelination. This could lead to a hyperactivation of autophagy that could lead to the enhanced myelin breakdown.

In contrast to developmental myelination, we did not find any functional role of OPA1 or MFN1/2 in remyelination of regenerating axons after nerve crush injury. This was surprising since nerve regeneration is associated with metabolic activation of repair Schwann cells, including an oxidative switch and a marked increase in mitochondrial respiration (Sundaram et al., 2023), as seen also in differentiating Schwann cells in developing perinatal nerves. These data suggest that mitochondria found in adult differentiating Schwann cells acquire additional mechanisms that can help compensate the metabolic deficits seen in mutant mice. Alternatively, another possibility is that the metabolic demands in generating myelin sheaths in regenerating nerves are much lower than in developing perinatal nerves, and that impairment in mitochondrial fusion and/or cristae remodelling would not necessarily lead to substantial metabolic deficiencies that would impact remyelination (Stassart et al., 2024).



1. The expression of “mitochondria-shaping proteins” MFN1, MFN2 and OPA1 is dynamically regulated during Schwann cell development and after nerve injury.
2. Genetic ablation of *Mfn1/2* or *Opa1* in Schwann cells during development results in mitochondrial alterations in both Schwann cells and axons
3. Loss of *Mfn1/2* or *Opa1* in Schwann cells leads to profound neuropathy characterized by marked myelination defects
4. In Schwann cells, MFN1 and MFN2 fulfil overlapping, functionally redundant roles during myelination
5. MFN1 exerts a greater functional role in Schwann cells than MFN2
6. Mitochondrial deficits, induced *Mfn1/2* or *Opa1* deletion, triggers compensatory stress signals in Schwann cells
7. Genetic deletion of either *Mfn1/2* or *Opa1* leads to alterations in OXPHOS components and mtDNA, compromising mitochondrial respiratory capacity.
8. OPA1 mediates the metabolic switch from glycolysis to oxidative phosphorylation necessary for Schwann cell differentiation
9. Loss of *Mfn2*, *Mfn1/2* or *Opa1* in Schwann cells accelerates myelin clearance following nerve injury
10. OPA1 and MFN1/2 do not play a major role in axonal regeneration or Schwann cell remyelination following nerve crush injury



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RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTO DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15012/2023/003

Data de inicio do expediente: 15.12.2022

Persoa solicitante: Anxo Vidal Figueroa

Procedemento: resolución de autorización

Forma de inicio: solicitude da persoa interesada

ANTECEDENTES

A persoa solicitante, como representante do Centro de Biomedicina Experimental CEBEGA, presentou con data 15.12.2022 unha solicitude para a realización do proxecto de experimentación animal (entrada no Rexistro Electrónico da Xunta de Galicia 2022/3091382), cuxos datos se detallan a continuación:

Denominación do proxecto: Mecanismos moleculares que regulan a mielinización e desmielinización de células de Schwann e patoloxías do nervio periférico

Nome do centro usuario: Centro de Biomedicina Experimental CEBEGA

Persoa responsable do proxecto: Ashwin Woodhoo

Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Centro de Biomedicina Experimental da USC (CEBEGA) (ES1507800292901)

Clasificación do proxecto: Tipo I ☐ Tipo II ☐ Tipo III ☒

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1 O Real decreto 53/2013, de 1 de febreiro (BOE 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2 O artigo 88 da Lei 39/2015, de 1 de outubro, do procedemento administrativo común das administracións públicas (BOE 236, do 2 de outubro de 2015) establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.





3 O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto realizada polo órgano habilitado, o Comité de Bioética de Experimentación Animal da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co Decreto 149/2018, do 5 de decembro, polo que se establece a estrutura orgánica da Consellería do Medio Rural e se modifica parcialmente o Decreto 177/2016, do 15 de decembro, polo que se fixa a estrutura orgánica da Vicepresidencia e das consellerías da Xunta de Galicia (DOG 235, do 11 de novembro).

De acordo con todo o indicado, RESOLVO:

1 Autorizar o proxecto solicitado.

2 O proxecto precisa someterse a unha avaliación retrospectiva aos tres anos dende o seu inicio.

3 A autorización deste proxecto terá unha duración de 5 anos e unha vez transcorrido este tempo, esta autorización deberá ser renovada.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpoñer un recurso de alzada ante o conselleiro de Medio Rural. O prazo comezará a contar dende o día seguinte ao da recepción desta resolución. Todo isto, segundo o disposto nos artigos 121 e 122 da citada Lei 39/2015.

Mediante este escrito notifícaselle a Anxo Vidal Figueroa esta resolución segundo o esixido no artigo 40.1 da antedita Lei 39/2015.

A Coruña, 17 de xaneiro de 2023

O xefe territorial

Jose Manuel Santos Maneiro





Centro de Biomedicina Experimental da
Universidade de Santiago CEBEGA
Antonio Rama, s/n
15782 Santiago de Compostela (A Coruña)

RESOLUCIÓN DE AUTORIZACIÓN DE AUTORIZACIÓN E INSCRICIÓN DUN CENTRO USUARIO DE ANIMAIS DE EXPERIMENTACIÓN

Expediente núm.: ES15012AE

Data de inicio: 9.7.2018

Persoa interesada: Anxo Vidal Figueroa

Forma de inicio: solicitude do interesado

Procedemento: resolución de autorización

ANTECEDENTES

1 A persoa interesada como representante do Centro de Biomedicina Experimental da Universidade de Santiago de Compostela, CEBEGA, presentou unha solicitude para a súa autorización e inscrición no Rexistro de centros de cría, subministradores e usuarios de animais de experimentación o 11.5.2018 a través de medios electrónicos.

2 O centro dependente da Universidade de Santiago está situado en Antonio Rama s/n, Santiago de Compostela.

3 O persoal veterinario do Servizo de Gandaría da Coruña realizou unha inspección ao citado centro o 19.6.2018 (acta núm. 68020) cun resultado favorable.

4 Este Servizo non encontra ningunha circunstancia ou requisito que puidese impedir a autorización solicitada, en canto ás competencias sectoriais en materia de gandería.

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1 O Real decreto 53/2013, do 1 de febreiro (BOE 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece nos artigos 16 e 17, a obriga de autorización e rexistro dos centros ou establecementos de cría, subministración ou uso de animais de experimentación. Nesta norma detállanse os requisitos relativos ao aloxamento e coidado dos animais albergados, así como outras obrigas no ámbitos dos movementos, identificación e rexistro destes. Tamén se detallan outros aspectos de carácter funcional dos centros e os requisitos específicos que afectan ao seu persoal.

2 O Decreto 296/2008, do 30 de decembro (DOG núm. 11, do 16 de xaneiro) polo que se regula a utilización de animais para experimentación e outros fins científicos, incluída a docencia e se crea o Rexistro dos centros de cría, de subministradores e usuarios e a Comisión Galega de Benestar dos Animais de Experimentación, establece no seu artigo 4 a creación deste rexistro, que queda estruturado nas seccións de centros de cría, centros subministradores e centros usuarios. No artigo 5, a obriga de que todos os centros de cría, subministración e usuarios de animais de experimentación localizados na Comunidade Autónoma de Galicia estean inscritos no citado rexistro.





3 O artigo 36.1 da Lei 8/2003, do 24 de abril, de sanidade animal (BOE núm. 99, do 25 de abril de 2003), establece que as explotacións de animais de nova instalación ou a ampliación das existentes, deberán de dispoñer da previa autorización da autoridade competente.

4 O artigo 3.8. do Real decreto 479/2004, polo que se establece e regula o Rexistro xeral de explotacións gandeiras (BOE núm. 89, do 13 de abril de 2004), establece que unha vez obtidos todos os permisos, autorizacións ou licenzas esixibles pola normativa vixente, ningunha nova explotación poderá iniciar a súa actividade sen estar rexistrada e ter recibido o correspondente código de identificación.

5 O artigo 88 da Lei 39/2015, do 1 de outubro, do procedemento administrativo común das Administracións Públicas (BOE núm. 236, do 2 de outubro de 2015) establece que a resolución que poña fin ao procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

6 O Servizo de Gandaría da Coruña revisou a documentación achegada por Anxo Vidal Figueroa, en representación do Centro de Biomedicina Experimental da Universidade de Santiago de Compostela CEBEGA e o informe elaborado polo persoal veterinario do Servizo de Gandaría da Coruña.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co artigo 11 do Decreto 245/2009 de 30 de abril, polo que se regulan as delegacións territoriais da Xunta de Galicia (DOG 84, do 1 de maio de 2009) e o Decreto 166/2015, de 13 de novembro, polo que se aproba a estrutura orgánica da Consellería do Medio Rural (DOG 220, do 18 de novembro de 2015).

De acordo con todo o indicado, RESOLVO:

Autorizar e acordar a inscrición solicitada polo centro no Rexistro de centros de cría, subministradores e usuarios de animais de experimentación, integrado no Rexistro xeral de explotacións gandeiras (Rega):

Razón Social: Universidade de Santiago de Compostela, Centro de Biomedicina Experimental CEBEGA

NIF: Q1518001A

Enderezo: Antonio Rama, s/n, Santiago de Compostela

Código Rega: ES150780292901

Este centro queda suxeito ao disposto nas disposicións vixentes en materia de protección animal en animais de experimentación, así como en materia de sanidade, identificación animal e outros aspectos competencia da Consellería de Medio Rural e ás seguintes CONDICIÓNs:

- A autorización ten unha validez de 10 anos a partir da data desta resolución, polo que unha vez transcorrido ese prazo deberá solicitar a súa renovación.
- A clasificación e rexistro unicamente son válidos para o solicitante e ampara o centro na situación actual, tal como figura no expediente. Ante calquera cambio significativo da estrutura ou actividades do centro que poida ter efectos negativos sobre o benestar dos animais, ou a cría, subministración ou uso de especies animais distintas das que constan á data de autorización, deberá solicitar a confirmación da súa autorización ao Servizo de Gandaría da Coruña.
- Igualmente, deberán notificar ao Servizo de Gandaría, no prazo dun mes desde que se produza, a modificación dos datos que figuren inscritos no rexistro derivada de ampliacións, reducións, traslados, cambios de persoal ou outras circunstancias, así como a suspensión, cesamento da actividade e cambio de titularidade.
- Deberán comunicar a este Servizo os proxectos que teñan previstos realizar, presentando o modelo de solicitude correspondente e a documentación requirida que consta nel.





- Deberán levar un rexistro de control dos animais, no que deberán anotar os datos que figuran no anexo VI do Real decreto 53/2013, do 1 de febreiro.

- A explotación está obrigada á comunicación do censo anual de animais que albergue, sempre antes do 1 de marzo e referido ao censo que tivera a 31 de decembro do ano anterior.

Esta autorización e rexistro non exime da obriga de obter as demais autorizacións sectoriais e permisos que poidan esixirse.

A cancelación da inscrición no rexistro terá lugar por solicitude do titular ou de oficio, cando se produzan incumprimentos do establecido na normativa ou cando se comprobe que o centro cesou a actividade.

Contra a presente resolución, que non lle pon fin á vía administrativa, poderá interpoñer un recurso de alzada ante a conselleira de Medio Rural no prazo dun mes que comezará a contar dende o día seguinte ao da recepción desta resolución. Todo isto, segundo o disposto nos artigos 121 e 122 da citada Lei 39/2015.

Mediante este escrito notifícaselle ao Centro de Biomedicina Experimental CEBEGA da Universidade de Santiago de Compostela esta resolución segundo o esixido no artigo 40.1 da antedita Lei 39/2015.

A Coruña, 9 de xullo de 2018

A xefa territorial

PA: a xefa do Servizo Xurídico-Administrativo

Ofelia Rivas Vázquez





Schwann cells are specialized glial cells of the peripheral nervous system that elaborate myelin sheaths along large diameter axons to potentiate fast conduction of action potentials and provide vital trophic and metabolic support to axons. Recent studies have shown that Schwann cells undergo metabolic reprogramming to execute the bioenergetic and biosynthetic demands of differentiation and repair during development and after nerve injury. In this study, we have delineated the functional and molecular role of mitochondrial fusion and cristae remodelling by targeting specific key genes (*Mfn1*, *Mfn2* and *Opa1*) using a variety of genetic models. In summary, we demonstrate that mitochondrial plasticity plays an essential role in the formation of peripheral myelin during development but a minimal one in injury response.