UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Investigating the Biological Role of PACAP, VIP and their Receptors in the Central Nervous System – Implications for Multiple Sclerosis

by Margo Jansen

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of Alessandro Castorina
co-supervisor: Alen Faiz

University of Technology Sydney
Faculty of Science

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Certificate of Original Authorship

I, *Margo Jansen* declare that this thesis, is submitted in fulfilment of the requirements for the award of *Doctor of Philosophy*, in the *Faculty of Science* at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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COVID-19 Impact Statement

Unfortunately, I commenced my PhD during the turbulent time of the COVID-19 pandemic. When I started my enrolment on the 31st of May 2021, life was slowly getting back to normal post the initial lockdown period and the first peak of the pandemic. However, from the end of June until October 2021, the lab was closed during the next wave of COVID-19 infections. This government health order resulted in significant disruptions during the first six months of my PhD, as I was unable to get training for the practical laboratory skills needed for my project. During this time, I focussed on developing my writing skills and was able to write two literature reviews included in my thesis. Additionally, I started teaching myself bioinformatics during this period and have been able to become skilled in R-based programming.

Despite the obstacles caused by the disruptions at the start of my PhD, the produced body of work in this thesis was possible due to the support of my supervisor, help of a previous PhD student Dr. Sarah Thomas Broome teaching me some of the protocols used in the lab, and the encouragement of my partner and family who have always motivated me to pursue my research passions.

Thesis format statement

I hereby declare that this thesis is submitted in the format of a 'thesis by compilation'.

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Abbreviation list

ACTH = adrenocorticotrophic hormone

ALS = Amyotrophic lateral sclerosis

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APC = antigen presenting cell

APC or CC1 = Quaking 7

AQP4 = aquaporin 4

ASPA = aspartoacylase

BBB = blood brain barrier

BCA = Bicinchoninic-Acid

BD = bipolar disorder

BDNF = brain derived neurotrophic factor

BNST = bed nucleus of the stria terminalis

Ca^{2+} = Calcium

Camk2a = Calcium/calmodulin-dependent protein kinase type II subunit alpha

cDNA = complementary DNA

CGRP = calcitonin gene-related peptide

cKO = conditional knock out

CNS = Central nervous system

CPZ = Cuprizone

CR3 = complement-receptor 3

CREB = cAMP-responsive element binding protein

CSF = cerebrospinal fluid

CxCL = C-X-C motif chemokine ligand

DAMPs = damage-associated molecular patterns

DAP12 = DNAX-activating protein of 12 kDa

DISC1 = disrupted-in-schizophrenia 1

DMT = disease modifying therapy

EAE = Experimental autoimmune encephalomyelitis

EBV = Epstein Barr virus

ECM = extracellular matrix

EPSP = excitatory postsynaptic potential

GAD1 = glutamate decarboxylase 1

GAD2 = glutamate decarboxylase 2

GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

Gal3 = Galectin-3

GFAP = glial fibrillary acidic protein

GPCR = G protein-coupled receptors

HLA = Human Leukocyte Antigen

HMGB1 = high mobility group protein B1

IBA1 = ionised calcium-binding adapter molecule 1

IFN = interferon

IL = interleukin

iNOS = inducible nitric oxide synthase

iPSC = inducible pluripotent stem cells

ISH = in situ hybridisation

IR = immunoreactivity

JAK = Janus kinase

KO = knockout

LFB = Luxol fast blue

LPS = lipopolysaccharide

LTD = long term depression

LTP = long term potentiation

MAX = Maxadilan

MBP = myelin basic protein

MHC = major histocompatibility complex

MIF = macrophage migration inhibitory factor

mitoCFP = mitochondria Cyan Fluorescent Protein

MMP9 = matrix metalloproteinase 9

MOA = mechanism of action

MOG = myelin oligodendrocyte protein

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRI = magnetic resonance imaging

MS = Multiple sclerosis

MyD88 = myeloid differentiation primary response 88

NAWM = normal-appearing white matter

NF-L = neurofilament light chain

NMDA = N-methyl-D-aspartate

NMOSD = Neuromyelitis Optica Spectrum Disorders

nNOS = neuronal nitric oxide synthase

NRG1 = neuregulin-1

NPY = neuropeptide Y

NSC = neural stem cells

OFT = open Field test

Olig2 = oligodendrocyte transcription factor 2

OL's = Oligodendrocyte lineage cells

OLT = object location task

OPCs = oligodendrocyte progenitor cells

OxPCs = oxidised phosphatidylcholines

PACAP = pituitary adenylate cyclase activating polypeptide

PAC1 = pituitary adenylate cyclase activating polypeptide receptor 1

PAMPs = pathogen-associated molecular patterns

PBS = phosphate buffered saline

PDL = poly-D-Lysine

PFA = paraformaldehyde

PKC = protein kinase C

PLP1 = proteolipid protein 1

PNS = peripheral nervous system

RA = rheumatoid arthritis

RIPA = radioimmunoprecipitation assay

ROI = region of interest

ROS = reactive oxygen species

RRMS = Relapse-remitting multiple sclerosis

RT = room temperature

RT-qPCR = real-time quantitative polymerase chain reaction

S1P = sphingosine-1-phosphate

SIRP-a = signal regulatory protein-a

SPMS = Secondary progressive multiple sclerosis

SRA = scavenger receptor-AI/II

Std chow = standard chow

Syk = spleen tyrosine kinase

Syph = synaptophysin

TGF = transforming growth factor

Thy1 = Thy-1 Cell Surface Antigen

TLR = toll-like receptor

TNF-α = Tumour necrosis factor alpha

TREM2 = triggering receptor expressed by myeloid cells 2

tPA = tissue plasminogen activator

TUJ1 = class III beta-tubulin

uPA = urokinase plasminogen activator

VIP = Vasoactive intestinal peptide

VISTA = V-type immunoglobulin domain-containing suppressor of T-cell activation

VPAC1 = Vasoactive intestinal polypeptide receptor 1

VPAC2 = Vasoactive intestinal polypeptide receptor 2

YFP = yellow fluorescence protein

Publications

Publications arising from thesis

[+] denotes joined first author

Under review

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Jansen MI[+], Thomas Broome S[+], Castorina A. Targeting the neurological comorbidities of multiple sclerosis: the beneficial effects of VIP and PACAP neuropeptides. *Journal for Integrative Neuroscience.* **2022**; 21(1):033. https://doi.org/10.31083/j.jin2101033

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Other publications

Jansen MI, Castorina A. Identification of Key Genes and Regulatory Pathways in Multiple Sclerosis Brain Samples: A Meta-Analysis of Micro-Array Datasets. *International Journal of Molecular Sciences*. **2023**; 24(11), 9361. https://doi.org/10.3390/ijms24119361

Lee J, Thomas Broome S, **Jansen MI**, Mandwie M; Logan GJ, Marzagalli R, Musumeci M, Castorina A. Altered Hippocampal and Striatal Expression of Endothelial Markers and VIP/PACAP Neuropeptides in a Mouse Model of Systemic Lupus Erythematosus. *International Journal of Molecular Sciences.* **2023**. *24*(13), 11118. https://doi.org/10.3390/ijms241311118

Piper JA, Al Hammouri N, **Jansen MI**, Rodgers KJ, Musumeci G, Dhungana A; Ghorbanpour SM, Bradfield LA, Castorina A. L-Proline Prevents Endoplasmic Reticulum Stress in Microglial Cells Exposed to L-azetidine-2-carboxylic Acid. *Molecules*. **2023**; *28*(12), 4808. https://doi.org/10.3390/molecules28124808

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Conference presentations

1. MS Frontiers UK– Invited Flash Talk (2024)
2. MS Frontiers UK– Presentation (2024)
3. Presentation at Federation of European Neuroscience Societies (FENS) (2024)
4. UTS student seminar night – *Invited Talk* (2024)
5. Presentation at 41th Annual Scientific Meeting Australasian Neuroscience Society (ANS) (2023)
6. Oral Presentation – Australian Society for Medical Research (ASMR) NSW Annual Scientific Meeting (2023)
7. Presentation at 40th Annual Scientific Meeting Australasian Neuroscience Society (ANS) (2022)

Awards & Prizes

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2. UTS Vice Chancellor's Conference Fund 2024 – Travel fund to attend the Federation of European Neuroscience Societies (FENS) 2024 in Vienna, Austria & MS Frontiers in Liverpool, United Kingdom conferences

3. 3MT™ Faculty Heats – Winner People's Choice Award *(*2023)
4. UTS Vice Chancellor's Conference Fund 2023 – Travel fund to attend the Australasian Neuroscience conference in Brisbane, Queensland

5. Best Oral presentation - International Student Congress Of (bio)Medical Sciences (ISCOMS) (2022)

Professional Experience

1. Industry Mentoring Network in STEM (IMNIS) initiative Participant (Sep-2022 – Sep-2023)
2. Researcher Exchange and Development within Industry (REDI) Mentee (Sep-2022 – Sep-2023)
3. NSW Representative Australasian Neuroscience Society, Student Committee (Jan-2022 – Jan-2024)
4. Mentor Honours students, University of Technology Sydney peer mentoring programme (Sep-2022 – ongoing)

Abstract

Multiple sclerosis (MS) is a chronic autoimmune and neurodegenerative disease of the central nervous system (CNS), characterized by demyelination and persistent neuroinflammation in the brain and spinal cord resulting in neuronal damage. As the exact cause of MS is unknown, there is currently no cure for the disease and available treatments are only able to delay disease progression. The complex interplay between neuroinflammation, white matter loss and axonal damage is considered a driving force of MS disease development. Therefore, disease-modifying therapies that target these three main contributors to MS pathology could potentially halt disease progression and/or reduce symptom severity.

The pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) neuropeptide system is associated with anti-inflammatory and neuroprotective signalling and shows beneficial effects in MS-associated peripheral inflammation. However, we currently lack in-depth knowledge of the role of neuropeptide system in the CNS within the context of MS. To address this gap, we explored the involvement of the PACAP/VIP system in the CNS, utilising post-mortem samples of people with MS and animal models of disease.

In post-mortem patient brain tissue, a disease subtype-specific dysregulation of the PACAP/VIP system was observed, with a striking decrease in the PACAP-preferred receptor PAC1 in rare primary progressive MS patients. Since a decrease in PAC1-mediated signalling is associated with inflammation and reduced neuroprotection, we next sought to examine if targeting the PACAP/VIP system *in vivo* could protect against CNS damage induced by the cuprizone model of MS. We found that targeting the PAC1 receptor specifically could prevent white matter loss, reduce astro- and microgliosis as well as decrease axonal damage, suggesting a broad protective effect of PAC1-mediated cellular signalling under demyelinating conditions. In turn, selectively removing PAC1 from a subset of neurons impaired neuronal functioning both under healthy and demyelinating conditions. Interestingly, removing neuronal PAC1 decreased the number of mature oligodendrocytes in the CNS, suggesting PAC1 may mediate neuron-oligodendrocyte communication. To explore this further, we generated a PAC1 conditional knockout model, allowing for inducible deletion of PAC1 in myelinating oligodendrocytes. Thus, offering new avenues for investigating PAC1 in oligodendrocytes specifically.

In conclusion, my findings suggest an important role for the PAC1 receptor in healthy CNS functioning and support the notion of targeting PAC1 as a potential therapeutic avenue in MS through its neuroprotective and immunomodulatory function.

Chapter 1

General Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic neurological disease of the central nervous system (CNS) characterized by the formation of lesions in the brain and spinal cord, resulting in inflammation, demyelination and axonal degeneration[1,2]. MS mainly affects people between the ages of 20 and 40 years old, with women being twice as likely to develop the disease compared to men[3]. Globally, cases of MS are rising with an estimated 2.8 million people currently living with the disease[3].

Symptoms of MS encompass a broad range of afflictions, including motor dysfunction, sensory disturbances, vision problems and cognitive impairment[2]. This heterogeneous clinical presentation of the disease makes diagnosis difficult, with diagnostic delay being linked to a worse disability score[4]. However, diagnosis time has been greatly reduced with recent revisions in the diagnostic criteria[5].

Clinically and traditionally, MS can be classified into three major subtypes of disease[6]. Relapse-remitting MS (RRMS) is the most common type of MS, with around 85% of patients receiving this diagnosis (Figure 1A). RRMS is characterized by episodes of disease worsening (relapse) followed by periods of disease recovery (remission)[7]. This often-incomplete recovery results in a slow stepwise worsening of symptoms. At some point, these periods of recovery will cease, and the disease takes on a more progressive nature. This is defined as secondary-progressive MS (SPMS; Figure 1B). Some patients do not experience episodes of recovery from disease onset, with symptoms steadily progressing. This subtype of MS is named primary-progressive MS (PPMS; Figure 1C)[8]. Around 10 to 20% of patients receive this diagnosis.

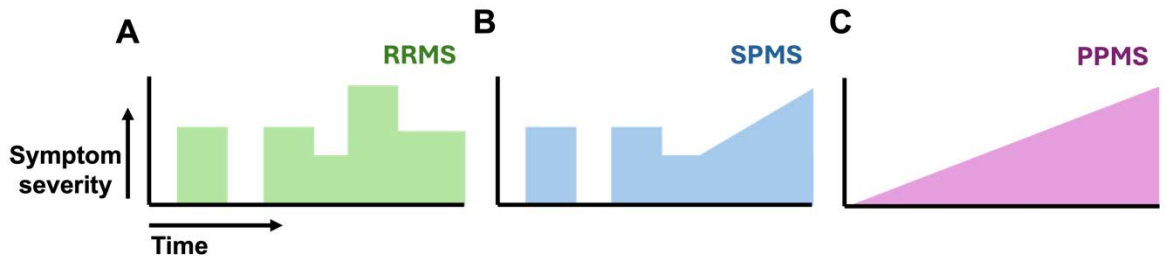

Figure 1.1 Schematic overview of multiple sclerosis clinical subtypes. *Schematic representation of clinical course for (A) relapse-remitting MS, (B) secondary-progressive MS and (C) primary-progressive MS. RRMS = relapse-remitting multiple sclerosis, SPMS = secondary-progressive multiple sclerosis, PPMS=primary-progressive multiple sclerosis*

However, in recent years there has been growing emphasis within the MS field to start considering the disease as a biological continuum rather than strictly adhering to discrete clinical classifications[9]. To elaborate, researchers argue that the underlying disease process of MS does not necessary reflect the patient's clinical trajectory. By solely characterising the disease based on clinical presentation you might hinder in optimal clinical care decisions and novel therapy development. For example, inflammation was initially marked as the starting point of MS, followed by neurodegeneration and myelin loss. However, we now know that neurodegeneration can occur at the same time as early signs of inflammation, as well as several other disease-inducing and compensatory mechanism that all contribute to clinical presentation [10,11]. Therefore, by considering a more holistic approach to MS disease development, more accurate clinical predictions could be made in future [9,12].

1.1.1 Aetiology of Multiple sclerosis

1.1.1.1 Environmental influence

MS disease aetiology is driven by a complex combination of genetic and environmental factors. Researchers quite early on noticed a geographical link between latitude and the frequency of MS cases. There is an increased prevalence of MS in regions farther from the equator and a lower risk closer to it[13,14]. This effect has been shown in Australia[15], New-Zealand[16], Europe[17], the US[18] and Japan[19]. Although some studies found no or an inverse relationship between latitude and MS prevalence, recent more comprehensive papers were able to find a significant correlation between latitude and MS prevalence[20-23]. This correlation gave rise to two main hypotheses on the type of environmental factors that could cause the development of MS.

One leading hypothesis on environmental factors and MS risk describes the impact of vitamin D_3 deficiency and MS. Exposure to sunlight, which is our main source of vitamin D_3, facilitates the conversion of 7-dehydrocholesterol into previtamin D_3. This was found to be inversely correlated to risk of MS[24,25]. Additionally, high serum levels of vitamin D are associated with a decreased risk of developing multiple sclerosis [26]. Fatty fish intake, high in vitamin D_3, might also reduce risk of MS[27]. However, several clinical trials have found no beneficial effect of vitamin D_3 supplementation on relapse rate in MS patients [28-30].

Another environmental risk factor for MS is Epstein-Barr virus (EBV) infection[31]. Around 90% of the world's population has been infected with this herpes virus, which can persist in a latent form in B-lymphocytes throughout the person's life[32]. Several studies have revealed a link between EBV and MS disease development. For example, a longitudinal study from Bjornevik *et al.* (2022) found a 32-fold increase in MS risk after EBV infection but not any other viral infection[33]. In fact, is it rare to be considered EBV-negative and also develop MS[34]. However, a direct explanation as to how EBV directly contributes to MS development is still unknown and several arguments challenge a direct causal role between EBV and MS.

First, EBV infection is near universal with a 90% infection rate in the global population as mentioned above, yet MS remains rare, implying that additional genetic or environmental factors are necessary for disease onset [32]. Secondly, while MS patients often exhibit abnormal EBV-specific immune responses, it is unclear whether this is a cause or consequence of broader immune dysfunction. Especially since other pathogens, including human herpesvirus 6 and human endogenous retroviruses, have also been implicated in MS [35,36]. Moreover, reports on the presence of EBV in the brain are inconsistent. While one study showed the presence of EBV lytic protein BZLF-1 in MS lesions, other studies have been unable to detect the presence of EBV in the MS brain[37,38,39]. Thus, aberrant viral control through a dysregulated immune response could be the factor that contributes to MS development, tying EBV infection to other environmental risk factors such as age and gender, and genetic susceptibility together [40]. So take together, while EBV may contribute to MS, its role as a primary trigger remains unproven, warranting further investigation into alternative and complementary disease mechanisms [31].

1.1.1.2 Genetic susceptibility

In addition to the clear importance of environmental factors influencing MS risk, several genetic associations have been described as well. To date, the strongest genetic link with MS is the human leukocyte antigen (HLA) gene cluster on chromosome six, which explains up to 10.5% of genetic variance underlying disease development[41]. HLA genes encode heterodimeric membrane glycoproteins that play a crucial role in antigen presentation and immune response, distinguishing between intracellular non-self-antigens (Class I HLA) and extracellular peptides (Class II HLA)[42]. Specifically, the Class II HLA-DRB1*15:01 allele is the strongest genetic risk for MS[43,44]. Despite it being unclear how this genetic cluster contributes to MS, a link

between HLA-DRB1*15:01 and EBV has been described, linking the proposed genetic and environmental risk factors together[45].

1.1.2 Introduction to pathogenesis of MS

The CNS has two main types of resident cells. Neurons, the signal relaying cells, are able to transduce electrical impulses through our body facilitating all biological functioning. The other main cell type of the CNS are glial cells, comprising of astrocytes, microglia and oligodendrocytes. Glial cells are the supporting cells of the brain, aiding in important functions such as homeostasis maintenance, signal transduction and response to inflammatory insults[46,47].

Oligodendrocytes produce myelin, the insulating sheath around axons. It is this myelin that gets damaged in MS. Where initially researchers believed MS was mostly defined by white matter pathology, it is now well-established that the CNS grey matter is also affected by the disease[48-50]. Given the strong inflammatory component of MS, research has focussed on this component in the last few decades. Giving rise to the commonly accepted "outside-in" theory that describes MS pathogenesis as an autoimmune inflammatory disorder where aberrant peripheral T- and B-cells infiltrate and attack the CNS, targeting myelin in particular[51,52]. This in turn causes the formation of multifocal lesions in the brain and spinal cord, characterized by further peripheral immune-cell infiltration, neuroinflammation, demyelination and axonal damage[53]. This theory has been the leading hypothesis in the field; however, a newly emerging hypothesis has gained significant status in recent years. Opposed to peripheral immune cell infiltration into the brain as a starting point for the disease, the "Inside-out" paradigm argues how the disease starts in the brain, with peripheral immune cell infiltration being secondary to the main pathological trigger[54].

To provide a thorough and comprehensive background on the pathogenesis of MS I have published a literature review article titled: 'Exploring the Pro-Phagocytic and Anti-Inflammatory Functions of PACAP and VIP in Microglia: Implications for Multiple Sclerosis.' This article, published in 2022 in the International Journal of Molecular Sciences (IF: 4.9), provides a detailed overview of the role of the immune system in MS and vice versa, the impact of demyelination in MS. Moreover, it highlights the interaction between neuroinflammation

and myelin debris engulfment in the central nervous system as an important therapeutic target to investigate.

In addition to examining MS and its pathogenesis, my review paper introduces the main neuropeptide system I will be studying in this thesis. The neuropeptides pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are part of a widely expressed neuropeptide signalling system that, as I will discuss in my review article, have both neuroprotective and anti-inflammatory properties. In the review article, I explore the roles of these neuropeptides in the CNS and highlight their therapeutic potential in MS. The review paper emphasises the role of these neuropeptides and their downstream receptors; type I adenylate cyclase activating polypeptide receptor (PAC1), vasoactive intestinal peptide receptor 1 (VIPR1), and VIPR2 in these effects and set the scene for my thesis where I will be exploring this neuropeptide system in the context of MS in great detail.

1.1.3 Exploring the Pro-Phagocytic and Anti-Inflammatory Functions of PACAP and VIP in Microglia: Implications for Multiple Sclerosis.

This section is published as:

Jansen MI, Thomas Broome S, Castorina A. Exploring the Pro-Phagocytic and Anti-Inflammatory Functions of PACAP and VIP in Microglia: Implications for Multiple Sclerosis. *International Journal of Molecular Sciences*. 2022; 23(9):4788. https://doi.org/10.3390/ijms23094788

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Signature of co-authors:

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Alessandro Castorina	Production Note: Signature removed prior to publication.

Work in this publication describes the role of the VIP/PACAP system in mediating neuroinflammation, focussing on its impact on microglia functioning.

Exploring the Pro-Phagocytic and Anti-Inflammatory Functions of PACAP and VIP in Microglia: Implications for Multiple Sclerosis

Margo I. Jansen[1], Sarah Thomas Broome[1], Alessandro Castorina[1]

1. Laboratory of Cellular and Molecular Neuroscience (LCMN), School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW 2007, Australia

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Abstract

Multiple sclerosis (MS) is a chronic neuroinflammatory and demyelinating disease of the central nervous system (CNS), characterised by the infiltration of peripheral immune cells, multifocal white-matter lesions, and neurodegeneration. In recent years, microglia have emerged as key contributors to MS pathology, acting as scavengers of toxic myelin/cell debris and modulating the inflammatory microenvironment to promote myelin repair. In this review, we explore the role of two neuropeptides, pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP), as important regulators of microglial functioning during demyelination, myelin phagocytosis, and remyelination, emphasising the potential of these neuropeptides as therapeutic targets for the treatment of MS.

Keywords: pituitary adenylate cyclase-activating polypeptide (PACAP); vasoactive intestinal peptide (VIP); microglia; phagocytosis; anti-inflammatory; multiple sclerosis; inflammation

1. Introduction

Multiple sclerosis (MS) is a debilitating disease affecting about 2.8 million people globally [3]. The disease is characterized by myelin damage due to an abnormal attack by immune cells, resulting in the appearance of white matter lesions in the central nervous system (CNS), neurodegeneration and focal neuroinflammation, which can lead to physical as well as neurological deficits[1,2,55,56]. There is currently no cure for MS, and the existing treatment options for MS patients primarily target the dysfunctional immune response, mainly by promoting the segregation of immune cells in peripheral compartments, thereby reducing the overall autoimmune damage to the CNS[57].

Microglia, the resident macrophages of the CNS, have emerged as key players in MS pathogenesis, with growing research interest focussed on unveiling new mechanisms to modulate some of its biological functions[58]. In MS, microglia demonstrate dichotomous profiles, as they exert either detrimental or beneficial effects[59-62]. Microglia are by nature immune modulatory cells, and respond with complex activation patterns to stress signals, either by facilitating the release of pro- and anti-inflammatory cytokines, chemokines, as well as by providing trophic support to neurons and/or scavenge toxic debris through phagocytosis[63,64].

In this review, we explore the role of microglia in MS, with specific emphasis on recent findings highlighting the critical role of these glial cells in myelin-debris clearance and anti-inflammatory properties in the CNS. More specifically, this work aims to highlight the role of two neuropeptides, pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP), as key regulators of microglial responses during CNS demyelination, portraying the potential of these molecules to become therapeutic targets for MS.

2. Multiple sclerosis

2.1. Autoimmunity in MS

MS is a complex, multifactorial disease with unknown origin, although bulk evidence suggests that disease onset might be triggered by a combination of contributing risk factors such as certain lifestyle habits (i.e. smoking), diet, genetic and environmental causes[65,66]. The exact pathogenesis of MS is also yet to be determined; however, it is widely accepted that most of the pathological events in MS patients are consequent to the infiltration of autoreactive peripheral immune cells into the CNS, with auto-reactive T cells being pivotal in promoting central demyelination[1,55,67]. Auto-reactive T lymphocytes are recruited to the CNS and cross the disrupted blood brain barrier (BBB). These immune cells – mostly CD4+ and CD8+ T cells – are believed to express myelin-specific surface antigens, hence are able to target myelin and

oligodendrocytes (OLs), the myelin-producing cells of the CNS, causing demyelination and OL depletion[67-70]. Demyelination results in the generation of myelin debris which in turn can get oxidised, creating oxidised phosphatidylcholines (OxPCs), here referred to as toxic myelin debris, that can have detrimental effects on cellular functioning[71-73]. When this occurs, microglia attempt to clean "the aftermath" by engulfing the toxic myelin debris[60,74,75]. Under these inflammatory conditions, these microglia are also believed to further stimulate the activation of the infiltrated immune cells due to their antigen presenting cells (APCs) properties and via the release of inflammatory factors. This activates a vicious cycle that leads to further infiltration of peripheral immune cells (CD4+ and CD8+ T-cells, macrophages, B-cells) and damage to the CNS white matter[76-78]. Altogether, these data pinpoint how the uncoordinated healing response of resident glia to an immune-mediated myelin attack can exacerbate the autoimmune response, leading to the formation (or expansion) of white matter lesions.

However, it is important to note that most of the data concerning the involvement of autoimmunity in MS has been gathered from pre-clinical models, such as the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. The EAE model is broadly used to model MS since it mimics certain aspects of the pathology, particularly its inflammatory component[79]. However, there are some essential differences with human MS, such as the lymphocyte subpopulations that are predominantly found in EAE vs. MS (CD4+ T-cells vs. CD8+ T cells, respectively), as well as the limited demyelination seen in these mice[79-81]. Nonetheless, although the model may not recapitulate the entire spectrum of pathological changes seen in MS, the EAE model has helped the scientific community gain essential insights on the role of autoimmunity and neuroinflammation in MS.

2.2. Demyelination in MS

Another major pathological hallmark of MS is the progressive loss of myelin in the CNS and OL cell loss. Myelin is necessary to provide insulation to axons in the brain and spinal cord, allowing proper conduction of electric impulses along the axons[82]. In healthy conditions, CNS myelin is produced by mature/fully-differentiated OLs, whereas OL precursor cells (OPCs) are a proliferative group of progenitor cells that can differentiate into mature OLs, allowing both adaptive myelination and the regeneration of myelin following injury or disease[83,84]. During MS, in addition to myelin loss, there is a remarkable depletion in the pool of both OPCs and mature OLs, which hinders the process of remyelination, the main cause of axonopathy and consequent neuronal loss[62,85-87]. In fact, it is the resulting neurodegeneration and the increased

lesion load, not demyelination (at least not directly), that correlates with the severity of disease-related disability[88,89].

Neurodegeneration in MS is not solely linked to myelin loss: it is also exacerbated by the hyper-inflammatory state of the CNS[11,90]. However, the notion that neurodegeneration in MS is always preceded by inflammation has been challenged in the field[91,92]. Since limited remyelination in the MS brain is considered a major contributor to lesion development, a "hot topic" in recent years has been to elucidate the molecular mechanisms underlying the de- and remyelination process within the CNS[93-95]. Both processes are often studied in mice using the cuprizone model. By feeding mice a diet supplemented with 0.2-0.3% of the copper chelator cuprizone [oxalic acid bis(cyclohexylidene hydrazide)] for more than three weeks, remarkable oligodendrocyte death and subsequent demyelination of the CNS can be induced in a reversible manner, allowing to study both the de- and remyelination process of the brain[96].

2.3 Microglia in MS

2.3.1. Role of microglia in MS

MS is characterised by both demyelination and extensive neuroinflammation, although the causal relationship between these two has yet to be determined. A crucial cell-type involved in determining the fate of both processes is microglia. In the CNS, microglia constantly survey the surrounding microenvironment, allowing for a rapid response upon the detection of a certain stressor (e.g. exposure to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs))[63,64,97,98]. Initially, the activation pattern of microglia was thought to be compartmentalised into two distinct activated phenotypes; M1 microglia, characterised by cells releasing pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-18, and M2 microglia, in which cells predominantly release anti-inflammatory cytokines (IL-4, IL-10, IL-13 and TNF-β)[98,99]. However, this neat subdivision of microglia phenotypes, although still in use, is progressively losing traction as more discoveries highlight the broad spectrum of dynamic temporal and spatial changes these cells undergo when stimulated [63,97].

In EAE mice, microglia activation promotes disease progression as well as reduces remyelination in interferon (IFN)-γ + lipopolysaccharide (LPS)-induced demyelination[100-102]. In the MS brain, microglial cells migrate to active lesion sites, where they represent the bulk of the initial pool of myeloid cells found around these lesions and display a typical pro-inflammatory phenotype[61,103-105]. Here, microglia act as the main APCs, and show increased

expression of major histocompatibility complex (MHC) I and II, CD40, CD80 and CD86 expression, contributing to the initiation and stimulation of T-cell activation[106-108]. In addition, they chemotactically recruit peripheral immune cells to the lesion sites through the release of chemokines such as CCL2, CXCL3, CCL12 and CCL13[109-111].

In contrast to the detrimental roles attributed to microglia during the active stages of MS (when there is a flare-up in the activity of immune cells against myelin), these cells also exert several beneficial functions during the stage of immune quiescence, hence it is critical to differentiate this division as microglial cells could become important cellular targets for the development of future MS therapies. For instance, microglia have been shown to mediate immunomodulation, and the promotion of neuronal and oligodendrocyte repair[60,112]. In fact, there is evidence suggesting that MS disease-associated microglia found surrounding demyelinating lesions slowly transition from a pro-inflammatory to more anti-inflammatory phenotype as the lesion becomes less inflamed, suggesting a controlled response to demyelination that aims for repair[105,113]. Microglia also promote the proliferation and differentiation of OPCs and neuronal progenitor cells via the release of certain trophic factors, such as brain-derived neurotrophic factor (BDNF) and transforming growth factor-β (TGF-β)[59,114-117].

Another critical function of microglia that is purportedly beneficial during MS is phagocytosis. Phagocytosis is the process by which cells engulf and digest large particles and debris in an attempt to maintain homeostasis[118]. Phagocytosis plays an important role in the brain, where it is known to be involved in synapse elimination, clearance of dying cells, and preventing an overflow of pro-inflammatory and/or anti-inflammatory cytokines[119,120]. Some research groups have reported that myelin phagocytosis could contribute to CNS demyelination[121,122]; however, there is stronger evidence *in vitro* and *in vivo* in support of the beneficial roles of microglia in the phagocytosis of damaged myelin in the CNS of MS patients[123,124]. Myelin-containing microglia and macrophages are found surrounding both active and chronic active lesions in MS patients[125]. Engulfment of myelin by microglia aims at facilitating the remyelination process by promoting the clearance of toxic myelin debris, whose accumulation around the damaged site prevents OPCs proliferation, therefore impeding their ability to repopulate the depleted pool of OLs[120,126,127]. However, over time, the CNS of MS patients becomes unable to remyelinate lesions. Whether this depends on the reduced phagocytic activity of microglia, the hindered OPC proliferation and/or OL maturation or their combination is still under scrutiny[62,93,94,128]. Nonetheless, it is clear why understanding which molecular mechanisms can reinstate remyelination is a focal point in MS research[95].

2.3.2. Phagocytic activity of microglia in the CNS white matter in MS

Phagocytosis plays an important role in the CNS, where it is known to be involved in synapse elimination, clearance of dying cells, and the control of pro-inflammatory and/or anti-inflammatory cytokines in the brain[119,120].

Evidence has suggested that myelin itself is able to regulate its own phagocytic fate by balancing pro- and anti-phagocytic signalling. On the one hand, myelin phagocytosis is believed to be stimulated through the activation of certain receptors including, the complement-receptor 3 (CR3), scavenger receptor-AI/II (SRA), the FCγ receptor, tyrosine kinase receptor MerTK, CD36 and triggering receptor expressed by myeloid cells 2 (TREM2)[129-136]. On the other hand, myelin was shown to downregulate myelin phagocytosis through CD47 and signal regulatory protein-a (SIRP-a) signalling[137]. In MS, the expression of phagocytosis-associated receptors (aka scavenger receptors) such as SRA and FCγ were found to be upregulated in lesions[138].

CR3 both activates and inhibits myelin phagocytosis in myeloid cells[139]. Myelin-mediated activation of CR3 signals F-actin/myosin contraction through Galectin-3 (Gal3)/K-RAS signalling, retracting the protruding filopodia, a signalling pathway that is also stimulated by SRA receptor activation[140]. CR3 activation also phosphorylates spleen tyrosine kinase (Syk), allowing for the downstream phosphorylation of cofilin (an actin-binding protein that regulates filament dynamics and depolymerization), remodulating F-actin stabilization and facilitating phagocytosis[139,141,142]. The FCγ receptor contributes to this process by also allowing for the activation of Syk, stimulating the Syk/cofilin/F-actin mediated engulfment of myelin[143].

MERTK, the gene encoding for the merTK receptor, has been described as a MS susceptibility gene, and substantial evidence supports its regulatory role in myelin phagocytosis by microglia[144-147]. However, the exact mechanism by which this occurs is still unclear. Two known ligands of merTK, Protein S and Gas6, have been found to tether the binding of apoptotic cells to merTK via binding to the phospholipid phosphatidylserine[129,148,149]. This interaction is an important “eat-me” signal in myeloid cells, which triggers phagocytosis[150]. Given how Gal6 and Protein S recognize phospholipids, added to the fact that degenerating myelin results in the accumulation of phospholipids at the damaged site, it can be hypothesised that merTK may bind to these myelin-derived phospholipids directly and contribute to phagocytic signalling. However, this mechanism is still hypothetical, as merTK signalling could also be instigated indirectly by other unknown signals yet to be unveiled.

There is an increasing interest towards comprehending the role of TREM2 and its involvement in lipid metabolism, especially in the field of MS research[136,151-154]. As the name suggests, TREM2 is expressed by immune cells, including infiltrating macrophages and resident microglia[155,156]. Although no direct risk factors for TREM2 and MS have been described, mutations in this receptor and its signalling partners are linked to the rare Nasu-Hakola disease, a disease characterised by frontal lobe dementia, CNS white matter lesions and widespread microglia activation[157-159]. In the CSF of MS patients, there are increased levels of soluble TREM2[160]. Moreover, TREM2 expression is increased in microglia and other phagocytes surrounding MS lesions[72,151].

TREM2 has been implicated in mediating microglial-mediated myelin clearance in MS, although it also plays a role in amyloid-β and apoptotic cell clearance, suggesting a possible involvement in Alzheimer's disease pathology[161]. TREM2 binds directly to phospholipids, triggering phagocytic activity in microglia through its interaction with the DNAX-activating protein of 12 kDa (DAP12)[134-136,161]. Activated TREM2 receptors have been shown to phosphorylate Syk through DAP12, triggering the stimulation of F-actin polymerisation, as highlighted above.

Following cuprizone-induced demyelination, TREM2$^{-/-}$ mice show reduced myelin phagocytosis and increased myelin degradation, two phenomena that in part are believed to be caused by the inability of microglia to activate lipid capture and lipid metabolism pathways[136,162]. This inability to metabolise myelin debris has been studied in detail in a recent study by Nugent et al. (2020), where TREM2$^{-/-}$ microglia were shown to pathologically accumulate myelin-derived cholesteryl ester lipids[153]. In the EAE model of MS, blocking TREM2 activity during the effector stage of the EAE model caused more severe pathological response and resulted in more diffuse demyelination pattern[163]. In fact, TREM2 is required to resolve the innate immune response upon detection of myelin debris[154]. Additionally, single-cell RNA sequencing of lesioned spinal cords demonstrated that, following injections of oxidised phospholipids (OxPCs) – common end-products found in MS lesions – into the mouse CNS, only microglia expressing high levels of TREM2 were responsive to OxPCs[72,164]. TREM2$^{-/-}$ mice had a more severe demyelinating response to OxPC injection compared to wildtype mice. In line with these findings, overexpression of TREM2 receptors seems to increase the phagocytic activity of microglia and reduces the release of pro-inflammatory cytokines[152,165]. Additionally, direct activation of TREM2 on microglia using antibodies was shown to promote myelin clearance, OL recruitment and maturation[151].

In conclusion, the signalling mechanisms contributing to myelin phagocytosis and the engulfment of other lipid-rich debris are complex and in great part overlapping. For clarity purposes, these mechanisms are summarised in **Fig. 1**. Furthermore, it should be highlighted that these signalling pathways also contribute to other important microglial functions, some of which will be explored in the next section. Understanding how these pathways control microglial responses to toxic myelin debris and regulate their metabolism may be of fundamental importance for the future development of therapeutic targets to treat demyelinating diseases, including MS.

2.3.3. Inflammatory signalling and myelin phagocytosis

Although phagocytosis can be triggered through the activation of lipid-sensing receptors expressed in the cell surface of microglia (**Fig. 1**), this biological process can also be activated by trophic factors and/or inflammatory mediators. A selection of these factors includes TNF-α, transforming growth factor β1 (TGF-β1), Activin-A and Gal3, as well as a range of inflammatory mediators that are secreted by lesion-associated microglial cells, and whose release also promotes OPC recruitment, proliferation and differentiation[116,166-170]. However, how these factors released by microglia can self-regulate myelin phagocytosis is a relatively understudied topic. Below, the most recent highlights from the literature are provided.

Myelin was found to stimulate cytokine expression and release through the activation of focal adhesion kinase (FAK)/PI3K/Akt/NFκB signalling in a process dependent on toll-like receptor (TLR)/myeloid differentiation primary response 88 (MyD88)[171-174]. This myelin-activated signalling pathway is critical for the facilitation of myelin phagocytosis. Specifically, previous evidence has shown that myelin induced the expression of TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-10, IL-17, macrophage migration inhibitory factor (MIF), matrix metalloproteinase 9 (MMP9), C-X-C motif chemokine ligand 10 (Cxcl10), Chemokine (C-C motif) ligand 3 & 4 (Ccl3 & Ccl4), whereas it downregulated TGF-β and IL-4 levels in bone marrow-derived macrophages[146,174]. This process appears to be partly regulated by the CR3 receptor. In fact, treatment with TNF-α but not IL-1 reduced the amount of myelin ingested by macrophages via their complement receptor type 3 (CR3), suggesting a regulatory role of this cytokine in CR3-dependent myelin phagocytosis[175]. However, it is noteworthy that there are indications where TNF-α, TGF-β and IFN-γ can stimulate phagocytosis in CR3-independent manner[129,171,172,176]. Since TNF-α is also an important signalling molecule in apoptosis, it is important that TNF-α induced stimulation of myelin phagocytosis does not result into

excessive phagocyte death[177]. Therefore, an important role of TGF-β is also to prevent apoptosis of myelin-laden microglia by limiting TNF-α expression and lowering oxidative stress[178]. Moreover, blocking IL-6 signalling using anti-IL-6 receptor antibodies increased expression of phagocytic markers, which coincided with a reduction of tissue damage and accumulation of myelin debris, suggesting a regulating role of this cytokine in microglial phagocytosis[179]. On the other hand, contradicting evidence has been published regarding the possible role of IL-4 in (myelin) phagocytosis. In fact, some studies report that IL-4 treatment reduced the expression of phagocytosis-related genes in microglia and dampened phagocytosis[171,180], whereas others indicate that both IL-4 and IL-10 stimulated phagocytosis in cultured microglia, likely through the upregulation TREM2[181,182]. It is possible that the discrepancy is explained by the different experimental approaches used. Nonetheless, it should be highlighted that other reports have found that the upregulation of IL-10 is lost in MS patient-derived macrophages, along with a decline in phagocytic capacity, which is restored after TGF-β treatment[147].

V-type immunoglobulin domain-containing suppressor of T-cell activation (VISTA) receptor is a known regulator of cytokine production (such as IFN-γ, TNF-α and IL-17) and suppresses CD4+ and CD8+ T-cell activation[183,184]. Evidence from VISTA KO microglia demonstrates a reduction of myelin phagocytosis[185]. Moreover, VISTA levels appear to be reduced in the EAE model, MS patient active and chronic active lesions but not in the normal-appearing white matter[185,186], further supporting a role of inflammatory mediators in regulating microglial phagocytic activity.

Based on the data reported, it seems plausible that manipulating pathways that regulate the release of inflammatory mediators with known pro-phagocytic effects may lead to the shift of microglia (and perhaps of other myeloid cells) towards a phenotype that is more prone to "clear" toxic myelin debris.

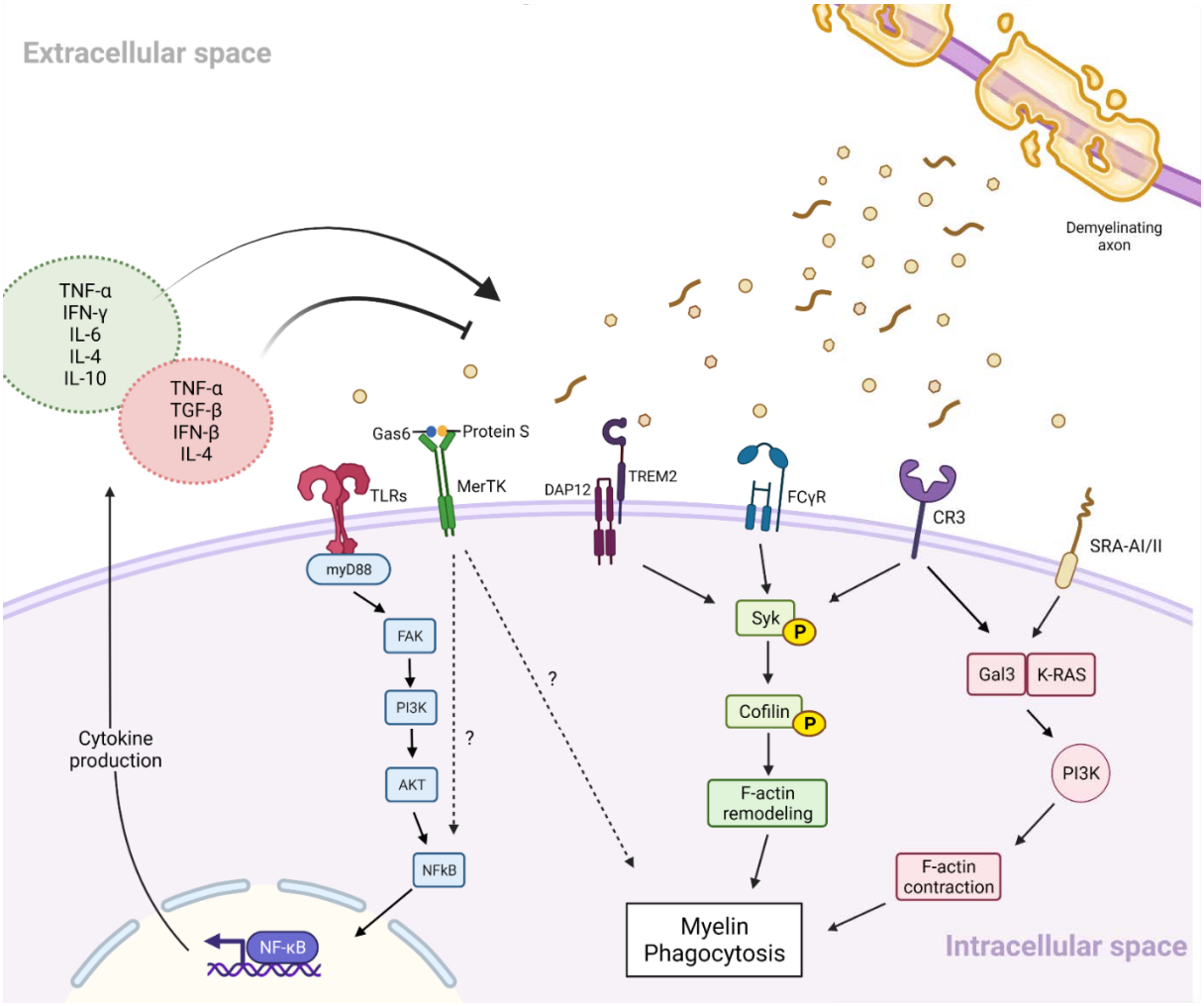

Figure 1. *Schematic overview of the main signalling pathways involved in myelin phagocytosis by microglia. In the figure, the molecular signalling pathways associated with microglial-mediated myelin debris phagocytosis are shown. Briefly, myelin debris is recognised by TLRs, MerTK, TREM2, FCγR, CR3 or SRA-AI/II receptors, which in turn stimulates the release of pro- and anti-inflammatory cytokines. Cytokines released in the extracellular space trigger the activation of several signalling pathways involved in the remodelling and contraction of the cytoskeleton, thereby facilitating the engulfment of myelin debris.*

3. PACAP and VIP

The neuropeptides pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are widely distributed throughout the central and peripheral nervous systems, where they exert pleiotropic neuroprotective and immune modulatory activities[187]. In view of their therapeutic potential, these naturally-occurring peptides have been linked to a range of neurodegenerative and neuroinflammatory diseases[188], including Parkinson's disease[189], cognitive and mood disorders[190] and MS[191]. PACAP has been implicated in maintaining a healthy CNS, as PACAP knockout (KO) mice display age-related

neurodegenerative signs much earlier than wild-type animals, including increased neuronal vulnerability, signs of systemic degeneration and a heightened inflammatory state[192]. In contrast, VIP has been emphasized more for its anti-inflammatory properties[193], with evidence showing its involvement in protecting both neurons and myelin, and to reverse motor defects and reduce lipid peroxidation in 6-OHDA parkinsonian rats[194].

PACAP and VIP exert their activities by activating three G-protein coupled receptors (GPCRs), PAC1, VPAC1 and VPAC2, each of which appears to mediate distinct biological and cell-specific functions[195]. This is of crucial importance for comprehending the activities of the PACAP/VIP system. In fact, despite all PACAP/VIP receptors recognize with high affinity both PACAP and VIP, the PAC1 receptor shows a much higher selectivity for PACAP, whereas both VPAC receptors exhibit equal high affinity for both PACAP and VIP[196]. Upon peptide binding, these receptors undergo conformational changes that allow the engagement of their intracellular domains with G proteins to either activate/inhibit a myriad of intracellular signaling cascades (**Fig. 2**). As mentioned, VPAC1 and VPAC2 are implicated in mediating most of the immune modulatory effects of the peptides, whereas PAC1 predominantly mediates growth/trophic factor release and other neuroprotective actions. Pathways that are commonly activated by these peptides include but are not limited to the adenylate cyclase/cAMP pathway, phospho-lipase C (PLC)/Calcium pathway, cAMP-responsive element binding protein (CREB) pathway and G-protein independent pathways such as PI3K and MAPK cascades[197].

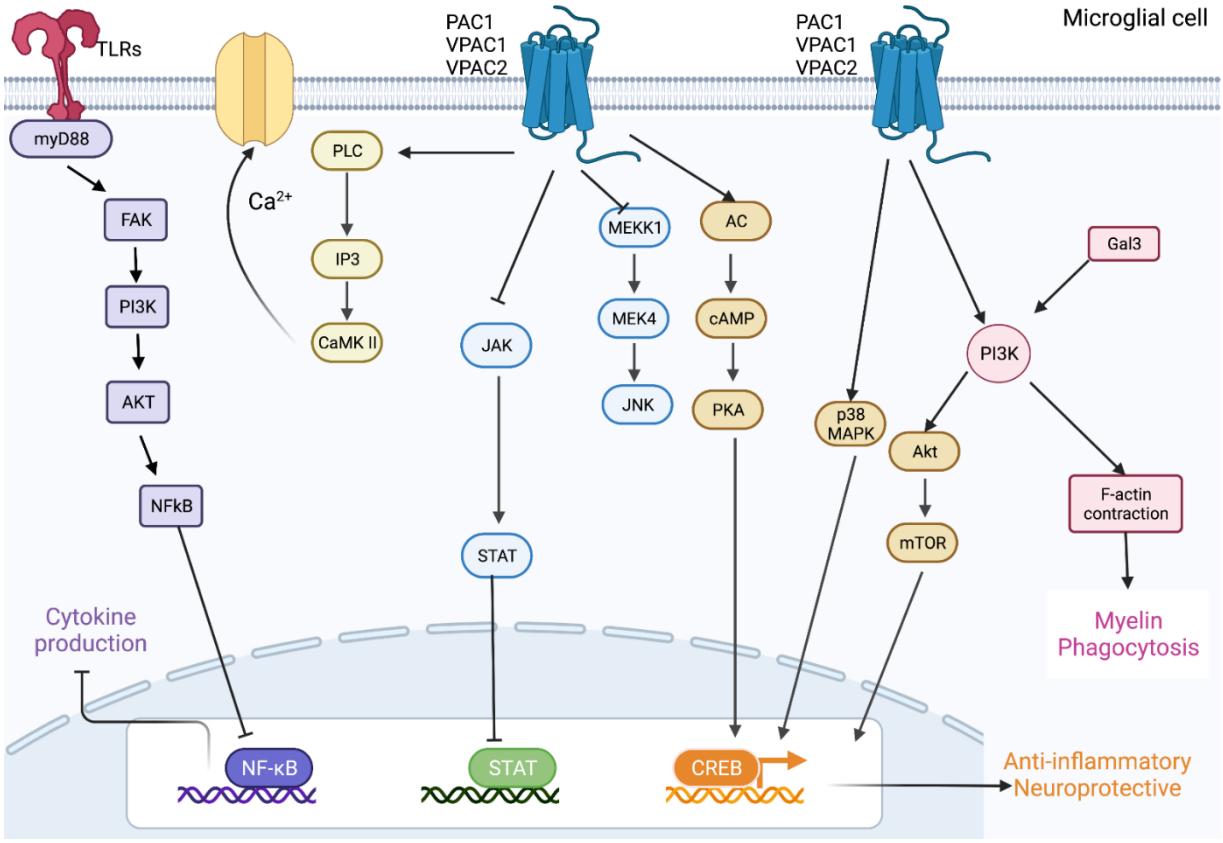

Figure 2. Schematic diagram of the signalling pathways activated by PACAP and VIP in microglia. *In the figure, the downstream molecular signalling pathways associated PAC1, VPAC1 and VPAC2 are visualised. Briefly, upon PAC1, VPAC1 or VPAC2 activation, the confirmational change induces activation of the classical GPCR pathways, including adenylate cyclase/cAMP pathway, phospho-lipase C (PLC)/Calcium pathway, cAMP-responsive element binding protein (CREB) pathway and G-protein independent pathways such as PI3K and MAPK cascades. This has as endpoint to activation of downstream transcription factors stimulating anti-inflammatory and neuroprotective properties as well as promoting myelin phagocytosis.*

3.1. PACAP and VIP in multiple sclerosis

The role of PACAP and VIP in MS has been extensively reviewed[187,191,193]. Furthermore, we have recently published a review article where we discussed the beneficial effects of these peptides in ameliorating several of the neurological co-morbidities of MS, outlining the potential usefulness of PACAP/VIP-based therapies as an all-in-one approach to counteract the disease by tackling distinct pathological domains[198]. However, whilst these studies have highlighted the ongoing efforts by several independent research groups in defining the proper strategy to maximize the therapeutic potential of these peptides in the context of MS and other neurodegenerative disorders, they have also emphasized the need for further investigations aimed at better characterizing their contributions to central myelin repair mechanisms.

Both neuropeptides can counteract several pathogenic mechanisms triggered by MS. Exogenous treatment with either peptides reduces both histopathological and clinical scores in preclinical models of MS. Studies using PACAP KO mice show that disease severity of MOG35-55-induced EAE was increased, and this correlated with enhanced Th1/Th17 and diminished Th2 responses[199]. Conversely, VIP KO mice were refractory to EAE-induced CNS inflammation, thus showing that immune cells failed to invade the CNS parenchyma[200]. These results provide indirect indications that PACAP and VIP are protective in the EAE model of MS. In addition, these studies have led to extensive research focusing on the roles of PACAP and VIP on T-cell function and the immune system in general[201-203].

Only a few studies using EAE (or other MS models) have investigated the potential therapeutic benefits of PACAP/VIP peptides during demyelination. Most recently, a pioneering study employing conditional PAC1 KO mice with targeted deletion of the gene (*Adcyap1r1*) in the retina, elegantly demonstrated that gene ablation exacerbated axonal pathology and increased microglia polarisation in the retina of mice undergoing EAE, highlighting the retinoprotective role mediated by the receptor in an MS model of optic neuritis[204]. Although important, the goal of the study was to investigate the neuroprotective effects mediated by this PACAP/VIP receptor, not on myelin repair.

As discussed in ***Section 2.2***, cuprizone-fed animals are largely used to model MS, particularly to study the demyelination occurring during MS and/or test the efficacy of drugs endowed with myelin repair properties[205]. In addition, the reversible nature of cuprizone-induced demyelination allows researchers to investigate spontaneous remyelination, a natural repair process that is impaired as the disease progresses[206]. Little work has been done using the cuprizone model to investigate the role of PACAP and VIP in de/remyelination and neuroprotection. We have preliminary evidence showing both peptides are able to prevent the locomotor deficits caused by a 4-week cuprizone diet (unpublished observations), therefore it cannot be excluded that the positive effects of PACAP and VIP might be due to either (1) protective effects causing increased OLs and OPCs survival and/or (2) enhanced myelin repair.

3.2. Role of PACAP and VIP in myelination

Although the focus of this review paper is to discuss the involvement of PACAP and VIP in regulating microglial phagocytosis and inflammatory responses during demyelination, given the relevance to MS pathology, for completeness we sought to introduce some of the state-of-the-art research relevant to PACAP/VIP roles in myelination.

It has been established that demyelination precedes axonal damage and neurodegeneration in MS. Therefore, strategies aimed at arresting this process and/or aimed at enhancing myelin repair mechanisms could be useful in preventing neurodegeneration and the progressive disability of MS sufferers[207]. Although we have recently re-iterated the importance of additional research to elucidate the exact involvement of these peptides in CNS myelination and in regulating OL functions[198], not the same can be said regarding PACAP/VIP activities on the homologous counterpart of the PNS – Schwann cells – and in peripheral neuropathies. We have shown that PACAP and VIP prevent apoptosis of Schwann cells *in vitro*[196], and found that PAC1 activation by PACAP promotes the proteolytic activity of these cells (critical for debris clearance)[208]. In addition, both peptides increase the expression of myelin related proteins in Schwann cell cultures[209]. More recently, our laboratory discovered that the antibiotics doxycycline and minocycline both promote similar proteolytic activity of Schwann cells as PACAP, perhaps acting as positive allosteric modulators of the PAC1 receptor[210]. Moreover, VIP and PACAP have been shown to both promote myelin gene expression and inhibit the release of pro-inflammatory cytokines by Schwann cells[211]. Although Schwann cells and OLs differ in embryologic origin, phenotype and are localized in different nervous system compartments (PNS vs CNS), the overlapping role between these two cell types, along with the pro-myelinating effects of PACAP and VIP promises well for future research on the possible use of PACAP/VIP analogues as boosters of myelin repair both in the PNS and CNS.

4. Effects of PACAP and VIP in microglia

In the MS brain, oxidative stress contributes to demyelination and neurodegeneration in a process that involves the oxidation of proteins, lipids, DNA, which also causes damage to mitochondria, resulting in energy deficits and further generation of reactive oxygen species (ROS)[212]. In this context, OPCs and OLs are hindered in their ability to proliferate, differentiate and repair myelin due to a toxic non-permissive CNS microenvironment. Microglia, as CNS scavengers, hold the potential to remove any toxic/oxidized myelin/cell debris and damaged mitochondria, contributing to the healthy microenvironment needed to allow these biological activities to occur. However, some of the mechanisms that regulate the activities of microglia, particularly phagocytosis, have not been fully investigated.

PACAP exerts essential regulatory functions on several biological activities of microglial cells, as evidenced by studies using PACAP deficient mice[213]. In a model of drug-induced retinal injury, an intravitreal injection of PACAP was sufficient to suppress retinal neuronal loss whilst significantly increasing microglia populations and triggering an anti-inflammatory

phenotype[214]. Similarly, VIP has been shown to inhibit pro-inflammatory mediators released by activated microglia *in vitro*[193]. Endogenous VIP was also shown to promote microglia proliferation and exert pro-neurogenic effects via VPAC1 activity during hippocampal neurogenesis[215]. This is further supported by evidence in preclinical models of brain trauma that show how VIP treatment prevents neuronal death by reducing the inflammatory burden caused by activated microglia[216]. These anti-inflammatory effects of VIP could also be mediated by the VPAC2 receptor, as a VPAC2 agonist, LBT-3627, reduced the subset of pro-inflammatory microglia and protected dopaminergic neurons in a parkinsonian rat model[217].

Delgado's research group has published several studies illustrating how PACAP and VIP significantly reduce the chemotactic activity of microglia[218] and inhibit the production of pro-inflammatory mediators, including TNF-α, IL-1β, IL-6 and NO in LPS-stimulated microglia[219]. These studies highlighted the immune modulatory role of these peptides, which favor the shift to anti-inflammatory phenotypes. In line with these results, in a recent study, we demonstrated that while both PACAP and VIP attenuate LPS-induced microglial activation and cytokine inflammatory profiles, each peptide triggered a phenotypic shift towards specific microglial populations with different morphology and migratory capacity[220]. More specifically, PACAP was more efficient to restore LPS-induced impairment of cell migration and expression of urokinase plasminogen activator (uPA) compared with VIP[220]. Proteolytic enzymes such as uPA are essential in the degradation of the extracellular matrix (ECM) and the removal of cell debris[221,222], therefore PACAP-induced uPA increase in microglia may point to a role in that direction. Further investigations are warranted to address this specific question.

In different works, rodent peritoneal macrophages have been employed to study the effects of both neuropeptides in phagocytosis. In one study, VIP increased the phagocytosis and ECM digestion of rat peritoneal macrophages[223], findings that were supported by another research that, instead, tested PACAP in mouse macrophages, with similar results[224]. More recently, Song et al. demonstrated that VIP markedly increased microglial phagocytosis via protein kinase C (PKC) signalling[225]. Moreover, in trophoblast cells, VPAC2 overexpression enhanced phagocytosis, which was associated with an anti-inflammatory microenvironment[226]. Additionally, PACAP enhanced phagocytosis of macrophages and inhibited the release of pro-inflammatory cytokines[227]. The indications that microglial release of anti-inflammatory mediators is paralleled by heightened phagocytic responses after PACAP or VIP treatment suggests that the peptides may trigger an anti-inflammatory phenotype in macrophages that, in turn, contributes to boost cell scavenging activities[228]. This theory is corroborated by additional

evidence showing that the switch to an anti-inflammatory phenotype increased the engulfment of apoptotic cells[229]. Based on the above, the general idea is that PACAP and VIP may promote the phagocytic activity of both peripheral and central myeloid cells, mainly via an indirect mechanism involving the initial transition of these cells towards an anti-inflammatory state. Once transitioned, peptide-stimulated microglia cells secrete anti-inflammatory molecules that act in a paracrine manner to promote phagocytosis. However, it is also possible that the peptides activate other more direct signalling mechanisms that are independent from the switch in microglial phenotype (**Fig. 2**), as some evidence indicates that pro-inflammatory signalling can also partake in the enhancement of phagocytic activities[172].

4.1. PACAP and VIP promote anti-inflammatory pathways in microglia

A great body of work has indicated that both PACAP and VIP activate anti-inflammatory signals in several *in vivo* and *in vitro* models, especially in microglia. Inhibitory effects of these peptides on the release of pro-inflammatory mediators like TNF-α and inducible nitric oxide synthase (iNOS) and the stimulation of the anti-inflammatory cytokine IL-10 are mediated by the VPAC1 receptor via cAMP dependent transduction pathways[230]. Additionally, both peptides have been shown to block interferon-γ (IFN-γ)-induced microglia inflammation by inhibiting the Janus kinase (JAK) / signal transducer and activator of transcription 1 (STAT1) pathway and control the gene expression of CD40, a critical mediator in the inflammatory cascade[231]. Furthermore, in a separate study the same group demonstrated that both PACAP and VIP inhibit the MEKK1/MEK4/JNK pathway, another intracellular mechanism involved in microglial activation[232]. It was also found that pretreatment of BV2 microglia with PACAP inhibited the activation of the TLR4/MyD88/NFkB signalling pathway and decreased inflammatory cytokine levels, thereby attenuating microglial polarisation in response to hypoxic injury[233]. This same phenomenon was observed in a rat model of traumatic brain injury (TBI), where PACAP exerted neuroprotection by inhibiting secondary inflammation via the same TLR4 pathway in both microglia and neurons, resulting in reduced neuronal death, overall inflammatory burden and improved recovery[234].

Furthermore, there is evidence that p38 MAPK activation is prevented by PACAP in microglia, and blockade of p38 MAPK activity inhibits iNOS induction[235]. In BV2 microglia, PACAP inhibition of IFN-γ-induced NO release is mediated by the increase in cAMP production, which inhibits p38 MAPK activation[236]. The structurally-related peptide VIP also seems to target similar pathways in microglia, as demonstrated in an animal model of Alzheimer's disease,

where the authors show that VIP inhibited inflammation by blocking the signalling of p38 MAPK, p42/p44 MAPK and NFkB intracellular cascades[237].

We have shown that both peptides promote the expression of myelin-related proteins via PAC1/VPAC2 receptor activation of PI3K/Akt signalling in Schwann cells[209]. PI3K/Akt signalling is also associated with the anti-inflammatory and neuroprotective functions of PACAP in the adult brain[197,238]. Interestingly, we also revealed that the PI3K/Akt pathway is activated in a model of high fat diet-induced brain inflammation, and metformin treatment restored both this pathway and the associated PACAP/VIP dysregulations, reducing the overall inflammatory burden[239]. Together, this suggests that the PI3K/Akt cascade is also part of the complex signalling that promotes the anti-inflammatory and protective functions in microglia.

It is important to note that several of these pathways result in the induction of CREB, a transcription factor involved in the transcription of genes containing a cAMP-responsive element, including IL-2, IL-6, IL-10 and TNF-α [240]. As discussed earlier, these factors contribute to microglia phagocytic activity, whilst stimulating the production of anti-inflammatory factors. CREB activation also promotes the expression of proteolytic enzymes, whose release facilitates microglial phagocytic function[241]. In prior work, we have shown that in Schwann cell lines, PACAP/PAC1 activation stimulated the expression of tissue plasminogen activator (tPA) in a PI3K/Akt/CREB dependent manner to promote proteolytic activity[209], activities that were mimicked brain derived neurotrophic factor (BDNF)[209].

4.2. PACAP and VIP activate protective pathways in neurons and microglia

The acquisition of an anti-inflammatory phenotype in microglia is accompanied by the release of neurotrophic factors that promote glial and neuronal survival via the activation of anti-apoptotic pathways[235].

There is a strong relationship between PACAP and VIP peptides and the expression/activity of the neurotrophic factor BDNF. We have previously shown that BDNF mimics the actions of PACAP on tPA expression and activity both via Akt and MAPK pathways[208] and there is additional evidence indicating that stereotactic injections of the PACAP peptide in specific CNS regions increase BDNF production[242,243].

Additionally, PACAP has been shown to inhibit apoptotic pathways in neuronal progenitor cells exposed to IFN-γ via a caspase 3-dependent mechanism[244]. However, in the BV2 microglial cell line, we reported that neither PACAP nor VIP reliably prevented cell loss induced by rotenone[245]. Altogether, these findings suggest that both peptides promote neuronal and,

although less efficiently, microglial survival in response to different types of insults. There is also indication for an involvement of an indirect inhibitory effect on the apoptotic pathways, which seems to be mediated by BDNF. However, the lack of pro-survival responses in rotenone-treated microglia suggests that the protective efficacy of PACAP and VIP might depend on the type of insult/challenge to which microglial and neuronal cells are exposed to.

4.3. PACAP and VIP regulate oxidative stress

Toxic myelin debris generated by demyelinating/degenerating neurons in conjunction with an overactive immune response are two mechanisms responsible for the accumulation of ROS, the main cause of oxidative stress. Recently, several studies have begun to focus on investigating the role of PACAP and VIP in oxidative stress and mitochondrial function. Waschek and collaborators postulated that the ability of PACAP to induce mitochondria biosynthesis might provide a plausible mechanism to explain how this peptide dampens the detrimental effects of oxidative insults in neurons and perhaps other glial cells (reviewed in[187]). This idea has been corroborated by several other investigations showing that PACAP treatment attenuates mitochondria-mediated oxidative stress and neuronal apoptosis[246] and inhibits the generation of ROS[247]. Interestingly, one study demonstrated that activation of the PACAP/PAC1 axis is protective against oxidative stress-induced cell death in astrocytes, whereas VIP was devoid of any anti-oxidant activity in this *in vitro* model[248]. Conversely, in a model of ulcerative colitis, VIP reliably restored mitochondrial function, with equivalent efficacy as super oxide dismutase and dimethyl sulfide, unveiling a novel free radical scavenging property of VIP[249]. Such novel function of VIP was also demonstrated in the CNS, as the peptide dose-dependently decreased the translocation of cytochrome c from mitochondria to the cytoplasm and prevented apoptosis in rat hippocampal stem cells[249]. Although there is still insufficient evidence to postulate that oxidative stress may hinder microglial phagocytosis, this needs to be taken into consideration as phagocytosis normally occurs in microenvironments where the redox balance is impaired. Therefore, it is possible that PACAP or VIP secreted by phagocytizing microglia or neighboring cells will help to mitigate the redox imbalance and aid in cell survival and debris engulfment.

5. PACAP and VIP modulate the biological activities of glial cells

Microglial cells communicate closely with other glia cells, including astrocytes and OLs in order to maintain CNS homeostasis. The two peptides PACAP and VIP are endogenously expressed in several glial cell types, and play regulatory functions in different biological

processes in astrocytes, OLs and microglia, both in healthy and pathological conditions, such as MS. This section will discuss current knowledge on the mechanisms through which PACAP and VIP regulate the activities of astrocytes and OLs and their crosstalk with microglia.

5.1. Astrocytes

Astrocytes are the most abundant glial cell type and are primarily involved in maintaining a healthy environment for neuronal signalling[250]. Activated astrocytes that release pro-inflammatory cytokines reduce myelination and promote axonal injury[251]. VIP, more so than PACAP, protects the white matter against excitotoxic insults and promotes axonal growth[252,253], likely through the activation of PKC survival pathways by supporting astrocytes[252]. PACAP protects astrocytes against H_2O_2 damage via PAC1 activation of PKA, PKC and MAPK pathways, which collectively contribute to prevent oxidative stress and preserve mitochondrial membrane integrity[254]. These overlapping functions seen in microglia and astrocytes demonstrate that these peptides promote an overall protective response of neuroglia, which also facilitates CNS repair after injury.

There is evidence that PAC1- and VPAC1/2-mediated activation of the PLC/calcium pathway promotes the secretion of neurotransmitters and neurohormones and may contribute to the neuromodulatory functions of the peptides[197]. It is known that PACAP and VIP can act as neuromodulators for several neurotransmitters, including dopamine, serotonin, glutamate etc.[255,256]. There is evidence of a strong interaction between these peptides and the dopaminergic and serotonergic systems. Pharmacological studies show that PACAP has neurotrophic and neuroprotective actions on dopamine and serotonin neurons[257]. Previous studies in D3R knockout (KO) mice revealed elevated hippocampal PACAP expression in the absence of the receptor, suggesting a possible link with PACAP and VIP in the processing of fear memories[258]. Moreover, the protective effect of PACAP administration correlated with increased dopamine levels[259]. In a rotenone model of PD, we reveal that buspirone, a 5HT1a agonist and D3R antagonist alters the expression profile of these peptides throughout the brain[260]. We have shown that D3Rs are expressed in microglia, and that D3R antagonism is anti-inflammatory[261]. Additionally, VPAC2 activation has been postulated to upscale astrocytic glutamate transport[262], thereby increasing neurotransmitter re-uptake. Interestingly, glutamate transmission is impaired in demyelinated lesions[263]. Consequently, the effect of these peptides on astrocytes could regulate glutamate release, preventing excitotoxic insults to OLs, OPCs and microglia.

5.2. Oligodendrocytes

The ability of PACAP and VIP to inhibit microglial production of IL-1β is a good indicator that both peptides may be effective in reinstating myelination. In fact, microglial derived IL-1β has been shown to hinder myelin production and repair processes, whilst suppressing OPC maturation[264]. It has now been verified that the crosstalk between microglia and OLs is an essential component of the myelin repair machinery in demyelinating diseases. PACAP exerts growth factor functions on OLs, and promotes the proliferation of OPCs[265]. However, its effects in configuring OLs myelinating functions is still understudied and partly controversial. In 2011, a study using PACAP KO mice found that these mice displayed a similar sequence of myelination as aged-matched wild-type mice, although the onset of myelination occurred earlier[266]. The authors concluded that endogenous PACAP exerted an inhibitory role on myelination *in vivo*. However, given the similar sequence of myelination seen in both genotypes and the comparable levels of myelin once mice reach adult age, it cannot be ruled out that PACAP inhibition of myelination is only a transient and developmentally conserved process that, as the same authors propose, favours neuronal maturation over myelin synthesis in critical stages of development. This hypothesis is supported by the evidence indicating that the PACAP-preferring PAC1 receptor, which is highly abundant in the CNS white matter[267], undergoes extensive alternative splicing during development to fine-tune neuronal activities[268]. Perhaps a similar PAC1-regulated myelination process, similarly to neurons, is essential for the correct maturation of the CNS and its insulating matter.

6. Conclusions

Microglia elicit complex roles in the CNS of MS patients, where these cells orchestrate both acute and chronic inflammatory responses as well as play an important role in the remyelination process of the brain[61,78,269]. In this review, we explored the role of microglia in the CNS, with special emphasis on their critical role as phagocytes of myelin debris, which, under demyelinating conditions, is a process that is necessary to promote remyelination[128,270,271]. Our deep dive into the molecular mechanisms behind myelin engulfment have highlighted the intricate network of signaling cascades involved in the controlled phagocytic response by microglia to myelin debris (**Fig. 1**). Moreover, we theorised the therapeutic potential of neuropeptides PACAP and VIP as "broad microglia modulators", able to stimulate an anti-inflammatory microglial phenotype with increased pro-phagocytic potential.

Although a there is still sparse evidence providing a clear/direct link between PACAP and VIP and microglial phagocytosis, we identified at least three main overlapping pathways commonly

activated by microglia (during phagocytosis) and in PACAP/VIP treated microglia. These include the MyD88/NFkB signalling, the induction of galectin-3 and the PI3K/Akt signalling[209,233,272]. Additionally, the shift towards an anti-inflammatory microglial phenotype triggered by PACAP and VIP treatment promotes the secretion of pro-phagocytic cytokines, including IL-10[181,273], suggestive of an indirect effect of the peptides. Finally, throughout the review, we also explored some of the broader effects of PACAP and/or VIP as potential therapeutics in models of neurodegeneration and traumatic injury, highlighting that the pleiotropic activities and in most cases beneficial activities of these neuropeptides extend beyond the CNS.

Altogether, the evidence presented here supports the hypothesis that both PACAP and VIP are potential inducers of microglial phagocytosis and could aid stimulating the removal of myelin debris in demyelinating diseases such as MS (**Fig. 3**).

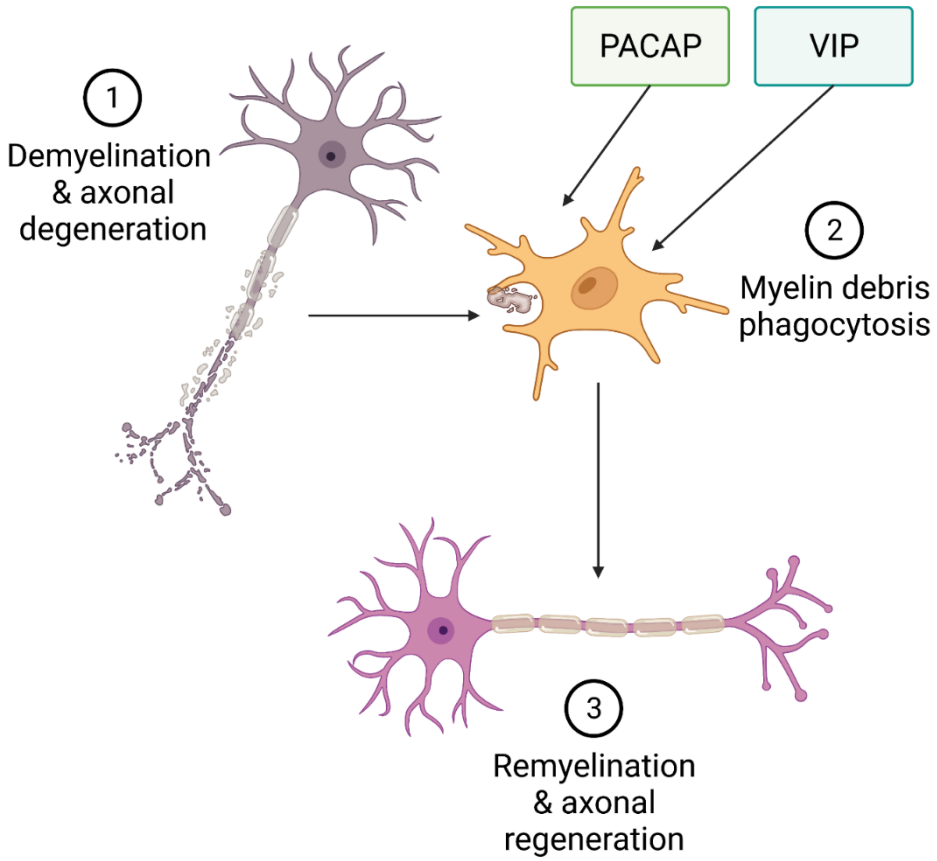

Figure 3. Proposed model for PACAP and VIP pro-phagocytic activities in microglia. *In this simplified model, myelinated fibres of the CNS undergo immune attack, resulting in myelin degradation, axonopathy and neuronal death (**1**). Both PACAP and VIP activate microglia to promote phagocytosis of myelin debris (**2**), creating a permissive microenvironment that allows myelin repair (**3**).*

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1.1.4 PACAP and VIP in comorbidities of multiple sclerosis

MS is characterised by a high incidence of disease comorbidity throughout its development. Gijsen and colleagues define comorbidity as the "total burden of illness other than the disease of interest"[274] and these comorbidities can have a significant impact on disease progression and the patient's quality of life [275]. Therefore, it is important that for new treatment development, the effect on common comorbidities be assessed as well. In the literature review article below, titled "Targeting the neurological comorbidities of multiple sclerosis: the beneficial effects of VIP and PACAP neuropeptides", I aim to explain how the VIP/PACAP system can be utilised in these common MS comorbidities. Aside from providing some additional details on the general mechanisms of the VIP/PACAP system in general and in MS specifically, this review mostly highlights the broad spectrum of potential impact this neuropeptide system can have and how it would be valuable to explore this multi-faceted target within the context of MS biology and its related comorbidities.

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1. Laboratory of Cellular and Molecular Neuroscience (LCMN), School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW 2007, Australia

* These authors equally contributed to the work

Contribution:

- Writing
- Manuscript preparation

Signature of co-authors:

Name	Signature
Margo Iris Jansen	Production Note: Signature removed prior to publication.
Sarah Thomas Broome	Production Note: Signature removed prior to publication.
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Targeting the neurological comorbidities of multiple sclerosis: the beneficial effects of VIP and PACAP neuropeptides

Margo I. Jansen[1,†], Sarah Thomas Broome[1,†], Alessandro Castorina[1]

1. Laboratory of Cellular and Molecular Neuroscience (LCMN), School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW 2007, Australia

† These authors equally contributed to the work

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Abstract

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are two widely expressed neuropeptides with important immunomodulatory and neuroprotective properties in the central nervous system (CNS). Both VIP and PACAP have been implicated in several neurological diseases and have shown favourable effects in different animal models of multiple sclerosis (MS). MS is a chronic inflammatory and neurodegenerative disease of the CNS affecting over 2.5 million people worldwide. The disease is characterised by extensive neuroinflammation, demyelination and axonal loss. Currently, there is no cure for MS, with treatment options only displaying partial efficacy. Importantly, epidemiological studies in the MS population have demonstrated that there is a high incidence of neurological and psychological comorbidities such as depression, anxiety, epilepsy and stroke among afflicted people. Hence, given the widespread protective effects of the VIP/PACAP system in the CNS, this review will aim at exploring the beneficial roles of VIP and PACAP in ameliorating some of the most common neurological comorbidities associated with MS. The final scope of the review is to put more emphasis on how targeting the

VIP/PACAP system may be an effective therapeutic strategy to modify MS disease course and its associated comorbidities.

Keywords: Multiple sclerosis, Vasoactive intestinal peptide, Pituitary adenylate cyclase-activating peptide, Comorbidities, Stroke, Epilepsy, Depression, Anxiety, Schizophrenia, Migraine

1. Introduction

1.1. Neuropeptides

Over the last couple of decades, general knowledge on the biological role of neuropeptides in the central nervous system (CNS) has substantially increased. Currently, more than a hundred different neuropeptides have been described in the CNS, most of which are involved in the modulation of different brain functions [276-280]. Neuropeptides are small, amino acid-based molecules that can influence neuronal activity, neuro-immune responses and whose dysregulations have been implicated in the pathogenesis of several mental illnesses such as Alzheimer's or Parkinson's disease, depression, anxiety, stroke, migraines, epilepsy and Multiple sclerosis [187,281-290].

It has become increasingly clear that certain neuropeptides such as neuropeptide Y (NPY), somatostatin, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) have an anti-inflammatory effect in the CNS [291]. This has resulted in research focusing on these neuropeptides as potential therapeutic targets for the treatment of neuroinflammatory diseases [191,292-295].

1.2. PACAP and VIP

The neuropeptides pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are widely distributed throughout the CNS and the peripheral nervous system (PNS) and are involved in neuroprotection and immunomodulatory functions [296-301]. The activities of PACAP and VIP are mediated by three G protein-coupled receptors (GPCRs), namely PAC1, VPAC1 and VPAC2 [195] (**Fig. 1**).

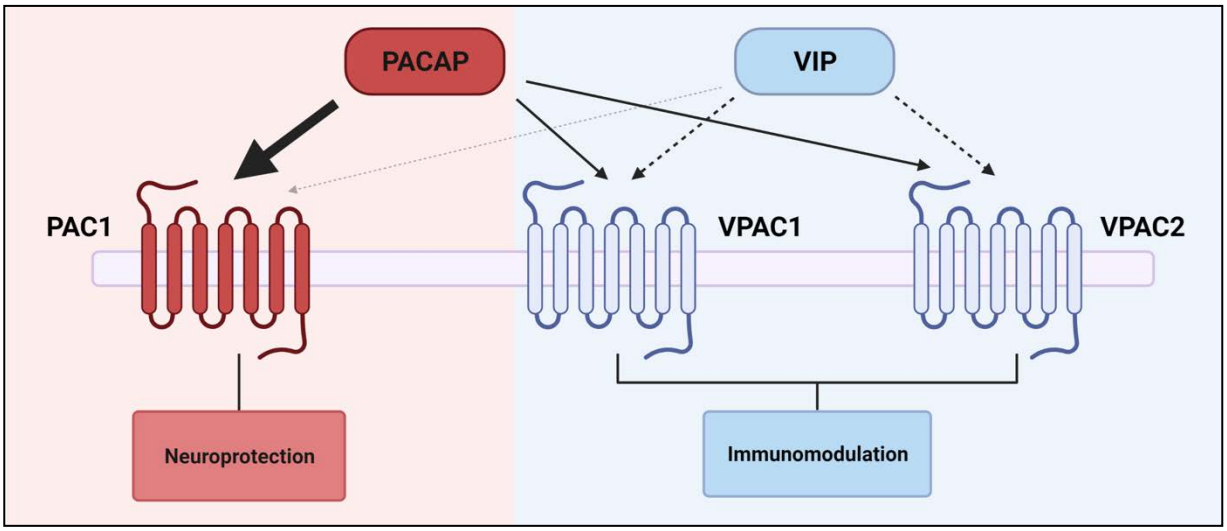

Figure 1. PACAP and VIP and their downstream effects. *A schematic overview of the binding of VIP and PACAP to the PAC1, VPAC1 and VPAC2 receptor with its main down-stream effect on cellular function.*

PACAP binds with high affinity to both PAC1, VPAC1 and VPAC2 receptors and its activity is believed to be predominantly neuroprotective [302-304]. For example, PACAP can prevent neuronal cell death after ischemia [305] and can even promote axonal regeneration after spinal cord injury [306]. In contrast, VIP binds less efficiently to PAC1 receptors, whereas its exhibits similar high affinities for VPAC1 and VPAC2 receptors as PACAP [196]. The latter two receptors' activities are thought to be mainly associated with immune modulatory roles in the CNS as well as in peripheral organs [193,307,308]. Given VIP binding preference towards VPAC receptor subtypes, this peptide has emerged as a potential anti-inflammatory target to treat Multiple Sclerosis (MS) and perhaps, other inflammatory diseases [309,310]. For example, in human rheumatoid arthritis (RA), VIP treatment downregulated chemokines production and interleukin-6 (IL-6) and decreased the levels of other pro-inflammatory mediators in RA patients [311]. Additionally, a singular cerebroventricular injection of VIP was able to attenuate microglial activation and prevent neurodegeneration in animal models of Parkinson's disease, [312].

1.3 VIP/PACAP system in Multiple sclerosis

1.3.1 Multiple Sclerosis

MS is a chronic neuroinflammatory disease of the CNS that is characterised by episodes of extensive demyelination within the CNS, with consequent axonal loss and gliosis. Both genetic vulnerability and/or exposure to certain environmental pollutants or unhealthy life styles are

considered risk factors for disease development [313,314]. The prevalence of MS has been increasing over the last decade with currently approximately 2.8 million people being predicted to suffer from the disease worldwide [3].

There is a clear involvement of the immune system in MS, with the infiltration of autoreactive T cells into the CNS believed to be a major pathophysiological event in disease aetiology [315]. Moreover, B cells, natural killer (NK) cells, astrocytes and microglia have also been shown to play a role in both disease onset and progression [67,316,317]. This heightened inflammatory state of the CNS in MS is believed to facilitate the loss of myelin and axonal degeneration [318]. A brief overview of current knowledge on the role of the VIP/PACAP system in neuroinflammation and myelination in MS is highlighted below.

1.3.2 VIP/PACAP and neuroinflammation in Multiple sclerosis

Given the known neuroprotective and immunomodulatory roles of the VIP/PACAP system in the body, they have been thoroughly investigated MS. In fact, MS patients were found to have reduced PACAP levels in their cerebrospinal fluid with a similar trend being seen for VIP [319]. Moreover, Waschek and colleagues have used global and conditional knockouts in VIP, PACAP and their receptors to dissect the neuroprotective and immunomodulatory actions of these peptides in acute monophasic MOG_{33-35} experimental autoimmune encephalomyelitis (EAE) models, a well-established mouse model of MS [199,201,320-322]. They revealed that mice lacking PACAP or VPAC2 displayed more severe and prolonged disease than wild type controls while VIP or VPAC1 knockout mice showed EAE resistance [201,202,321,322].

When examining the effects of VIP and PACAP on a cellular level in MS models, a clear immunomodulatory influence can be observed. Extensive research has focused on the effect of PACAP and VIP on T-cell function, but clear effects on other immune cells have been described as well. VIP and PACAP have been shown to regulate Th1 and Th17 profiles, stimulating a more anti-inflammatory phenotype, while also assisting in the recruitment of anti-inflammatory Th2 and Treg cells [191,201,310,320,321,323,324]. Additionally, PACAP-/- and VPAC2-/- mice, subjected to MOG_{33-35} induced EAE show increased immune cell infiltration in the CNS, whereas reduced infiltration was seen in VIP-/- and VPAC1-/- mice [199,201,202,322]. *In vitro* analysis also showed that both PACAP and VIP treatment of cultured cells and in EAE models resulted in reduced levels of pro-inflammatory cytokines, chemokines, chemokine receptors and inducible nitric oxide synthase (iNOS) produced by T-cells, macrophages and microglia [218,219,310,325-328]. This could potentially play a neuroprotective role under inflammatory conditions through the upregulation of cell survival markers [329]. Moreover, PACAP treatment

was also shown to reduce antigen presenting cell function in the EAE model, which is an important mediator of T-cell differentiation [203,323].

Taken together, both VIP/PACAP play a clear role in the modulation of the inflammatory response of the CNS and show clear anti-inflammatory effects *in vitro*. However, there are some contradicting results on the role of VIP and PACAP in MS mouse models. This highlights the need for additional research to further breakdown the differential effects of these peptides on the immune system.

1.3.3 VIP/PACAP and myelination

On top of the immunomodulatory effects of VIP and PACAP in the CNS, there is evidence of a role of the VIP/PACAP system in oligodendrocyte development as well [330]. Oligodendrocytes are the myelin producing cells of the CNS and the main cell-type affected in MS. PACAP is a known stimulator of oligodendrocyte progenitor cell proliferation and can also delay the myelination process [265,331]. This role is further corroborated by the fact that during development, PACAP-deficient mice begin the myelination process at an earlier stage, resulting in increased density of myelin in the brain [266]. These mice also show impaired axon regeneration upon nerve injury [332]. VIP, on the other hand was shown to play a more differentiating role in oligodendrocyte progenitor cells. A VIP injection near transected nerves was shown to promote early myelination and remyelination of the damaged nerves [333]. Moreover, VIP/VPAC1 signalling was shown to reduce the severity of ibotenate-induced white matter lesions under inflammatory conditions [334]. There are also some indications of a myelin deficit in VIP-deficient mice, although proper quantification is needed before this can be concluded [330]. Thus, there are indications of a differential effect of VIP and PACAP in oligodendrocyte maturation and myelination. Although it is beyond the scope of this review to further dive into the details of the role of VIP and PACAP in oligodendrocyte and myelin development, there are some excellent reviews by Maugeri *et al.* (2020) and Maduna *et al.* (2016) summarizing current understanding of the field [330,335].

1.4 Multiple sclerosis and associated comorbidities

Recent studies have highlighted a higher incidence and prevalence of comorbidities in MS patients compared with the healthy population [275,336]. Mounting evidence indicates that MS is associated with comorbidities that are often seen in people suffering from either vascular and/or metabolic diseases, as well as certain neurological and psychiatric disorders [337-341].

Epidemiological studies in MS patients have demonstrated how afflicted people also have a higher chance of developing epilepsy [340], migraines [341], as well as affective/emotional disturbances such as depression or anxiety [336]. In view of the comorbidities often seen in MS patients and the critical role of neuropeptides in many pathological domains of these comorbid diseases, exploring the mechanisms and the extent at which both PACAP and VIP peptides can contribute to ameliorate the comorbid events associated to MS pathology is becoming a hot topic. For this purpose, this review will summarise literature on the role of neuropeptides, focussing on the role of VIP and PACAP in the neurological comorbidities of MS, and how these neuropeptides could contribute to improve the clinical presentation of MS and its disease course.

2. The role of PACAP and VIP neuropeptides in comorbidities of MS

2.1. Depression, Anxiety and Bipolar disorder

MS is a devastating disease that comes with physical as well as psychological hardship, which may ultimately lead to the development of mood disorders or facilitate its onset in vulnerable people. One of the most prevalent comorbidities seen in MS patients is depression (23.7%), followed by anxiety (21.9%) and bipolar disorder (BD; 5.83%) [336]. Interestingly, these disorders are all associated with neurochemical evidence of CNS inflammation, supporting a pathological link with MS [287,342-345]. Moreover, depression, anxiety and BD are all known to be influenced and triggered by stress, which is able to affect oligodendrocyte's health [346-350]. Here, the role of PACAP and VIP in depression, anxiety and BD is highlighted.

Several neuropeptides such as NPY, somatostatin, galanin and orexin, at different extents, have been implicated in the pathology of affective disorders and have been suggested as potential therapeutic targets [295,351-358]. The VIP/PACAP system has also been implicated with depression and anxiety, although there are some conflicting findings. On the one hand, knockout studies using PACAP-deficient mice have reported that PACAP gene ablation increased depressive-like and anxiety-like behaviour under stress conditions [359-361]. On the other hand, a study by Lehmann and colleagues found reduced anxiety and no depressive-like behaviour in PACAP$^{-/-}$ mice [362]. However, this study used social defeat to trigger chronic stress whereas the other studies either used naïve mice or mice exposed to mild form of stress. Thus, PACAP$^{-/-}$ mice might exhibit a whole spectrum of anxiety-like and/or depressive-like behaviours, depending on the initial source of stress and duration. In a clinical study, VIP serum levels were found to be negatively correlated with depression and anxiety state and were positively correlated with brain volume of the left amygdala [363]. Similarly, decreased serum

levels of VIP were detected in a rat depression model [364]. Moreover, single nucleotide polymorphisms (SNPs) in the *VIPR2* gene and *VIP* gene were found to be associated with unipolar major depression and BD, respectively [365]. Also, a VIP injection into the CA1 region of the hippocampus of rats showing anxiety-like behaviours attenuated the affective symptoms [366], although, for unexplained reasons, VIP antagonist was not able to abolish VIP-mediated behavioural improvements.

Corticosterone, one of the hormones produced by the adrenal gland as part of the hypothalamic-pituitary-adrenal axis (HPA-axis), is known to play a critical role in the development of depression, anxiety and bipolar disorder [349,367,368]. Under stress conditions, PACAP-deficient mice exhibited an attenuated corticosterone response, which occurred irrespectively of the development of depressive-like behaviours [362,369]. Moreover, PACAP injections into the amygdala and bed nucleus of the stria terminalis (BNST) have been found to reliably increase corticosterone levels and anxiety-like behaviours in rodents [370-372]. Interestingly, under chronic stress conditions there was a brain region-specific increase of PACAP and PAC1, but not VIP and VPAC1-2 levels in the BNST [243]. However, VIP has also been implicated in the control of glucocorticoid hormones release, although in a work focused on studying its effects in relationship to circadian rhythmicity [373]. Thus, there appears to be a link between PACAP, stress and the HPA axis, at least for certain affective disorders.

In summary, the VIP/PACAP system appears to be a key player in the modulation of mood and other affective disturbances. Either peptides exert intrinsic regulatory functions in brain homeostasis, and it is not surprising that dysfunctional regulation of PACAP or VIP in specific brain regions or cell populations may be critical for the development of conditions such as depression, anxiety and bipolar disorder. Future research is therefore warranted to explore if targeting this neuropeptide system can be used as an effective therapeutic strategy to treat mood disorders.

2.2 Psychotic disorders – Focus on schizophrenia

Psychotic disorders encompass a broad range of mental illnesses such as schizophrenia, affective psychosis, delirium and drug-induced psychosis. Approximately 2-4% of MS patients have reported to experience psychotic episodes at some point during the course of the disease, which is a considerably higher rate than in the general population [374]. In line with MS pathology, neuroinflammation and increased oxidative stress events in the CNS are pathogenic events that are associated with the occurrence of certain psychotic disorders [375,376].

Several neuropeptides have shown to activate signalling pathways that are implicated in the genesis of psychosis. Clear associations have been found between schizophrenia and neuropeptide Y, neurotensin, somatostatin and oxytocin and the number of psychotic episodes (reviewed in [377,378]). Moreover, a genetic link between neuregulin-1 (NRG1), cholecystokinin A and schizophrenia has been described [379-383]. Similarly, genetic polymorphisms of genes encoding PACAP peptide or its receptors have also been correlated with schizophrenia [384-388], although for the former gene target (PACAP gene, aka *Adcyap1*), a replication study failed to reproduce the same findings [389].

There are certain indications that neuroinflammation can lead to the development of schizophrenia and psychotic episodes. In schizophrenia and first-time psychosis patients, studies have reported increased levels of pro-inflammatory cytokines and decreased levels of the anti-inflammatory cytokine IL-2 in the CNS [390,391]. Additionally, increased microglia activation has been observed in recent-onset schizophrenic patients [392,393]. However, since no comprehensive animal model exists for psychiatric diseases, methods to study neuroinflammation at a molecular level in these diseases are still challenging. Furthermore, since neuroinflammation in schizophrenia is a relatively novel concept, the exact mechanisms through which it could contribute to disease aetiology remain unclear. However, it would be interesting to explore the anti-inflammatory activities of the VIP/PACAP system could potentially attenuate the CNS inflammation seen in people with psychotic disorders.

There is a leading hypothesis featuring schizophrenia as a neurodevelopmental disorder [394,395]. In *post mortem* brain tissue from schizophrenia patients, the pool of neural stem cells (NSC) is reduced, suggesting decreased NSC proliferative activity [396]. Moreover, schizophrenia is characterised by abnormal connectivity among brain regions and axonal abnormalities [397-399]. A noteworthy link between brain development, schizophrenia and white matter can be seen for NRG1. NRG1 signals through disrupted-in-schizophrenia 1 (DISC1) and interestingly, PACAP has been found to affect DISC1 signalling as well [400]. In addition, both NRG1 and DISC1 are involved in neuronal migration, axon ensheathment and oligodendrocyte maturation [401,402], suggesting a role for PACAP in modulating NRG1-mediated activities. Moreover, PACAP, PAC1 and DISC1 are known to be essential components of the cellular machinery regulating neurite outgrowth [400,403,404]. PACAP was found to stimulate NSC proliferation in mice and prevented the reduction of NSCs in a ketamine-induced schizophrenia-like mouse model via PAC1 receptor activation [405,406].

Taken together, these data suggest that PACAP (and perhaps other PAC1 receptor agonists) could aid in the treatment of schizophrenia. Based on the reported pro-mitotic

activities of PACAP in NSCs, it is reasonable to believe that PACAP treatment could replenish the depleted pool of NSCs in schizophrenic patients, hence promoting neurogenesis. Additionally, it could also reduce the chronic CNS inflammation that seems to also contribute to the development of psychotic disorders. Whilst additional investigations into this topic are still warranted, there is already some indication that the beneficial actions of known neuroleptic drugs can happen by restoring a dysfunctional VIP/PACAP signalling in the brain [407].

2.3 Epilepsy

Epilepsy is a chronic neurological disorder that is characterised by recurring and unprovoked seizures [408]. Seizures occur when the balance between excitatory and inhibitory signals in the brain are disrupted [408]. Epilepsy is one of the most common neurological disorders that is disproportionately prevalent in MS patients compared to the general population [409]. It has been reported that the prevalence of epilepsy is six times higher in MS patients than in the healthy population [410]. This is not surprising, as both lesions, inflammation and neurotransmitter imbalances within the CNS of people with MS may trigger such disabling ailment, with evidence also suggesting that the frequency of seizures tends to increase as the disease progresses [411].

As mentioned, an imbalance in neuronal activity is a critical neurochemical feature of seizure episodes [408]. It is well-documented that neuropeptides can contribute to reset this imbalance, including PACAP and VIP [412,413]. Despite the emerging evidence, to date only one neuropeptide-based hormone, adrenocorticotropic hormone (ACTH), is currently being tested in clinical trials for the treatment of seizures in a rare disease that affects infants (West syndrome), whereas investigations on the efficacy of other neuropeptides in epilepsy has been limited to preclinical studies [408,414].

PACAP and VIP have both shown to be released upon high neuronal firing activity [415,416]. VIP exerts an overall excitatory effect on synaptic transmission, which is mediated by VPAC1 and VPAC2 receptors [417,418]. In hippocampal surgical samples of patients suffering from human temporal lobe epilepsy, both VPAC1 and VPAC2 receptors were shown to be up-regulated [290] and up-regulation of VPAC receptor subtypes has been associated with increased neuronal survival [417]. These data support the idea that hippocampal VPAC receptors are increased as a homeostatic mechanism to prevent excessive neuronal damage/death caused by epilepsy.

Research into the role of PACAP in epilepsy has been centred on its ability to modulate microglia and glutamate transmission [419-421]. The expression of PACAP increases after kainic

acid-induced seizures in rats, with many suggesting that this seizure-induced increase in PACAP may help to reduce excitotoxicity and promote overall neuroprotection to protect the CNS from damage [419]. Specifically, PACAP acts on microglia to promote the release of anti-inflammatory factors that polarise microglia towards an anti-inflammatory M2 phenotype, whilst concurrently increasing the expression of glutamate transporters that promote glutamate re-uptake, resulting in two parallel protective mechanisms [422,423].

Magnetic resonance imaging and pathological studies have proposed that cortical inflammation, demyelination and grey matter damage in MS patients may be responsible for the onset and development of epileptic seizures [411]. However, epilepsy is an active process, so it is difficult to determine if any aspect of MS pathology may specifically promote or trigger seizure episodes, especially given that MS patients often present with unique pathogenic profiles and distributions of lesion within the CNS. This should be considered as a further incentive to invest more in researching the efficacy of PACAP and VIP as therapeutic targets for epilepsy and MS, as both conditions are often comorbid and targeting these peptides or their receptors may prove to be effective to ameliorate epilepsy associated to MS.

2.4 Stroke

Stroke is the leading cause of adult disability, with one in four people globally experiencing a stroke event in their lifetime [424]. Stroke occurs when there is a long-lasting interruption or severe reduction of cerebral blood flow, which triggers a myriad of pathological cascades including excitotoxicity, oxidative stress, neuronal cell death and blood brain barrier (BBB) leakage [425]. Inflammatory processes including the autoimmune activities of MS are thought to induce endothelial dysfunction and atherosclerosis, which may contribute to promote the development of micro- and macro-vascular dysfunctions that culminate in ischaemic stroke. As such, it should not be surprising that compared with the general population, people with MS are at increased risk of experiencing a stroke, and if they do, they tend to suffer more severe symptoms [426].

It has been shown that administration of PACAP in animal models of stroke is neuroprotective and causes a reduction of both neurological deficits and the degree of pathological change of the CNS tissue of the ischaemic brain area [300]. There is an increase in inflammation post-stroke [288,427], and PACAP and VIP are well-known anti-inflammatory agents in the CNS [187]. Both peptides are expressed in different types of immune cells, including microglia and astrocytes [193,428]. Masmoudi-Kouki et al. suggested that the neurotrophic and neuroprotective effects of PACAP and VIP can be partly accounted for by their effects on

astrocytes [429]. This is supported by the increase in both astrocytic PACAP and PAC1 expression immediately following cerebral ischemia [430]. Additionally, exposure of cultured astrocytes to PACAP was found to be capable of up-regulating glutamate uptake mechanisms via PAC1-mediated signalling, suggesting that PACAP can reduce post-stroke excitotoxicity due to excessive glutamate [423].

In a study, treatment with VIP has shown to reduce brain damage and to promote neurogenesis following ischemic injury in the rat brain [431], although it should be highlighted that most of the neuroprotective effects reported for experimental stroke are related to PACAP and not VIP. Indeed, studies in PACAP knockout mice suggest that PACAP prevents post-ischemic neuronal cell death [432,433]. In animal models of stroke, intranasal administration of PACAP reduced infarct volume and improved functional recovery [434]. Additionally, PACAP-dependent polarisation of microglia towards a M2 anti-inflammatory phenotype resulted in improved functional recovery in mice post-ischemia [435].

These studies provide evidence that both PACAP and VIP have potential therapeutic validity for the treatment of stroke and may find application to aid in the recovery of neuronal injury post-stroke due to their neuroprotective and anti-inflammatory functions.

2.5 Neuropathic pain

Neuropathic pain is defined as pain caused by damage to the somatosensory nervous system [436]. WHO defines MS as one of the most important conditions that causes central neuropathic pain, together with spinal cord or brain injury and stroke, with neuropathic pain reported in 86% of MS patients [437]. Neuropeptides are key regulators of inducing and maintain peripheral nociception and contribute to the central sensitisation of pain. In many cases, chronic inflammation, ion channel imbalance and a lack of inhibition in the dorsal horn of the spinal cord maintains pain [438]. PACAP and VIP are expressed across key anatomical regions that are important in somatosensory processing and are also associated with pain [439]. In the peripheral nervous system, this includes the dorsal root ganglion of the spinal cord and in the central nervous system, these peptides are found in key pain processing regions, including the thalamus, periaqueductal grey (PAG) and amygdala [440]. Despite the anatomical relevance of PACAP and VIP in neuropathic pain pathways, their distinct roles in the pathophysiology of neuropathic pain remains to be identified. Studies using PACAP knockout mice have indicated that PACAP plays an excitatory role in pain transmission, with these mice displaying an absence of neuropathic and inflammatory pain [441]. This was confirmed in subsequent studies

that demonstrated PACAP was increased in the dorsal horn which is be critical for the development of neuropathic pain [442].

Several studies have shown that PACAP signalling causes both hyperalgesia and allodynia, which are common presentations of neuropathic pain. For example, intrathecal injection of PACAP induces hyperalgesia in mice [443]. A novel small molecule antagonist of the PAC1 receptor was able to inhibit PACAP and nerve injury induced allodynia, suggesting a role of PACAP-PAC1 interaction in the induction of pain [444]. Furthermore, intrathecal PACAP administration resulted in prolonged allodynia and sustained activation of astrocytes [445]. The overlap between pain and inflammatory signalling has been extensively studied and reviewed [445]. Additionally, an association between axonal regeneration and pain has been put forward, with accelerated regeneration ameliorating pain, while permanent expression of key regeneration markers maintaining pain signalling [438]. PACAP and VIP have well-described roles in promoting axonal regeneration after peripheral nerve injury [196,210]. For example, RNA sequencing data revealed ADCYAP1, the gene encoding PACAP, was the most differentially expressed gene associated with regeneration post-surgery in carpal tunnel syndrome patients [446].

Most studies have suggested PACAP has a more dominant role in pain pathophysiology compared to VIP. However, intradermal injections of VIP into the forearm caused more pronounced cutaneous pain and increased blood flow compared to PACAP, suggesting this peripheral nociceptive effect is mediated by the VPAC receptors. Moreover, the relationship between inflammation and pain suggest both VPAC receptors play a pivotal role in the pathophysiology of neuropathic pain [302]. Chronic neuropathic pain is known to affect behaviour and it has been shown that PACAP/PAC1 signalling in the amygdala contributes to pain and behavioural responses [371]. Moreover, we have shown that PACAP and VIP are altered in the PAG in response to neuropathic injury [296]. As discussed previously, PACAP and VIP are involved in the pathophysiology of behavioural disorders, suggesting these neuropeptides are attractive therapeutic targets to relieve neurological dysfunction across pathologies.

2.6 Migraine

Migraine is a complex and debilitating headache disorder that affects one in seven people worldwide [447]. Despite clinically manifesting as recurrent attacks of headache, migraines are associated with a range of symptoms and are linked to other conditions like depression, anxiety,

sleep disorders, chronic fatigue and cognitive dysfunction [448]. The role of neuropeptides in migraine in an active area of research. The most studied neuropeptide in relation to migraine is CGRP [449]. CGRP administration has been shown to induce migraine like headaches that are indistinguishable from spontaneous migraine attacks [450,451]. This has led to the discovery and use of CGRP antagonists that are effective in treating migraine symptoms [4514].

PACAP has been shown to act in a similar way to CGRP, with infusion of PACAP inducing headaches in healthy volunteers [452]. Similarly, plasma levels of CGRP and PACAP are elevated following a migraine attack [453]. Interest in PACAP has a therapeutic target for migraine, stems from the discovery that PACAP-induced migraine did not results in an increase in CGRP, suggesting an alternate route of migraine initiation [454]. Human provocation studies have provided the most robust evidence describing the involvement of PACAP in migraine [4544]. Intravenous administration of PACAP produced immediate headache in healthy participants, and delayed migraine in migraine sufferers [454]. Additionally, PACAP induced pronounced dilations of extracranial arteries [455]. This dilation was further confirmed using magnetic resonance angiography that reported dilation of the middle meningeal artery in participants that reported migraine after PACAP infusion [456].

Despite sharing similar anatomical distribution and vasodilation properties to PACAP, the role of VIP in migraine remains controversial. Previously, it was suggested that VIP was unable to induce migraine [443]. Additionally, Bertels and colleagues, investigated the impact of PACAP and VIP on functional brain connectivity using functional magnetic resonance imaging, and revealed that PACAP, but not VIP, altered connectivity that coincided with the development of migraine in migraine patients [454]. However, recent studies have reported contrasting findings. Pellesi and colleagues, demonstrated that a 2-hour infusion of VIP could induce migraine attacks in 71% patients with migraine without aura, similar to PACAP [457]. They revealed that this induction was caused by dilation of cranial arteries mediated by VPAC1 and VPAC2 receptors, contradicting previous notions that migraine was induced via PAC1 receptor activation [457]. This could explain why novel therapeutics targeting PAC1 have had low efficacy in preventing migraine, in clinical trials [454,458,459]. Therefore, more research is needed to elucidate the specific role of each receptor in migraine induction and maintenance. For example, recent studies have linked mast cell degranulation to contributing to migraine pathology in a VPAC1/2 receptor mediated fashion [460]. Chronic inflammation has been shown to promote the maintenance of pain states. As such, both migraine and neuropathic pain demonstrate the delicate balance of targeting PACAP and VIP in neurological disease due to their pleiotropic functions and global expression.

4.PACAP and VIP as a broad-spectrum therapy for MS and its associated comorbidities

The broad beneficial functions of PACAP and VIP in the CNS make them ideal neuroprotective agents, capable of promoting neuronal survival, function and protect neurons against inflammation. These peptides can be useful in a myriad of neurological diseases. In addition, as highlighted in this review, the VIP/PACAP system is clearly implicated in ameliorating several neurological comorbidities associated with MS (**Fig. 2**).

As highlighted in this review, in MS the VIP/PACAP system has shown to partly counteract autoimmunity. The neuropeptide system causes a shift from autoreactive T cells to anti-inflammatory state of T-cells by promoting the recruitment of anti-inflammatory Th2 and Treg cells as well as several other immunomodulatory effects (see 1.3.2 ;reviewed in [461]; [324]). During the active stages of the disease, the MS brain shows signs of white matter inflammation in lesioned areas, which is believed to cause not only myelin damage, but also oligodendrocyte cell loss [318]. As discussed above, an increased inflammatory *milieu* in the CNS is also a major trigger of the comorbidities associated with MS. Strikingly, CNS white matter pathology has recently been suggested as a contributing factor to disease pathology in schizophrenia, epilepsy and stroke [346,393,462-468]. Thus, despite the different clinical presentations and domains of some of the pathologies discussed in this review, the underlying similarities at a molecular level highlight a potential link and good target for therapeutic intervention that could span these disorders.

This idea is further substantiated by the overlapping positive effects of the VIP/PACAP system in MS and its comorbidities. The multiple actions of this neuropeptide system could mean that various downstream pathways are likely to be targeted. We predict that targeting this system could potentially ameliorate MS symptoms both by protecting oligodendrocytes, likely through a reduction of the pro-inflammatory CNS microenvironment, while also positively affecting comorbid conditions arising with the disease. For example, for those MS patients who also experience strokes, targeting the VIP/PACAP system could stimulate neuroprotection and neurogenesis, inhibit apoptosis and promote axon regeneration, while also triggering an anti-inflammatory phenotype of activated glial cells, which could help preventing the ongoing oligodendrocyte death [187,469,470].

PACAP and VIP have been in clinical trials as therapeutics for a range of peripheral disorders such as arthritis [471], in which when injected intraperitoneally, the peptides reduced the incidence and severity of arthritis, even when administered in the late stage of disease [311]. Moreover, since data from the aforementioned migraine studies show that stimulating the VIP/PACAP system can actually lead to an increased incidence of migraines, several

researchers are exploring the option of blocking PACAP activity in the brain to help migraine patients. Recent studies saw the development of two different humanized monoclonal antibodies against PACAP (ALD1910) and PAC1 (AMG301) with antagonizing activity [472,473]. These antibodies are currently in preclinical and clinical trials as well to study their safety and efficacy in migraine prevention. While the results of ALD1910 and migraine prevention have not been published yet. Recently, the results of a phase II clinical trial using AMG301 to aid in migraine prevention were published, finding no significant differences in migraine prevention in participants exposed to ALD1910 compared to a placebo [474]. Although no significant beneficial effects were found, it does highlight the confidence of the field that targeting the VIP/PACAP system can offer potential treatment opportunities. Noteworthy, a recent study described the potential of two well-known broad-spectrum antibiotics as a potential compound to target the PAC1 receptor as well. Doxycycline and Minocycline were shown to act as positive allosteric modulators of PAC1, stimulating axonal regeneration activities in cultured Schwann cells [475,476]. It would be interesting to see if a potential future *in vivo* experiment could replicate these findings.

To achieve potential beneficial effects of PACAP and VIP in the CNS, passage through the blood brain barrier (BBB) must be improved. To overcome this issue, recently Yu and colleagues generated a VIP-TAT construct with enhanced efficiency to cross the BBB, which showed increased neuroprotection compared with VIP alone in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease [477,478]. Therefore, in view of the current advances in biotechnology, it is reasonable to anticipate more research into PACAP and VIP and more opportunities for these peptides to be used as therapeutics for neurological and cognitive diseases.

However, it is important to recognize that targeting the VIP/PACAP system can come with its downsides. Since PACAP and VIP are pleiotropic molecules and can target different cell types within the CNS and periphery, it would require specialised administration strategies to deliver the peptides so that they can target the desired cell population or CNS region. Adeno-associated virus and/or other personalised gene therapy approaches are becoming closer to achieving targeted administration of therapeutics to the brain via systemic administration [479]. This could limit potential off-target effects by limiting the availability of the peptides to for example, the PAC1 receptor in cardiac cells [479].

Altogether, despite the fact that direct administration of the peptides to brain would limit peripheral side effects, it might still lead to noteworthy adverse CNS side effects considering how stimulating the VIP/PACAP system is known to trigger migraines. However, the plethora

of positive effects of the VIP/PACAP system the CNS means that targeting this system could create an overall beneficial environment in the MS brain spanning core-pathology as well as most associated comorbidities.

5. Conclusion & future directions

The VIP/PACAP system appears to have protective benefits in MS and most of its associated neurological comorbidities, making it an attractive therapeutic target to pursue (**Fig. 2**). With recent advancements in CNS-targeted drug administration, there is more flexibility in treatment options as new ways of overcoming the challenge of delivering drugs past the BBB or impeding rapid degradation are being developed. As such, future research can now explore the potential of PACAP and/or VIP as targets for a range of neurological diseases that involve inflammation and consequently, neurodegeneration.

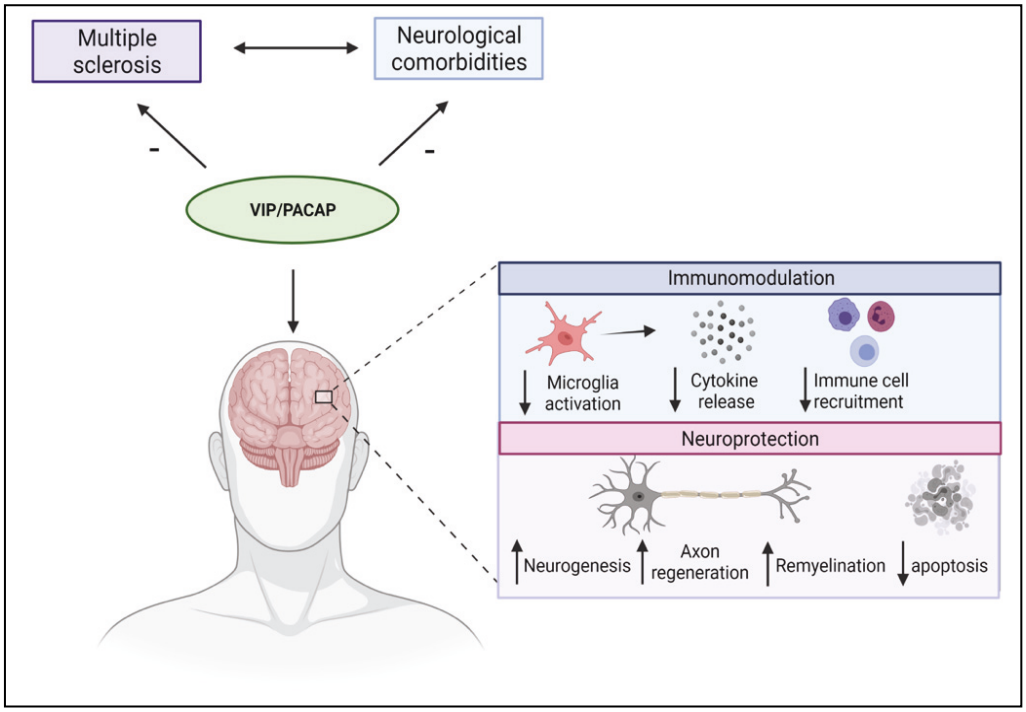

Figure 2. Immunomodulation and neuroprotection functions of PACAP and VIP in Multiple Sclerosis and neurological comorbidities. *Both PACAP and VIP are promising neuropeptides that can aid in MS and the associated neurological comorbidities of MS through the potent immunomodulatory actions and neuroprotective effects of both these peptides.*

Abbreviations

ACTH, adrenocorticotrophic hormone; BBB, blood brain barrier; BD, bipolar disorder; BNST, bed nucleus of the stria terminalis; CNS, central nervous system; CGRP, calcitonin gene-related peptide; DISC1, disrupted-in-schizophrenia 1; EAE, experimental autoimmune encephalomyelitis; GPCR, G protein-coupled receptors; HPA, hypothalamic-pituitary-adrenal; IL, interleukin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MRI, magnetic resonance imaging; MS, multiple sclerosis; NK, natural killer cells; NPY, neuropeptide Y; NSC, neural stem cells; NRG1, neuregulin-1; PACAP, pituitary adenylate cyclase-activating polypeptide; PNS, peripheral nervous system; SNPs, single nucleotide polymorphisms; RA, rheumatoid arthritis; VIP, vasoactive intestinal peptide; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

1.1.5 Modelling multiple sclerosis in mice

To understand the pathogenesis and pathophysiology of MS as well as study new therapeutic options, it is important to recapitulate MS in experimental models of disease. Since MS is a highly complex and heterogeneous disease, there is no "perfect" model that mimics all features of disease. However, with the use of mouse models, researchers can study aspects of MS pathology in detail.

Two popular experimental models of MS used in preclinical research are the experimental autoimmune encephalomyelitis (EAE) model and the cuprizone (bis-cyclohexanone oxaldihydrazone) intoxication model (CPZ model)[81].

The EAE model in rodents relies on the external administration myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 with a set of adjuvants. This induces an immune response in the rodent's body where autoreactive myelin-recognising T-cells migrate to the CNS triggering an inflammatory cascade causing tissue injury[480,481]. This model of MS recapitulates the autoimmune and neuroinflammation component of the disease, and has helped the field make tremendous progress into understanding the role of the immune system in MS pathogenesis and the development of MS treatments[482].

However, there are many issues with the EAE model that make it not ideal to use in all scenarios. For example, lesions develop most predominantly in the white matter of the spinal

cord in EAE mice, whereas in people with MS, lesions are mostly located in the white matter and cortex of the cerebrum[80,81]. Additionally, the EAE model elicits a very strong $CD4^+$ T cell response while research has shown the importance of $CD8^+$ T cells and B cells in MS patients. Moreover, since we do not yet know the origin of MS pathology, utilising this model runs at the risk of omitting the oligodendrocyte and neuronal contribution to disease development as well. These problems become especially apparent when using the EAE model to study treatments, where it has been proven difficult to use this model to predict success DMT in MS [79,483]. In the practical sense, another critical problem of this model is its relative high cost, and difficulty establishing and reproducing the experimental outcomes due to batch differences in reagents used[484].

The CPZ model mimics oligodendrocyte damage in MS, where it induces white matter damage first with subsequent neuroinflammation [96,485]. By feeding mice with CPZ, progressive loss of oligodendrocytes can be observed in the brain leading to a hypomyelinated state reflecting type III or IV lesions in people with MS[486,487]. This model of MS helps our understanding of how oligodendrocytes and their precursors are potentially affected and function under injury. This tool would thus allow for studies on potential therapies that target oligodendrocytes and myelination[485].

Upon 4-week exposure to 0.2% CPZ, demyelination can be observed throughout the corpus callosum and in cortical areas of the brain. Astrogliosis, microgliosis and axonal damage is also reflected in this model. Although mechanistically we do not fully understand why oligodendrocytes are particularly vulnerable to CPZ, metabolic stress is believed to be a driving factor of the molecular mechanism[487]. For instance, CPZ is a copper chelator and could therefore alter the copper homeostasis in the brain of mice[488]. This altered metabolic state is believed to impact mitochondrial functioning, triggering ROS production, mitochondrial swelling and altered energy metabolism, leading to oligodendrocyte apoptosis[489]. Outside of CPZ eliciting intrinsic oligodendrocyte damage, reactive astrocytes and microglia are also crucial players for CPZ-induced demyelination where their cytokine release contributes to a toxic microenvironment further aggravating oligodendrocyte pathology[490].

Thus, CPZ-model is an excellent tool to study demyelination, reflecting many pathological signs associated with MS. However, another great feature of this model is the spontaneous remyelination that occurs in the brain upon ceasing the CPZ diet, allowing for research into the

molecular pathways driving remyelination in the brain. This makes the CPZ toxicity the most ideal model to study the de-myelination and re-myelination process in detail in a reproducible and easy-to-use manner.

1.1.6 Current treatment approaches

There are currently no cures available for MS. However, a selection of disease-modifying therapies (DMTs) are available for clinical use[491]. Most of these therapies are approved for use in RRMS or SPMS with only limited treatment options available for people with PPMS.

Given the strong inflammatory component of MS, first-line treatments have traditionally been immunomodulatory, with patients often receiving high-dose interferon-β-1a or glatiramer acetate. These therapies target T-cell activity, pushing them to more anti-inflammatory Th2 phenotypes[492,493]. Although these strategies have been modestly effective in reducing relapse rates and disease severity, they are associated with debilitating side effects[494].

More recently, a selection of new medications got FDA-approval for people with relapsing forms of MS, targeting immune cells other than T-cells. Sphigosine-1-phosphate (S1P) receptor modulators have shown beneficial effects in people with relapsing MS and several modulators are now used in the clinic (e.g. fingolimod, siponimod, ozanimod, and ponesimod)[495]. S1P receptor modulators work by preventing lymphocyte egress from lymph nodes, effectively reducing the number of activated immune cells entering the CNS. Additionally, they are believed to promote the presence of regulatory T cells and naïve B cells, thereby impacting the aberrant immune response in MS[496]. However, several interesting off-target effects have also been implicated in its unique mechanism-of-action (MOA)[497]. Other drugs recently approved in relapsing MS, such as Teriflunomide and Cladribine inhibit T-and B-cell proliferation albeit through different MOAs[498,499]. For people with PPMS, treatment options are extremely limited. The only FDA-approved drug currently available is ocrelizumab, an anti-CD20 monoclonal antibody that selectively target B-cells which has shown to lower clinical and MRI progression[500]. Progress into DMT's that benefit PPMS patients has been limited by difficulties in patient recruitment due to the rarity of the diagnosis, lack of suitable outcome measures and lower involvement of inflammation in PPMS [501,502].

The approach for MS treatment has always been to target the aberrant immune response to prevent disease progression. However, while this targets one aspect of the disease, it fails to address the underlying demyelination and neurodegeneration. In fact, neuronal loss is the strongest correlate with MS symptoms [503]. Additionally, at some point all available treatment options cease to work, and the patient will experience progressive worsening of symptoms. On top of that, available therapies have severe debilitating side-effects and quality of life is just as important to people with MS as slowing down disease progression[504]. This shows how there is a need for therapies to do more than just slow progression and should also have patient quality of life in mind. Future therapeutic approaches should thus aim to not only modulate the immune response, but also promote neuroprotection and remyelination, possibly using neuroprotective agents or regenerative medicine techniques. To summarise, there is a clinical need for MS-therapies that can target both the inflammatory response as well as prevent the loss of myelin and neurons in the CNS.

1.2 Aims and Hypothesis

The overarching theme of my thesis is to dissect the specific roles of the VIP/PACAP peptides and their related receptors PAC1, VPAC1 and VPAC2 on brain functioning in the context of MS and demyelination. While researchers have started to examine the role of this neuropeptide system and the peripheral immune system in MS, I recognise that much remains unknown about the contributions of these neuropeptides and their receptors during the demyelination process, as well as their effects on neuronal functioning and neuroinflammation.

Specifically, I will start by examining the VIP/PACAP system in brain tissues from MS donors. Additionally, I will unravel the impact of PACAP and VIP on preventing demyelination in the CPZ model, investigating their potential use as a therapeutic agent in MS. Based on these findings, I will focus on understanding the role of neuronal PAC1 in MS and initiate our capability to study the role of PAC1 in oligodendrocytes specifically, setting the stage for future research into the cell-type specific roles of PAC1.

To study these concepts, I will pursue four specific aims:

Aim 1: Determine if the expression and distribution of VIP/PACAP neuropeptides, as well as that of PAC1, VPAC1 and VPAC2 receptors are altered in the normal-appearing white matter (NAWM) of MS donors with different clinical histories of disease progression.

For this, I examined:

1. The differential transcript levels of VIP, PACAP, PAC1, VPAC1 and VPAC2 in the NAWM of MS donors with differing disease subtype using real-time quantitative PCR (RT-qPCR).
2. Changes in the protein expression of PAC1, VPAC1 and VPAC2 in the NAWM and lesion sites of within the white matter of MS donors using immunohistochemistry.

Hypothesis 1: There are MS subtype-specific alterations in the expression of the VIP/PACAP neuropeptides and receptors in the NAWM of people with MS.

Aim 2: Assess if PACAP, VIP or maxadilan (MAX) treatment have beneficial effects on locomotion and molecular markers of disease during CPZ-induced demyelination.

To pursue this aim, I provided:

1. In-depth analyses of how CPZ-induced demyelination and treatment with PACAP, VIP or MAX affect general locomotion.
2. Thorough molecular analyses of the beneficial effects of PACAP, VIP and MAX treatment on neuroinflammation, axonal damage and expression of myelin markers.

Hypothesis 2: Treatment with PACAP, VIP and MAX will prevent CPZ-induced locomotor impairments, dampen neuroinflammation and axonal damage, as well as prevent myelin loss.

Aim 3: Dissect the contribution of neuronal PAC1-loss in neuronal functioning both under healthy and demyelinating conditions.

1. Provide a comprehensive behavioural and molecular analysis of the inducible neuronal PAC1 knockout mouse model (Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$) under healthy and demyelinating conditions induced by the CPZ model.

Hypothesis 3: The PAC1 receptor is essential in neuronal functioning and protects the CNS after a demyelinating challenge.

Aim 4: Generate a tamoxifen-inducible conditional mouse model with targeted PAC1 deletion in mature oligodendrocytes.

1. Develop a PLP-driven inducible PAC1 knockout mouse model using the advanced CreERT-LoxP system.

Hypothesis 4: Induction of PAC1 knockout in the PLP-CreERT$^{+/-}$ PAC1$^{flox/flox}$ mouse model is expected to lead to a specific reduction in PAC1 expression levels within white matter.

Chapter 2:

VIP/PACAP receptor expression in NAWM of MS donors

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1. Laboratory of Cellular and Molecular Neuroscience (LCMN), School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW 2007, Australia
2. Department of Biomedical and Biotechnological Sciences, Human Anatomy and Histology Section, School of Medicine, University of Catania, 95123, Catania, Italy

Contribution:

- Experiments
- Analysis
- Writing
- Manuscript preparation

Signature of co-authors:

Name	Signature
Margo Iris Jansen	Production Note: Signature removed prior to publication.
Guiseppe Musumeci	Production Note: Signature removed prior to publication.
Alessandro Castorina	Production Note: Signature removed prior to publication.

Differential Expression of PACAP/VIP Receptors in the Post-mortem CNS White Matter of Multiple Sclerosis Donors

Margo Iris Jansen [1], Giuseppe Musumeci [2] and Alessandro Castorina [1,*]

1. Laboratory of Cellular & Molecular Neuroscience (LCMN), School of Life Sciences, Faculty of Science, University of Technology Sydney, P.O. Box 123, Sydney, NSW 2007, Australia; margo.jansen@student.uts.edu.au
2. Department of Biomedical and Biotechnological Sciences, Human Anatomy and Histology Section, School of Medicine, University of Catania, 95123 Catania, Italy; g.musumeci@unict.it

*Correspondence: alessandro.castorina@uts.edu.au

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two neuroprotective and anti-inflammatory molecules of the central nervous system (CNS). Both bind to three G protein-coupled receptors, namely PAC1, VPAC1 and VPAC2, to elicit their beneficial effects in various CNS diseases, including multiple sclerosis (MS). In this study, we assessed the expression and distribution of PACAP/VIP receptors in the normal-appearing white matter (NAWM) of MS donors with a clinical history of either relapsing–remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS) or in aged-matched non-MS controls. Gene expression studies revealed MS-subtype specific changes in PACAP and VIP and in the receptors' levels in the NAWM, which were partly corroborated by immunohistochemical analyses. Most PAC1 immunoreactivity was restricted to myelin-producing cells, whereas VPAC1 reactivity was diffused within the neuropil and in axonal bundles, and VPAC2 in small vessel walls. Within and around lesioned areas, glial cells

were the predominant populations showing reactivity for the different PACAP/VIP receptors, with distinctive patterns across MS subtypes. Together, these data identify the differential expression patterns of PACAP/VIP receptors among the different MS clinical entities. These results may offer opportunities for the development of personalized therapeutic approaches to treating MS and/or other demyelinating disorders.

Keywords: pituitary adenylate cyclase-activating polypeptide; vasoactive intestinal peptide; multiple sclerosis; normal-appearing white matter; relapsing-remitting MS; secondary progressive MS; primary progressive MS; demyelination

1. Introduction

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS) characterized by ongoing myelin loss, which leads to the formation of white matter lesions responsible for the subsequent neurodegeneration and functional impairment [1,2,55]. For yet undetermined reasons, the prevalence of MS is rising worldwide with an estimated 3 million people currently living with this diagnosis [3]. While the MS etiology remains partly unclear, genetic, environmental and lifestyle factors have been established as contributors to disease onset and development [66,505].

Historically, MS has been categorized into three main subtypes based on its clinical course, and although this classification is now becoming somewhat outdated, it is still in use in view of its validity for differentiating progressive forms of MS of unknown etiology from those secondary to milder cases at onset. Under this classification, the most common (and less aggressive) MS subtype is relapsing–remitting MS (RRMS), which accounts for approximately 87% of all MS cases [506]. RRMS is characterized by the recurring exacerbation of symptoms (relapses) that are usually followed by some degree of recovery and the absence of clinical symptoms (remissions). In most cases, RRMS will progress into secondary progressive MS (SPMS), a stage in which patients will no longer experience episodes of recovery and the progressive worsening of symptoms becomes a prevalent clinical feature [6]. The third clinical subtype of MS is primary-progressive MS (PPMS). This form of MS is considered the most severe and is characterized by the relentless deterioration of symptoms after disease onset [507]. Currently, there is no cure for MS, and most disease-modifying therapies (DMTs) aim to tackle the dysfunctional immune system responsible for the myelin loss and/or attempt to ameliorate the large inflammatory component of the disease, especially in the earliest stages. However,

despite the ever-growing arsenal of DMTs, not all MS patients respond well to currently available drugs, and many prefer symptomatic treatment over DMTs due to the severe adverse effects experienced with treatment [504]. This has prompted further studies regarding the identification of new therapeutic targets and the development of more effective treatment strategies.

A key pathological feature of MS is the chronic overactivation of the immune system against specific components of CNS myelin, which, combined with an inefficient reparative response by oligodendrocytes and their precursors, culminates in the formation of multi-focal scars/lesions, the typical hallmarks of an MS brain in diagnostic imaging [8]. In this context, the white matter that appears "normal" is referred to as normal-appearing white matter (NAWM). However, there is a growing body of evidence suggesting that, even in these seemingly unaffected areas, there may be subtle microscopic changes, such as axonal damage or alterations in cell density [508,509], which may not be easily detected by standard magnetic resonance imaging (MRI) techniques. In addition, there are reports suggesting that the NAWM of MS patients may present with subtle pathological and molecular signatures, such as signs of mild inflammation [510] and alterations in tight junctions [511-513], that are often difficult to capture using standard detection methods.

Vasoactive intestinal peptide (VIP) and pituitary adenylate-cyclase-activating peptide (PACAP) are two small neuropeptides produced and secreted by a multitude of CNS cells [187,195]. VIP and PACAP elicit their biological activities by binding to three G protein-coupled receptors (GPCRs) called VPAC1, VPAC2 and the PAC1 receptor. VIP, like PACAP, binds with high affinity to all three GPCRs; however, PACAP shows a higher affinity to PAC1 receptors than VIP (about 100-folds higher), making it a preferential PAC1 agonist [514,515]. Both VIP and PACAP are known for their anti-inflammatory and neuroprotective properties in the CNS and have been proposed as potential therapeutic targets in MS and associated disorders, such as optic neuritis [191,193]. Indeed, decreased serum VIP and PACAP levels have been detected in human blood and cerebrospinal fluid (CSF) samples, respectively [319,516], suggesting that these neuropeptides may also find application as biomarkers of disease and/or be utilized to monitor MS progression.

In the context of preclinical studies using experimental autoimmune encephalomyelitis (EAE), a well-established rodent model of MS, researchers have described divergent roles for some

VIP/PACAP receptors and peptides. In fact, both PACAP- and VPAC1-deficient mice showed heightened resistance to EAE and reduced symptomatology, whereas VPAC2-deficient mice showed an exacerbated EAE pathology and more severe symptoms [201,202,321]. In addition, treatment with either PACAP or VIP ameliorated EAE severity, whereas unpublished data from our laboratory indicate that PACAP is most effective in preventing myelin loss in the cuprizone demyelination model. These data, despite being obtained from animal observations, pinpoint the potential distinctive beneficial roles of the two neuropeptides' receptors with respect to different pathological domains of MS [310,323], and warrant further investigations on VIP/PACAP receptor expression and distribution in the human MS brain.

In the present study, we utilized a combination of post-mortem tissue sections (fixed) and case-matched fresh–frozen samples obtained from local brain tissue banks to explore the different expression levels and distribution of PAC1, VPAC1 and VPAC2 receptors in the NAWM of MS brains versus aged-matched non-MS controls. Furthermore, to define the pattern of changes in VIP/PACAP receptor levels across different MS clinical entities, analyses were also stratified based on the disease subtype. Finally, the study also investigated the distribution of VIP/PACAP receptors around and within lesioned areas of selected cases encompassing at least one chronically demyelinating lesion, unless otherwise stated.

2. Results

2.1. PACAP and VIP Gene Expression in the Normal-Appearing White Matter of Multiple Sclerosis Cases Reveals Subtype-Specific Changes

Prior to examining the expression levels of the VIP/PACAP system in human normal-appearing white matter (NAWM), we conducted a preliminary evaluation of the NAWM integrity to identify gross structural alterations and locate lesioned areas within the tissue sections (Figure 1). To achieve this, we utilized Luxol Fast Blue (LFB) staining, a common histological technique that specifically stains myelin (in blue) and aids in the localization of lesions, which appear as discolored areas. This approach also enabled the definition of the regions of white and grey matter in the CNS adjacent to lesions, where present (WM and GM, respectively; Figure 1A and Figure 1D). As shown in the representative images (Figure 1B–D), at least one lesion was identified in each section representing the different MS clinical subtypes. No obvious differences in the LFB staining patterns were noticed in the NAWM amongst the selected cases.

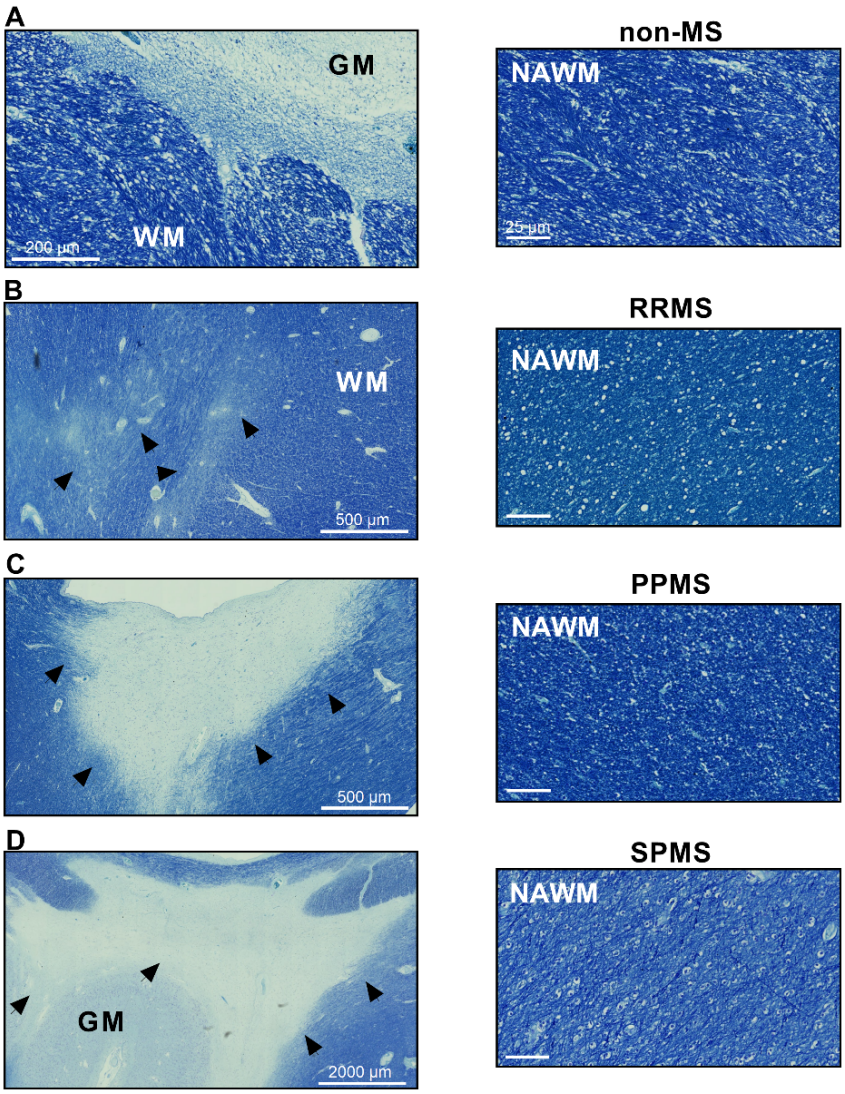

Figure 1. Representative lesions and normal-appearing white matter in human brain sections from donors with different MS subtypes. *Luxol Fast Blue (LFB) staining shows the intense blue staining of myelinated fibers in the white matter (WM) of (**A**) non-MS donors, differentiating it from the less myelinated grey matter (GM). Evident discoloring of lesioned areas (indicated by black arrowheads) can be appreciated in sections from (**B**) RRMS, (**C**) PPMS and (**D**) SPMS cases. Myelin is stained blue, resulting in a clear distinction between GM and WM. The sites of demyelination in each MS subtype are indicated by black arrowheads. Scale bar in (**A**) 200 µm, (**B**,**C**) 500 µm, (**D**) 2000 µm and NAWM (panels on the right) 25 µm. MS = multiple sclerosis, RRMS = relapsing–remitting MS, PPMS = primary-progressive MS, SPMS = secondary-progressive MS, GM = grey matter, WM = white matter.*

Using real-time quantitative polymerase chain reaction (RT-qPCR), we measured the relative expression levels of the neuropeptides PACAP and VIP in the NAWM of MS patients and the non-MS control tissue samples (Figure 2). Upon the examining the PACAP (gene name *ADCYAP1*) and VIP expression in non-MS versus MS cases, no statistically significant differences were found (Figure 2A). However, the further stratification of data based on the clinical subtype demonstrated a significant increase in PACAP expression in SPMS cases (t_{10} = 4.790; *** p = 0.0007; Figure 2A‴) but not in RRMS and PPMS (p = 0.066 and p = 0.257, respectively; Figure 2A′ and Figure 2A″). VIP expression was significantly increased in RRMS (t_{12} = 2.454; * $p < 0.05$; Figure 2B′) and strongly downregulated in the NAWM of PPMS cases (t_{12} = 2.195; *p=0.0533***; Figure 2B″). No statistically significant changes were observed in the NAWM of SPMS cases (t_{10} = 1.069; p = 0.3104; Figure 2B‴).

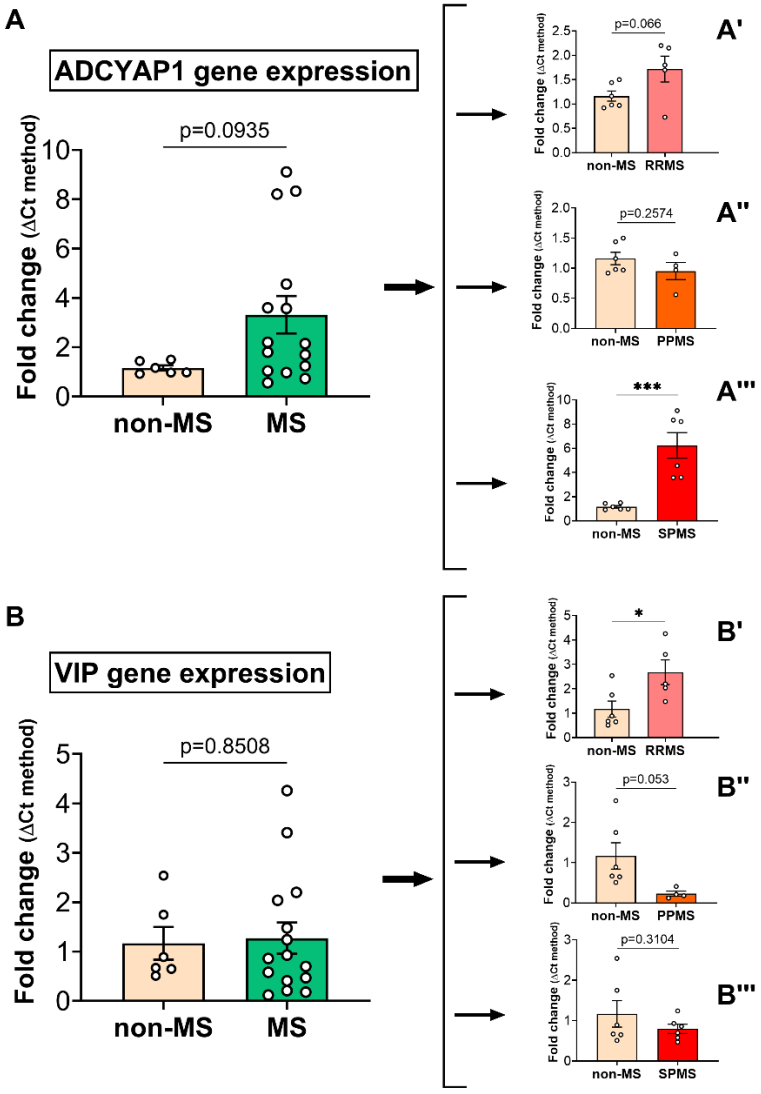

Figure 2. Differential expression of PACAP and VIP neuropeptide genes in the normal-appearing white matter of MS donors. *(**A**) PACAP (gene name = ADCYAP1) expression was measured using RT-qPCR, comparing non-MS and MS cases. Further stratification of cases by clinical course, showing the expression levels of ADCYAP1 in (**A'**) non-MS vs. RRMS, (**A"**) non-MS vs. PPMS and (**A‴**) non-MS vs. SPMS. (**B**) VIP gene expression in non-MS vs. MS cases. A stratification similar to that in A demonstrates relative changes in the transcript levels between (**B'**) non-MS and RRMS, (**B"**) non-MS and PPMS and (**B‴**) non-MS and SPMS. The data shown are the mean fold change ± SEM, obtained from n = 6 (non-MS), n = 5 (RRMS), n = 6 (SPMS) and n = 4 (PPMS) cases. p-values > 0.05 are also shown. * p < 0.05 or *** p < 0.001 vs. non-MS, as determined by unpaired t-test. VIP = vasoactive intestinal peptide, PACAP = pituitary adenylate cyclase activating polypeptide, MS = multiple sclerosis, NAWM = normal-appearing white matter, RRMS = relapsing–remitting MS, PPMS = primary progressive MS, SPMS = secondary progressive MS.*

2.2. Differential Gene Expression Levels of the PACAP/VIP Receptors in the NAWM of MS Patients

Upon examining the gene expression levels of the PAC1 (*ADCYAP1R1*), VPAC1 (*VIPR1*) and VPAC2 (*VIPR2*) receptor genes in the NAWM of all MS patients combined, no statistically significant differences were observed ($p = 0.357$, $p = 0.2987$ and $p = 0.6558$, respectively; Figure 3A–C). However, comparisons of the MS cases based on the clinical course of the disease revealed clinical subtype-specific differences in the expression of each PACAP/VIP receptor. Specifically, while *ADCYAP1R1* expression was still not significantly affected in the NAWM of RRMS cases vs. non-MS cases ($t_9 = 0.125$; $p = 0.9036$; Figure 3A′), the transcript levels were significantly downregulated in PPMS ($t_8 = 2.681$; * $p < 0.05$; Figure 3A″). In contrast, they were remarkably increased in SPMS cases ($t_{10} = 5.709$; *** $p < 0.001$; Figure 3A‴).

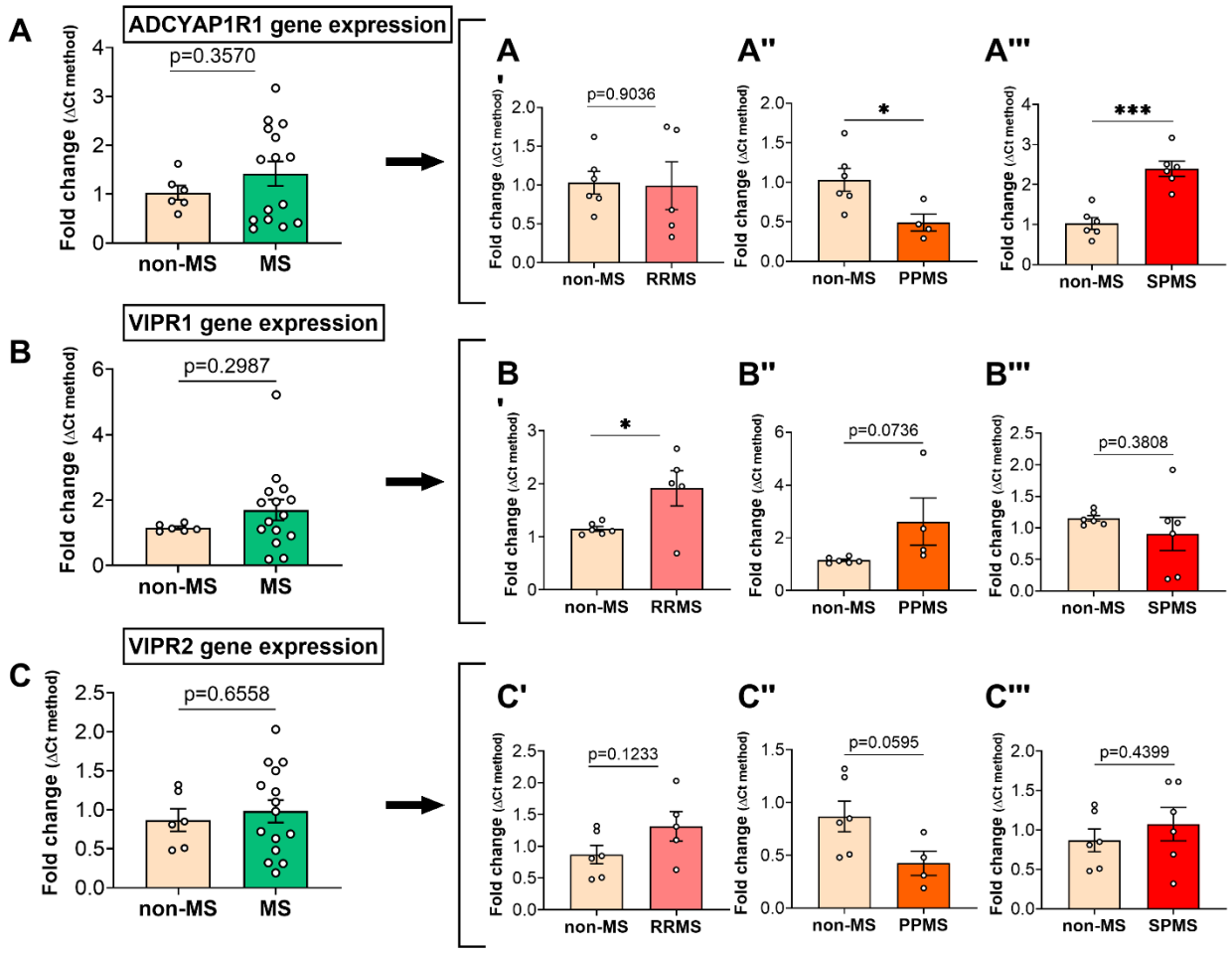

Figure 3. Differential expression of PAC1, VPAC1 and VPAC2 genes in the normal-appearing white matter of MS donors. *Gene expression of (**A**) ADCYAP1R1 (aka PAC1), (**B**) VIPR1 (VPAC1) and (**C**) VIPR2 (VPAC2) in the NAWM of non-MS vs. MS donors. Upon stratification based on the clinical MS course, the gene expression levels of ADCYAP1R1, VIPR1 and VIPR2 were determined for (**A'**–**C'**) RRMS, (**A''**–**C''**) PPMS and (**A'''**–**C'''**) SPMS cases. The data shown are the mean fold change ± SEM, obtained from n = 6 (non-MS), n = 5 (RRMS), n = 6 (SPMS) and n = 4 (PPMS) cases. p-values > 0.05 are also shown. * p < 0.05 or *** p < 0.001 vs. non-MS, as determined by unpaired t-test. ADCYAP1R1 = Pituitary adenylate cyclase-activating polypeptide type I receptor, VIPR1 = Vasoactive intestinal polypeptide receptor 1, VIPR2 = Vasoactive intestinal polypeptide receptor 2, MS = multiple sclerosis, NAWM = normal-appearing white matter, RRMS = relapsing–remitting MS, PPMS = primary progressive MS, SPMS = secondary progressive MS.*

VIPR1 expression was significantly increased in the NAWM of RRMS cases ($t_9 = 2.527$; * $p < 0.05$; Figure 3B′), as well as in PPMS cases, although not at a statistically significant level ($t_8 = 2.058$; $p = 0.074$; Figure 3B‴). In contrast, no changes were identified when comparing the *VIPR1* expression in SPMS vs. non-MS cases ($t_{10} = 0.917$; $p = 0.3801$; Figure 3B″).

No significant changes were identified when analyses of the *VIPR2* gene expression were narrowed to RRMS cases ($t_9 = 1.7$; $p = 0.123$; Figure 3C′). In contrast, gene expression was reduced in the NAWM of PPMS cases, bordering statistical significance ($t_8 = 2.195$; $p = 0.0595$; Figure 3C″). No statistically significant changes were observed when analyzing the *VIPR2* gene expression in SPMS cases ($t_{10} = 0.804$; $p = 0.44$; Figure 3C‴).

2.3. Differential Expression and Distribution of PAC1 Receptors in the NAWM and Lesions of MS Donors

Further to our gene expression studies, we sought to determine if the observed changes in transcript levels were mirrored by comparable changes in PAC1 protein expression, and we also assessed cellular/tissue localization. For this purpose, we conducted immunohistochemistry in brain tissue sections containing NAWM in selected MS cases (RRMS, PPMS and SPMS), as well as in aged-matched non-MS controls.

As shown in the representative sections shown in Figure 4A, PAC1 immunoreactivity (IR) was distinctively segregated to the perinuclear and cytoplasmic compartments of cells that exhibited either euchromatic or heterochromatic nuclear patterns, typical histological features of oligodendrocytes and oligodendrocyte progenitor cells (OPCs), respectively. In addition, most of these $PAC1^+$ cells demonstrated enlarged nuclei, especially in progressive MS cases (Figure 4A, left and right lower panels), an indication of underlying myelin pathology [517]. Stereological analyses determined that the average cell counts were not significantly different among MS subtypes ($F_{3,57} = 1.3$, $p = 0.283$; Figure 4B), suggesting that, at least in the NAWM, despite the signs of myelin pathology, myelin-producing cells are spared from any obvious cell loss. In contrast to mRNA measurements, PAC1-IR (normalized by the number [#] of cells) was remarkably decreased in RRMS ($F_{3,57} = 11.2$, *** $p < 0.001$ vs. non-MS; Figure 4C). Instead, in PPMS cases, the PAC1 immunosignals correlated well with the transcript levels and were significantly reduced compared with non-MS cases (**** $p < 0.0001$); however, this was not seen in SPMS cases, as PAC1-IR was similar to non-MS controls ($p = 0.121$).

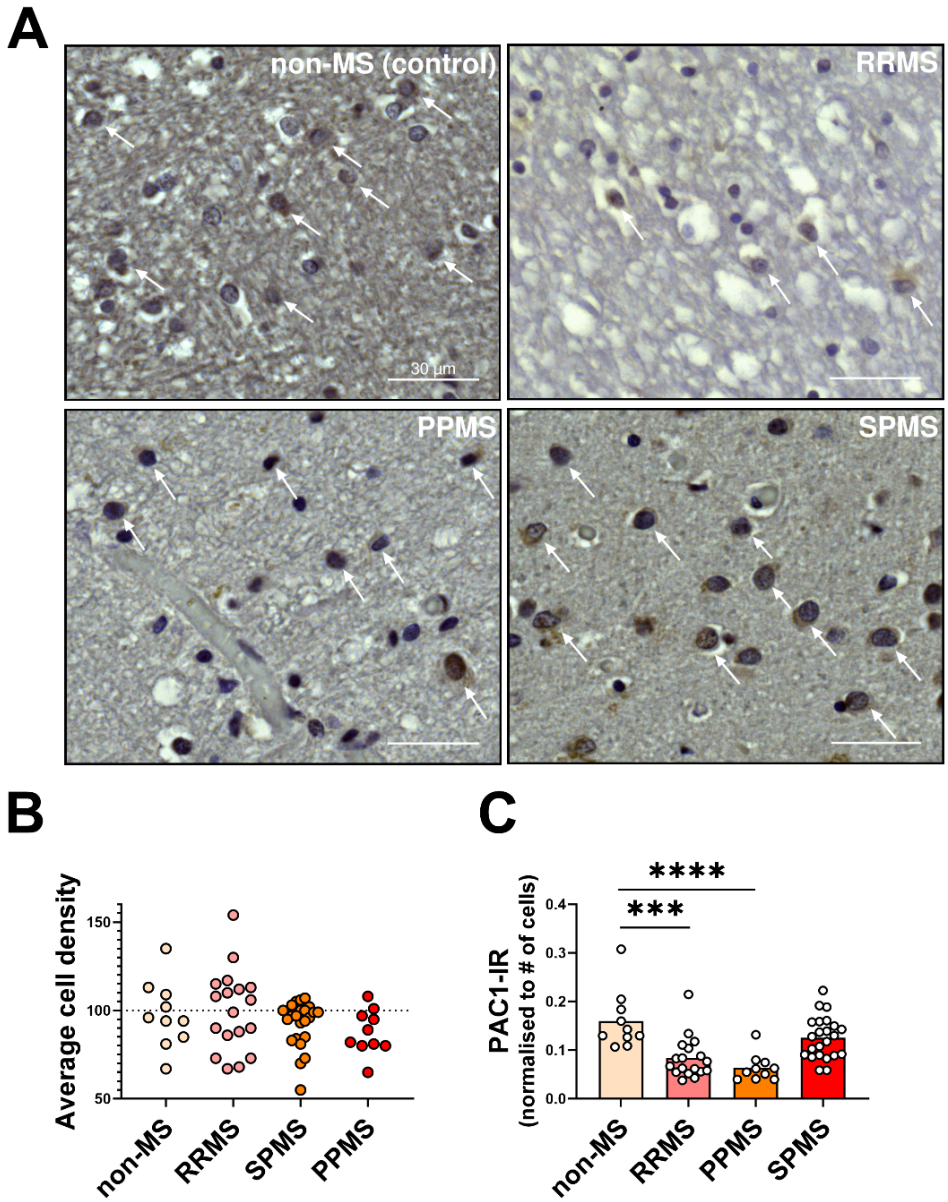

Figure 4. PAC1 immunoreactivity in the normal-appearing white matter of RRMS, PPMS and SPMS cases. *(**A**) Representative images showing PAC1 immunoreactive cells in the NAWM of MS donors with a clinical history of RRMS, PPMS or SPMS and non-MS control cases. White arrows in each panel point to PAC1$^+$ cells, which exhibit chromatin-dense and rounded/oval shaped nuclei, consistent with the oligodendrocyte/OPC morphology. (**B**) The average cell density (total # of cells per region of interest (ROI); ROI area = 1.23 mm^2) was calculated using 2–4 ROIs from n = 5 (non-MS), n = 4 (PPMS), n = 6 (RRMS) and n = 6 (SPMS) cases. (**C**) The PAC1 immunoreactivity in cells was determined by normalizing the mean PAC1 staining intensity/average # of cells counted within the same ROIs/cases as in B. *** $p < 0.001$ or **** $p < 0.0001$ vs. non-MS cases, as determined by one-way ANOVA followed by Sidak's post hoc test. Scale bar = 30 µm. OPC = Oligodendrocyte progenitor cell, PAC1 = Pituitary adenylate cyclase-activating polypeptide type I receptor, MS = multiple sclerosis, NAWM = normal-appearing white matter, RRMS = relapsing–remitting MS, PPMS = primary progressive MS, SPMS = secondary progressive MS.*

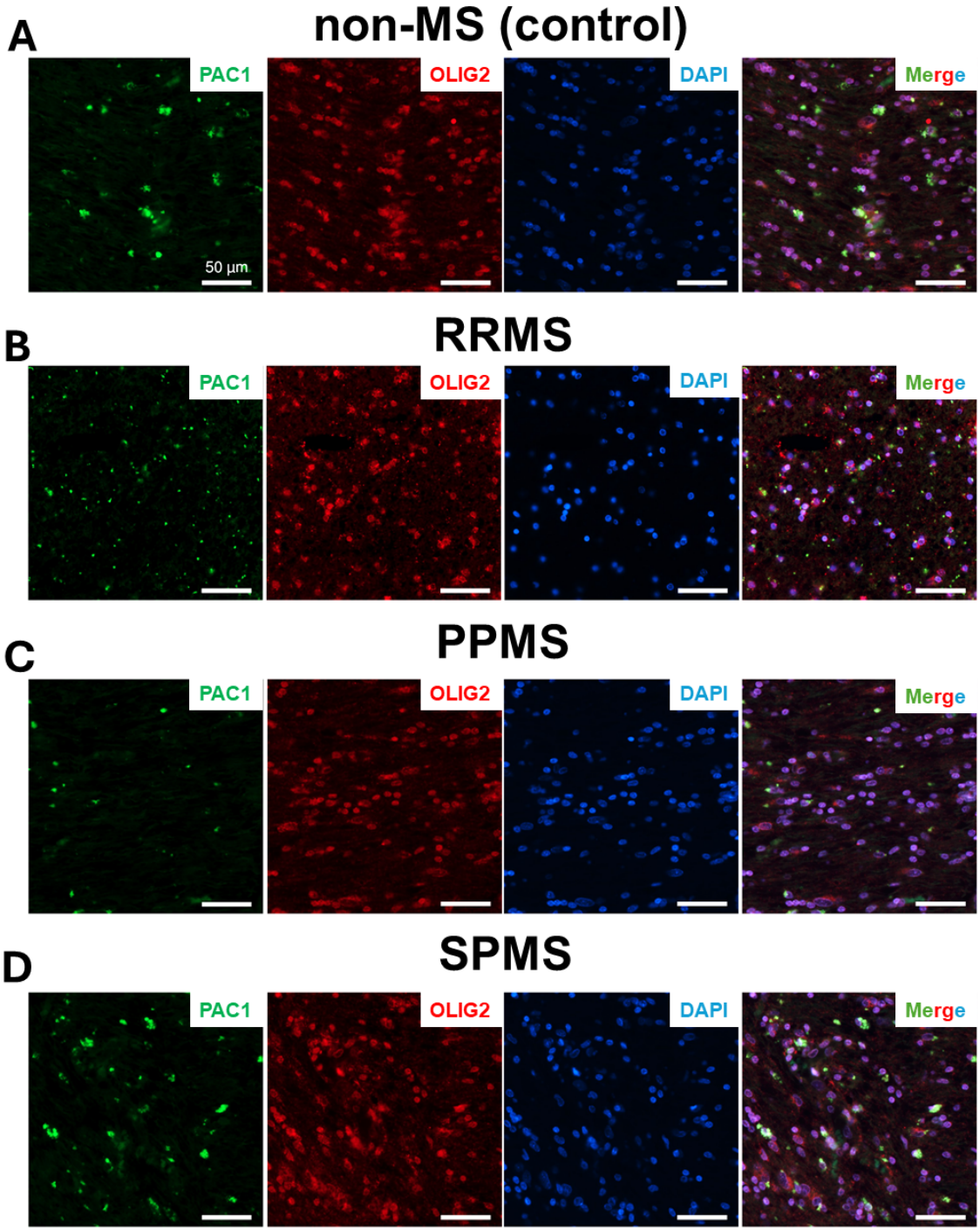

Figure 5. PAC1 co-localizes to OLIG2^{+} cells in the normal-appearing white matter of RRMS, PPMS and SPMS cases. *Representative images showing PAC1 (green)/OLIG2 (red) colocalization in the NAWM of (**A**) non-MS, (**B**) RRMS, (**C**) PPMS or (**D**) SPMS donors. Nuclei were counterstained with DAPI.*

Additional co-immunolocalization experiments were conducted in selected RRMS, PPMS, SPMS and non-MS control cases to confirm whether PAC1 was mostly confined to cells belonging to the oligodendroglial lineage. As such, NAWM sections were co-stained with PAC1 and OLIG2 (Figure 5).

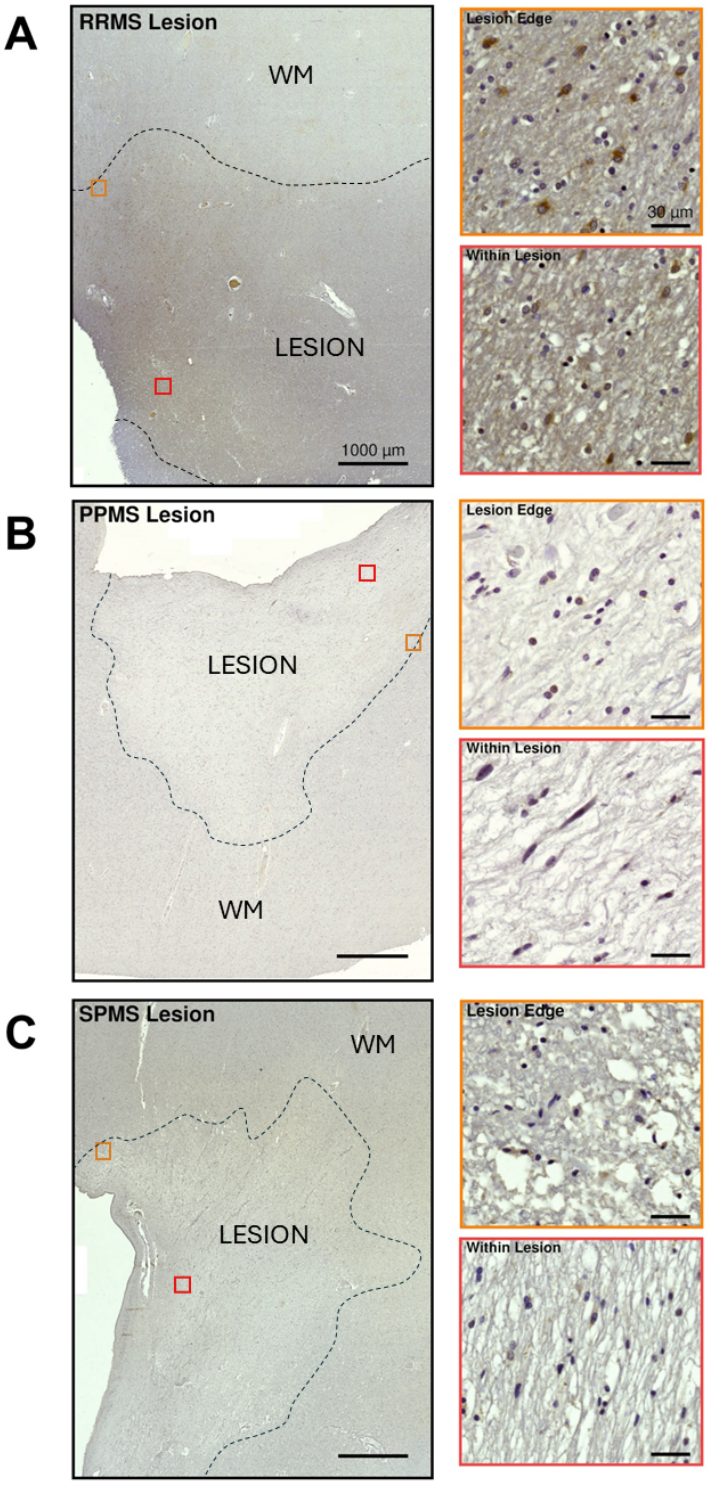

Figure 6. PAC1 immunoreactivity in representative white matter lesions in selected MS clinical cases. *(**A**–**C**, left panels) Low-magnification images showing PAC1 immunoreactivity in a lesion taken from one RRMS, PPMS or SPMS-exemplary case. Lesion borders are demarcated by the black dashed lines. Scale bar = 1000 µm. (Insets in **A**–**C**) High-power images of ROIs in the left panels (orange and red squares) demonstrating PAC1$^+$ staining around the lesion edge (top inset) and within the lesion (bottom inset) of the selected RRMS, PPMS and SPMS cases. Scale bar = 30 µm. WM = white matter.*

To further characterize PAC1 expression and localization in MS lesions, we also conducted immunohistochemical analyses in lesioned areas in at least one representative MS case selected from each MS clinical subtype (Figure 6). The first example shows a chronically active and moderately regenerating white matter lesion from an RRMS case (Figure 6A). Both intense and diffuse PAC1-IR was found within the lesion core and the peri-lesional areas, respectively (Figure 6A). When viewed at a higher magnification, strong PAC1 immunosignals were found along the lesion borders, which were mostly restricted to a subset of cells exhibiting hypochromatic, swollen nuclei and finely granular chromatin patterns, typical phenotypic features of gemistocytic (reactive) astrocytes [518]. In contrast, the majority of PAC1-IR was segregated to oligodendrocytes (i.e., cells with small dense nuclei surrounded by a clear halo) within the lesion center, although some PAC1$^+$ astrocytes were still noticeable (insets in Figure 6A). Given the remarkable and well-defined PAC1 positivity seen in astrocyte-appearing cells found around the portrayed regenerating lesion in our exemplary RRMS case, we sought to conduct co-immunolocalization studies using the astrocytic marker glial fibrillary acidic protein (GFAP) and PAC1 to confirm cell specificity. Experiments confirmed that PAC1+ cells were mostly abundant around the contours of the lesion rather than within the lesion core (Supplementary Figure S1).

Similar experiments were carried out in a selected PPMS lesion presenting overt histological signs of chronic demyelination (i.e., reduced cellularity and strong myelin loss). The lesion demonstrated a global downregulation of PAC1-IR and a well-defined area of discoloration (Figure 6B). The lesion edges displayed some weak PAC1 positivity, more frequently in cells with small, tubular-shaped nuclei (possibly microglia), as well as in scattered cells with nuclei showing the typical “clock-face” or “cartwheel” appearance of infiltrating plasma cells. Within the lesion, there were few PAC1$^+$ cells exhibiting the morphological features of foamy macrophages and rare undefined cells with a spindle-like morphology, perhaps oligodendrocytes (insets in Figure 6B).

In the chronic demyelinating SPMS lesion, the distribution of PAC1-IR was similar to the PPMS case, with rather weak PAC1 immunosignals seen both within and around the lesioned area (Figure 6C). A few $PAC1^+$ cells resembling microglia/macrophages were also found both around the edge and within the lesion, but there was no obvious evidence of $PAC1^+$ oligodendrocytes, especially in the lesion center (insets in Figure 6C).

2.4. Differential Expression and Distribution of VPAC1 Receptors in the NAWM and Lesions of MS Donors

The immunohistochemistry performed to detect VPAC1 protein expression and distribution within the NAWM demonstrated that this PACAP/VIP receptor could not be localized to oligodendrocytes, its precursors, or other cell types, corroborating previous findings in the CNS white matter of rats and non-human primates [267,519]. Yet, moderate to strong VPAC1 immunosignals were observed in what appeared to be axonal bundles in RRMS, PPMS and SPMS cases (black arrowheads in Figure 7A′–D′). Notably, comparative analyses of VPAC1-IR did not reveal any significant differences amongst the different MS subtypes ($F_{3,46} = 1.225$, $p = 0.3112$; Figure 7E). Observations of VPAC1-IR within the adjacent grey matter (GM) of selected MS cases (black arrowheads in Figure 7B″–D″) showed moderate cytoplasmic reactivity in neurons and no staining of GM oligodendrocytes, providing some degree of assurance regarding the specificity of the antibody used for VPAC1 detection.

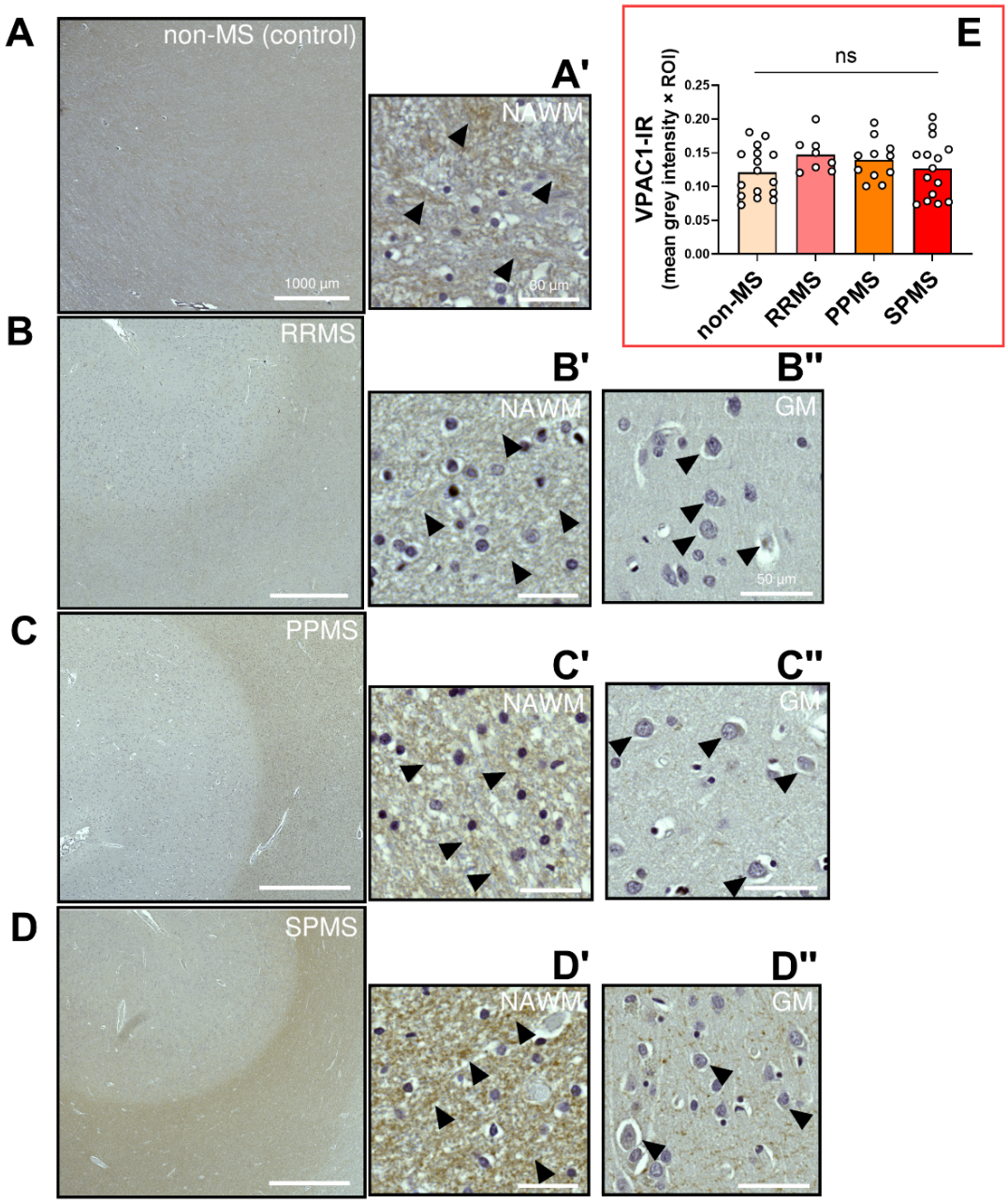

Figure 7. VPAC1 immunoreactivity in the normal-appearing white matter of RRMS, PPMS and SPMS cases. *(**A**–**D**) Representative images depicting VPAC1 immunoreactive sites in the NAWM of MS donors with a clinical history of RRMS, PPMS or SPMS and non-MS control cases. Scale bar = 1000 µm. (**A'**–**D'**) Insets of the NAWM taken at a higher magnification. Black arrowheads point to VPAC1$^+$ axonal fibers. Scale bar (NAWM) = 30 µm (**B"**–**D"**). Insets showing VPAC1$^+$ in the grey matter of the selected cases. Black arrowheads indicate VPAC1$^+$ neurons. Scale bar (GM) = 50 µm. (**E**) Bar graph showing the average VPAC1 immunoreactivity (IR) in the NAWM. The data shown are the mean grey intensity ± SEM and were calculated by averaging the grey intensity of 2–4 ROIs from n = 5 (non-MS), n = 4 (PPMS), n = 6 (RRMS) and n = 6 (SPMS) cases. Each ROI area = 1.23 mm^2. No statistical significance was found using one-way ANOVA. Ns = Not significant. VPAC1 = Vasoactive Intestinal Peptide/Pituitary Adenylate Cyclase Activating*

Polypeptide Receptor 1, MS = multiple sclerosis, NAWM = normal-appearing white matter, WM = white matter, GM = grey matter, RRMS = relapsing–remitting MS, PPMS = primary progressive MS, SPMS = secondary progressive MS.

Within and surrounding the lesions of our selected RRMS, PPMS or SPMS cases, VPAC1 protein was also distributed along axonal bundles, although VPAC1-IR was much weaker in damaged WM sites (Figure 8) than in the surrounding NAWM (Figure 7).

In our representative chronically active RRMS lesion, moderate to strong VPAC1 reactivity was localized in axons, but not in any glial, resident or infiltrating immune cells within or surrounding the lesion (Figure 8A). Interestingly, the insets of the lesion at a higher magnification demonstrated reduced VPAC1-IR along the lesion rim, with an almost complete discoloration (lack of IR) within the lesion core (insets in Figure 8A).

Immunohistochemical analyses of another chronic demyelinating lesion—although from a PPMS case—demonstrated moderate and axon-specific VPAC1-IR around the edges of the lesion, but less so within the lesion itself, where there was strong discoloration (Figure 8B). The lesion rim displayed mildly elevated cellularity, with several $VPAC1^-$ cells exhibiting a small, rounded nucleus (presumably OPCs or oligodendrocytes) and a few weakly $VPAC1^+$ cells with phenotypic features of microglia. As shown in the inset of the lesion core seen at a higher magnification, there were clusters of $VPAC1^+$ microglial-like cells surrounding some intralesional small vessels, whereas the cells that configured as astrocytes were found in close apposition to the vessel walls and were all $VPAC1^-$ (insets in Figure 8B).

The representative case shown in Figure 8C shows a typical chronic demyelinating lesion from a SPMS donor. The lesion presented little to no sign of regeneration. As in RRMS and PPMS cases, moderate VPAC1-IR was localized in axons passing through the white matter and adjacent to the lesion border, whereas IR was almost totally absent inside the lesion, with only a few glial cells displaying mild VPAC1 positivity (possibly microglia).

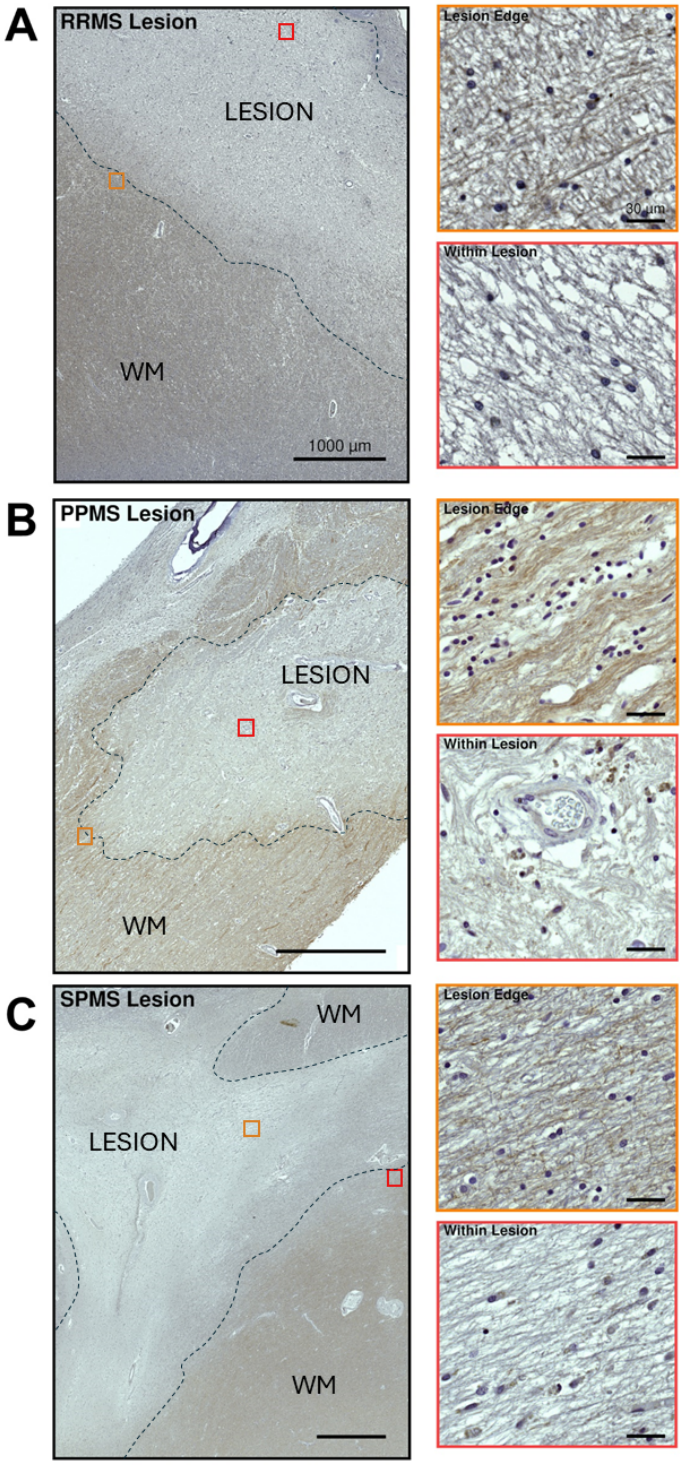

Figure 8. VPAC1 immunoreactivity in white matter lesions from selected MS clinical cases. *(**A–C**, left panels) Low-magnification images showing VPAC1 immunoreactivity in a lesion taken from one RRMS, PPMS or SPMS-exemplary case. Lesion borders are demarcated by the black dashed lines. Scale bar = 1000 µm. (Insets in **A–C**) High-power images of ROIs in left panels (orange and red squares) demonstrating VPAC1^{+} staining around the lesion edge (top inset) and within the lesion (bottom inset) of the selected RRMS, PPMS and SPMS cases. Scale bar = 30 µm. WM = white matter.*

2.5. Differential Expression and Distribution of VPAC2 Receptors in the NAWM and Lesions of MS Donors

The immunohistochemistry for VPAC2 in brain tissue sections of donors with differing clinical disease courses (i.e., RRMS, PPMS or SPMS) did not reveal any cell-specific staining within the NAWM (Figure 9A). However, sparse and weak VPAC2-IR was identified in what appeared to be the walls of some small vessels infiltrating the NAWM (white arrowheads in Figure 9C′). Some mild/moderate cytoplasmic VPAC2 staining was detected in neurons (black arrowheads in Figure 9B″–D″), as well as in axons of the presented SPMS case (white arrowheads in Figure 9D″). Semi-quantitative analyses of VPAC2-IR identified a robust and statistically significant increase in VPAC2 staining in the NAWM of RRMS cases ($F_{3,57}$ = 17.17, **** $p < 0.0001$; Figure 9E), but not in PPMS or SPMS cases.

In the reported RRMS case, depicting a chronically active and demyelinating lesion with minimal signs of regeneration, $VPAC2^+$ cells displayed a scattered distribution both along the edges and within the lesion, where we also detected some isolated $VPAC2^+$ cells with astroglial appearance (insets in Figure 10A).

In the chronically demyelinated PPMS case presented below, $VPAC2^+$ cells exhibited the same histological features seen in RRMS cases, including the scattered distribution of IR astrocytes along the lesion edges (Figure 10B); however, the cell nuclei here appeared slightly smaller (top inset in Figure 10B), suggesting a non-reactive/resting phenotype. In contrast, $VPAC2^+$ cells with swollen nuclei and organized in isolated clusters were found within the lesion core (bottom inset in Figure 10B).

Finally, in a chronically active SPMS lesion with moderate myelin loss and minimal evidence of remyelination (Figure 10C), focal VPAC2-IR was detected in isolated cells with microglial resemblance, both at the edge and within the center of the lesion (insets in Figure 10C). The immunohistochemical analysis of VPAC2-IR within and surrounding lesions demonstrated consistent staining in cells exhibiting a hypochromatic nucleus with astrocytic resemblance, specifically in representative RRMS and PPMS cases (Figure 10A, B).

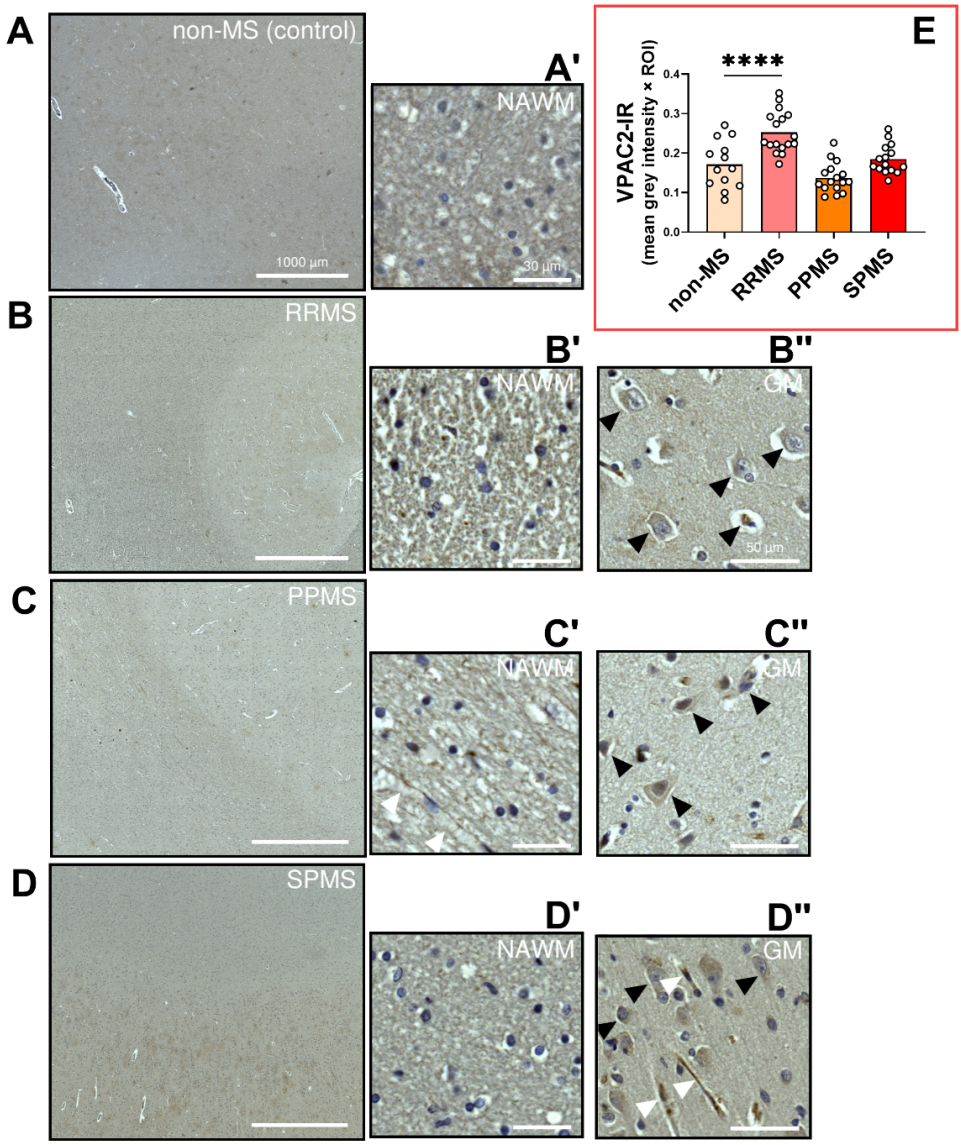

Figure 9. VPAC2 immunoreactivity in the normal-appearing white matter of RRMS, PPMS and SPMS cases. *(**A**–**D**) Representative images depicting VPAC2 immunoreactive (IR) cells in the NAWM of MS donors with a clinical history of RRMS, PPMS or SPMS and non-MS controls. Scale bar = 1000 µm. (**A'**–**D'**) Insets of the NAWM taken at a higher magnification. White arrowheads in C' show VPAC2$^+$ vessel walls. Scale bar (NAWM) = 30 µm. (**B"**–**D"**) Insets showing VPAC2$^+$ in the grey matter of the selected cases. Black arrowheads indicate VPAC2$^+$ neurons, whereas white arrowheads show VPAC2-IR in axons. Scale bar (GM) = 50 µm. (**E**) Bar graph showing the average VPAC1 immunoreactivity (IR) in the NAWM. The data shown are the mean grey intensity ± SEM and were calculated by averaging the grey intensity of 2–4 ROIs from n = 5 (non-MS), n = 4 (PPMS), n = 6 (RRMS) and n = 6 (SPMS) cases. Each ROI area = 1.23 mm^2. **** p < 0.0001 vs. non-MS (control) cases, as determined by one-way ANOVA and Sidak's post hoc test. VPAC2 = Vasoactive Intestinal Peptide/Pituitary Adenylate Cyclase Activating Polypeptide Receptor 1, MS = multiple sclerosis, NAWM = normal-appearing white matter, GM = grey matter, RRMS = relapsing–remitting MS, PPMS = primary progressive MS, SPMS = secondary progressive MS.*

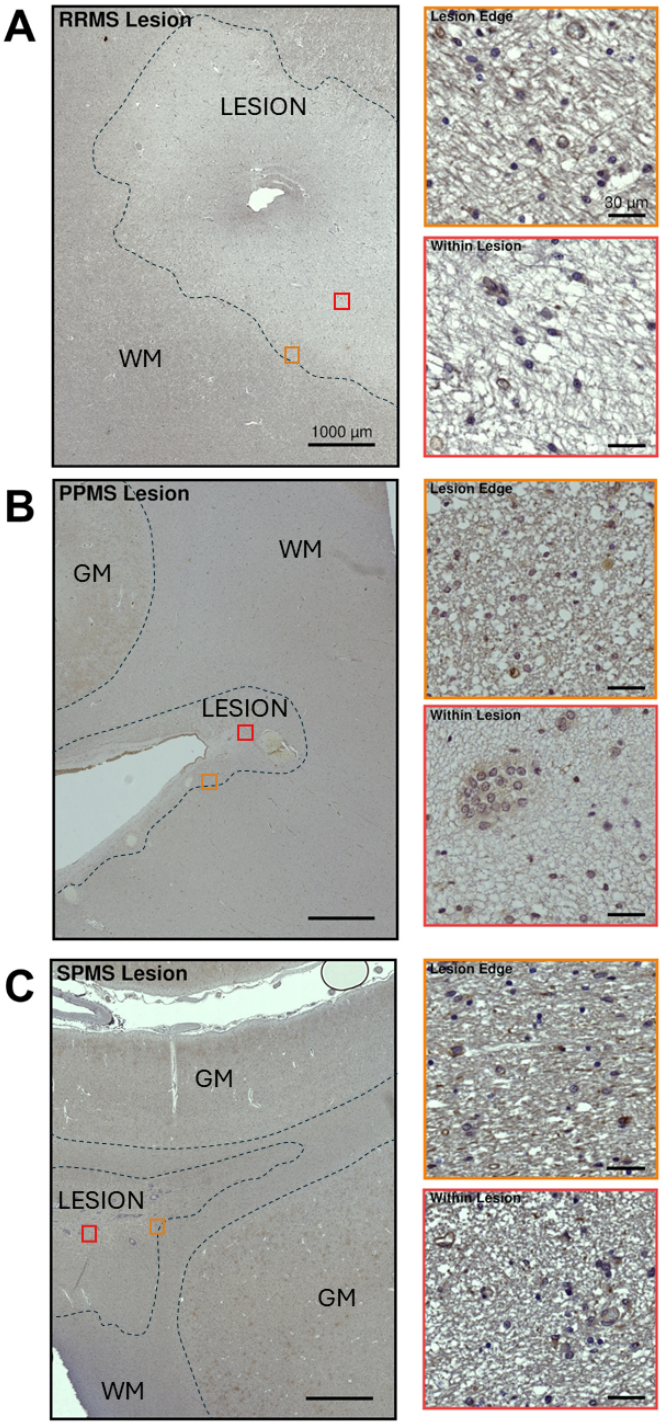

Figure 10. VPAC2 immunoreactivity in white matter lesions from selected MS clinical cases. *(**A**–**C**, left panels) Low-magnification images showing VPAC2 immunoreactivity in a lesion taken from one RRMS, PPMS or SPMS-exemplary case. Lesion borders are demarcated by the black dashed lines. Scale bar = 1000 µm. (Insets in **A**–**C**) High-power images of ROIs in left panels (orange and red squared) demonstrating VPAC2 staining around the lesion edge (top inset) and within the lesion (bottom inset) of the selected RRMS, PPMS and SPMS cases. Scale bar = 30 µm. GM = grey matter; WM = white matter.*

3. Discussion

In this study, we aimed to provide a comprehensive overview of the changes in the expression and distribution of PACAP and VIP receptors in the CNS white matter of MS patients. To our knowledge, there are no studies portraying the differential expression levels of PACAP/VIP receptors in the human brain, let alone in the brain of MS patients. In fact, only a handful of investigations describing the expression of these neuropeptide receptors in the rodent and non-human primate brain have been published so far [267,519,520]. Therefore, this study provides novel and important findings on the differential expression of PACAP/VIP receptors in the NAWM and provides some insights from the analyses of representative lesions from donors with different clinical courses of MS.

After stratifying data according to the clinical disease progression, we identified several disruptions in the expression of PACAP/VIP receptors, some of which appeared to be dependent on the clinical progression of the disease. Indeed, both the PACAP and PAC1 gene expression levels were unaltered in the NAWM of RRMS patients; however, the quantification of immunosignals demonstrated that PAC1-IR was decreased in these same cases. Instead, in SPMS cases, PAC1 transcripts were increased in the NAWM, although this could not be confirmed at the protein level by immunolocalization studies. Only in PPMS cases was there complete congruence between gene and protein expression, as the PAC1 transcripts and immunoreactivity intensities were reduced in both cases.

PAC1 reactivity in the NAWM of MS patients appears to mainly be seen in subpopulations of oligodendrocytes and OPCs. These discoveries were supported by co-immunolocalization studies demonstrating the presence of varying numbers of $PAC1^{+}/OLIG2^{+}$ cells within the NAWM across the different MS subtypes. Although it is important to note that this was not further quantified, and future research ought to explore this notion. However, this observation aligns with previous work performed in rats and non-human primates, further confirming that PAC1 is the only PACAP/VIP receptor to be expressed in the CNS white matter, and specifically in myelin-producing cells [267,519]. Indeed, in vitro data have shown the expression of functional PAC1 receptors in OPCs and mature oligodendrocytes [265]. Interestingly, the PAC1-preferring agonist PACAP exerts opposite effects in central vs. peripheral myelin cells. In OPCs, PACAP treatment delays cell maturation but stimulates proliferation, whilst in peripheral myelin cells (i.e., Schwann cells), it promotes cell differentiation and enhances the expression of myelin markers [196,208,209,265]. In the CNS, PACAP-mediated signalling is

therefore thought to be crucial for the temporal control of myelin production [266]. As such, the reduced PAC1 expression in the NAWM of RRMS and SPMS cases might signify an increased demand for differentiation/myelination at the expense of OPC proliferation in chronically damaged WM.

Around the lesion edges, PAC1 and VPAC2 reactivity was mostly seen in cells resembling microglia as well as infiltrating cells, including plasma cells. Whilst not a prevalent feature of early MS lesions, it is not uncommon to identify plasma cells infiltrates in chronic lesions of advanced MS cases [521]. However, at this stage, we are unable to determine the significance of PAC1 or other PACAP/VIP receptors in plasma cells, although we cannot rule out the possible involvement of the receptors in regulating antibody production and downstream inflammatory cascades. Preclinical data from EAE mice showed that the loss of PAC1 or VPAC2 aggravated symptoms in comparison to wild-type mice [199,202,321]. Since inflammation is a key component of MS pathology, our results highlight the potential importance of increased PAC1 and/or VPAC2 expression in reactive glial cells surrounding lesions [220,522]. Both PACAP and VIP have been hypothesized to stimulate phagocytosis in the CNS [523]. Since the effective phagocytosis of myelin debris around lesions is a crucial step in fostering a healthy microenvironment that promotes the maturation of OPCs and enhances remyelination [151,524], $PAC1^+$ or $VPAC2^+$ microglia in and surrounding lesions might have a role in regulating the phagocytosis of myelin debris [525] and/or the active regenerative processes driven by reparative astrocytes [526]. Both around and within the lesion of the RRMS case presented in this study, we also observed several $PAC1^+$ cells with obvious histological features of reactive astrocytes; this was confirmed by co-immunofluorescence using the astrocyte marker glial fibrillary acidic protein (GFAP) (Figure S1). Reactive astrocytes in this example of a mildly active lesion might be there to promote repair; therefore, increased PAC1 expression in these cells might be of potential therapeutic significance [526,527]. Future studies addressing the importance of astrocyte-specific PAC1 receptor expression in remyelination may help shed more light on the role of these glial cells in myelin repair, especially in patients with progressive MS. Furthermore, our interpretations regarding the cell-specific expression of different PACAP/VIP receptors should be taken with caution, as our results were obtained from an individual RRMS case. Therefore, more comprehensive assessments of PACAP/VIP receptors at the cell-resolution level and in diverse lesion types (i.e., active, inactive, smoldering, shadow plaques, remyelinating lesions, etc.) are warranted to capture the full spectrum of changes needed to comprehend how these

neuropeptide receptors contribute to glial cell functionality during lesion development or at different stages of recovery.

Our analyses of VPAC1 gene expression demonstrated robust increases in RRMS cases and only marginal changes in PPMS cases. Although the immunohistochemistry performed did not demonstrate significant increases in VPAC1 expression among the different MS subtypes, VPAC1 immunosignals were distributed in a diffuse pattern along the axonal bundles traversing the NAWM, and less than in any other local or infiltrating cells. At a glance, our VPAC1 staining resembled background staining; however, staining within the grey matter was neuron-specific, increasing our confidence regarding the specificity of the antibody.

Preclinical studies in VPAC1-deficient mice have shown reduced disease severity following EAE, suggesting that the receptor may play a role in regulating or perhaps sustaining the inflammatory response during experimental demyelination [201]. Therefore, it was surprising to observe an increase in VPAC1 expression in the NAWM of RRMS and PPMS cases, especially considering that, in these donors, most lesions were chronically demyelinating, with scarce evidence of inflammation. However, VPAC1 signaling is also linked to neuroprotection [312]; therefore, it cannot be excluded that the increased VPAC1 expression in the reported MS cases reflects the homeostatic neuroprotective response of stressed axons trying to retain functionality in a non-physiological CNS microenvironment, such as in the NAWM of MS patients.

With regards to VPAC2 expression, we did not find significant changes in gene expression amongst the different MS cases; however, there were trends towards increased mRNA expression in RRMS cases that were corroborated by robust increases in VPAC2-IR. In contrast, the VPAC2 gene and IR were reduced in PPMS cases. VPAC2 is normally upregulated under neuroinflammatory conditions [528] as it is generally considered an inflammatory sensor of the CNS [529]. From an immunohistochemical standpoint, VPAC2-IR was mostly expressed in endothelial cells of small infiltrating vessels present in the NAWM of PPMS cases. The expression of VPAC2 in infiltrating vessels might be the result of a subtle microvascular pathology or may represent a physiological adjustment of the vascular compartment to preserve blood–brain barrier (BBB) maintenance, since VIP treatment has previously been shown to reduce BBB permeability [530]. Altered blood-brain barrier (BBB) permeability around veins in MS patients has been linked to perivascular astrocytosis [531]. Since VPAC2 expression is known to be upregulated in reactive astrocytes, it is plausible that

increased astrogliosis surrounding the central vein contributes to the observed VPAC2+ immunoreactivity[532]. However, as we did not find similar staining patterns in other MS subtypes, including PPMS cases, future research is warranted to determine the role of the VIP/VPAC2 axis in BBB homeostasis.

It is important to address a few limitations of the current study. Firstly, our cell identification was based on the morphological appearance and certain histological features of CNS cells. Whilst this approach may not be completely accurate and can be prone to interpretation biases, it remains an acceptable solution when performing histopathological evaluations of CNS tissues. However, a way to circumvent these limitations would have been to conduct experiments following a more integrated approach involving the co-staining of sections with specific glial or immune cell markers (which were conducted in certain instances; please see Figure 5 and Figure S1) concurrent with other investigative modalities (i.e., Western blot and other molecular diagnostic tools). However, given the limited tissue availability, we based most of the identification of cell types on prototypical morphological features. It should also be noted that the main goal of this study was to assess the expression and distribution of PACAP/VIP receptors in the NAWM of donors with different clinical subtypes of MS, whereas our immunohistochemical studies in exemplary lesions were solely conducted with the purpose of providing some viable examples of how PACAP/VIP receptor distribution would vary across cell types in and surrounding damaged WM. In this regard, most of the assessed lesions were chronically active demyelinating lesions with minimal signs of regeneration (except for our exemplary RRMS case, which showed moderate signs of remyelination). Therefore, additional studies should address how these neuropeptides and related receptors are affected in non-remyelinating vs. remyelinating lesions; this would represent an important advancement in this specific field of research. Further limitations were related to the heterogeneity of the age of our cohort and staining across each subcategory of MS cases, which increased the intragroup variability when attempting semi-quantifications of IR and may have introduced a sampling bias. Further work using much larger cohorts with narrower age differences are needed to expand our current findings. Nonetheless, despite these technical challenges, we were glad that our PACAP/VIP receptor antibodies were rather specific, as demonstrated by the cytoplasmic staining of neurons in the GM. Additionally, the implementation of conditional knockout animal models, perhaps associated with a high-throughput single-cell RNA sequencing and/or in situ hybridization study, may represent a future goal of our research, as these studies will

help establish the role of the VIP/PACAP receptors in individual cell types both in the NAWM and in MS lesions.

Overall, this study demonstrated a heterogenous pattern of expression and distribution in PACAP/VIP receptors in the NAWM of MS patients, which seemed to depend on the MS and receptor subtype. In this regard, it is well established that relapsing and progressive MS cases display several pathological differences, including the degree and duration of inflammation, the regenerative activity and more [502,533]. These are likely to extend far beyond the lesion site [534] and it is not unreasonable to believe that these factors may cause subtle alterations in the NAWM, including the changes in VIP/PACAP receptors reported in this study. As such, this additional information could be useful to better understand how this neuropeptide system operates in the CNS of people with different clinical courses of MS.

4. Materials and Methods

4.1. Human Postmortem Brain Tissue

The MS and non-MS control tissue was supplied by the MS Research Australia Brain Bank (Tissue Transfer Deed—CT31920, approved on 21 June 2021) and the Victorian Brain Bank (Material Transfer Deed—VBB.19.07, approved on 16 January 2020). Snap-frozen tissue blocks (~100 mg each) were obtained and used for RNA isolation, cDNA synthesis and subsequent real-time qPCR analyses. For immunohistochemistry, fixed tissue sections cut at 5 µm and encompassing at least one lesion per case were provided for downstream analyses. Tissue sections were collected from a total of 6 individuals with RRMS, 6 with SPMS and 4 with a diagnosis of PPMS. Five age-matched cases from non-MS donors were included as controls in each experiment. A detailed overview of the demographic and clinical history of the donors is shown in Table 1.

Table 1. Demographic information of non-MS and MS donors. PMI = post-mortem interval. Adapted from [535]

Group	Age (Years)	Place of Birth	Sex	PMI (Hours)	MS Duration (Years)	Lesion Type
Control	79	Australia	Female	59	N/A	N/A
Control	82	England	Female	25	N/A	N/A

Control	83	Australia	Male	27	N/A	N/A
Control	73	Australia	Male	22	N/A	N/A
Control	73	Australia	Female	26.5	N/A	N/A
RRMS	70	Australia	Male	21	43	Chronic active
RRMS	80	Australia	Male	14	21.3	Chronic active
RRMS	40	Australia	Male	5	8	Chronic active
RRMS	72	Australia	Female	31	20	Chronic active
RRMS	79	New Zealand	Female	24	29.5	Chronic active
RRMS	82	Australia	Female	19	33.1	Chronic active—minimal regeneration
SPMS	57	Australia	Female	26.8	17.9	Chronic active—minimal regeneration
SPMS	68	Australia	Female	15	33.5	Chronic active—minimal regeneration
SPMS	69	New Zealand	Female	8.5	38	Chronic active—minimal regeneration
SPMS	84	Australia	Female	15	42	Chronic active—minimal regeneration
SPMS	47	Australia	Female	20.8	25.8	Chronic active—minimal regeneration
SPMS	55	Australia	Male	7	40.1	Chronic active—minimal regeneration
PPMS	36	Australia	Female	24	13	Chronic active
PPMS	83	Australia	Female	16	16	Chronic active
PPMS	73	Australia	Male	25	15.6	Chronic active—moderate regeneration
PPMS	73	England	Male	24	41	Chronic active—minimal regeneration

4.2. Normal-Appearing White Matter Dissection and RNA Extraction

The RNA extraction of NAWM samples was performed as described previously [535]. Briefly, total RNA was extracted from micro-dissected snap-frozen WM shavings (each weighing about 120 mg) under RNase-free conditions using TRIreagent (Sigma-Aldrich, Castle Hill, NSW,

Australia). Given that there is no universal agreed-upon distance to determine the diffusion of a lesion's pathology, we empirically considered the NAWM in those tissue samples that were placed at a distance of >0.7 mm from the closest boundary of a lesion. After a tissue homogenization step, the samples were centrifuged (12,000× *g* at 4 °C) in the presence of 200 µL of chloroform (Sigma-Aldrich, Castle Hill, NSW, Australia). The RNA fraction was precipitated using 2-propanol (Sigma-Aldrich, Castle Hill, NSW, Australia) and spun down. The obtained RNA was treated with DNase I (Thermo Fisher Scientific, Scoresby, VIC, Australia), followed by a clean-up step using the RNeasy Micro Kit (Qiagen, Clayton, VIC, Australia). The RNA concentrations were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia).

4.3. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Single-stranded cDNA was synthesized from the isolated total RNA using the Tetro cDNA synthesis kit (Bioline, Australia), according to the manufacturer's instructions. RT-qPCR was performed to analyze the mRNA levels of *ADCYAP1* (PACAP), *VIP*, *ADCYAP1R1* (PAC1), *VIPR1* (VPAC1) and *VIPR2* (VPAC2). Ribosomal protein S18 (*RPS18*) was used as a housekeeping gene. A detailed overview of the primer sequences can be found in Table 2. The RT-qPCR for each reaction was performed on CFX96 Real-Time System (C1000 Touch Thermal Cycler) using iTaq Universal SYBR Green Master Mix (Biorad, Australia). Each reaction contained a final concentration of 100 ng of cDNA, 400 nM of forward and reverse primer, and 5 µL of iTaq Universal SYBR Green Master Mix. The relative gene expression changes were calculated using the ΔΔCt method, as described previously [261].

Table 2. Overview of the genes tested using RT-qPCR.

Accession #	Gene	Primer Sequences (5′-3′)	Product Size (bp)
NM_001099733.2	Pituitary adenylate-cyclase-activating peptide (PACAP; *ADCYAP1)*	TAACGAGGCCTACCGCAAAG GTGAAGATCCCGTCCGAGTG	150
NM_003381.4	Vasoactive intestinal peptide (*VIP*)	AATAAGGCCCAGCTCCTTGTG TGTCACCCAACCTGAGAGCA	106

NM_001199635.2	Pituitary adenylate-cyclase-activating peptide receptor 1 (PAC1; *ADCYAP1R1*)	TTGGCATTATCGTCATCCTTGT AATGGTGGACAGTTCTGACATC	152
NM_004624.4	Vasoactive intestinal peptide receptor 1 (VPAC1; *VIPR1*)	TAAGCCTGAAGTGAAGATGGTC CATTGAGGAAGCAGTAGAGGAT	86
NM_003382.5	Vasoactive intestinal peptide receptor 2 (VPAC2; *VIPR2*)	CTCGCCCCCGTGAACAG GCACGTGATGTTGTCCCAGA	141
NM_022551.3	Ribosomal protein S18 (RP*S18*)	GAGGATGAGGTGGAACGTGT GGACCTGGCTGTATTTTCCA	115

4.4. Immunohistochemistry

The tissue slides obtained from the Victorian Brain Bank and MS brain bank from non-MS and MS donor samples were deparaffinated in xylene and rehydrated through decreasing ratios of ethanol to water. A mild-heat antigen retrieval step (10 mM of sodium citrate, 0.05% Tween 20, pH 6.0; 15 min) was performed to unmask antigenic epitopes and improve antibody binding. The following antibodies were used for staining: Rb-αPAC1 (1:250, GeneTex Cat# GTX30026, RRID:AB_3097721), Rb-αVIPR1 (1:250, Sigma-Aldrich Cat#SAB4503084, RRID:AB_10751033), Rb-αVIPR2 (1:250, Millipore Cat#AB2266, RRID:AB_10807709). Immunoreactivity was detected using the Rabbit-specific HRP/DAB (ABC) Detection IHC Kit (Abcam Cat# ab64261, RRID:AB_2810213), according to the manufacturer's instructions. Counterstaining using Hematoxylin (Lillie Mayer's, POCD healthcare) was performed to visualize the cell nuclei. The slides were subsequently dehydrated with increasing concentrations of ethanol and xylene and mounted using VectaMount Express Mounting medium (H-5700-60, Abacus DX). Images were taken using the ZEISS AxioScan Z1 (Carl Zeiss Australasia, Australia) at ×20 magnification. Image analysis was performed using ImageJ (version 1.53k). Briefly, each image was deconvolved using the deconvolution function of ImageJ, by selecting the DAB deconvolution from the available options. This function splits the image into separate channels showing either DAB staining or hematoxylin counterstaining. Then, a threshold was applied to the DAB channel to remove any background staining. Next, 2–4 ROIs (area = 1.23 mm^2) per case were randomly selected from the NAWM, added to the ROI manager, and saved for subsequent grey intensity measurements. Using the Analyze > Measure function, the intensities of each ROI were calculated and exported to a spreadsheet. For the calculation of the normalized staining intensity (based on # of cells), the hematoxylin channel was also used to determine the total number of nuclei per ROI. The mean grey intensity

was then divided by the total number of cells. The intensity values shown in graphs refer to each ROI (pseudoreplicates) to better reflect the heterogeneity of staining within a given section/case. The exact number of cases (biological replicates) that were analyzed is reported in each corresponding Figure legend. Images were generated using OMERO.Figure (v4.2.0).

4.5. Immunofluorescence

Immunofluorescence staining was performed in 2–3 representative sections taken from non-MS, RRMS, PPMS and SPMS. Briefly, the NAWM sections were deparaffinized and rehydrated using xylene and decreasing ratios of ethanol to water. Following a mild antigen retrieval step (10 mM of citric acid, 0.05% Tween-20, pH 6.0; 15min), an auto-fluorescence quenching step was performed using 0.25% NH_3 in 70% ethanol for 1 h at RT. Slides were washed in PBS-T (0.05% Tween-20 in PBS) and permeabilized for 20 min (0.4% Triton-X100 in PBS). To block endogenous peroxidase activity, the sections were submerged in 3% hydrogen peroxide (3% H_2O_2 in methanol; 15 min), followed by a washing step. The sections were blocked for 1 h in 5% BSA (0.2% gelatin, 0.25% Triton-X100 in PBS). Primary antibody dilutions were prepared in 1% BSA (0.2% gelatin, 0.25% Triton-X100 in PBS) and incubated overnight (4 °C). After washing, the slides were incubated with the appropriate secondary antibody for 1 h at RT (1% BSA, 0.2% gelatin, 0.25% Triton-X100 in PBS) and counterstained with Hoechst-33258 for 15 min (1 µg/mL in PBS; 94403, Sigma-Aldrich). The slides were mounted using Anti-Fade Fluorescence Mounting Medium (AB104135, Abcam). The primary antibodies used for this co-localization experiment were rabbit-anti-PAC1 receptor (1:250, GeneTex Cat# GTX30026, RRID:AB_3097721), mouse-anti-GFAP (1:250, Cell Signaling Technology Cat# 3670, RRID:AB_561049), goat-anti-Olig2 (1:250, R&D Systems Cat# AF2418, RRID:AB_2157554), Goat anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) (1:500, Cell Signaling Technology Cat# 4412 (also 4412S), RRID:AB_1904025), Goat anti-mouse IgG H&L (Alexa Fluor® 594) (1:500, Abcam Cat# ab150116, RRID:AB_2650601), Donkey Anti-Rabbit IgG H&L (Alexa Fluor 647; 1:500, (Abcam Cat# ab150063, RRID:AB_2687541) and Donkey Anti-Goat (Alexa Fluor 488; 1:500, Thermo Fisher Scientific Cat# A-11055, RRID:AB_2534102). A pilot Western blot experiment was performed on protein lysates from exemplary non-MS and PPMS cases to determine the suitability of the PAC1 antibody in human brain tissues (data not shown). All sections were imaged using the Zeiss Axioscan Z1 (20×, Zeiss). Figures were generated using OMERO.figure (v4.2.0).

4.6. Statistical Analysis

All data were analyzed and graphs generated using GraphPad Prism (v9.3.1). For pairwise comparisons (i.e., non-MS vs. MS), statistical significance was determined using the unpaired *t*-test. For comparisons involving three or more groups, statistics were computed using a one-way analysis of variance (ANOVA) followed by Sidak's post hoc tests. *p*-values < 0.05 were considered statistically significant.

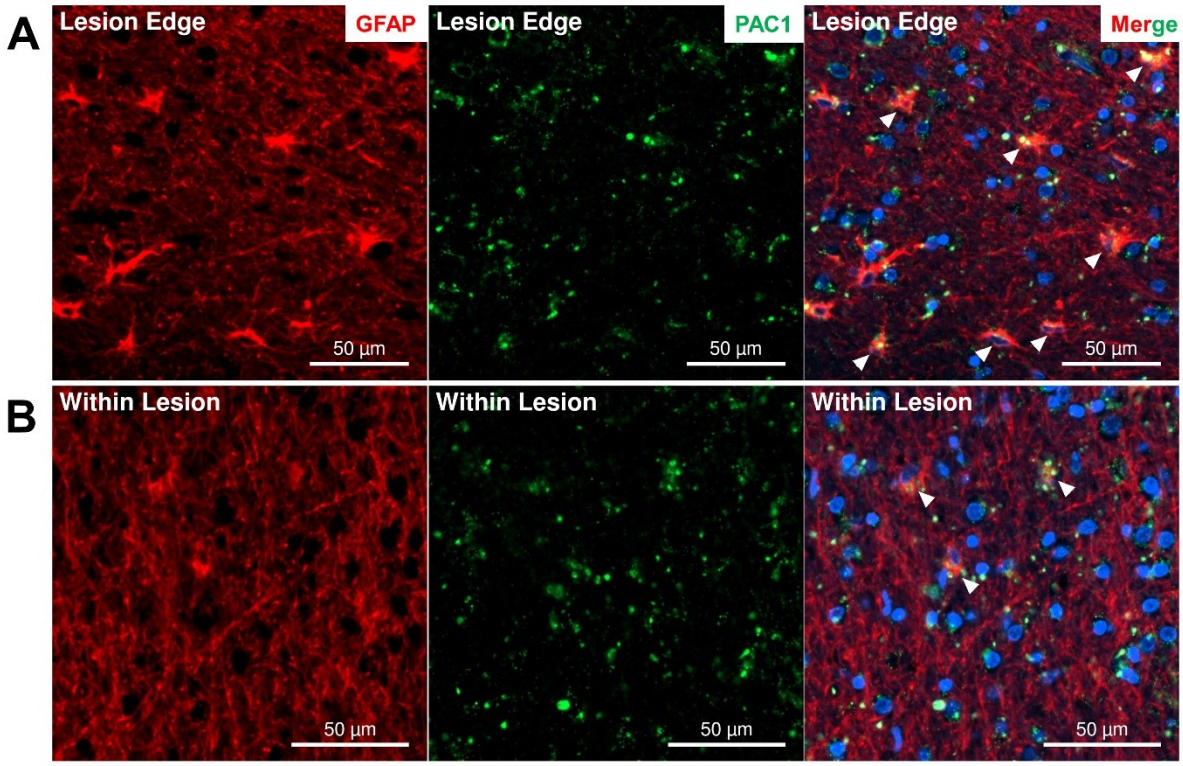

Figure S1. PAC1 colocalization in astrocytes in representative white matter lesions from a selected RRMS case*. (A, top panels) Co-immunofluorescence demonstrating strong colocalization of PAC1 (green) in GFAP+ cells (red) along the lesion edges of an exemplary RRMS case. (B, bottom panels) Within the lesion centre of the same clinical case, PAC1$^+$ astrocytes are less abundant. White arrowheads point to PAC1$^+$ astrocytes both in A and B. Scale bar = 50µm.*

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25168850/s1.

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Bridging section: From dysregulation to exploration of the VIP/PACAP system as a treatment for MS

The conclusion from my 'Chapter 2 - VIP/PACAP receptors in NAWM of MS donors outlines how the VIP/PACAP system is dysregulated in the NAWM of MS patients, demonstrating clear differences between MS subtypes. Thus, we can infer that there is altered VIP/PACAP signalling in the white matter of these patients. Since VIP/PACAP system has previously been shown to be protective in the brain, we next aimed to explore whether activation of the neuropeptides' receptors could provide protection during the demyelination process in the CNS.

To investigate this, we decided to utilise the CPZ mouse model for demyelination (see 1.1.5 Modelling multiple sclerosis in mice). The next chapter presents a published manuscript detailing this work, which addresses my second aim.

Chapter 3:

Targeting PAC1 receptors mitigates degradation of myelin and synaptic markers and diminishes locomotor deficits in the cuprizone demyelination model

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1. Laboratory of Cellular and Molecular Neuroscience (LCMN), School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW 2007, Australia
2. Semel Institute for Neuroscience and Human Behavior/Neuropsychiatric Institute, Intellectual and Developmental Disabilities Research Center, University of California, Los Angeles, Los Angeles, California, USA

Contribution:

- Experiments
- Analysis
- Writing
- Manuscript preparation

Signature of co-authors:

Name	Signature
Margo Iris Jansen	Production Note: Signature removed prior to publication.
Yasir Mahmood	Production Note: Signature removed prior to publication.
Jordan Lee	Production Note: Signature removed prior to publication.
Sarah Thomas Broome	Production Note: Signature removed prior to publication.

James A. Waschek	Production Note: Signature removed prior to publication.
Alessandro Castorina	Production Note: Signature removed prior to publication.

Targeting the PAC1 receptor mitigates degradation of myelin and synaptic markers and diminishes locomotor deficits in the cuprizone demyelination model

Margo I. Jansen[1], Yasir Mahmood[1], Jordan Lee[1], Sarah Thomas Broome[2], James A. Waschek[2], Alessandro Castorina[1*]

1. Laboratory of Cellular and Molecular Neuroscience, School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney 2007, Australia
2. Semel Institute for Neuroscience and Human Behavior/Neuropsychiatric Institute, Intellectual and Developmental Disabilities Research Center, University of California, Los Angeles, Los Angeles, CA, USA.

* Correspondence: alessandro.castorina@uts.edu.au; Tel.: +61-2-9514-5028

ORCID:

- Margo I. Jansen - 0000-0003-0721-4055
- Yasir Mahmood – N/A
- Jordan Lee – N/A
- Sarah Thomas Broome - 0000-0002-5444-0920
- James A. Waschek – 0000-0003-2727-9876
- Alessandro Castorina - 0000-0001-7037-759X

Abstract

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) with a strong neuroinflammatory component. Current treatments principally target the immune system but fail to preserve long-term myelin health and do not prevent neurological decline. Studies over the last two decades have shown that the structurally related neuropeptides VIP and PACAP (vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide, respectively) exhibit pronounced anti-inflammatory activities and reduce clinical symptoms in MS disease models, largely via actions on their bivalent vasoactive intestinal peptide (VIP) receptor type 1 and 2. Here, using the cuprizone demyelination model, we demonstrate that PACAP and VIP, and strikingly the PACAP-selective receptor PAC1 agonist maxadilan, prevented locomotor deficits in the horizontal ladder and open field tests. Moreover, only PACAP and maxadilan were able to prevent myelin deterioration, as assessed by a reduction in the expression of the myelin markers proteolipid protein 1, oligodendrocyte transcription factor 2, quaking-7 (APC) and Luxol Fast Blue staining. Furthermore, PACAP and maxadilan (but not VIP), prevented striatal synaptic loss and diminished astrocyte and microglial activation in the corpus callosum of cuprizone-fed mice. *In vitro,* PACAP or maxadilan prevented lipopolysaccharide (LPS)-induced polarization of primary astrocytes at 12-24h, an effect that was not seen with maxadilan in LPS-stimulated microglia. Taken together, our data demonstrates for the first time that PAC1 agonists provide distinctive protective effects against white matter deterioration, neuroinflammation, and consequent locomotor dysfunctions in the cuprizone model. The results indicate that targeting the PAC1 receptor may provide a path to treat myelin-related diseases in humans.

Keywords

Multiple sclerosis; Pituitary adenylate cyclase-activating polypeptide; vasoactive intestinal polypeptide; maxadilan; demyelination; neuroinflammation

Abbreviations: CNS = central nervous system; CC1 = adenomatous polyposis coli, EAE=experimental autoimmune encephalomyelitis; GFAP = glial fibrillary acidic protein; IBA1 = ionised calcium-binding adapter molecule 1; MBP=myelin basic protein; MOG= myelin oligodendrocyte protein; MS = Multiple sclerosis; Olig2 = oligodendrocyte transcription factor 2; PACAP = Pituitary-adenylate cyclase activating polypeptide; PAC1 = pituitary adenylate cyclase-activating receptor 1; PLP1=proteolipid protein 1; RRID = Research Resource Identifier, SYP = Synaptophysin; VIP = vasoactive intestinal polypeptide;

VPAC1 = vasoactive intestinal polypeptide receptor 1; VPAC2 = vasoactive intestinal polypeptide receptor 2

Introduction

Multiple sclerosis (MS) is a chronic neuroinflammatory and demyelinating disease characterized by the appearance of multifocal lesions in the central nervous system (CNS) white matter, peripheral immune cell infiltration and subsequent axonal degeneration [8]. The aetiology of MS is unknown; however, both genetic and environmental factors play a significant role in disease onset and progression [536]. Demyelination and inflammation go hand in hand in MS pathogenesis, being two main factors contributing to the exacerbation of the condition. Currently, the most widely stated hypothesis of MS aetiology maintains that the infiltration of autoreactive peripheral immune cells into the CNS is the primary pathological event to initiate the disease. In this context, the invasion of immune cells into the CNS drives demyelination, followed by oligodendrocyte cell loss, gliosis and finally neurodegeneration [8]. However, there is controversy around whether neuroinflammation, in fact, initiates the MS pathogenic cascade or if it is secondary to the initial oligodendrocyte cell death and myelin loss [56,486,537].

Irrespective of its temporal involvement in MS pathogenesis, chronic neuroinflammation remains a critical hallmark of the disease, especially during relapses. In fact, persistent inflammation limits the ability of the CNS to spontaneously regenerate damaged myelin and inhibits glial and neuronal survival mechanisms [538]. At present, there is no cure for MS. Currently available disease-modifying treatments (DMTs) target the peripheral immune system and treated patients are subject to adverse effects due to the immunosuppressive functions [55,57,539]. Efforts into developing drugs able to preserve myelin integrity and promote remyelination are underway; however, none of these drugs have, as yet, made it past phase II clinical trials [540,541]. Therefore, it is of importance to identify new potential DMTs able to preserve myelin health.

Pituitary-adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) neuropeptides are known for their broad anti-inflammatory and neuroprotective properties [187,193,523]. Three heterotrimeric G-protein-coupled receptors are known to mediate the actions of these peptides. Pituitary adenylate cyclase-activating receptor 1 (PAC1) interacts only with PACAP with high affinity, whereas vasoactive intestinal

polypeptide receptor 1 (VPAC1) and vasoactive intestinal polypeptide receptor 2 (VPAC2) can interact with either VIP or PACAP with high affinity. By taking advantage of the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, researchers have started exploring the role of the PACAP/VIP system as a treatment strategy to halt the development and progression of MS, highlighting the potential therapeutic application of these neuropeptides [191,193,523]. In addition, evidence from PACAP-deficient mice showed worsened clinical course and increased mortality rate in the EAE model [321]. *In vitro* studies demonstrated that PACAP and VIP exhibit anti-inflammatory activities on microglia cells, the resident immune cells of the CNS, and can stimulate myelin-related proteins and prevent apoptosis in schwannoma cells [196,209,220].

Whilst results with the EAE model have provided essential clues on the immune modulatory effects of these neuropeptides, this preclinical model does not address the certain pathogenic mechanisms of demyelination, such as whether these endogenous molecules can prevent myelin loss, as observed in the CNS of MS patients [81]. Therefore, alternative preclinical models addressing these pathogenic features should be utilised to investigate this aspect of the disease. One such model is the cuprizone intoxication model of demyelination [487]. This animal model takes advantage of the demyelinating properties of the copper-chelator cuprizone (administered via the diet) to create a reversible demyelination associated with a secondary neuroinflammatory response in the CNS at sites of damage (white matter) within four weeks of treatment [542,543]. Cuprizone fed mice usually develop locomotor deficits and myelin loss, mimicking several behavioural and molecular features of MS pathology [544-546].

In this study, we aimed to investigate – for the first time – if targeting the PAC1 receptor could prevent white matter gliosis and preserve myelin integrity and associated locomotor deficits in the cuprizone model of MS. In addition, we performed *in vitro* experiments in isolated primary astrocytes or microglia challenged with lipopolysaccharide (LPS) to determine the relative contribution of PAC1 receptors to the anti-inflammatory properties elicited by the neuropeptides in each glial cell population.

Material and Methods

Animals

Fifty-six male C57BL/6 mice (7-week-old) weighing approximately ~20gr were purchased from the Animal Resources Centre (ARC; Perth, WA, Australia; RRID:IMSR_JAX:000664). Upon arrival, mice were allowed to acclimatise for 1 week prior to the experimental protocol and were then divided into five treatment groups: (1) Standard chow + saline intraperitoneal [i.p.], (2) cuprizone (CPZ) diet + saline i.p., (3) CPZ + PACAP, (4) CPZ + VIP and (5) CPZ + Maxadilan (n=12/group; Figure 1A). Mice were housed in individually ventilated cages (4 mice/cage) under a 12 h light/dark cycle, with access to food and water *ad libitum*. All experiments were carried out with the approval of the University of Technology Sydney (ETH17-1991) and conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Experimental protocol

The experimental protocol involved feeding mice either a standard chow or a diet supplemented with 0.2% CPZ for 28 days (n=10-12/group; Figure 1B). No randomization was performed to allocate animals in the study. CPZ diet was prepared daily by mixing standard rodents' pellets (grounded into powder) with 0% (control) or 0.2% CPZ (C9012-25G, Sigma-Aldrich), since previous evidence demonstrates that the addition of CPZ to grounded chow increases microglia and axonal damage in the corpus callosum and guarantees more consistent and reproducible demyelination compared to CPZ in pellets [547]. In addition, this approach ensured that food and CPZ was provided fresh every day. Working concentrations of PACAP (Sigma-Aldrich; A1439), VIP (AusPep) and PAC1-agonist Maxadilan trifluoroacetate (Bachem AG) were prepared in sterile saline [546]. Every other day, each mouse received either a saline, or PACAP (90.68 ng/kg), VIP (66.52 ng/kg) or Maxadilan (137.32 ng/kg) i.p. in a final volume of 0.1 mL, corresponding to a final dose of 5 nmol/mouse. PACAP and VIP dosages were based on previous work [548], whereas that of maxadilan was based on the similar affinity of both peptides for PAC1 receptors [546]. Mice weights were recorded daily to monitor overall well-being. Every 7 days, starting from day 0, mice were assessed for changes in locomotor behaviours. The open field test was used to examine general ambulation and exploratory behaviour [549], which is a tool often used to assess locomotor behaviour *in vivo* [544,549-551]. The horizontal ladder test was utilised to assess locomotor coordination [552]. On day 29, mice were sacrificed using CO_2 asphyxiation (EA-33000TS SMARTBOX® Prodigy, Total CO_2 3.01 l/min). Pedal plus eye

reflexes were checked to verify death of the animal followed by a cardiac bleed. Brains were collected and snap frozen for molecular analyses or fixed for 24 hours in 4% paraformaldehyde (by immersion) for immunohistochemical analyses.

Horizontal Ladder Test

To assess motor coordination, we utilised the horizontal ladder test, following the protocol described in Metz & Withaw (2009) [552]. The apparatus consists of a plexiglass tunnel of 60 cm equipped with sixty adjustable metal rungs, each with a diameter of 2 mm and 1 cm spacing in-between. The width of the tunnel/ladder was set at 5 cm and elevated to 30 cm above the bench using a neutral cage on one side, and the animal home cage on the other. Mice were allowed to familiarise with the apparatus before the actual testing. For these sessions, rungs were regularly spaced. In contrast, during the testing session, rungs were adjusted in at least five different irregular patterns to prevent mice from learning the rungs arrangements. On the testing day, each animal was allowed to cross the ladder 3 times in the same direction (from the neutral to the home cage) and each passage was filmed at a lateral angle (30 frames/sec). Recorded videos were analysed using VirtualDub (v1.10.4; 50 frames/sec). The foot-fault scoring system developed by Metz & Whishaw [552,553] was then applied to determine the correct (or faulty) positioning of both the front- and hind limbs of mice. An average error-score was also calculated from the three trials and used for analysis. Additionally, we averaged the time required to complete the ladder test, to which we subtracted the immobility time.

Open Field Test

The open field test was utilised to assess mobility and exploratory behaviour in mice [550]. Prior to testing, mice were allowed to acclimatise to the behaviour room for 30 minutes in the dark. Afterwards, mice were placed in a dark grey square Perspex box (40 cm × 40 cm × 50cm) and allowed to freely explore the open space in the dark for 5 minutes, whilst being filmed from the top using an infrared video camera (Sony FDRAX53 4K Full HD Handycam, 1/50fps, in the dark) held by a Manfrotto 190X tripod. Location tracking of the footage was performed using ezTrack (3 minutes/mouse) [554].

Real Time-quantitative PCR

To assess the effects of experimental treatments on the expression of neuropeptides' receptors, core myelin genes and glial activation markers, the corpus callosum (rich in myelin) was micro-dissected from frozen brains under a stereoscopic microscope (10× magnification) under

RNAse-free conditions. Briefly, the brain was removed from the skull by making an incision along the midline using dissecting scissors. Then, it was separated from the skull using fine forceps and placed it in a Petri dish containing cold PBS. Under a dissecting microscope, any remaining meninges or blood vessels attached to the surface of the brain were gently removed. Using a scalpel, the brain was cut coronally at a level corresponding to about +0.5-1.0 mm from Bregma and a second cut was performed at -1.0-1.5mm, to obtain a block encompassing the callosal structures located above the striatum. The corpus callosum was then carefully separated from the surrounding grey matter using two 23-gauge needles, which were used as scalpels. This approach allowed increased dissection accuracy. Dissected callosal structures (rich in white matter) were then placed in ice-cold PBS and collected for storage/downstream analyses. Total RNA was extracted using TRI-reagent (T9424-200ML, Sigma Aldrich) and precipitated using 2-propanol as previously described [239]. Complementary DNA (cDNA) was generated using the Tetro cDNA Synthesis kit according to manufacturer's instructions (BIO-65043, Bioline). Each RT-qPCRs reaction contained 5 µL iTaq Universal SYBR Green Supermix (Cat. No. #1725124, Bio-rad), 3 µL of cDNA (100 ng), 0.8 µL forward (5 nM) and reverse primer (5 nM). Ribosomal protein S18 was used as a reference gene. Table 1 illustrates the oligonucleotides sequences of the RT-qPCR optimised primer sets used in this study. For the analysis, relative mean fold changes in gene expression were calculated using the ΔΔCt method as described previously [261]. Primer specificity was determined by running a melting curve analysis at the end of each PCR amplification.

Table 1. Primer sets used in RT-qPCR analyses.

Accession #	**Gene**	**Primer sequence (5'-3')**	**Length (bp)**
NM_009625.3	*Adcyap1*	Fwd: GATCAGACCAGAAGACGAGG Rev: GCTGGATAGTAAAGGGCGTAAG	129
NM_011702.3	*Vip*	Fwd: GACCACCTTCTGTAGTGAGTAG Rev: TTTCTGCTAAGGGATTCTGCAA	117
NM_007407.4	*Adcyap1r1*	Fwd: TATCCACCATTACTCTACGGCT Rev: TCTGGAGAGAAGGCAAATACTG	91
NM_011703.4	*Vipr1*	Fwd: CCCTCTGTTTGGAGTTCACTAT Rev: TACGACGAGTTCAAAGACCATT	88
NM_009511.2	*Vipr2*	Fwd: ATTTCATAGATGCGTGTGGCTA Rev: TGCTTCCTGTTGTAAGAGACAT	126

NM_011123.4	*Plp1*	Fwd: ATGCCAGAATGTATGGTGTTCT Rev: TTTAAGGACGGCGAAGTTGTAAG	200
NM_010814.2	*Mog*	Fwd: CTTCTTCAGAGACCACTCTTACC Rev: CCCAATAGAAGGGATCTTCCAC	71
NM_001025251.2	*Mbp*	Fwd: TATAAATCGGCTCACAAGGGATT Rev: TGTCTCTTCCTCCCAGCTTA	85
NM_016967.2	*Olig2*	Fwd: AAAGACAAGAAGCAGATGACTGA Rev: AGCATGAGGATGTAGTTTCGC	200
NM_001361501.1	*Iba1*	Fwd: ACGTTCAGCTACTCTGACTTTC Rev: GTTGGCCTCTTGTGTTCTTTG	107
NM_001131020.1	*Gfap*	Fwd: GAGATTCGCACTCAATACGAGG Rev: CTGCAAACTTAGACCGATACCA	79
NM_011296.2	*S18*	Fwd: CCCTGAGAAGTTCCAGCACA Rev: GGTGAGGTCGATGTCTGCTT	145

Luxol Fast Blue staining

Half of the brains from each experimental group were fixed in 4% paraformaldehyde (PFA; 158127-500G, Merck) for 24h before tissue processing (Excelsior™ AS Tissue Processor) and embedding in paraffin (The Epredia™ HistoStar™ embedding workstation). Serial coronal sections (each 5µm thick) spanning the antero-posterior extension of the corpus striatum (between +1.0 and -1.5 mm from Bregma) were cut using a microtome (Epredia™ HM 325 microtome) and were collected on silane-coated slides (StarFrost, QLD, Australia). To assess myelin integrity, sections were stained with Luxol Fast Blue (LFB) staining (0.1% LFB in 95% ethanol; S3382-25G, Sigma-Aldrich). To perform this histological evaluation, sections were deparaffinised in xylene (3× 5 min), followed by a 100% ethanol step (2× 5 min) and a 95% ethanol step (5 min) before overnight incubation with LFB staining solution at 58°C. The next day, sections were rinsed in 95% ethanol prior to a brief differentiation step in Lithium Carbonate (0.05% in ddH_2O, 255823-100G, Sigma Aldrich) and a further rinse in 70% ethanol. Afterwards, sections were counterstained using 0.1% Cresyl Violet solution (10 min at RT, C5042-10G, Merck). After a rinse in ddH_2O and a differentiation step in 95% ethanol, slides were dehydrated, mounted using VectaMount Express Mounting medium (H-5700-60, Abacus DX) and cover slipped. Stained sections were imaged using the Zeiss Axioscan Z1 (20×, ZEISS). Images were analysed in ImageJ/FIJI (version 1.53k). Myelin intensity in the corpus callosum was quantified by separating the LFB staining from the Cresyl Violet counterstaining

using the colour deconvolution2 plugin (v2.1) followed by thresholding and measuring the mean grey value.

Immunohistochemistry

Serial coronal sections taken at the same antero-posterior coordinates as for LFB staining were deparaffinised in xylene and rehydrated using decreasing concentrations of ethanol to H_2O. Following an antigen-retrieval step of 15 min (10mM citric acid, 0.05% Tween-20, pH 6.0), Proteolipid protein (PLP) (1:2000, Abcam Cat# ab254363, RRID:AB_3095302), Olig2 (1:200, Cell Signaling Technology Cat# 65915, RRID:AB_2936997) or anti-adenomatous polyposis coli (APC, aka Quaking 7 or CC1) (1:100, Sigma-Aldrich Cat# SAB4501438, RRID:AB_10762093) immunoreactivity was assessed using the Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam Cat# ab64261, RRID:AB_2810213) according to manufacturer's instructions. Hematoxylin (Lillie Mayer's, POCD healthcare) was used to counterstain nuclei. Following this, sections were dehydrated using increasing concentrations of ethanol and xylene and mounted using VectaMount Express Mounting medium (H-5700-60, Abacus DX). Stained sections were imaged using the Zeiss Axioscan Z1 (20×, Carl Zeiss). Images were analysed with ImageJ/FIJI (version 1.53k). The average intensity of DAB staining was measured in at least three selected sections per mouse (n=6 per group) taken along the rostro-caudal axis of the corpus callosum, which were used to assess alterations in white matter PLP and Quaking 7 immunoreactivity. Briefly, following a colour deconvolution step, a universal threshold was set for all images and the mean grey intensity values in the corpus callosum were measured. To measure the number of $Olig2^+$ nuclei, regions of interest (200 × 200 μm = 40,000 μm^2) selected from the corpus callosum of each mouse were colour de-convolved. Thereafter, universal threshold was set, and the number of cells and size of nuclei were determined using the particle analysis plug-in of ImageJ/FIJI after a thresholding step. To calculate the average # of cells / mm^2 we applied the following formula:

$$Cells/(mm\text{^}2) = ((average\ \#cells\ in\ each\ ROI\ *\ 10\text{^}6))/\ ROI\ area.$$

Immunofluorescence

Immunofluorescence staining was performed based on a protocol published by Kajimura et al. [555] with minor modifications. Briefly, sections were deparaffinised and rehydrated using xylene and decreasing concentrations of ethanol. After an antigen-retrieval step of 15 min (10 mM citric acid, 0.05% Tween-20, pH 6.0), an auto-fluorescence quenching step was performed by submerging the sections in a solution containing 0.25% NH_3 in 70% ethanol for 1h at RT, followed by a permeabilisation step of 20 min (0.4% Triton-X100 in PBS). A brief hydrogen peroxide step (3% H_2O_2 in methanol, 15 min) was performed to block endogenous peroxidase activity. Sections were blocked for 1 h in 5% BSA (0.2% gelatin, 0.25% Triton-X100 in PBS). Primary antibody dilutions were prepared in 1% BSA (0.2% gelatin, 0.25% Triton-X100 in PBS) and incubated overnight (4 °C). Slides were incubated with secondary antibodies for 1h at RT in the 1% BSA solution, before being mounted using Vectashield® Antifade Mounting Medium containing DAPI (H-1200-10, Abacus DX). Primary antibodies used for these experiments were Rabbit-anti-GFAP (1:250, Abcam Cat# ab68428, RRID:AB_1209224), Mouse-anti-IBA1 (1:250, GeneTex Cat# GTX632426, RRID:AB_2888314), Rabbit-anti-SYP (1:200, Santa Cruz Biotechnology Cat# sc-9116, RRID:AB_2199007), Mouse-anti-TUJ1 (1:200, BioLegend Cat# 801201 (also 801202, 801213), RRID:AB_2313773), Rabbit-anti-NEFL (1:250, Thermo Fisher Scientific Cat# MA5-14981, RRID:AB_10984147), Goat anti-mouse IgG H&L (Alexa Fluor® 594) (1:500, Abcam Cat# ab150116, RRID:AB_2650601) and Goat anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) (1:500, Cell Signaling Technology Cat# 4412 (also 4412S), RRID:AB_1904025). All stained sections were imaged using the Zeiss Axioscan Z1 (20×, Zeiss). Images were analysed in ImageJ/FIJI (version 1.53k). For fluorescence intensity measurements, a universal threshold setting was applied to all images and the mean intensity of each channel was measured specifically in the corpus callosum and plotted relative to the intensity in control mice. $GFAP^+$ or IBA^+ cell counts in the corpus callosum were measured as described above for $Olig2^+$ nuclei. Figures were generated using OMERO.figure (version 4.2.0).

Primary glia cultures & treatments

Primary cultures of astrocytes or microglial cells were prepared as per the protocol described by [556,557] with minor modifications. Briefly, whole brains of P0-P2 C57BL/6 mice (ARC; Perth, WA, Australia; RRID:IMSR_JAX:000664) pups were isolated (4-5 pups per prep/three independent repeats) and dissected under sterile conditions using filtered Hanks' balanced salt solution (H9394, Sigma-Aldrich). Cortices were pooled and collected for mixed glia cell

isolation. After a trypsin/EDTA digestion (0.25% trypsin/EDTA for 30 min at 37 ◦C; Sigma-Aldrich) and DNase I treatment step (10 mg/mL, Sigma-Aldrich), the cell suspension was centrifuged (300 rpm, 5 min) followed by a triturated step to further dissociate the tissue and obtain a single cell solution. Cells were plated in a T75 flask (10–15 × 10^6 cells) for 10–11 days (or until 80–90% confluent), with media replacements on alternate days. After 10 days, to isolate microglial cells, the mixed glial cultures were placed on an oscillator (200 rpm × 2 h, RT). Supernatants containing enriched microglia were then centrifuged at 1200 rpm × 5 min and plated on poly-D-Lysine-coated (PDL; P6407, Sigma-Aldrich; diluted in milliQ H_2O) glass ViewPlate 96-well plates for immunocytochemistry (PerkinElmer). The remaining culture consisting of astrocytes and OPCs was further dissociated using an oscillator (240 rpm × 4 h, RT), followed by vigorous tapping for 1 min to remove any residual OPCs, which were discarded. The final astrocyte monolayer was then detached from the flask using 2.5% trypsin/EDTA and seeded on poly-D-lysine (PDL)-coated glass 96 well plates for immunocytochemistry. Cells were allowed to grow for 3-4 days prior to downstream experiments to reach a 60-70% confluence. Using this protocol, a purity of >90% was attained for both primary astrocyte and microglia cultures. Full growth media only, media containing lipopolysaccharide (LPS; 500 ng/mL; L2630-10MG, Sigma-Aldrich) or LPS + each of the neuropeptides (PACAP, VIP or Maxadilan, 100 nM) was added to the wells for 12 or 24 h prior to fixation.

Immunocytochemistry

Untreated or treated astrocytes or microglial cells were fixed in 4% PFA (in PBS; 158127-500G, Merck) for 15 min (37◦C) and washed three times with PBS. After a brief permeabilisation step (0.1% Triton X-100 in PBS, 10 min at RT), cells were blocked in a 5% BSA solution (in PBS, 3h at RT). Primary antibody dilutions were prepared in 5% BSA (in PBS, A2153-10G, Sigma Aldrich) and incubated overnight (4 °C). After a washing step, cells were incubated with secondary antibodies for 2h at RT in the 5% BSA solution, followed by a nuclear staining step using DAPI (1:500, in PBS, 1h at RT, D1306, Sigma-Aldrich). Following thorough washing, stained cells were kept in 100 µL PBS until imaging. Antibodies used in these experiments were: mouse-anti-GFAP (1:200, (Thermo Fisher Scientific Cat# 14-9892-82, RRID:AB_10598206)), mouse-anti-IBA1 (1:250, GeneTex Cat# GTX632426, RRID:AB_2888314), Goat-anti-Mouse FITC (1:250, Abcam Cat# ab6785, RRID:AB_955241). All images were acquired on the Leica Stellaris 8 confocal fluorescence microscope (Leica Microsystems) equipped with a 40× oil-immersion objective (NA =1.1, Z-

stack=0.4µm optical slices). Images were analysed in ImageJ/FIJI (version 1.53k) using maximum intensity projection images.

Data analyses

All statistical analyses and graphs were generated using GraphPad Prism (version 9.3.1). Data are reported as the mean ± SD. One-way repeated measures ANOVA followed by Tukey's multiple comparisons tests was used to analyse both Open Field and Horizontal Ladder results. For RT-qPCR analyses, immunohistochemistry and immunofluorescence, we utilised one-way ANOVA followed by Sidak's multiple comparisons tests. For the immunocytochemistry experiments, we used two-way ANOVA followed by Tukey's multiple comparisons test. P-values ≤ 0.05 were considered statistically significant.

Results

PACAP, VIP and Maxadilan prevent locomotor deficits in cuprizone-fed mice.

In this set of experiments, mice on a normal dietary regime or on a 0.2% CPZ-diet received injections of saline, PACAP, VIP or the specific PAC1 agonist Maxadilan on alternate days over the course of four weeks (Figure 1A & B) [546]. Maxadilan was used in view of its selective agonist activity towards the PAC1 receptor, since both PACAP and VIP have high binding affinity to PAC1, VPAC1 and VPAC2 receptors.

Upon monitoring the weight of these mice, a global weight reduction was recorded in mice intoxicated with CPZ (****p<0.0001 vs Control; Figure 1C). A similar weight loss was also seen in intoxicated mice treated with PACAP (****p<0.0001 vs Control), VIP (***p=0.0008 vs Control) and Maxadilan (****p<0.0001 vs Control). As shown, the average daily weight of all mice fed a CPZ diet was decreased, irrespective of the peptide administered, throughout the four weeks of treatment (Figure 1D).

Using the horizontal ladder test – also called ladder rung walking test – we assessed the effects of the neuropeptides on motor coordination and stepping accuracy in animals subjected to a CPZ diet with or without receiving neuropeptides treatment. A significant reduction of foot fault scores (indicative of deteriorated coordination) was observed in the forelimbs of mice intoxicated with 0.2% CPZ diet after two weeks (Figure 1E) (***p=0.0002 vs Control at Day 14), which partly worsened during the remaining two weeks of testing (*p<0.05 and

****p<0.0001 vs Control at Day 21 and 28, respectively). Treatment with Maxadilan prevented the loss of forelimbs coordination caused by the CPZ diet from day 7 onwards, whereas signs of ameliorated coordination were observed after two weeks in the PACAP-treated groups. VIP treatment was able to prevent loss of forelimb coordination only after 4 weeks of treatment (#p<0.05 vs CPZ at Day 28).

Analyses of hindlimbs demonstrated similar locomotor deterioration in CPZ-fed mice (Figure 1F), with statistically significant reductions of foot fault scores in comparison with controls (****p<0.0001, **p=0.0027, ***p=0.0002 and ****p<0.0001 vs Control at Day 7, 14, 21 and 28, respectively). As for the forelimbs, PACAP, VIP and Maxadilan treatments all ameliorated hindlimbs coordination, with a functional recovery comparable to controls as early as 1 week after the commencement of treatments.

In Figure 1G and H, the average error rate is shown for both the forelimbs and hindlimbs. The error rate is defined as the percentage of errors made per step. A significant increase in the error rate was seen in mice exposed to CPZ, starting from day 14 onwards for the forelimbs (**p=0.0063, p=0.0607 and ***p=0.0002 vs Control at Day 14, 21 and 28, respectively) and from day 7 onwards from the hindlimbs (****p<0.0001, **p=0.0051, ***p=0.0002, ***p=0.0002 vs Control at Day 14, 21 and 28).

Treatment with PACAP or Maxadilan rescued the behavioural deficits caused by CPZ as early as 14 days into the diet for the forelimbs. For the hindlimbs, treatment with PACAP, VIP or Maxadilan all ameliorated CPZ-induced deficits from 7 days onwards.

The open field test is an excellent and well-established tool utilised to study mice exploratory and locomotion behaviour in mice undergoing CPZ intoxication [550,551,558,559]. In the open field test, CPZ-fed mice showed a reduction in the total distance travelled starting after 2 weeks on the diet (Figure 1I & J; *p<0.05 vs Control at Day 14 and 21 and **p=0.0093 vs Control at Day 28). Treatment with either PACAP or Maxadilan prevented the decrease in total distance travelled caused by the CPZ diet, whereas treatment with VIP was only partly effective. In terms of exploratory behaviour (measured as the distance covered by each mouse in the centre quadrant), although slightly improved by PACAP treatment at day 21 (Figure 1K; #p<0.05 vs CPZ), none of the neuropeptides mitigated the reduced exploration in mice subjected to CPZ

diet. Overall, neither neuropeptide effectively improved the CPZ-induced reduction of exploration in the centre of the open field apparatus (Figure 1K).

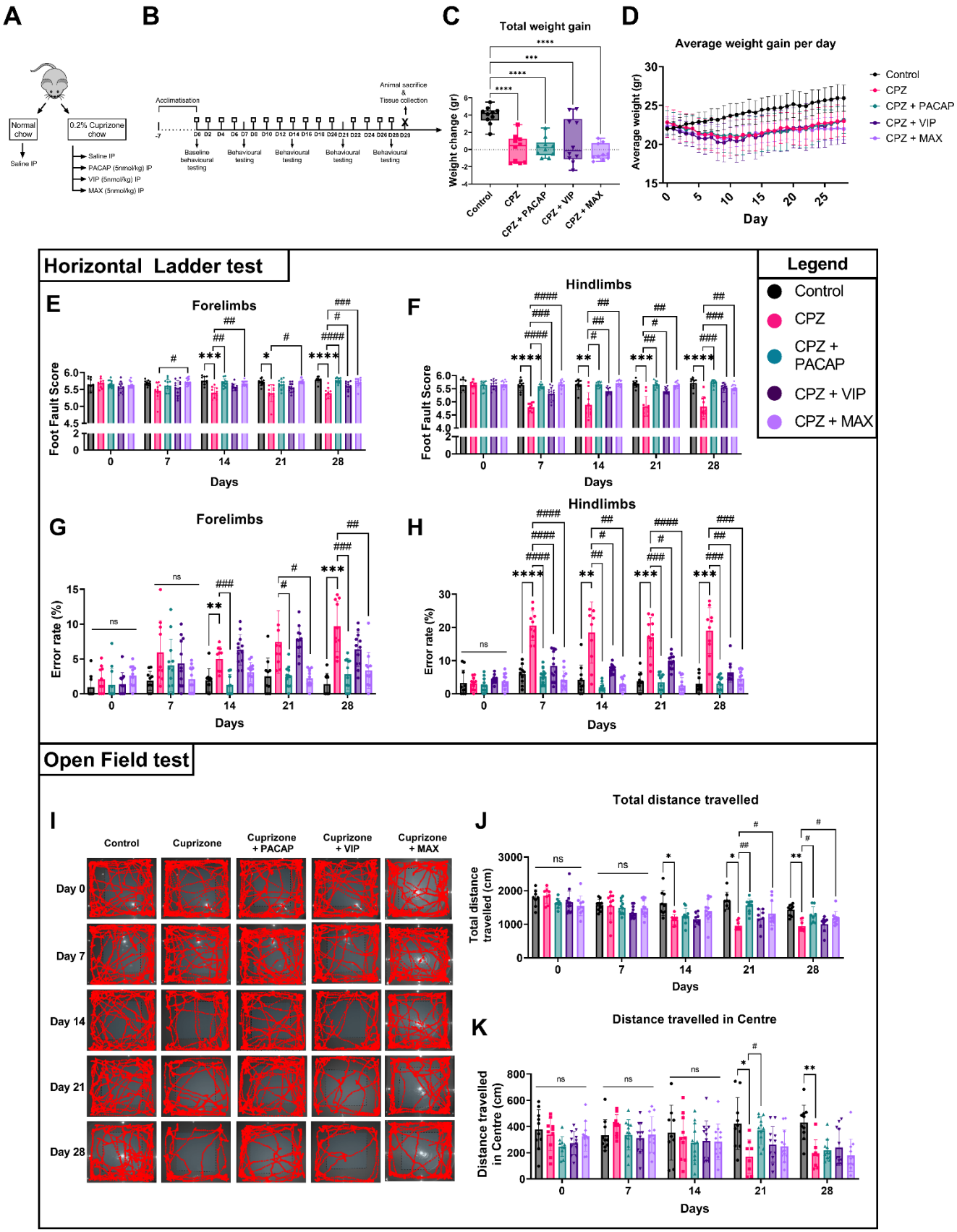

A
B
C
D
Normal chow
0.2% Cuprizone chow
Saline IP
Saline IP
PACAP (5nmol/kg) IP
VIP (5nmol/kg) IP
MAX (5nmol/kg) IP
Acclimatisation
Animal sacrifice & Tissue collection
Baseline behavioural testing
Behavioural testing
Total weight gain
Weight change (gr)
Control
CPZ
CPZ + PACAP
CPZ + VIP
CPZ + MAX
Average weight gain per day
Average weight (gr)
Day
Horizontal Ladder test
Legend
Control
CPZ
CPZ + PACAP
CPZ + VIP
CPZ + MAX
E
Forelimbs
Foot Fault Score
Days
F
Hindlimbs
Foot Fault Score
Days
G
Forelimbs
Error rate (%)
Days
H
Hindlimbs
Error rate (%)
Days
Open Field test
I
Control
Cuprizone
Cuprizone + PACAP
Cuprizone + VIP
Cuprizone + MAX
Day 0
Day 7
Day 14
Day 21
Day 28
J
Total distance travelled
Total distance travelled (cm)
Days
K
Distance travelled in Centre
Distance travelled in Centre (cm)
Days

Fig. 1. PACAP, VIP or Maxadilan treatment prevents the locomotor deficits caused by cuprizone. *Schematic overview of (A) the experimental groups and (B) timeline for treatments and behavioural assessments. Squares indicate days in which mice received intraperitoneal peptides' injections. The cuprizone (CPZ) and control diets commenced on day 1 and continued until the day of tissue collection (day 29). (C) Shows the overall weight change in response to diet/treatment. (D) Line graph showing the daily average weight of mice throughout the experimental timeline. Average foot fault scores measured in the (E) forelimbs and (F) hindlimbs of mice. (G) Error rates (shown as %) for the forelimbs and (H) hindlimbs of mice in each group. (I) Representative linetracks showing the trajectory of mice while exploring the open field apparatus. Bar graphs showing the (J) total distance travelled and (K) distance travelled in centre. Statistics in C were computed using one-way ANOVA followed by Sidak's multiple comparison tests, showing significant changes with respect to control ***p<0.001 or ****p<0.0001 or cuprizone (# p<0.05). n=10-12 mice per group for weight. Behavioural data was analysed using mixed-effects ANOVA followed by Tukey's multiple comparison's test. Results are the mean +/- SD. **p<0.01, ***p<0.001, ****p<0.0001 vs Control. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs Cuprizone. n=10-12 mice per group.*

PACAP, VIP, and Maxadilan treatment fails to prevent overall downregulation of neuropeptides and receptors genes but rescues VPAC2 expression in the white matter of cuprizone-intoxicated mice.

In view of the behavioural improvements afforded by PACAP and Maxadilan in CPZ-fed mice, we sought to determine if the demyelinating diet disrupted the expression PACAP, VIP and related receptors in the white matter. Additionally, we evaluated if exogenous administration of either neuropeptide prevented any gene expression changes caused by CPZ. RT-qPCR experiments revealed that the levels of PACAP and VIP genes were significantly diminished in the corpus callosum of CPZ mice (****p<0.0001 vs Control for both PACAP and VIP genes; Figure 2A&B). Exogenous administration of PACAP, VIP or Maxadilan did not prevent the downregulation in mRNA expression caused by CPZ (Figure 2A&B). PAC1 gene expression was reduced by the CPZ diet (**p=0.0022, Control vs CPZ; Figure 2C) and did not return to control levels after treatment with any of the neuropeptides tested (*p<0.05, Control [with both PACAP and VIP]; **p=0.0025 [Maxadilan]). CPZ also significantly lowered VPAC1 receptor mRNA levels compared with a normal diet (****p<0.0001 vs Control), and similarly to PAC1, none of the peptides were able to rescue the effects of CPZ on gene expression (Figure 2D). In contrast to PAC1 and VPAC1 transcripts, VPAC2 expression was increased by the CPZ diet (****p<0.0001 vs Control; Figure 2E). This effect was ameliorated by PACAP, VIP and Maxadilan treatment (####p<0.0001 vs CPZ [both PACAP and VIP], #p<0.05 vs CPZ [Maxadilan]).

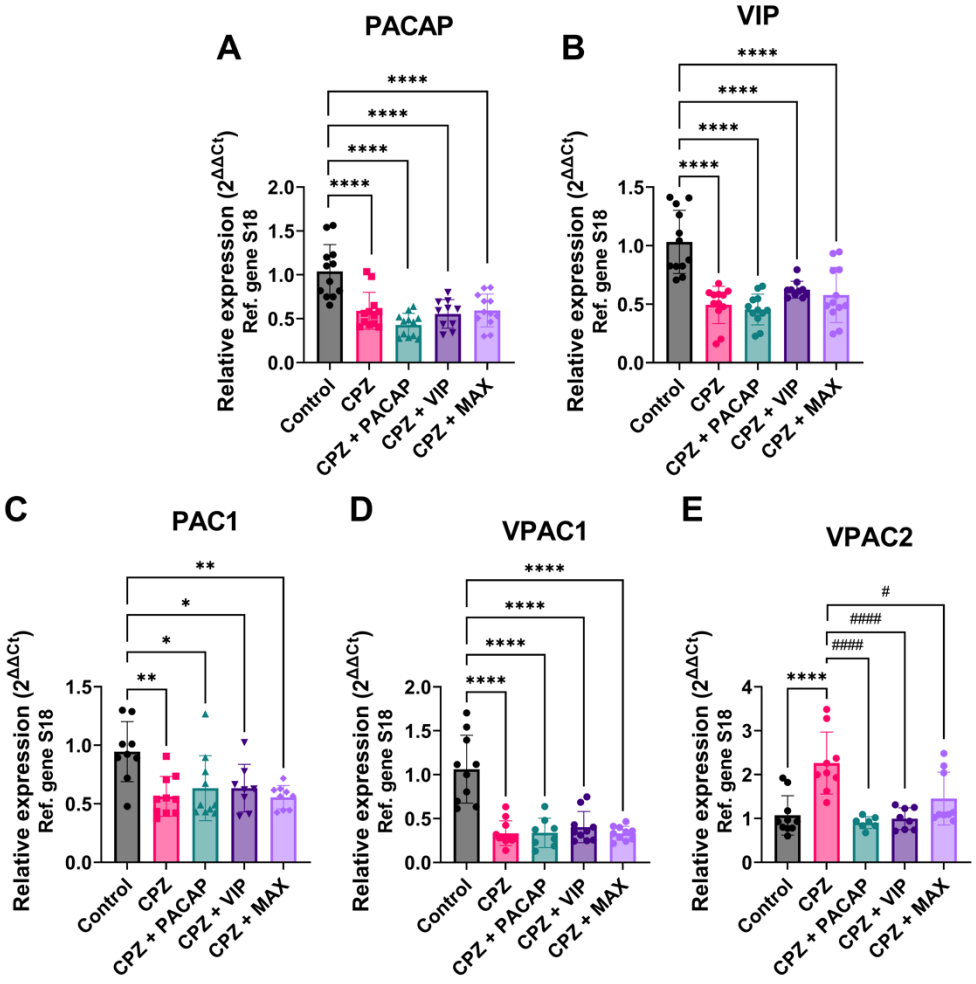


Fig. 2. Expression levels of PACAP, VIP and related receptors in the CNS white matter of mice fed a CPZ diet for 4 weeks. *Real-time quantitative PCR showing the differential expression levels of (A) Adcyap1 or PACAP, (B) Vip or VIP, (C) Adcyap1r1 or PAC1, (D) Vipr1 or VPAC1 and (E) Vipr2 or VPAC2. n= 7-12 mice per group. Fold changes were calculated using the ΔΔCt method relative to ribosomal protein S18, the reference gene. Data shown are the mean ± SD (n=7-12 mice). Statistics was computed using one-way ANOVA followed by Sidak's multiple comparisons test. *p<0.05, **p<0.01, ****p<0.0001 vs Control. #p<0.05 or ####p<0.0001 vs Cuprizone. PACAP, pituitary adenylate cyclase-activating peptide; VIP, vasoactive intestinal peptide; PAC1, pituitary adenylate cyclase-activating peptide receptor 1; VPAC1, vasoactive intestinal polypeptide receptor 1; VPAC2, vasoactive intestinal polypeptide receptor 2*

The PAC1-preferring ligands PACAP and Maxadilan prevent myelin cell loss in CPZ mice. Once we established that treatment with the neuropeptides PACAP, VIP and Maxadilan prevented the locomotor deficits caused by the CPZ diet, we aimed to determine if these changes were linked to alterations in the mRNA expression of core myelin genes in the white

matter. RT-qPCR analyses revealed that CPZ induced a robust reduction in the expression levels of myelin markers *PLP1* (****p<0.0001 vs Control; Figure 3A), myelin basic protein (*MBP)* (****p<0.0001 vs Control; Figure 3B), myelin oligodendrocyte glycoprotein (*MOG)* (****p<0.0001 vs Control; Figure 3C), and oligodendrocyte transcription factor 2 (*Olig2)* (****p<0.0001 vs Control; Figure 3D). Treatment with Maxadilan, but not PACAP or VIP, partially prevented the reduction of *PLP1* (#p<0.05 vs CPZ), *MOG* (#p<0.05) and *Olig2* (#p<0.05), but not *MBP* (Figure 3A-D).

PLP is the most abundant protein of CNS myelin, which plays a critical role in myelin stability and maintenance of compact lamellar structure [560]. Immunohistochemical analyses were performed in coronal sections of the corpus callosum spanning the rostro-caudal axis of the striatum (Figure 3A) highlighted substantial differences of PLP immunoreactivity in mice that received CPZ alone in comparison with animals that had also been injected with the peptides (Figure 3E-J). Semi-quantification of the average PLP immunoreactivity obtained from the analyses of regions of interest (ROIs) randomly selected along the medio-lateral axis of the white matter demonstrated a remarkable reduction of signal intensity in CPZ-fed mice (****p<0.0001 vs Control; Figure 3K). Amongst the neuropeptides treated groups, PLP intensity was partly preserved in CPZ mice injected with PACAP (#p<0.05 vs CPZ) and, concordant with mRNA data, these effects were more pronounced in Maxadilan-injected mice (###p=0.001 vs CPZ; Figure 3K). Further to these findings, we also performed Luxol Fast Blue staining in the same brain region, which demonstrated a robust reduction in the density of intact/myelinated fibres in CPZ-fed mice (****p<0.0001 vs Control; Supplementary Figure 1A-F). Treatment with PACAP or Maxadilan partially prevented CPZ-induced demyelination in the corpus callosum (#p<0.05 vs CPZ; Supplementary Figure 1A-F), whereas VIP showed no appreciable effects.

Oligodendrocyte transcription factor 2 (OLIG2) is a protein that plays a critical role in the development and differentiation of oligodendrocytes and it commonly used as a marker to identify cells committed to the oligodendrocyte lineage during development [561,562]. Stereological analyses demonstrated that $Olig2^+$ cells were decreased in the corpus callosum of CPZ-fed mice (****p<0.0001 vs Control; Figure 3L, M). Higher magnification images also showed increased cellularity in CPZ fed mice (Figure 3L, white arrowheads in lower panels). PACAP and Maxadilan, but not VIP, significantly increased the number of $Olig2^+$ cells in the corpus callosum (#p<0.05 [PACAP] & ##p=0.0071 [Maxadilan] vs Cuprizone). Moreover,

cellularity was decreased in animals receiving PACAP and, to a lesser extent, in those receiving Maxadilan.

As cell swelling/blistering of both the cytoplasm and nuclear compartment is a common phenomenon in damaged oligodendrocytes and/or OPCs [563,564], we also sought to measure the mean surface size of $Olig2^+$ nuclei to provide an additional indication on the extent of damage to the oligodendroglia cell lineage. We found that $Olig2^+$ nuclei were increased in size both in CPZ-fed mice (*p<0.05 vs Control; Figure 3N) and CPZ-fed mice receiving VIP treatment (**p=0.0014 vs Control) but not in mice receiving PACAP or Maxadilan injections.

Comparative analyses of the expression of quaking 7, the binding target of the anti-adenomatous polyposis coli (APC) antibody, were performed in the corpus callosum of demyelinated mice at a level spanning the same antero-posterior bregma interval as in Figure 3E [565]. Quaking 7 is a reliable marker for the detection of mature (myelinating) oligodendrocytes [565]. quaking 7 immunoreactivity was significantly reduced in CPZ-fed mice and CPZ-fed mice receiving VIP treatment (**p=0.0022 & *p<0.05 vs Control; Figure 3O, P). In CPZ-fed mice treated with either PACAP or Maxadilan, both successfully prevented the attenuation of quaking 7 staining, which reached levels to comparable to control mice (##p=0.0097 [PACAP] & ##p=0.0022 [Maxadilan] vs Cuprizone).

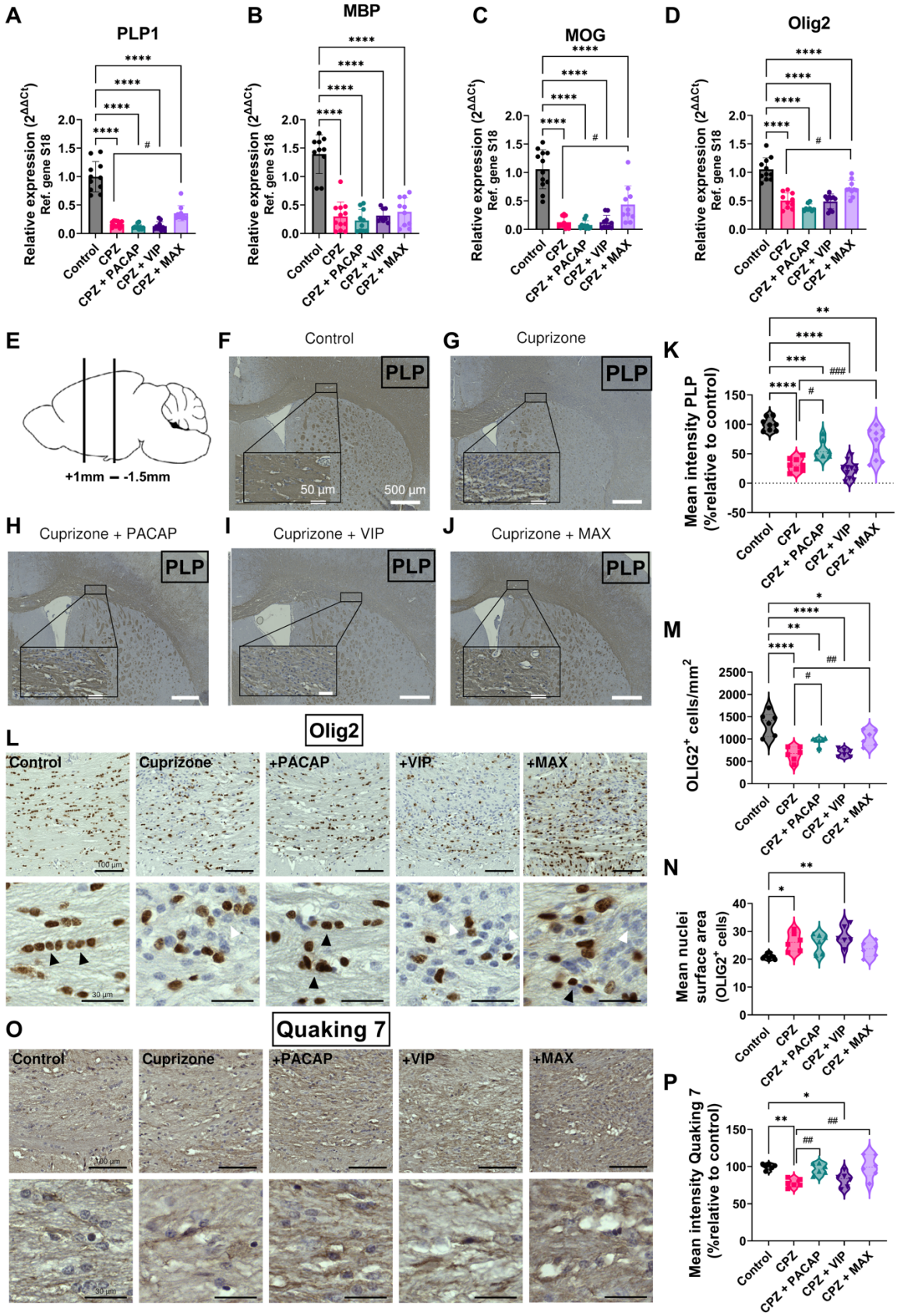

A
PLP1
B
MBP
C
MOG
D
Olig2
Relative expression (2^ΔΔCt)
Ref. gene S18
Control
CPZ
CPZ + PACAP
CPZ + VIP
CPZ + MAX
E
+1mm – -1.5mm
F
Control
G
Cuprizone
PLP
50 µm
500 µm
H
Cuprizone + PACAP
I
Cuprizone + VIP
J
Cuprizone + MAX
K
Mean intensity PLP (%relative to control)
L
Olig2
Control
Cuprizone
+PACAP
+VIP
+MAX
100 µm
30 µm
M
OLIG2+ cells/mm2
N
Mean nuclei surface area (OLIG2+ cells)
O
Quaking 7
P
Mean intensity Quaking 7 (%relative to control)

Fig. 3. Effects of PACAP, VIP and Maxadilan on myelin health after a demyelinating insult. *RT-qPCR analyses demonstrating decreased gene expression of the myelin markers (A) PLP1, (B) MOG, (C) MBP, (D) Olig2 in mice fed a 0.2% cuprizone diet for a period of 4 weeks. As shown in A, C and D, Maxadilan only partly prevented CPZ-driven downregulation of myelin genes. n= 7-12 mice per group. Fold changes were calculated using the ΔΔCt method, using the ribosomal protein S18 as reference gene. RT-qPCR data are shown as mean +/- SD. For histology, (E) shows the approximate rostro-caudal coordinates (relative to Bregma) used for the sampling of coronal brain tissue sections. Representative images depicting PLP immunoreactivity in the corpus callosum of (F) Control, (G) Cuprizone, (H) Cuprizone + PACAP, (I) Cuprizone + VIP and (J) Cuprizone + Maxadilan mice. Scale bar in F, G, H, I and J = 500 µm. Scale bar in insets = 50 µm. (K) Violin plot showing the mean intensity of PLP staining measured, expressed as the percentage of control. (L) Representative low- (upper panels) and high- magnification images (lower panels) showing Olig2+ cells in the corpus callosum. Black arrowheads indicate one or more sites with at least three or more regularly aligned Olig2+ cells, whereas white arrowheads point to areas with increased cellularity due to glial cell infiltration. Stereological measurements of Olig2+ cells in the corpus callosum showing the (M) average Olig2+ cell count and (N) cell surface area. n = 6 mice per group. (O) Representative callosal tissue sections depicting Quaking 7 staining in the corpus callosum and (P) and semi-quantification of the mean intensity levels (relative to control). Scale bars in L and O = 100 µm (upper panels) and 30 µm (lower panels). Data were analysed using a one-way ANOVA followed by Sidak's multiple comparisons tests. *p<0.05, **p<0.01 and ****p<0.0001 vs Control; #p<0.05, ##p<0.01, ###p<0.001 vs Cuprizone.*

PLP, proteolipid protein, MOG, myelin oligodendrocyte protein; MBP, myelin basic protein; Olig2, oligodendrocyte transcription factor 2; Quaking 7, adenomatous polyposis coli

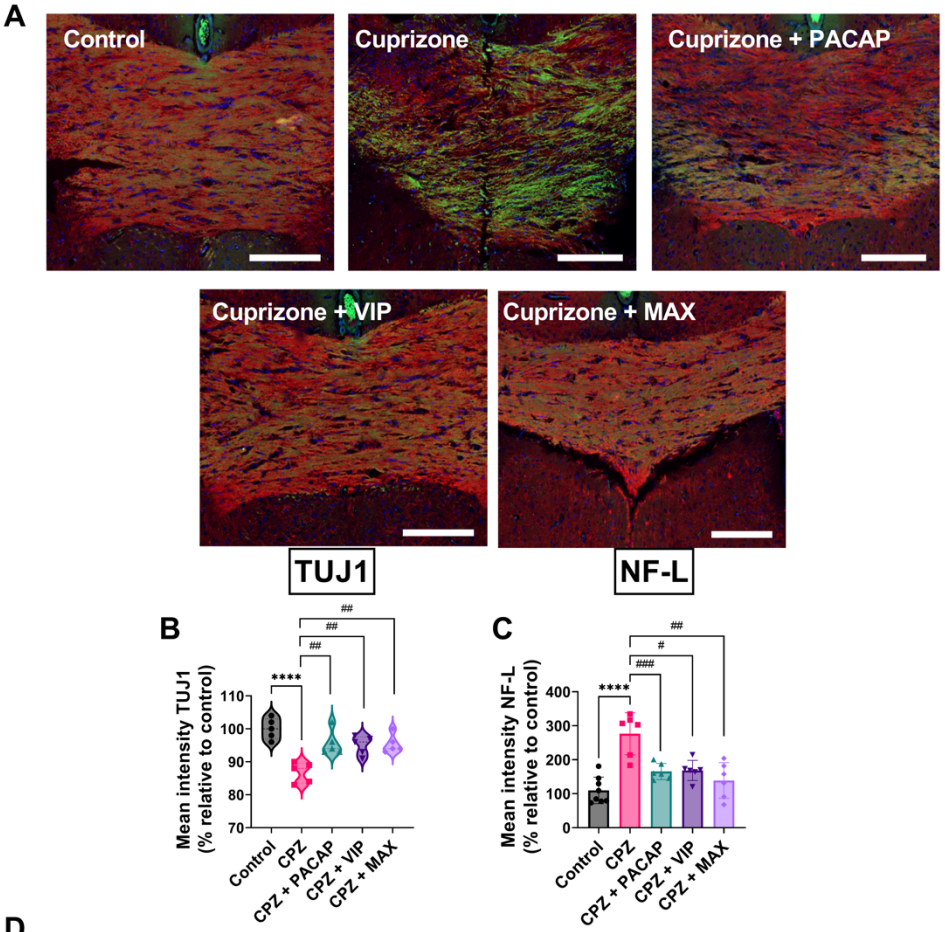
PACAP, VIP and Maxadilan attenuate cuprizone-induced axonopathy, neuronal and synaptic loss.

The major contributing factor to clinical disability in MS is the progressive axonal damage and neuronal loss in afflicted people [566,567]. This pathological feature has also been observed in EAE mice and in the CPZ demyelination model [568,569].

Here we assessed if PACAP, VIP or Maxadilan treatment prevented axonopathy, neuronal damage and synaptic loss in mice subjected to the CPZ diet (Figure 4). In both the corpus callosum and striatum, the CPZ diet caused a robust reduction in the staining intensity of the class III beta-tubulin (aka TUJ1) – a pan-neuronal/axonal marker [570] – in comparison with control mice (****p<0.0001 vs Control [Corpus callosum], ****p<0.0001 vs Control [Striatum]; Figure 4A,B,D,E). Interestingly, all three neuropeptides equally prevented the reduction of TUJ1 staining caused by the demyelinating diet both in the corpus callosum

(##p=0.002 [PACAP], ##p=0.004 [VIP] and ##p=0.0025 [Maxadilan] vs CPZ; Figure 5A, B) and striatum (###p=0.001 [PACAP], ###p=0.0009 [VIP], ####p<0.0001 [Maxadilan] vs CPZ; Figure 4D, E). To complement our findings with TUJ1, we also assessed levels of Neurofilament protein light chain (NF-L). NF-L is commonly used to indicate axonal degeneration and can be quantified directly through immunofluorescence as a proxy for neuronal damage [571-573,574]. NF-L staining intensity increased in CPZ-fed mice (****p<0.0001 vs Control; Figure 4C), with neuropeptide treatment reducing NF-L levels back to control levels (###p=0.001 [PACAP], ##p=0.0014 [VIP], ####p<0.0001 [Maxadilan] vs CPZ; Figure 4C).

To assess if CPZ intoxication would lead to striatal synaptic loss, striatal sections were co-stained with synaptophysin, a marker used to identify synaptic terminals [575]. Semi-quantification of synaptophysin immunoreactivity showed a clear loss of synaptic density in the striatum of CPZ-fed mice (****p<0.0001 vs Control; Figure 4C, D), an effect that was prevented by either PACAP or Maxadilan (####p<0.0001 [PACAP], ####p<0.0001 [Maxadilan] vs CPZ), but not VIP.


A
Control
Cuprizone
Cuprizone + PACAP
Cuprizone + VIP
Cuprizone + MAX
TUJ1
NF-L
B
Mean intensity TUJ1 (% relative to control)
C
Mean intensity NF-L (% relative to control)
Control
CPZ
CPZ + PACAP
CPZ + VIP
CPZ + MAX

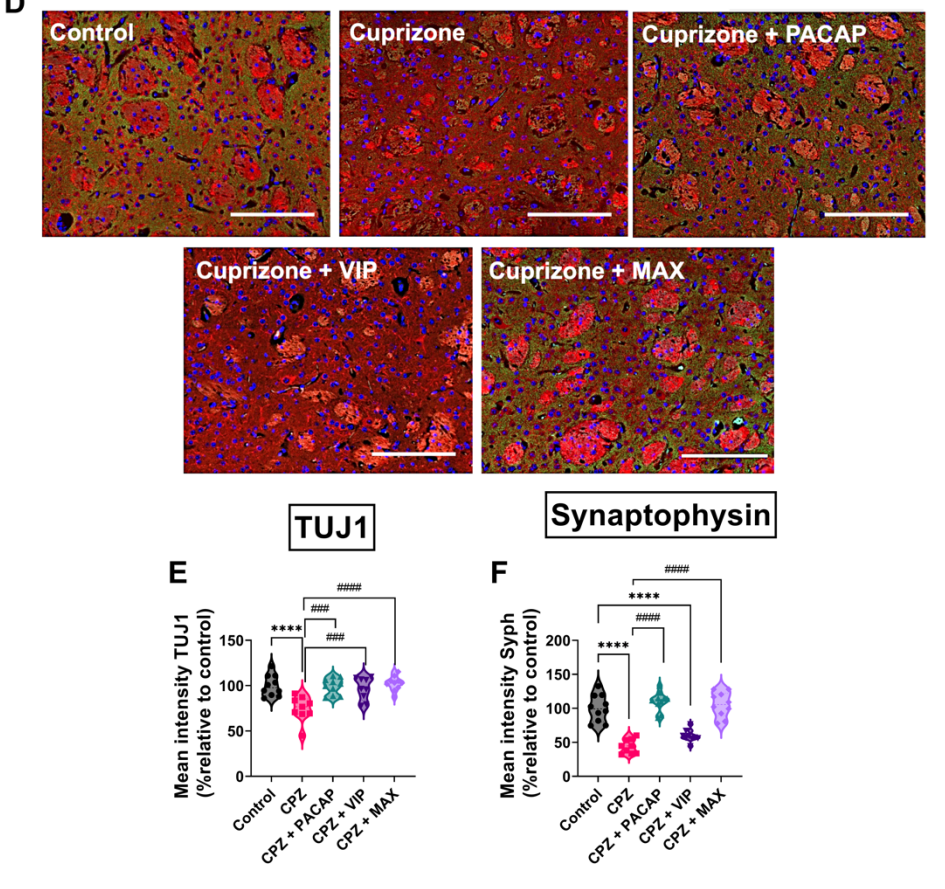

D
Control
Cuprizone
Cuprizone + PACAP
Cuprizone + VIP
Cuprizone + MAX
TUJ1
Synaptophysin
E
Mean intensity TUJ1 (%relative to control)
F
Mean intensity Syph (%relative to control)
Control
CPZ
CPZ + PACAP
CPZ + VIP
CPZ + MAX

Fig. 4. Protective effects of the neuropeptides against cuprizone-induced axonopathy, neuronal and synaptic loss. *(A) Representative images of TUJ1 (red) and NF-L (green) co-staining in the corpus callosum of CPZ-fed mice after 4 weeks. Nuclei were counterstained with DAPI (blue). Scale bar = 200 µm. n = 5-9 mice per group. Violin plot showing the (B) average TUJ1 staining intensity and (C) NF-L staining intensity of axons in the corpus callosum of each treatment group are presented. (D) Representative images of striatal sections (antero-posterior +1 mm - -1.5 mm from Bregma) co-stained with Synaptophysin (green) and TUJ1 (red). Scale bar = 100 µm. n = 9-10 mice. Quantification of (E) synaptophysin and (F) TUJ1 staining intensities in the corpus striatum. Data shown are the mean fluorescence intensities (relative to control), expressed as percentages. ****p<0.0001 vs Control. ##p<0.01, ###p<0.001 or ####p<0.0001 vs Cuprizone, as determined by one-way ANOVA and Tukey post-hoc test. TUJ1, class III beta-tubulin; NF-L, Neurofilament light chain; Syph, synaptophysin.*

Treatment with PACAP or Maxadilan decreases the expression of the inflammatory markers GFAP and IBA1 in the corpus callosum of cuprizone-intoxicated mice.

Glial fibrillary acidic protein (*GFAP*) gene encodes for a protein that is highly abundant in astrocytes and whose expression is induced upon an inflammatory challenge [576]. Our analyses revealed that the CPZ diet caused a robust increase in *GFAP* gene expression (****p<0.0001 vs Control; Figure 5A), suggestive of astrogliosis. Treatment with either PACAP, VIP or Maxadilan prevented *GFAP* gene induction caused by the CPZ diet (####p<0.0001 vs CPZ [PACAP]; #p<0.05 [VIP]; ####p<0.0001 [Maxadilan]).

Similarly to GFAP, ionized calcium-binding adapter molecule 1 (*IBA1*) is a well-established marker of microglial cells, indicating both pro-inflammatory and anti-inflammatory phenotypes [577], although in this instance it likely reflects pro-inflammatory activation. As expected, upon receiving the CPZ diet, *IBA1* transcripts were significantly increased (****p<0.0001 vs Control; Figure 5B). Treatment with PACAP and Maxadilan, but not VIP, significantly reduced *IBA1* mRNA levels (#p<0.05 vs CPZ [PACAP]; ####p<0.0001 [Maxadilan] vs Cuprizone).

To corroborate our mRNA findings, predominantly suggesting that the beneficial effects of PACAP and Maxadilan in astrocytes and microglia were PAC1-mediated, we conducted additional immunofluorescence experiments in the corpus callosum of mice subjected to the same experimental conditions (Figure 5C-E). In line with RNA data, CPZ-fed animals showed robust astrocytic activation, as indicated by the significant increase in $GFAP^+$ cells in the corpus callosum (****p<0.0001 vs Control; Figure 5C&D). PACAP treatment partly prevented CPZ-

driven GFAP up-regulation (####p<0.001 vs CPZ). These beneficial effects were also seen in Maxadilan-treated mice (###p=0.0002 vs CPZ). In contrast, VIP treatment failed to prevent astrocytic activation, as the number of $GFAP^+$ cells in the corpus callosum did not differ from CPZ mice.

$IBA1^+$ cell count was also robustly increased in the corpus callosum of CPZ-fed mice (****p<0.0001 vs Control; Figure 5C&E). PACAP and Maxadilan significantly reduced the number of IBA1+ cells in the corpus callosum (##p=0.001 vs CPZ [PACAP]; ##p=0.0024 [Maxadilan]; Figure 5E), whereas VIP did not (Figure 5C&E).

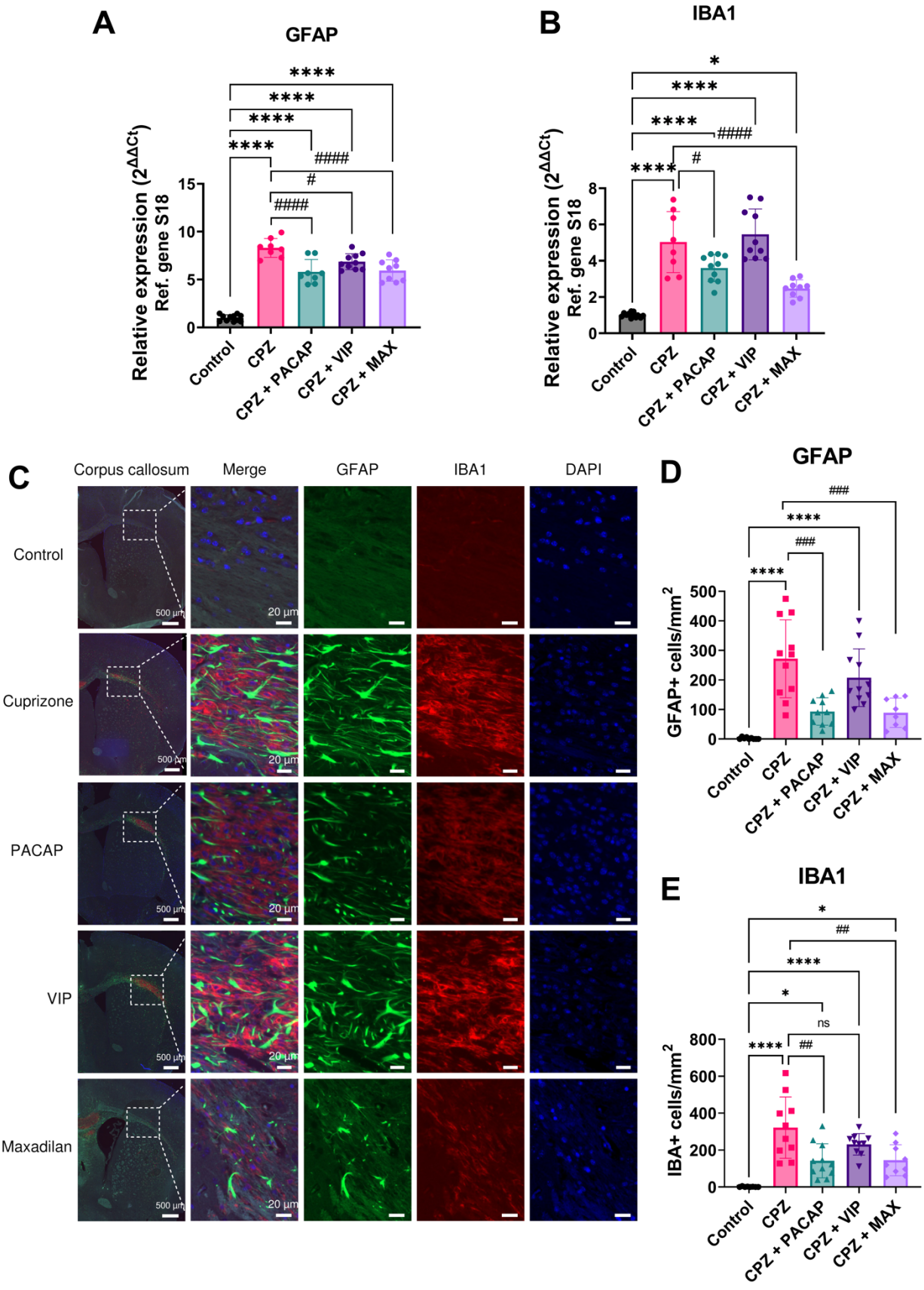

A
GFAP
Relative expression ($2^{\Delta\Delta Ct}$) Ref. gene S18
Control
CPZ
CPZ + PACAP
CPZ + VIP
CPZ + MAX
B
IBA1
C
Corpus callosum
Merge
GFAP
IBA1
DAPI
Control
Cuprizone
PACAP
VIP
Maxadilan
500 µm
20 µm
D
GFAP
GFAP+ cells/mm^2
E
IBA1
IBA+ cells/mm^2

Fig. 5. PACAP and Maxadilan attenuate astrogliosis and microgliosis in the corpus callosum of CPZ-fed mice after 4 weeks. *Relative mRNA expression of (A) GFAP and (B) IBA1 was determined using RT-qPCR. Fold changes were calculated using the ΔΔCt method, using ribosomal protein S18 as the housekeeping gene. Results are presented as mean fold change values ± SD, using 10-12 mice per group. (C) Representative photomicrographs of the corpus callosum (antero-posterior +1 mm - -1.5 mm from Bregma) co-stained with GFAP and IBA1 from mice fed a standard diet (Control) or a 0.2% CPZ diet and injected with either saline, PACAP, VIP or Maxadilan. Number of (D) $GFAP^+$ and (E) $IBA1^+$ cells per mm^2 are depicted in bar graphs (n=10-12 mice). One-way ANOVA followed by Sidak's multiple comparison's test was used to assess statistical significance. *p<0.05, ****p<0.0001 vs control; ## p<0.01, ### p<0.001, ####p<0.0001 vs CPZ. GFAP, glial fibrillary acidic protein. IBA1, ionized calcium-binding adapter molecule 1.*

PAC1 receptor-mediated anti-inflammatory effects stem predominantly from astrocytes.

Given that CPZ has no direct effects on astrocytes or microglia, our next step was to dissect the individual contributions of these glial cell populations to the anti-inflammatory responses triggered by the neuropeptides. For this purpose, primary astrocyte and microglial cultures were challenged with the inflammatory mimetic lipopolysaccharide (LPS) and co-treated with each neuropeptide for either 12 or 24 h. Representative photomicrographs and related quantifications are depicted in Figure 6.

In line with our *in vivo* findings, PACAP and Maxadilan, but not VIP reduced LPS-induced astrocytosis in primary cultures after 12 h (####p<0.0001 for both PACAP and Maxadilan vs LPS at 12 h; Figure 6A, B). After 24 h LPS exposure, all three neuropeptides were able to dampen astrocyte activation (####p<0.0001 [PACAP], ##p=0.0048 [VIP], ####p<0.0001 [Maxadilan] vs LPS at 24 h).

In primary microglia, LPS-induced microgliosis was prevented solely by PACAP treatment at the 12 h time point (####p<0.0001 vs LPS at 12 h; Figure 6D, E). After 24 h, both PACAP and VIP, but not Maxadilan, diminished IBA1 intensity (####p<0.0001 [PACAP], ####p<0.0001 [VIP] vs LPS at 24 h).

These results imply that the anti-inflammatory effects of the neuropeptides in isolated microglial cultures may also involve the co-activation of VPAC1 and VPAC2 receptors, in addition to PAC1 receptors.

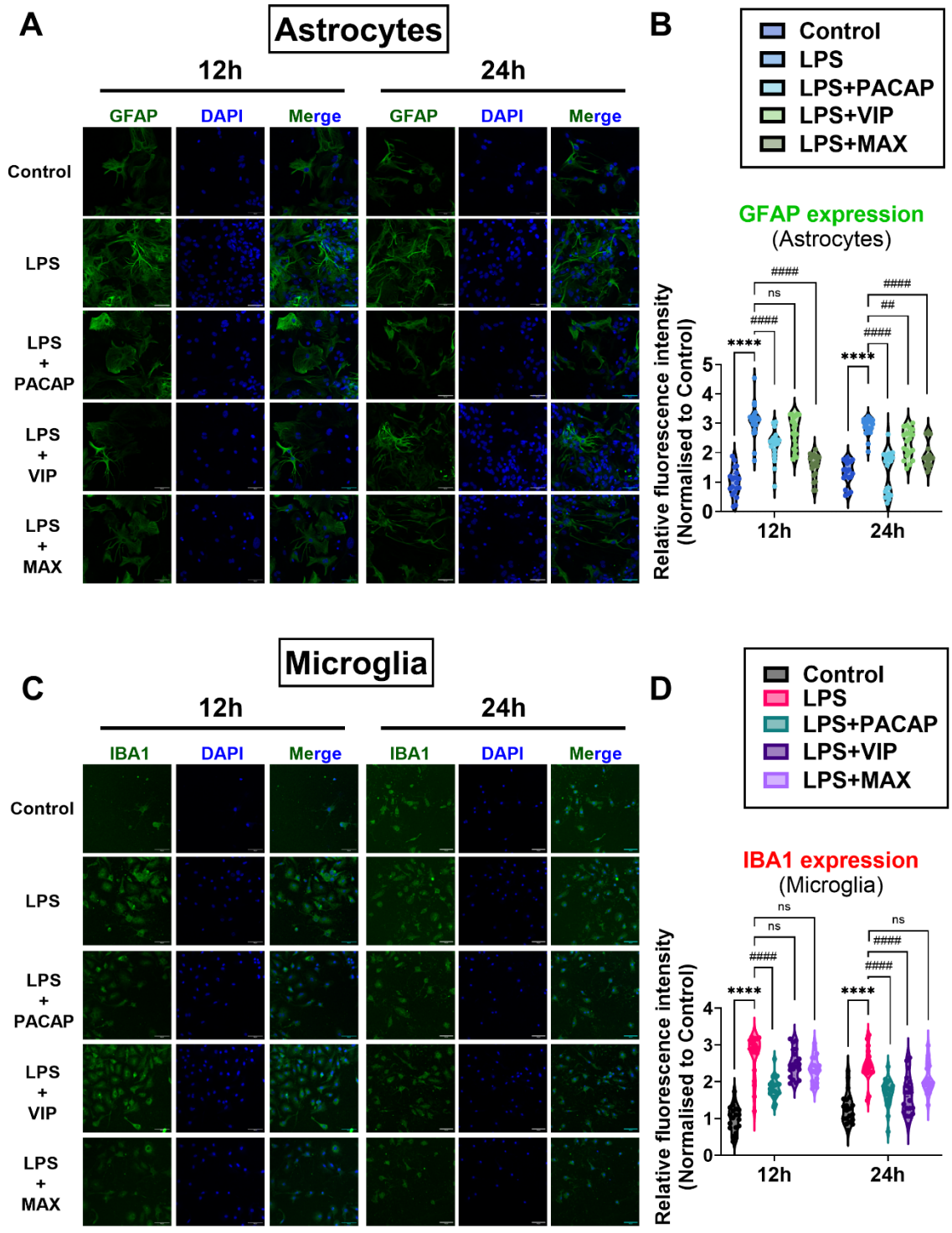


Fig. 6 Anti-inflammatory effects of PACAP, VIP and Maxadilan in primary glia cells challenged with lipopolysaccharide (LPS). *(A) Representative photomicrographs of primary murine astrocytes treated with LPS (500 ng/mL) alone, LPS + PACAP, +VIP or +Maxadilan (all used at 100 nM concentration) after 12 or 24 h and stained for GFAP (green) and DAPI (blue). Untreated cells were used as controls. Scale bar = 50 µm. (B) Quantification of GFAP fluorescence (n=9-15 ROIs / three independent experiments). (C) Representative photomicrographs of primary murine microglia exposed to the same treatments/conditions as in A, but stained for IBA1 (green) and nuclei counterstained with DAPI (blue). (D) Quantification of IBA1*

*fluorescence (n=7-14 ROIs / three independent experiments). Data in B and D was analysed using two-way ANOVA followed by Sidak's multiple comparison's test. ****p<0.0001 vs control; ##p<0.01, ####p<0.0001 vs LPS at the corresponding time point. GFAP, glial fibrillary acidic protein. IBA1, ionized calcium-binding adapter molecule 1.*

Discussion

In this study, we explored the potential therapeutic benefits of treatment with either PACAP, VIP or the PAC1-specific agonist Maxadilan in the cuprizone mouse model of demyelination. Our experiments demonstrated consistent improvements in locomotor performance and motor coordination in animals treated with either PACAP or Maxadilan, and less so in animals treated with VIP. These findings were corroborated by histopathological/immunohistochemical evidence indicating robust protective activities of the two PAC1-preferring ligands against cuprizone-induced myelin loss. We also observed reversal of astro- and microgliosis in the demyelinating corpus callosum after treatment with these neuropeptides. However, further *in vitro* studies revealed that PAC1-driven ameliorative effects were mostly seen in astrocytes, rather than in microglia, suggesting a novel and perhaps overlooked cell-specific responsiveness to this class of neuropeptides.

Since PACAP and Maxadilan elicited equipotent ameliorative effects, our results emphasise the role of the PAC1 receptor in mediating a protective response within the CNS white matter of mice undergoing experimentally induced demyelination. To our knowledge, these findings pinpoint for the first time the importance of targeting PAC1 receptors as a possible therapeutic strategy to prevent myelin loss and reduce CNS white matter inflammation following a primary acute demyelinating event.

Maxadilan is a selective PAC1 receptor agonist with comparable affinity to PACAP [578]. Given its similar receptor binding properties, it is reasonable to use equivalent dosages of maxadilan and PACAP in in vivo studies. Indeed, prior studies have established that PAC1 receptor activation by both peptides leads to overlapping intracellular signalling cascades, including cAMP and ERK1/2 activation, which mediate their neuroprotective and anti-inflammatory effects[579,580]. Although detailed pharmacokinetic and pharmacodynamic (PK/PD) data for maxadilan remain limited compared to PACAP, its efficacy has been demonstrated in various in vivo models. For example, maxadilan has been shown to induce vasodilation and anti-

inflammatory effects via PAC1 activation in models of neuroinflammation and immune regulation [581-583]*. Furthermore, in studies assessing neuroprotection, maxadilan and PACAP have shown comparable effects in modulating astrocyte reactivity and promoting neuronal survival* [470]*. Given these similarities, the same dosing regimen applied to PACAP has been used for maxadilan in this study to ensure a consistent and biologically relevant activation of PAC1 receptors. Future studies evaluating maxadilan-specific PK/PD properties in the CNS will further refine its therapeutic potential in demyelinating disease models*

Currently, several animal models of MS have been developed in the attempt to uncover disease complexity and identify possible cures, each with their own benefits and limitations [80,81]. The cuprizone model is recognised as a suitable preclinical model of demyelination, as animals on a four week cuprizone dietary regime develop white matter loss consistent with type III MS lesions in humans, whilst also offering the opportunity to understand the dynamic interplay of demyelination/remyelination that is seen in clinic [542,543,584,585]. In this study, we focussed on the role of PACAP and VIP treatment during demyelination as we were interested in unveiling any mitigating effects of these neuropeptides during a damaging insult to CNS myelin. Future research investigating whether targeting PAC1 (or perhaps other PACAP/VIP receptors) may also play a role in promoting spontaneous myelin recovery may offer additional insights on the spectrum of beneficial activities elicited by these neuropeptides. However, this remains beyond the scope of our current study.

Another commonly used animal model of MS is the model of experimental autoimmune encephalitis (EAE). Using this model, researchers have shown that PACAP ameliorated clinical symptoms and improved disease course via its anti-inflammatory properties [323]. Additionally, PACAP-deficient mice showed worsened EAE, further emphasising the immune- and possibly neuro-and myelo-protective activities of this peptide in other MS models [199,321]. Moreover, several studies have also shown the beneficial impacts of PACAP in stimulating myelin regeneration in the peripheral nervous system [196,209,332]. *In vitro*, endogenous PACAP and exogenously applied PACAP stimulate the proliferation of oligodendrocyte progenitor cells, although it inhibits cell maturation [265,266]. The results from our study are in line with these findings, as PACAP-treated mice show some degree of myelin preservation – likely caused by its peripheral anti-inflammatory effects – in the cuprizone demyelination model. Moreover, we have reason to believe that the effects seen are mediated via the agonistic activity on the PAC1

receptor, since administration of the selective receptor agonist Maxadilan produced similar, if not more potent protective effects than PACAP in the CNS white matter.

To our surprise, whilst we also observed some locomotor improvements in CPZ mice injected with VIP, we did not detect any significant histological or molecular signs of myelin preservation. These findings partly align with previously reported effects in VIP-deficient mice subjected to EAE, where animals showed impaired parenchymal CD4 T cells infiltration and reduced CNS inflammatory burden, suggesting that VIP plays an unanticipated permissive and/or proinflammatory role in the propagation of the inflammatory response in the CNS [200]. The fact that VIP treatment was still able to prevent neurological deterioration in CPZ mice is likely due to its protective activities in neurons. In this respect, we report that all the three neuropeptides tested, including VIP, were able to preserve TUJ1 and NF-L expression at levels similar to controls in axons travelling through the corpus callosum, suggesting reduced axonal damage. Furthermore, although VIP was unable to maintain synaptic density in the striatum of CPZ-fed mice, it prevented TUJ1 downregulation in neurons/axons, which could explain why clinical, and locomotor performance was partly improved even in mice treated with VIP. Additionally, the VIP peptide is known to promote neuronal disinhibition, through activation of VPAC1 receptors, or enhanced pyramidal cell excitability, through activation of VPAC2 receptors [417]. Therefore, it cannot be excluded that in VIP-treated animals, some of the persisting locomotor deficits may have been hidden by the excitatory activities elicited by the peptide.

A further point worth considering is that VIP targets both VPAC receptors with similar affinity; however, studies using immunised VIP receptor knock out mice showed opposing results [201,202,586]. As mentioned, VPAC1-deficient mice displayed resistance to EAE induction whereas VPAC2-deficiency exacerbated EAE pathology. In addition, from a pharmacokinetic perspective, VIP blood-brain-barrier permeability seems to be lower than PACAP [587,588]. Although our results suggest that the main effect of the peptides is likely to be peripheral, as rapid systemic degradation interferes with CNS bioavailability [589], the potent central neuroprotective effects of the neuropeptides still support this theory. In fact, Maxadilan, which does not have any significant sequence homology with PACAP and therefore may evade systemic enzymatic inactivation [590], produced more robust therapeutic effects than PACAP, especially in terms of myelin protection.

Interestingly, *in vitro* studies in primary astrocytes and microglia revealed some cell-specific differences in the responses to these neuropeptides under inflammatory conditions. Specifically, both PACAP and Maxadilan strongly reduced astrocytic polarisation in cells challenged with LPS within 12 hours, suggesting a PAC1-mediated effect. In contrast, PACAP and VIP (but not Maxadilan) reliably prevented polarisation in LPS-treated primary microglia, suggesting the involvement of VPAC type receptors in the anti-inflammatory response of the neuropeptides.

However, further research is warranted to dissect both the cell-specific contributions to the ameliorative properties of these neuropeptides, as well as assessing the central vs peripheral differences in the activities of both neuropeptides in the context of demyelinating pathologies, such as in MS. Moreover, additional studies using conditional/inducible mice models may help dissecting the cell-specific contributions of PAC1 receptors to myelin protection and regeneration.

In conclusion, this study provides novel evidence that targeting PAC1 receptors is sufficient to mitigate myelin loss and locomotor deficits during an acute demyelination challenge. The study highlights the potent anti-inflammatory and neuroprotective potential of this receptor subtype in the CPZ model of MS. Convergent behavioural and histopathological data emphasised a protective activity by this class of peptides, which adds important knowledge that may be utilised to instigate novel investigations aimed to exploit this therapeutic target as a novel DMT for demyelinating disorders.

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Competing Interests

The authors declare no competing interests.

Author Contributions

Study conception and design: Alessandro Castorina. Investigations: Margo I. Jansen, Yasir Mahmood, Jordan Lee and Sarah Thomas Broome. Data analysis: Margo I. Jansen, Yasir Mahmood and Jordan Lee. First draft of manuscript: Margo I. Jansen. Revision and editing of the final manuscript: Margo I. Jansen, Alessandro Castorina and James. A. Waschek. Funding acquisition: Alessandro Castorina.

Data Availability

Raw data and images can be made available upon reasonable request to authors.

Ethics Approval

All animal experiments were carried out with the approval of the University of Technology Sydney (ETH17-1991 and ETH22-7182) and conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Bridging section: Delving into the role of neuronal PAC1 *in vivo*

The main findings from the previous chapter highlight the importance and potential therapeutic benefit of targeting the PAC1 receptor specifically in the context of MS. At both the behavioural and molecular level, we observed significant improvements in several indicators of pathology. In the brain, we noted beneficial effects of PAC1 activation in both neurons and glial populations in response to CPZ-induced demyelination. Given that PAC1 expression is widespread throughout the brain and that axonal damage is associated with MS progression [503], our next aim was to examine the role of PAC1 specifically in neurons. To achieve this, we collaborated with the laboratory of Associate Prof. Allan McKenzie-Graham at the University of Los Angeles, California to obtain an inducible neuron-specific conditional PAC1 knockout mouse model. These Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$ mice provided us with the unique opportunity to study the effects of a targeted deletion of the PAC1 receptor specifically in a subpopulation of excitatory neurons (Camk2a^{+}) that are abundant in the cortex and hippocampus, while sparing other cell types in the CNS. In the manuscript below, we have focussed our research on understanding the impact of Camk2a^{+} PAC1 loss in neuronal function.

Chapter 4:

PAC1 Deletion in Camk2a expressing Neurons Impairs Hippocampal Plasticity

Status: Under review

Neuronal PAC1 Deletion Impairs Structural Plasticity

Margo I. Jansen[1], Haley Hrncir[2], Allan MacKenzie-Graham[2], James A. Waschek[3], Judith Brinkman[1], Laura A Bradfield[4], Giuseppe Musumeci[5], Velia D'Agata[5], Alessandro Castorina[1]

Affiliations:

1. Laboratory of Cellular and Molecular Neuroscience, School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW, Australia
2. Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA
3. Semel Institute for Neuroscience and Human Behavior/Neuropsychiatric Institute, Intellectual and Developmental Disabilities Research Center, University of California, Los Angeles, Los Angeles, CA, USA.
4. Brain and Behaviour Group, School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW, Australia.
5. Department of Biomedical and Biotechnological Sciences, Section of Anatomy, Histology and Movement Science, School of Medicine, University of Catania, Catania, Italy.

* Correspondence: alessandro.castorina@uts.edu.au; Tel.: +61-2-9514-5028

Contribution:

- Experimental design
- Experiments
- Analysis
- Writing
- Manuscript preparation

Name	Signature
Margo I. Jansen	Production Note: Signature removed prior to publication.
Haley Hrncir	Production Note: Signature removed prior to publication.
Allan MacKenzie-Graham	Production Note: Signature removed prior to publication.
James A. Waschek	Production Note: Signature removed prior to publication.
Judith Brinkman	Production Note: Signature removed prior to publication.
Laura A. Bradfield	Production Note: Signature removed prior to publication.
Guiseppe Musumeci	Production Note: Signature removed prior to publication.
Velia D' Agata	Production Note: Signature removed prior to publication.
Alessandro Castorina	Production Note: Signature removed prior to publication.

Abstract

Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is an endogenous neuropeptide of the central nervous system (CNS), whose biological activities are mediated via three G protein-coupled receptors PAC1, VPAC1, and VPAC2. PACAP has been extensively studied for its role in neuroprotection and development; however, studies are only beginning to explore its contribution to neuronal plasticity. Moreover, the role of the PAC1 receptor-specific influences on structural plasticity of excitatory pyramidal CNS neurons remains under-investigated.

This study aimed to elucidate the specific role of PAC1 in excitatory pyramidal neurons in the hippocampus and cortex. To achieve this goal, we utilised a tamoxifen-inducible transgenic mouse model with conditional deletion of the PAC1 receptor gene (*Adcyap1r1*) in Camk2a^{+} neuronal subpopulations. The mice harboured two additional transgenes, *Thy1-YFP* and *Thy1-mitoCFP*, providing valuable tools for high-resolution imaging of pyramidal neurons and mitochondria following PAC1 deletion.

PAC1 ablation in Camk2a^{+} neurons impaired locomotion and spatial memory. This was accompanied by a hippocampal CA1-specific increase in neuronal nitric oxide synthase (nNOS) and glutamate decarboxylase 65/67 (GAD65/67). Additionally, PAC1 loss reduced spine density of more 'mature' spines in apical dendrites, which was associated with reduced phosphorylation of CREB^{S133} and mitochondrial density in the hippocampal CA1 region. Taken together, our data suggest a crucial role for neuronal PAC1 signalling in maintaining proper hippocampal synaptic plasticity, function and energy dynamics.

Keywords:

PACAP; PAC1; CamK2a; hippocampus; synaptic plasticity; spine density

Introduction

In the central nervous system, neuropeptides play a critical role in cell-to-cell communication, physiology, and homeostasis [591,592]. So far, over 100 different neuropeptides have been described in the human brain, exerting a wide range of biological functions [593]. Two key neuropeptides endogenously produced in our central nervous system are the Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and the Vasoactive Intestinal Peptide (VIP). These are part of the secretin/VIP peptide family and exert their biological activities through three G protein-coupled receptors called PAC1, VPAC1 and VPAC2, though with different binding affinities [195,515]. PAC1 has a higher affinity for PACAP than for VIP, whereas VPAC1 and VPAC2 receptor subtypes have a similarly high binding affinity for both PACAP and VIP [594,595].

Disturbances of the PACAP/VIP neuropeptide system – which includes both neuropeptides and PAC1/VPAC type receptors – have been associated with several neurological conditions such as Post-Traumatic Stress Disorder, Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (MS) [187,190,198,596-598]. The neuropeptides have also shown to mediate potent anti-inflammatory and neuroprotective signalling pathways, contributing to the regulation of immune responses and promoting neuronal survival [187,190,198,215,597].

Abundant data indicates that the VPAC1/VPAC2 signalling arms exert potent immunomodulatory actions, whereas PACAP/PAC1 signalling is strongly linked to the maintenance of neuronal homeostasis and neuroprotection [193,198,204,305,599,600]. For example, PACAP treatment prevents cell death upon ischemic insult in cultured neurons and promotes axonal regeneration after spinal cord injury [599,601]. Additionally, selective loss of PAC1 in retinal neurons has been associated with increased axonal damage and decreased dendritic arborisation in a chronic model of MS-associated optic neuritis [204]. This highlights the importance of PAC1 signalling in preserving neuronal function.

PACAP/PAC1 signalling has been directly implicated in hippocampal function [602]. For example, experiments in which the neuropeptide PACAP was delivered directly into the CA1 region of the hippocampus, rich in Camk2a^{+} neurons, enhanced the consolidation of contextual fear conditioning [603]. A similar infusion of PACAP, but in the dentate gyrus, provided immediate anti-depressive effects via a Camk2a-dependent mechanism [602]. PACAP treatment

also ameliorates spatial memory impairments in mice [604]. PACAP signalling enhances neuronal excitability and facilitates hippocampal synaptic plasticity via long-term potentiation [605-609].

Most of these studies have primarily focused on investigating the role of the PACAP-PAC1 signalling axis in neurons through the exogenous administration of PACAP. While PAC1 receptors interact with PACAP with an affinity 100-fold higher than VIP, it is crucial to consider that PACAP and VIP activate VPAC1 and VPAC2 receptors with a similar high affinity. [610]. These latter two receptors are known to play a critical role in axonal outgrowth and dendritic arborisation and could thus explain some of the findings highlighted above [417,611,612]. Finally, PACAP and VIP receptors are expressed in both neuronal and glial cells throughout the central nervous system [187]. Consequently, global knockout (KO) models targeting PACAP or PAC1 disrupt multiple central nervous system cell types, complicating the efforts to delineate the specific role of the PACAP-PAC1 signalling axis in neurons.

To our knowledge, the cell type-specific roles of the PAC1 receptor in synaptic plasticity have only recently been explored [604,613] . This study aims to investigate how the selective ablation of the PACAP-preferring PAC1 from Camk2a expressing (Camk2a^{+}) neurons influences hippocampal-dependent behaviour and plasticity, utilising inducible/conditional Camk2a-CreERT2$^{+/-}$ PAC1$^{flox/flox}$ mouse line that also harbour YFP^{+} and CFP^{+} transgenes under the control of the *Thy1* promoter to allow in-depth analyses of both neurons and mitochondria.

Methods

Animals and Breeding Strategy

The strategy for the generation of transgenic Camk2aCreERT2$^{+/-}$ -PAC1$^{flox/flox}$ Thy1-YFP-mitoCFP mice involved the crossing and breeding of different transgenic mouse lines backcrossed onto the C57BL/6J background, as follows:

- *Thy1-YFP* transgenic mice (B6.Cg-Tg(Thy1-YFP)HJrs/J; RRID:IMSR_JAX:003782, Jackson Laboratories). This strain that expresses YFP (Yellow Fluorescent Protein) under the Thy1 promoter, marking neurons for fluorescence imaging.
- *Thy1-mitoCFP* mice (B6.Cg-Tg(Thy1-CFP/COX8A)S2Lich/J; RRID:IMSR_JAX:007967) is a strain that expresses CFP (Cyan Fluorescent Protein) specifically in mitochondria under the *Thy1* promoter.
- C57BL/6N-Atm1Brd Adcyap1r1tm1a(KOMP)Wtsi mice. This mouse strain was commissioned to the National Institutes of Health Knockout Mouse Project (KOMP) by Profs. Waschek and Prof. Joseph Pisegna to obtain mice with loxP sites flanking the PAC1 gene (*PAC1$^{flox/flox}$*).
- *Camk2aCreERT2* mice (B6;129S6-Tg(Camk2a-cre/ERT2)1Aibs/J; RRID:IMSR_JAX:012362, Jackson Laboratories) are transgenic mice that express a tamoxifen-inducible Cre recombinase under the control of the mouse *Camk2a* (calcium/calmodulin-dependent protein kinase II alpha) promoter.

Initially, *Thy1-YFP$^{+/+}$* and *Thy1-mitoCFP$^{+/+}$* transgenic mice were crossed to obtain homozygous double transgenic *Thy1-YFP^{+}mito-CFP^{+}* mice. Double transgenic mice were then crossed in parallel with either *PAC1$^{flox/flox}$* and *Camk2aCreERT2* mice to obtain offspring harbouring *PAC1$^{flox/-}$-Thy1-YFP^{+}mito-CFP^{+}* mice and *Camk2aCreERT2$^{+/-}$-Thy1-YFP^{+}mito-CFP^{+},* respectively. The latter two generations of triple transgenic mice were interbred to obtain *Camk2aCreERT2$^{+/-}$-PAC1$^{flox/-}$-Thy1-YFP^{+}mito-CFP^{+}* mice. Finally, these animals were backcrossed with *PAC1$^{flox/-}$-Thy1-YFP^{+}mito-CFP^{+}* mice to generate both our desired quadruple transgenic *Camk2aCreERT2$^{+/-}$-PAC1$^{flox/flox}$-Thy1-YFP^{+}mito-CFP^{+}* mice, as well as *Camk2aCreERT2$^{-/-}$-PAC1$^{flox/flox}$-Thy1-YFP^{+}mito-CFP^{+}*, which will be called *PAC1$^{flox/flox}$* from here onwards and used as controls.

Our quadruple transgenic mouse line, which for simplicity will be named *Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$* throughout the text, combines all relevant alleles, producing mice that can be used to study the conditional effects of PAC1 deletion in Camk2a^{+} neurons while tracking neuron-specific and mitochondrial fluorescent markers. These animals were housed under

environmentally controlled conditions in a 12-h light/dark cycle with access to food and water *ad libitum*. All experiments were carried out with the approval of the University of Technology Sydney (ETH21-6281) and conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Breeding and characterisation of these transgenic mice was approved by our institutional Biosafety committee (ETH21-6743, approved on the 11th November 2022).

Tamoxifen injections

4-week-old *PAC1*$^{flox/flox}$ or *Camk2a-CreERT2*$^{+/-}$*-PAC1*$^{flox/flox}$ received either corn oil (vehicle) or 75 mg/kg tamoxifen (in corn oil) injections over a period of four consecutive days as outlined in the schematic shown for each experimental section. Since tamoxifen is an estrogen-receptor agonist, a three-week washout was implemented to ensure tamoxifen was no longer bioactive prior to the commencement of any behavioural or biochemical assessment.

Genotyping

At 4weeks of age, mice were ear-notched and DNA was extracted using the KAPA Express Extract kit (KK7302, Roche) according to manufacturer's instructions. Briefly, DNA was extracted from tissue samples using 10µL 10X KAPA Express Extract Buffer, 2µL 1 U/µL KAPA Express Extract Enzyme in a total volume of 100µL by incubating the samples for 15 min at 75°C followed by 5 min at 95°C. After DNA extraction from the ear-notches, polymerase chain reactions (PCR) were set up to: (1) determine the allelic presence of the PAC1-flox construct and (2) resolve the presence of the Camk2a-CreERT2 construct in our mice. Each reaction (final volume of 25µL) was set up as follows: 12.5µL 2X KAPA2G Fast (HotStart) Genotyping Mix with dye2, 1.25µL of forward and reverse primer (10µM), 1µL template DNA from the DNA extraction and topped up with 9µL ddH_2O.

Primer sequences used for genotyping the PAC1 flox gene are: Forward: 5'-ACGTGCTCTCTTGGACCTTAGTAGC-3' and reverse: 5'-ATGGAGAGTAGGTGAATGAGCGACC-3'. Primer sequences to resolve the presence of the Camk2a-CreERT2 construct in our mice are: Forward: 5'-GACCTGGATGCTGACGAAG-3' Reverse: 5'-AGGCAAATTTTGGTGTACGG-3'. PCR settings were as outlined in Table 1. PCR products were resolved on a 2% agarose gel (Sigma-Aldrich in 1× Tris/Borate/EDTA buffer; 1× SYBR Safe DNA stain; ThermoFisher Scientific) using HyperLadder™ 1kb (Bioline) as a marker. Images were acquired using the Bio-Rad ChemiDoc MP Imaging System

(Bio-Rad). A product of 818bp was expected if the mice harboured the PAC1flox construct in both alleles (homozygous) and a 638bp product was expected for any wild-type PAC1 alleles. The heterozygous presence of Camk2a-CreERT2 was confirmed by the detected 200bp product.

Table 1. PCR protocol (Genotyping)

Step	Temperature	Duration	Cycles
Initial denaturation	95ºC	3 min	1
Denaturation	95ºC	15 sec	35–40
Annealing	60ºC	15 sec	
Extension	72ºC	15 sec/kb	
Final extension	72ºC	1 min/kb	1

Open Field test

The open field test was performed as outlined in [597]. Briefly, mice were acclimatised to the behaviour room for 30 min prior to performing the Open Field test in the dark. The Open Field arena consisted of a dark grey square Perspex box (40 × 40 × 50 cm), where recordings of the mice locomotor behaviour were filmed for 5 min from the top using an infrared video camera (Sony FDRAX53 4K Full HD Handycam, 1/50fps) held by a Manfrotto 190X tripod. Location tracking of the footage was performed using ezTrack (3 min/mouse) [554].

Object Location task

The Object Location task (OLT) was performed to assess short-term spatial memory in mice. For this test, we adapted a protocol from [614]. Mice were allowed to acclimatise to the room for 30 min in the light prior to commencing the behavioural task. Mice were then put in the same arena used for the Open Field test in the presence of two identical objects that were placed in two distinct spots within the box and mice were allowed to explore these objects for 5 min. Thereafter, mice were taken back to their home cage for a 5 min retention period and the box and objects were cleaned with 70% ethanol to remove any olfactory cues. Afterwards, objects were placed back into the box; however, one of the two objects was moved to a new location. Mice were then returned to the box for a 3 min test period. The exploration and the actual test were filmed using the Sony FDRAX53 4K Full HD Handycam (1/50fps) held by a Manfrotto

190X tripod. Videos were then analysed using ODLog (V2.7.2; Macropod Software) and the exploration ratio was calculated as follows:

$$Exploration\ ratio = \frac{novel\ exploration\ time}{(novel + familiar\ exploration\ time)}$$

This metric reflects spatial memory, as a higher ratio indicates that the animal recognises the object in the new location and preferentially explores it, suggesting intact spatial recognition. A ratio lower than 0.5 implies the animal exhibits no clear preference for the displaced object, indicating impaired or less discriminative spatial memory.

Rotarod test

General motor coordination was tested using the Rotarod apparatus (Ugo Basile, Milan, Italy) which, for the purpose, was programmed with a speed ranging from 4 to 40 revolutions per minute (RPM) with progressive acceleration for a total of 300 sec. The average time in seconds, also called latency, that each mouse spent on the rotating rod (average of three separate trials with a 5 min interval between each trial) was measured. A drop or full rotation of the mouse counted as the end of the trial.

Brain dissections and tissue collection

At the conclusion of each experimental protocol, brain tissues were collected for further molecular or histological analyses. For real-time quantitative PCR (RT-qPCR) or Western blot, fresh brain tissues were extracted under RNAse free conditions and snap-frozen using liquid nitrogen. For hippocampal tissue micro-dissections, samples were isolated from hemi-brains that were placed on an ice-cold dissection tray under a stereoscopic microscope (16× magnification) with the medial surface facing up. A spatula was then used to gently lift the midbrain/brainstem structures, exposing the hippocampus underneath. A 23-gauge needle was used as a small scalpel to carefully remove the overlaying meninges covering the hippocampus. After meninges were removed, the spatula was used to gently peel the hippocampus away from the surrounding tissue, paying attention that the hippocampal sample retained its natural anatomical curvature along the lateral ventricle. Once detached, the hippocampus was placed in ice cold PBS, and any residual white matter attached to the contours was carefully removed using the needle. This simple technique ensures accurate isolation of the hippocampus, with minimal contamination (if any) from neighbouring structures.

For immunofluorescence, mice were sacrificed by CO_2 asphyxiation, followed by a cardiac perfusion using cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS; pH 7.5). Afterwards, brains were collected and exposed to an overnight post-fixation step (in 4% PFA) at 4°C. Then, brains were embedded in Tissue Plus OCT Compound (ProSciTech) and 25µm thick coronal sections were cut using a cryostat (Cryostar NX70, Thermo Scientific), with the temperature set at -20°C. Sections were collected in a cryoprotectant solution (30% Ethyl Glycol, 20% Glycerol in 0.05M PB) and stored at -20°C until use.

RNA extraction, cDNA synthesis and Real-Time quantitative PCR

RNA from the dissected brain samples was isolated using TRI-Reagent (Sigma Aldrich) and precipitated using 2-propanol as described in [220,597]. Complementary DNA was generated using the Tetro cDNA synthesis kit (BioLine) as per manufacturer's instructions using 1000µg of input RNA. RT-qPCR was performed and analysed as described in previous studies using the ΔΔCt method [261,597]. Primers sets used for gene expression studies are shown in Table 2.

Table 2. Primer sets used in RT-qPCR. Forward and reverse primers were selected from the 5' and 3' region of each gene mRNA. The accession number reflects the mRNA sequence used for primer design. The expected length of each amplicon (in base pair = bp) is indicated in the right column.

Accession #	**Gene name**	**Primer sequences (5'-3-')**	**Product length (bp)**
NM_008712.3	NOS1	Fwd: AAGCCCTGGTGGAGATTAAC Rev: TTTGATGAAGGACTCGGTGG	98
NM_007540.4	BDNF	Fwd: CGAGTGGGTCACAGCGGCAG Rev: GCCCCTGCAGCCTTCCTTGG	160
NM_001313894.1	HMGB1	Fwd: GGGAGGAGCACAAGAAGAAG Rev: TTGTCAGCCTTTGCCATATCT	133
NM_008077.5	GAD1	Fwd: AATTGCACCCGTGTTTGTTC Rev: TGACCATCCAACGATCTCTC	70
NM_008078.2	GAD2	Fwd: CAGCCTTAGGGATTGGAACA Rev: CACTCACCAGGAAAGGAACA	132
NM_011296.2	S18	Fwd: CCCTGAGAAGTTCCAGCACA	145

		Rev: GGTGAGGTCGATGTCTGCTT	

Protein isolation and Western blot

Hippocampal tissues were homogenised in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) in the presence of a protease inhibitor cocktail (cOmpleteTM, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich) using a sterile pestle. Following sonication (three bursts of 10 sec interrupted by 30 sec on ice, 50% power intensity, QSonica Sonicators), proteins were clarified by centrifugation at 10,000 × g for 10 min at 4 ˚C. Supernatants were then used to determine protein concentrations using the Pierce BCA Protein Assay kit (Thermo Scientific). Protein lysates (30 µg) were loaded on 4-20% TGX Stain free mini-Gels (Criterion 15-well Mini-Protean SFX, BioRad) together with a Pre-stained HyperLadder Precision Plus Protein Ladder (BioRad), which was used as a molecular weight reference. After the gel was transferred onto a 0.2mm PVDF membrane (BioRad) using the Trans-blot Turbo system (BioRad), membranes were blocked for 1 h in 5% milk (in TBS + 0.1% Tween-20; TBST). Blots were then incubated overnight at 4°C in a primary antibody solution (in 5% milk in TBST). An overview of the primary antibodies used in this study is shown in Table 3. Following washes in TBST, membranes were incubated with secondary antibody (1:10000, Abcam Cat# ab6721, RRID:AB_955447) and bands were detected using Clarity Western ECL Blotting Substrate (Biorad). Membranes were imaged using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad). Densitometric analysis of detected bands was performed using ImageJ2 (V2.9.0/1.53t). Densities were normalised to those of GAPDH, here used as loading control.

Immunofluorescence

Brain tissue sections were washed in 1× PBS (3 × 10 min) to remove the cryoprotectant solution and then permeabilised for 30 min in 1× PBS + 0.1% Triton-X100 (0.1% PBST). Sections were blocked in 3% bovine serum albumin (BSA) (in 0.1% PBST, Bovogen) for 1 h followed by an overnight incubation with the primary antibody (1:250 in 3% BSA). The next day, sections were washed 3× in 0.1% PBST and incubated for 3 h with the appropriate secondary antibody. To counterstain nuclei, sections were incubated with DAPI (1 µg/ml; Sigma-Aldrich) for 30 min followed by a further washing step in PBST prior to mounting using the VECTASHIELD Vibrance® Antifade Mounting Medium. Antibodies used in immunofluorescent staining can be found in Table 3.

Table 3. Primary antibodies used. RRID = Research Resource Identifiers, IF = immunofluorescence

Company	Catalogue number	Antibody	Dilution	RRID	Use
GeneTex	GTX30026	PACAP receptor	1:1000	AB_3097721	Western blot
Cell signalling Technology	4691	Total-Akt	1:1000	AB_3492054	Western blot
Cell signalling Technology	4060	p-Akt (Ser473)	1:1000	AB_2315049	Western blot
Cell signalling Technology	9197	Total-CREB	1:1000	AB_3492056	Western blot
Cell signalling Technology	9198	p-CREB (Ser133)	1:1000	AB_2561044	Western blot
BioRad	VPA00187	GAPDH	1:2000	AB_3431937	Western blot
Thermo Fisher Scientific	61-7000	n-NOS	1:250	AB_2313734	IF
Abcam	ab183999	GAD65/67	1:250	AB_3662875	IF
Abcam	ab150063	Donkey Anti-Rabbit IgG H&L Alexa Fluor 647	1:500	AB_2687541	IF

Image acquisition & analyses

Twenty microns-thick Z stacks of stained brain tissue sections were acquired using a Zeiss Axioscan Slide scanner (20× magnification) and analysed with 'extended depth of focus' images generated through the contrasts or max intensity projection algorithms from the Zeiss Zen Software (version 2.6). For the analysis of dendritic spines, images were taken using the Leica Stellaris confocal microscope and employing the lightning deconvolution mode (63.5×, ~22µm Z-stack, NA=1.4). Mean grey intensity and % Area of the acquired 20× images were analysed using ImageJ software (v2.9.0). YFP^+ cell count was performed using the Analyse Particles module in ImageJ after thresholding each image using the "Otsu" method and applying the watershed function. IMARIS (v9.6.0) Filament tracer (spine diameter = 0.15µm,

max spine length = 3µm) was used to analyse dendritic spine morphology and density by harnessing the Golgi-like resolution of Thy1-YFP^{+} neurons in the hippocampus and cortex. For our spine classification, we implemented the descriptors used in other studies, with minor modifications [615,616]. Specifically, spines were classified as follows: Filopodia: spine length > 2µm, long thin: spine length > 1µm, mushroom: diameter of spine head > 1.5× diameter of spine neck, stubby: spine length < 1µm. For mitochondria analyses, we utilised IMARIS (v9.6.0) software 'surface' module to first create a 3D rendering of each individual neuronal cell body (surface grain size = 0.5µm), measuring cell soma volume or dendrite volume. Next, we performed our mitochondria analysis using the 'spots' module (estimated xy-diameter = 0.593µm, estimated z-diameter = 0.6µm) in IMARIS.

Statistical analyses

Data is presented as mean ± standard deviation (SD). All statistical analyses were computed using GraphPad Prism 9.3.1, which was also used to prepare graphs and assemble figures. Representative images of fluorescence staining were generated using OMERO Figure. To determine if data was normally distributed, quantile-quantile (Q-Q) plots were generated to compare the quantiles of the dataset to those of a theoretical normal distribution. Normality was evaluated based on the degree of alignment between the data points and the 45-degree reference line, which represents the expected distribution under normality. Once normal distribution was confirmed, pairwise comparisons were analysed using an unpaired two-tailed *t*-test. For the time course experiment, we used a two-way ANOVA followed by Sidak's multiple comparisons test to assess statistical significance. *p*-values ≤0.05 were considered statistically significant.

Results

Validation of PAC1 gene knockout in Camk2a^{+} neurons

Prior to commencing our experimental work, we first set out to induce the conditional PAC1 gene (*Adcyap1r1*) deletion in Camk2a^{+} excitatory neurons, and assessed if there was significant reduction of PAC1 expression in the hippocampus, a brain region in which Camk2a expression is known to be highly abundant [617]. Figure 1A provides a schematic overview of the gene alterations bred into the mice used in this study. Examples of genotyping results to identify the Camk2a and PAC1-floxed transgenes are depicted in Figure 1B and show that mice #4 and #7 are Camk2a-CreERT2$^{-/-}$-PAC1$^{flox/flox}$ (Cre$^{-/-}$), whereas all the others are Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$ (Cre$^{+/-}$). Four weeks after the pups were born, PAC1$^{flox/flox}$ or Camk2aCreERT2$^{+/-}$PAC1$^{flox/flox}$ mice were injected with tamoxifen to induce Cre translocation to the nucleus and the consequent molecular excision of the PAC1 gene (*Adcyap1r1*) from Camk2a^{+} neurons (Figure 1C). Following a 3-week washout period, we isolated the hippocampus from these mice to perform protein studies. As visible in Figure 1D-E, one-week post-induction, we observed a noticeable reduction in PAC1 protein expression (**p=0.0099, Cre$^{-/-}$ vs Cre$^{+/-}$ at week 1). As expected, this reduction was stable, as shown three weeks post-tamoxifen induction (*p<0.0485, Cre$^{-/-}$ vs Cre$^{+/-}$ at week 3).

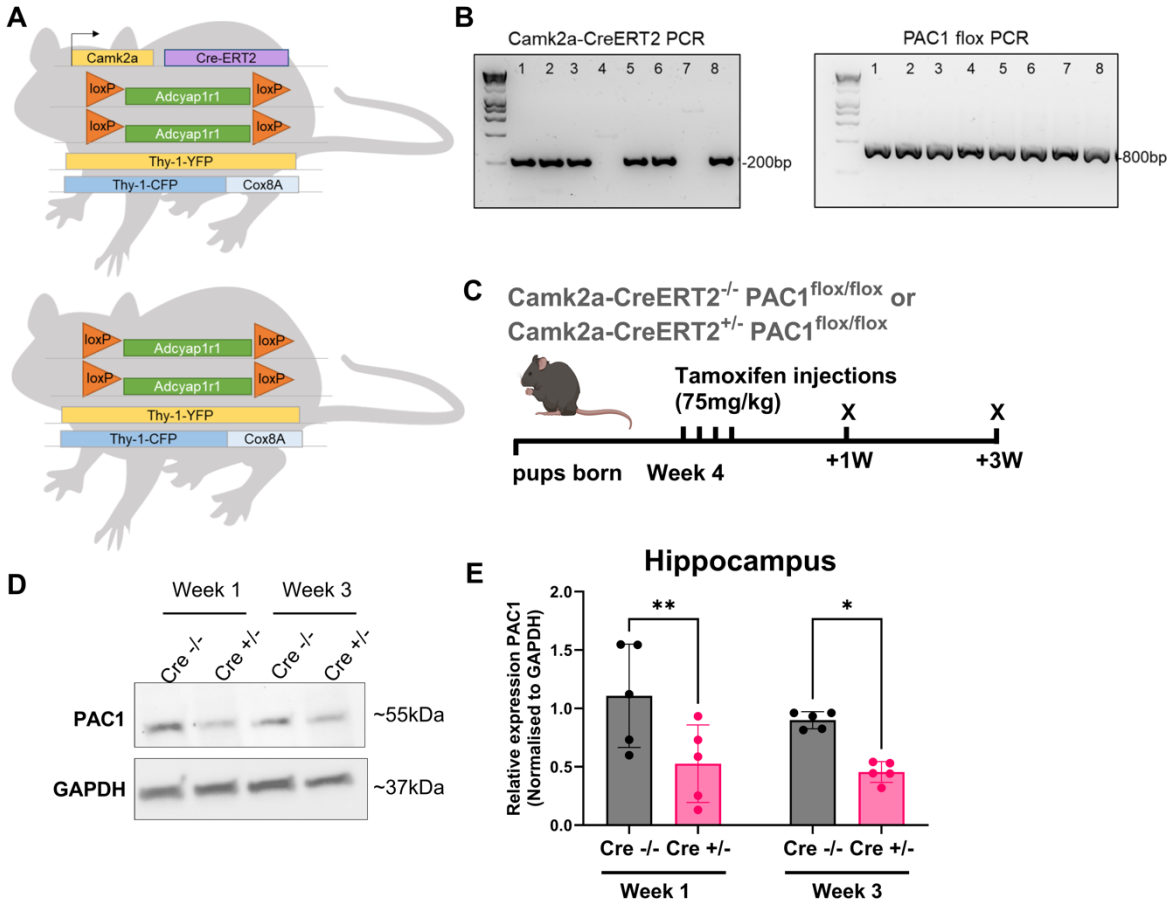


Figure 1. Validation of PAC1 deletion in our Camk2aCreERT2$^{+/-}$ PAC1$^{flox/flox}$ mouse model. *(A) Schematic overview of the Camk2a construct and PAC1-flox alleles in our mouse model on a genetic background of mice expressing Thy1-YFP and Thy1-mitoCFP. (B) Exemplary genotyping results for the detection of the Camk2a-CreERT2 construct and floxed PAC1 gene. (C) Timeline of the experimental plan for our validation experiments. (D) Representative Western blot and (E) quantification of PAC1 protein expression in the hippocampus of PAC1$^{flox/flox}$ (Cre$^{-/-}$) or Camk2aCreERT2$^{+/-}$ PAC1$^{flox/flox}$ (Cre$^{+/-}$) at 1- or 3-weeks after tamoxifen induction (75mg/kg intraperitoneal [i.p]). Data shown is mean relative fold change ± SD, obtained using n=5 different mice per genotype and timepoint. *p<0.05, **p<0.01 as determined by two-way ANOVA followed by Sidak's multiple comparisons test.*

PAC1 = pituitary adenylate cyclase activating polypeptide receptor 1, GAPDH = Glyceraldehyde 3-phosphate dehydrogenase Protein

PAC1 loss in Camk2a^{+} neurons disrupt spatial memory and locomotion

To study the effects of PAC1 deletion in Camk2a^{+} neurons, from here on referred to as PAC1 conditional knockout (cKO) mice, 4-week-old mice were injected with tamoxifen or vehicle for 4 days consecutively, and then subjected to behavioural analyses after a 3-weeks washout period (Figure 2A). Since the PACAP/PAC1 axis has been implicated in locomotion [597], we

were interested in determining if a targeted PAC1 gene ablation in this neuronal subset would elicit any effect on locomotor behaviour.

Both the Rotarod and Open Field tests are considered the gold standards for the assessment of general locomotor behaviours (i.e. ambulation, coordination, and balance) in rodents [549-551,618]. Here, we first used the Rotarod test to assess motor coordination/balance by measuring the ability of mice to withstand rotations for a fixed period (Figure 2B) [618]. As shown, deletion of PAC1 from Camk2a^{+} neurons did not affect the latency of mice to fall off the rods, indicative of intact motor coordination/balance (Figure 2C). In contrast, the Open Field test demonstrated that general ambulation was reduced in PAC1 cKO mice, as these animals travelled significantly less than control mice in the Open Field arena (Figure 2D-E; t_{46}=3.521, ***p=0.001). However, we did not observe any significant change in the time transgenic mice spent in the centre of the Open Field arena, suggesting a preserved exploratory behaviour of PAC1 cKO mice (Figure 2F).

Since Camk2a and PAC1 expression is relatively high in the hippocampus and PACAP-PAC1 signalling axis is critical for spatial memory formation and recovery [604,619], our next step was to examine if PAC1 cKO mice exhibited defects in hippocampal-dependent spatial memory function (Figure 2G). Therefore, both control and PAC1 cKO mice were subjected to the Object Location test, a test used to assess this specific type of memory [620]. We found a moderate though statistically significant reduction of the exploration ratio when comparing the performance of control versus PAC1 cKO mice, suggesting an impairment of hippocampal-dependent spatial memory in transgenic mice (Figure 2H; t_{45}=2.131, *p=0.0386).

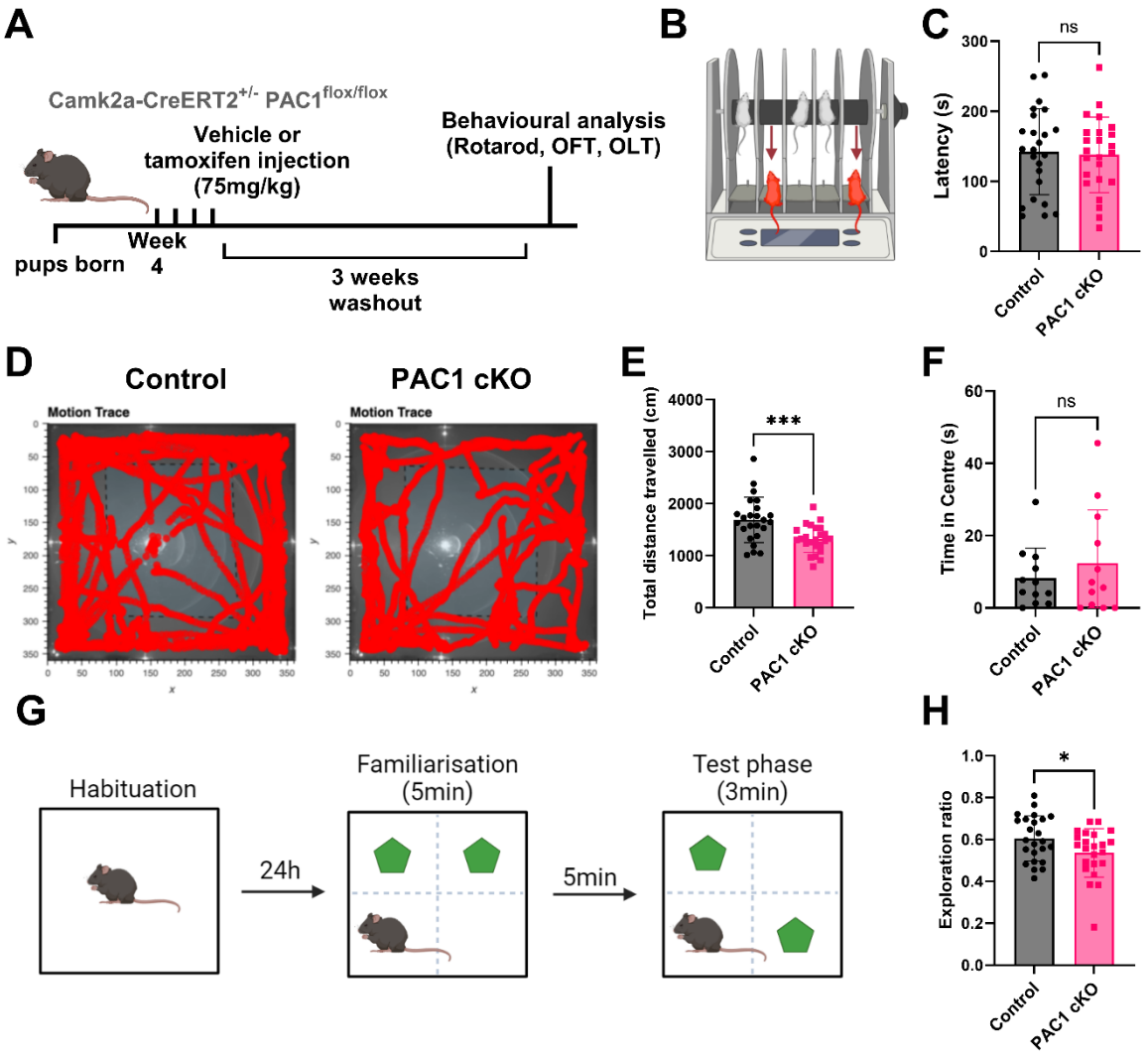


Figure 2. Loss of PAC1 in Camk2a^{+} neurons causes spatial memory impairments and reduces overall locomotion in mice. *(A) Overview of the timeline of our experimental design. (B) Schematic image of Rotarod machine. (C) Latency measurements of Control and PAC1 cKO mice. (D) Representative line tracks showing the trajectory of mice while exploring the open field apparatus. (E) Mice displayed reduced overall locomotion activity in PAC1 cKO mice in the Open Field test but (F) no change in the total time spent in the centre. (G) Schematic overview of the object location task paradigm used to study the mice' spatial memory capabilities. (H) A statistically significant drop in exploration score is seen in PAC1 cKO mice. *p<0.05, ***p<0.001, n=23-24 mice per group. Data is presented as mean ± SD and analysed using the unpaired t-test.*

OFT = Open Field test, OLT = Object Location test, PAC1 cKO = PAC1 conditional knockout.

Targeted deletion of PAC1 in Camk2a$^+$ neurons cause Hippocampal CA1-specific increase in Neuronal Nitric oxide synthase and Glutamate Decarboxylase

Since our transgenic mice are bred with a genetic background incorporating the Thy1-YFP construct and Thy1$^+$ cells represent a subpopulation of Camk2a$^+$ neurons (see Supplementary Figure 1 for details), we harnessed Thy1-YFP$^+$ neurons as a proxy to assess the effects of conditional PAC1 deletion on the health of Camk2a$^+$ neurons, axons and dendrites [621,622].

Given our observations indicating a reduction in hippocampal-dependent spatial memory, we sought to conduct further molecular analyses aimed at revealing any changes in genes/proteins involved in regulating hippocampal-dependent synaptic plasticity [623-625], memory function [625] and neuronal communication [626,627] in PAC1 cKO mice.

Following drug washout, whole hippocampi of vehicle or tamoxifen-treated Camk2aCre-ERT2$^{+/-}$-PAC1$^{flox/flox}$ mice were harvested and used for further downstream analysis (Figure 3A-B). First, we explored the gene expression levels of a set of important neuronal markers using RT-qPCR (Figure 3C-G). The *NOS1* gene encodes for neuronal nitric oxide synthase (nNOS), which synthesises nitric oxide in the brain, an important secondary messenger involved in learning and memory, and synaptic plasticity [628]. *NOS1* levels were increased in PAC1 cKO mice compared to control mice (t_8=2.846, *p=0.0216; Figure 3C). *BDNF*, encoding for brain-derived neurotrophic factor (BDNF), another critical mediator of hippocampal plasticity, was not altered upon Camk2a$^+$ PAC1 loss (Figure 3D) [629]. Neither was the expression of High Mobility Group Box 1 protein (*HMGB1*), a damage-associated molecular pattern molecule associated with inflammation (Figure 3E) [630]. Interestingly, both *GAD1* and *GAD2* genes, which encode for Glutamate decarboxylases (GADs) 67 and 65 respectively – the enzymes that convert glutamate into γ-aminobutyric acid (GABA) – were both significantly increased in PAC1 cKO mice (*GAD1*; t_8=2.811, *p=0.0228 and *GAD2*; t_8=5.500, ***p=0.0006; Figure 3F-G) [631].

To complement our gene expression studies, we performed immunofluorescence experiments to establish the topographical distribution of nNOS (Figure 3H-K) or GAD65/67 immunoreactivities (Figure 3L-O) along the main anatomical subdivisions of the hippocampus [632]. We observed an increase of nNOS immunoreactivity in the CA1 region of PAC1 cKO mice only (Figure 3I; t_{10}=3.674, **p=0.0043), but not in CA2/3 (Figure 3J). In contrast, nNOS expression was reduced in the dentate gyrus (DG; Figure 3K; t_{10}= 2.820, *p=0.0182). GAD65/67 fluorescent levels were robustly increased in CA1 (Figure 3M; t_{10}=0.0075,

**p=0.0075). Both in the CA2/3 region and the DG, no statistical difference between controls and PAC1 cKO mice was identified ($p>0.05$; Figure 3N-O).

Conditional PAC1 depletion in mice at seven weeks of age had no effect on the total number of Thy1-YFP$^+$ cells (measured at the end of the experiments) in any of the hippocampal regions (Supplementary Figure 2A-C), nor in the somatosensory cortex (Supplementary Figure 3).

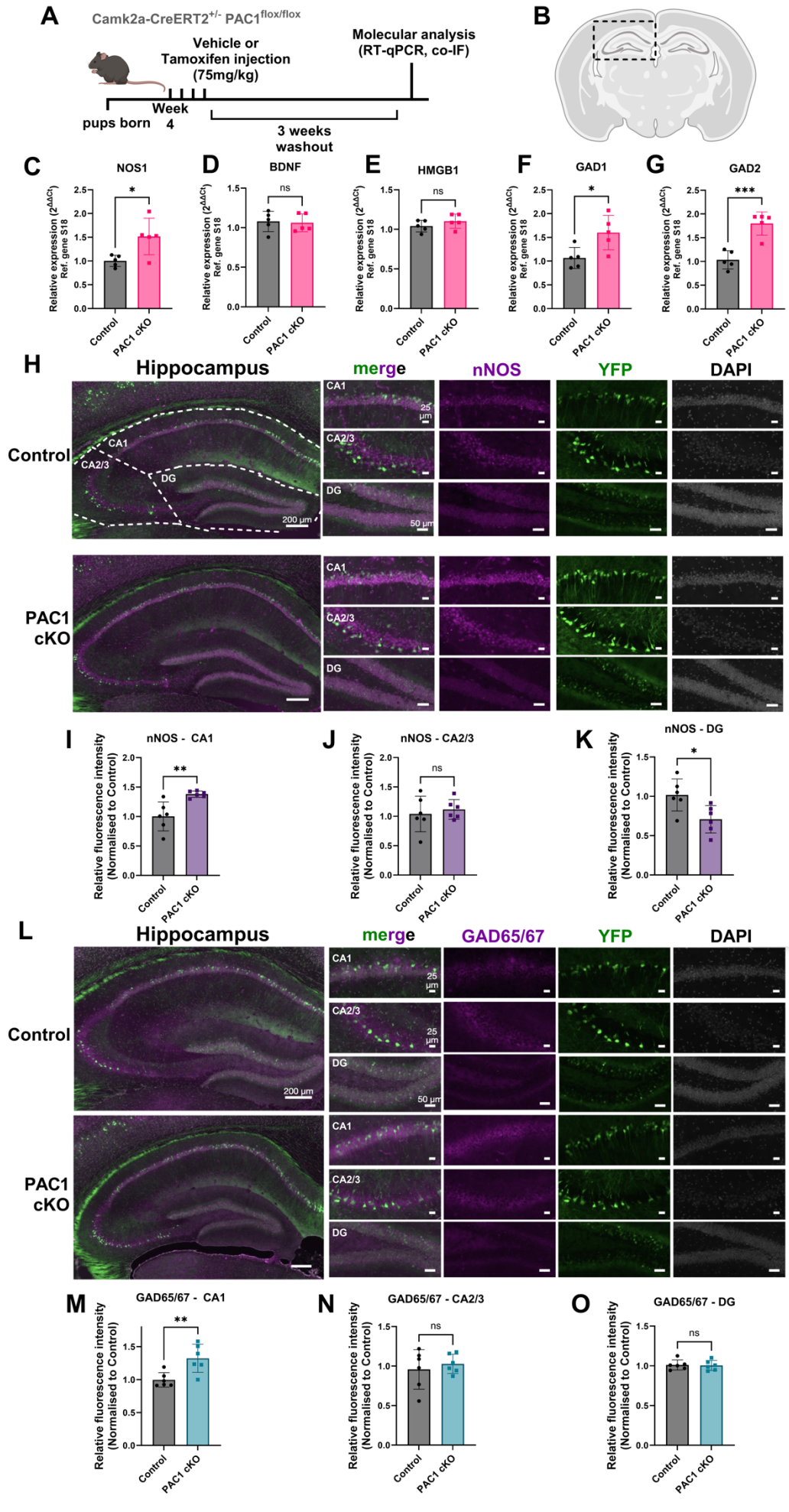

A
Camk2a-CreERT2+/- PAC1flox/flox
Vehicle or
Tamoxifen injection
(75mg/kg)
Molecular analysis
(RT-qPCR, co-IF)
pups born
Week
4
3 weeks
washout
B
C
NOS1
D
BDNF
E
HMGB1
F
GAD1
G
GAD2
Relative expression (2ΔΔCt)
Ref. gene S18
Control
PAC1 cKO
H
Hippocampus
merge
nNOS
YFP
DAPI
Control
PAC1
cKO
CA1
CA2/3
DG
200 µm
50 µm
25 µm
I
nNOS - CA1
J
nNOS - CA2/3
K
nNOS - DG
Relative fluorescence intensity
(Normalised to Control)
L
Hippocampus
merge
GAD65/67
YFP
DAPI
M
GAD65/67 - CA1
N
GAD65/67 - CA2/3
O
GAD65/67 - DG

Figure 3. Targeted PAC1 depletion in hippocampal Camk2a$^+$ neurons induce CA1-specific increase in neuronal nitric oxide synthase and GAD65/67 levels. *(A) Schematic overview of experimental timeline and approach. (B) Schematic drawing of the mouse brain at the level of the hippocampus, the region of interest used in our molecular analyses. Gene expression levels of (C) NOS1, (D) BDNF, (E) HMGB1, (F) GAD1 and (G) GAD2 are measured using RT-qPCR. Representative fluorescent staining of hippocampal nNOS can be seen in (H). Relative fluorescence intensity levels of nNOS in the (I) CA1, (J) CA2/3 and (K) Dentate Gyrus. The representative image of GAD65/67 staining is shown in (L). Quantification of GAD65/67 is plotted for the (M) CA1, (N) CA2/3 and (O) Dentate Gyrus. n=5/group for RT-qPCR. n=6/group for immunofluorescence analyses. Data is the mean ± SD. Pairwise comparisons were analysed using the unpaired t-test. *p<0.05, ***p<0.001.*

PAC1 cKO = PAC1 conditional knockout, NOS1 = neuronal nitric oxide synthase 1, BDNF = brain-derived neurotrophic factor, HMGB1 = high-mobility group box 1 protein, GAD1 = Glutamate decarboxylase 1, GAD2 = Glutamate decarboxylase 2.

Loss of dendritic spine density in the hippocampal CA1 region of mice with targeted deletion of PAC1 in Camk2a$^+$ neurons

Here, we utilised the Thy1-YFP expression in the hippocampus to examine the impact of PAC1 depletion on neurons in more detail. PACAP-PAC1 signalling has been linked to enhanced synapse plasticity and accelerated hippocampal remodelling after injury [604,609,633]. Additionally, Thy1-YFP mice have successfully been used to study dendrite spine morphology in the hippocampus [634,635] and our data identified localised changes in the CA1 region of PAC1 cKO mice (Figure 3). Therefore, our next aim was to study if PAC1 depletion in Camk2a$^+$ neurons caused any effects on dendritic spine remodelling, morphology and density in the CA1 region of the hippocampus (Figure 4A).

No changes in dendritic spine length (Figure 4B), total spine volume (Figure 4C), volume of the head of the spine (Figure 4D), and volume of the neck of the spine (Figure 4E) were identified in PAC1 cKO mice. However, a statistically significant reduction in dendritic spine density was observed in the CA1 region of the hippocampus of these mice (Figure 4F; t_{19}=2.348, *p=0.0299). This is an effect we also see replicated in cortical Thy1$^+$ neurons (Supplementary Figure 4A, F; t_{19}=3.073, **p=0.0063).

Based on these results, we next sought to determine if the CA1-specific spine loss caused by PAC1 gene deletion was confined to a specific typology of spines on apical dendrites (Figure

4G). Our analyses demonstrated that loss of PAC1 in Camk2a^{+} neurons did not influence the density of immature filopodia-like spines in apical dendrites of CA1 hippocampal neurons (Figure 4H). However, we observed a decrease in the density of long-thin spines (Figure 4I; t_{21}=2.253, *p=0.0351). The more mature stubby spines also showed a trend towards a decrease, albeit it was not statistically significant (p=0.0612; Figure 4J). Finally, the density of mature mushroom spines, which are key for long-term memory storage [636], were significantly reduced in dendrites of neurons within the CA1 region of PAC1 cKO mice (Figure 4K; t_{21}=2.538, *p=0.0191). These results suggest that loss of PAC1 expression in neurons may have contributed to delay the remodelling of apical dendritic spines in the CA1 region of the hippocampus.

Given these findings, we next aimed to explore whether PAC1 ablation in hippocampal neurons would disrupt key pathways involved in synaptic plasticity, with the goal of uncovering the intracellular mechanisms contributing to the reduced dendritic spine density observed. [607,637,638]. While phosphorylation levels of Akt at serine 473 residue (Ser473) were not affected (Figure 4L-M), whereas a considerable decrease in the phosphorylation levels of CREB at serine residue 133 (Ser133) were observed in the hippocampus of PAC1 cKO mice (Figure 4L,N; t_{4}=4.506, *p<0.0108).

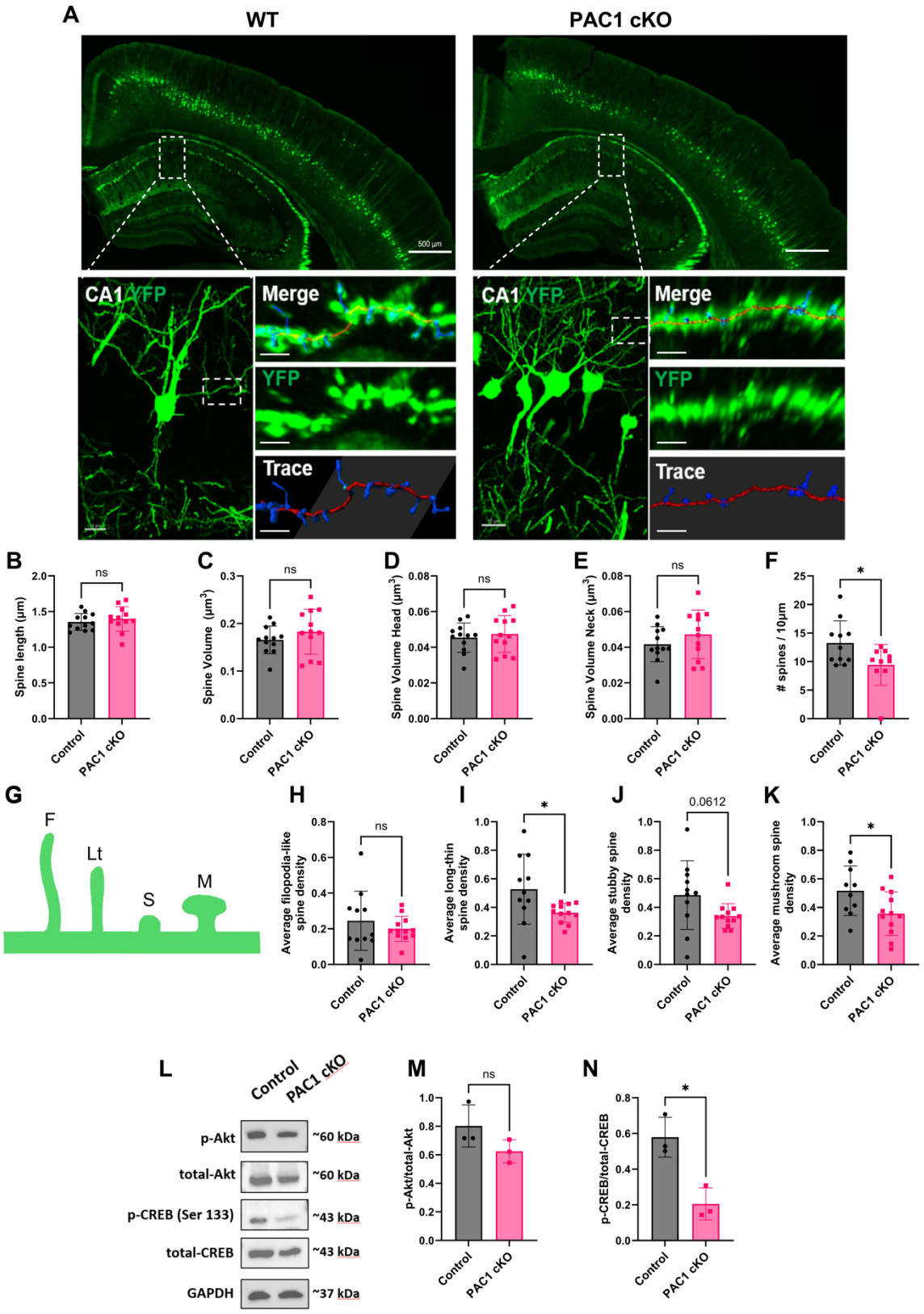
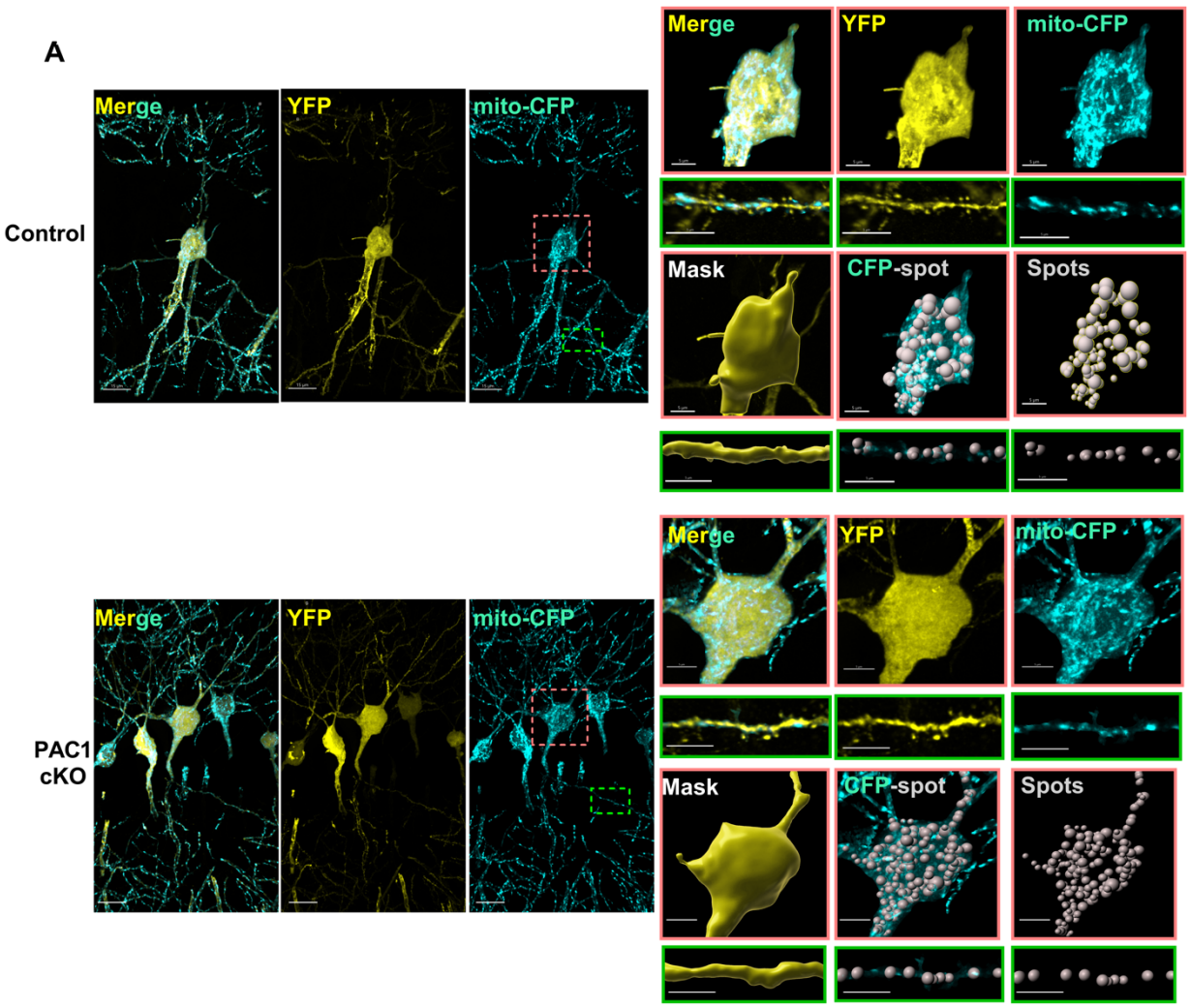

A
WT
PAC1 cKO
500 µm
CA1 YFP
Merge
YFP
Trace
B
Spine length (µm)
C
Spine Volume (µm³)
D
Spine Volume Head (µm³)
E
Spine Volume Neck (µm³)
F
spines / 10µm
ns
*
Control
PAC1 cKO
G
F
Lt
S
M
H
Average filopodia-like spine density
I
Average long-thin spine density
J
Average stubby spine density
0.0612
K
Average mushroom spine density
L
Control
PAC1 cKO
p-Akt
~60 kDa
total-Akt
~60 kDa
p-CREB (Ser 133)
~43 kDa
total-CREB
~43 kDa
GAPDH
~37 kDa
M
p-Akt/total-Akt
N
p-CREB/total-CREB

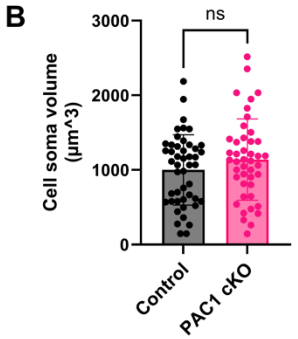
Figure 4. Targeted PAC1 deletion in neurons causes dendritic spine density loss in the hippocampal CA1 region and reduced CREB$^{(Ser133)}$ phosphorylation. *(A) Representative image of a CA1 hippocampal neuron in control and PAC1 cKO mice. No change in (B) dendritic spine length, (C) spine volume, (D) volume of the head of the spine and (E) volume of the neck of spines were identified in hippocampal neurons from PAC1 cKO mice. (F) Dendritic spine density levels were reduced in neurons lacking the PAC1 gene. (G) Schematic overview of dendritic spine types. (H) Filopodia spine density, (I) Long-thin spine density, (J) stubby spine density and (K) mushroom spine density in the hippocampal CA1 region of control and PAC1 cKD mice. (L) Representative Western blots showing p-Akt and p-CREB in control and PAC1 cKO hippocampal tissue. Quantification of (M) p-Akt normalised to total-Akt and (N) p-CREB normalised to total-CREB. n=9-12 filaments/3 mice for IMARIS filament tracing. n=3 mice for the Western blots. Data is plotted as mean ± SD and analysed using the unpaired t-test. *p<0.05. CA1 inset (scale bar) = 15μm, dendrite inset (scale bar) = 2μm.*

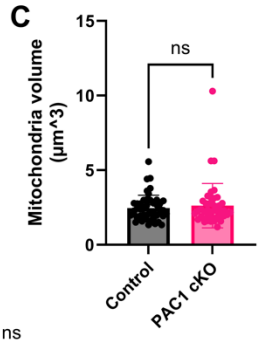
PAC1 cKO = PAC1 conditional knockout, YFP= yellow fluorescent protein, F=filopodia, Lt = long-thin, M=mushroom, S=stubby.

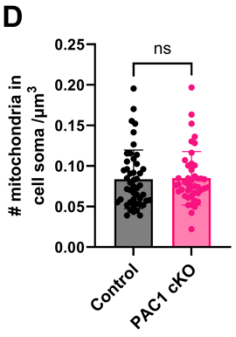
PAC1 depleted neurons exhibit CA1-specific loss of mitochondria

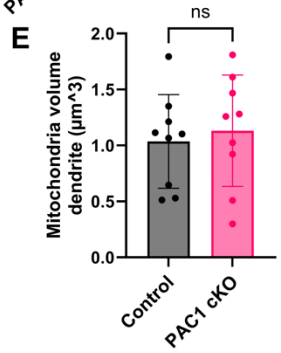
Amongst the several biological effects elicited by PACAP, the neuropeptide has also been implicated in mitigating mitochondrial stress in neurons [246,639]. Additionally, mitochondria density in dendrites is known to directly correlate with spine density [640]. Here, we utilised the Thy1-mitoCFP construct in our genetic mouse model to study mitochondria morphometrics more in depth [641]. No difference in cell soma size (Figure 5A-B), volume of mitochondria (Figure 5A,C) or mitochondria density (Supplementary Figure 5A,D) were identified in the cell soma of CA1 hippocampal neurons when comparing control versus PAC1 cKO mice. Similarly, mitochondrial volume was unchanged in the dendrites of these neurons (Figure 5A,E). However, mitochondrial density was significantly reduced in the dendrites of PAC1-depleted hippocampal neurons (Figure 5A,F; t_{16}=2.271, *p=0.0373).


A
Control
PAC1 cKO
Merge
YFP
mito-CFP
Mask
CFP-spot
Spots


B
Cell soma volume (µm^3)
3000
2000
1000
0
ns
Control
PAC1 cKO


C
Mitochondria volume (µm^3)
15
10
5
0
ns
Control
PAC1 cKO


D
mitochondria in cell soma /µm3
0.25
0.20
0.15
0.10
0.05
0.00
ns
Control
PAC1 cKO


E
Mitochondria volume dendrite (µm^3)
2.0
1.5
1.0
0.5
0.0
ns
Control
PAC1 cKO

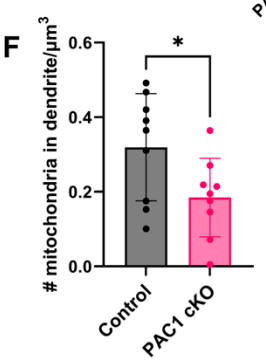

F
mitochondria in dendrite/µm3
0.6
0.4
0.2
0.0
*
Control
PAC1 cKO

Figure 5. Cell soma mitochondria analysis of control and PAC1 cKO mice in the CA1 region of the hippocampus. *(A) Representative images of mitochondria analysis in control and PAC1 cKO mice in both the cell body and a selected dendrite. (B) Cell soma volume, (C) mitochondria volume and (D) mitochondria density in the cell body of CA1 hippocampal neurons in both control and PAC1 cKO mice. (E) Mitochondria volume and (F) mitochondria density in the dendrites of CA1 hippocampal neurons. n=44-47 cells/4 mice for cell soma analysis. n=9 filaments/4 mice for mitochondria analysis. Data is plotted as mean ± SD and analysed using the unpaired t-test. **p<0.01. Scale bar (lower magnification) = 15µm, (Inset at higher magnification) = 5µm.*

PAC1 cKO = PAC1 conditional knockout, YFP = yellow fluorescence protein, mito-CFP = mitochondrial cyan fluorescent protein.

Discussion

Considerable data indicate that PACAP signalling plays an important role in hippocampal-dependent synaptic plasticity, transmission and function [606,609,642-644], although the receptor subtypes and specific actions are not cell defined. Considering the abundance of Camk2a$^+$ neurons in this region (Supplementary Figure 1) [645-647], and the importance of PAC1 in PACAP signalling [515,597], we examined the effects of PAC1 depletion in Camk2a$^+$ neurons. For this purpose, we utilised a conditional PAC1 knockout model developed in mice on a genetic background that allows the concurrent visualisation (at Golgi-like resolution) of Thy1$^+$ neurons, as well as their structural (dendrites, axons) and subcellular components (mitochondria).

Our data show that loss of PAC1 in Camk2a$^+$ neurons impaired spatial memory. The effect was associated with increased expression of nNOS and GAD65/67 and reduced dendritic spine density in the hippocampus. It is possible that PAC1 depletion in this subset of neurons generates a redox imbalance in favour of nitrosative stress, and that increased expression of the GABA-producing enzymes (GAD1 and GAD2) might be a compensatory response to increase the inhibitory tone and reduce the neuronal stress burden. An alternative explanation could be that, since the PAC1 receptor modulates the neuronal excitability and synaptic plasticity of Camk2a$^+$ neurons [644], its deletion might result in abnormal hyperexcitability or excitotoxicity in these neuronal subpopulations. In this scenario, the increased nNOS expression would be a natural consequence to the increased oxygen demand or calcium influx due to heightened neuronal activation [648,649], whereas the increased levels of GAD65/67 could represent a homeostatic response to modulate the excessive neuronal activation in the effort to preserve

hippocampal function in the absence of PAC1. However, it is important to note our PAC1 cKO model is not strictly confined to the hippocampus. Camk2a is also expressed in the cortex and several other subcortical brain regions, such as the amygdala and basal ganglia, which could impact our behavioural outcomes [520,645].

Nonetheless, PACAP treatment has previously been shown to facilitate synaptic transmission in the CA1 region through N-methyl-D-aspartate (NMDA)-mediated signalling, an effect assisted by nNOS activation, whose expression levels positively correlated with hippocampal spine density [649,650]. Despite these possible compensatory mechanisms at play, our stereological analyses still revealed strong reductions in the number of dendritic spines in the CA1 region of PAC1 cKO mice, which has also been reported in PACAP-deficient mice [642]. Furthermore, most of the dendritic spine loss pertained to the more mature stubby and mushroom-shaped spines, which are generally more stable and play a critical role in long-term memory storage [651-653]. Cumulatively, our data does suggest that the observed compensatory mechanisms activated after PAC1 gene deletion may not be sufficient to prevent optimal dendritic spine maturation, at least in the CA1 region of the hippocampus.

The formation of long-term, mature synaptic connections depends on multiple downstream pathways and gene expression changes, including the phosphorylation of the transcription factor CREB, a crucial regulator of neuronal and synaptic plasticity [654-657]. Evidence shows that constitutively activated CREB increases dendritic spine density, whereas in $CREB^{S133A}$ mutant mice, in which the serine 133 residue cannot be phosphorylated, it leads to reduced spine density [658,659]. Since we observed a strong reduction in p-$CREB^{S133}$ levels, our results provide a rationale for the possible contribution of the PACAP/PAC1/CREB signalling pathway as a key driver of dendritic spine remodelling in the hippocampus independent of nNOS.

To complement these findings, our subcellular analyses identified a loss of mitochondria in hippocampal CA1 dendrites, whose presence is needed to sustain the energy requirements for neuronal communication at dendritic spines [660]. Loss of dendritic mitochondria is associated with synapse and spine loss [661,662]. This suggests that the reduction in mitochondrial density in neurons lacking PAC1 receptors may have contributed to dendritic spine loss, although a causal relationship remains to be determined. However, mitochondria in the CA1 region are also rapidly redistributed based on energy demands [663], thus the loss of mitochondria in dendrites

could be a response to impaired spine formation rather than the cause of this effect. Future research should examine the connection between PAC1, dendritic spines, and mitochondrial energy metabolism, especially since PACAP treatment in trigeminal ganglion cell cultures has been shown to impact mitochondrial function [664].

In this study, we demonstrate that PAC1 receptors in Camk2a^{+} hippocampal neurons are crucial for synaptic plasticity and memory. PAC1 deletion resulted in impaired spatial memory, decreased dendritic spine density, increased expression of nNOS and GAD65/67, and altered mitochondrial morphology. Some of the effects may represent compensatory mechanisms to mitigate the effects of PAC1 loss. Despite these adaptations, we observed a marked reduction in mature, stable dendritic spines, essential for long-term memory storage. Additionally, a loss of dendritic mitochondria in the CA1 region may contribute to spine reduction, though the directionality of this effect remains to be clarified. In any case, our findings underscore the role of PAC1 in sustaining synaptic integrity, likely through pathways involving CREB phosphorylation and mitochondrial support.

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Competing Interests

The authors declare no competing interests.

Author Contributions

Study conception and design: Alessandro Castorina. Investigations: Margo I. Jansen, Haley Hrncir, Judith Brinkman. Data analysis: Margo I. Jansen, Judith Brinkman. First draft of manuscript: Margo I. Jansen. Revision and editing of the final manuscript: Alessandro

Castorina, Allan MacKenzie-Graham, Laura A. Bradfield, Giuseppe Musumeci, Velia D'Agata and James. A. Waschek. Funding acquisition: Alessandro Castorina.

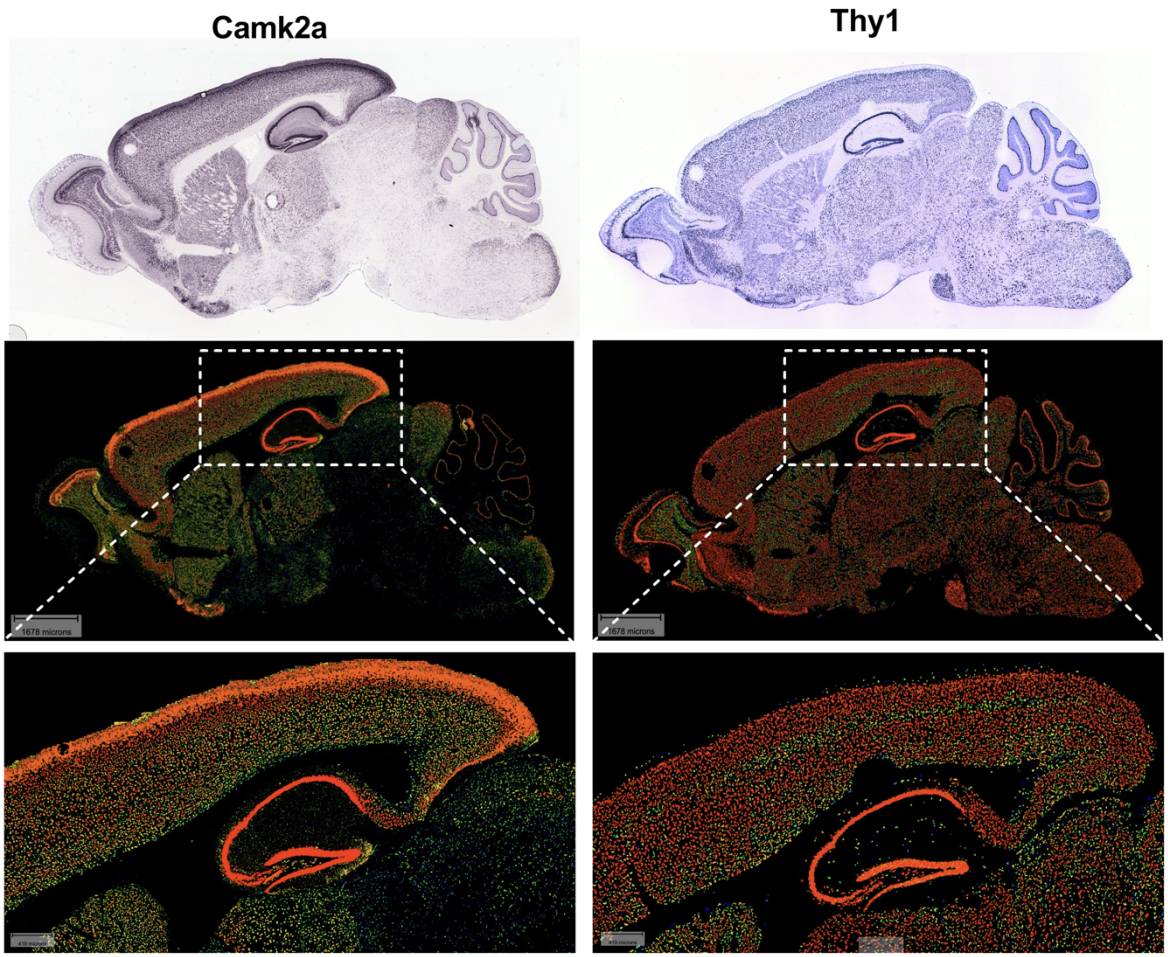
Data Availability

Raw data and images can be made available upon reasonable request to authors.

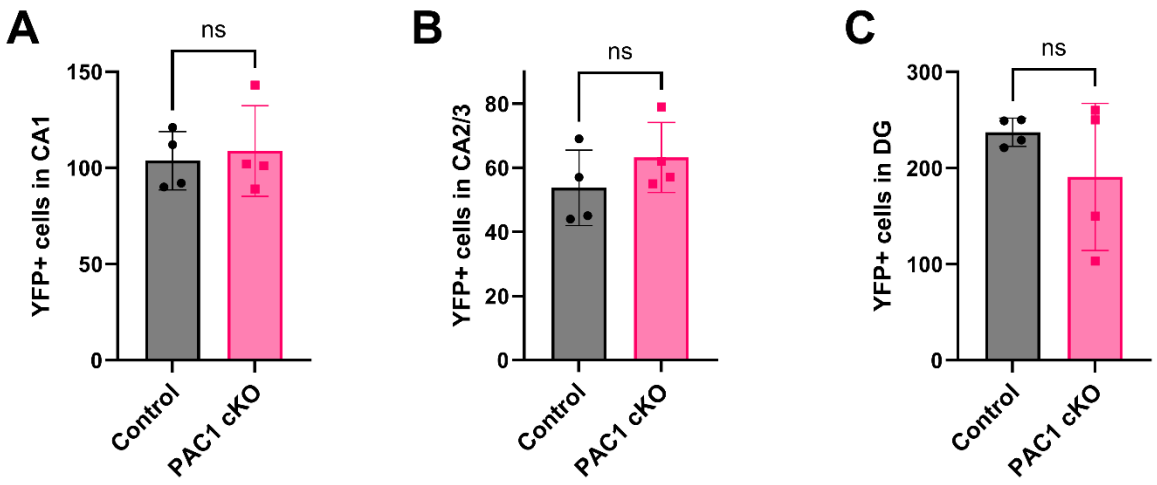
Ethics Approval

All animal experiments were carried out with the approval of the University of Technology Sydney Animal Care and Ethics Committee (ETH21-6281) and institutional Biosafety Committee (ETH21-6743) and conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Supplementary Data

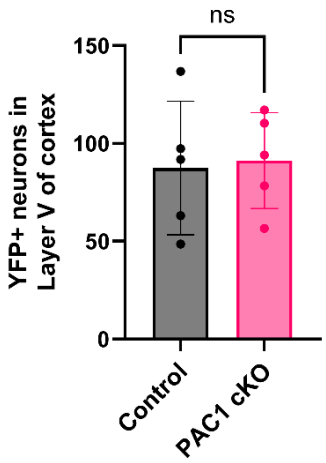


Supplementary Figure 1. *Representative in situ hybridisation (ISH) images, taken from the Allen Mouse Brain Atlas, demonstrating high abundance and similar hippocampal distribution pattern of both Camk2a and Thy1 mRNAs in sagittal brain sections of adult C56BL/6J mice. Images were taken from Allen Mouse Brain Atlas, mouse.brain-map.org at the same position [665]. ISH and Expression data for Camk2a in C57BL/6J was obtained from: https://mouse.brain-map.org/experiment/show/79360274 [666]. ISH and Expression data for the Thy1 gene in C57BL/6J mice was obtained from: https://mouse.brain-map.org/experiment/show/68798025 [667]. Scale bar (top panels = 1678µm, bottom panels = 419µm).*



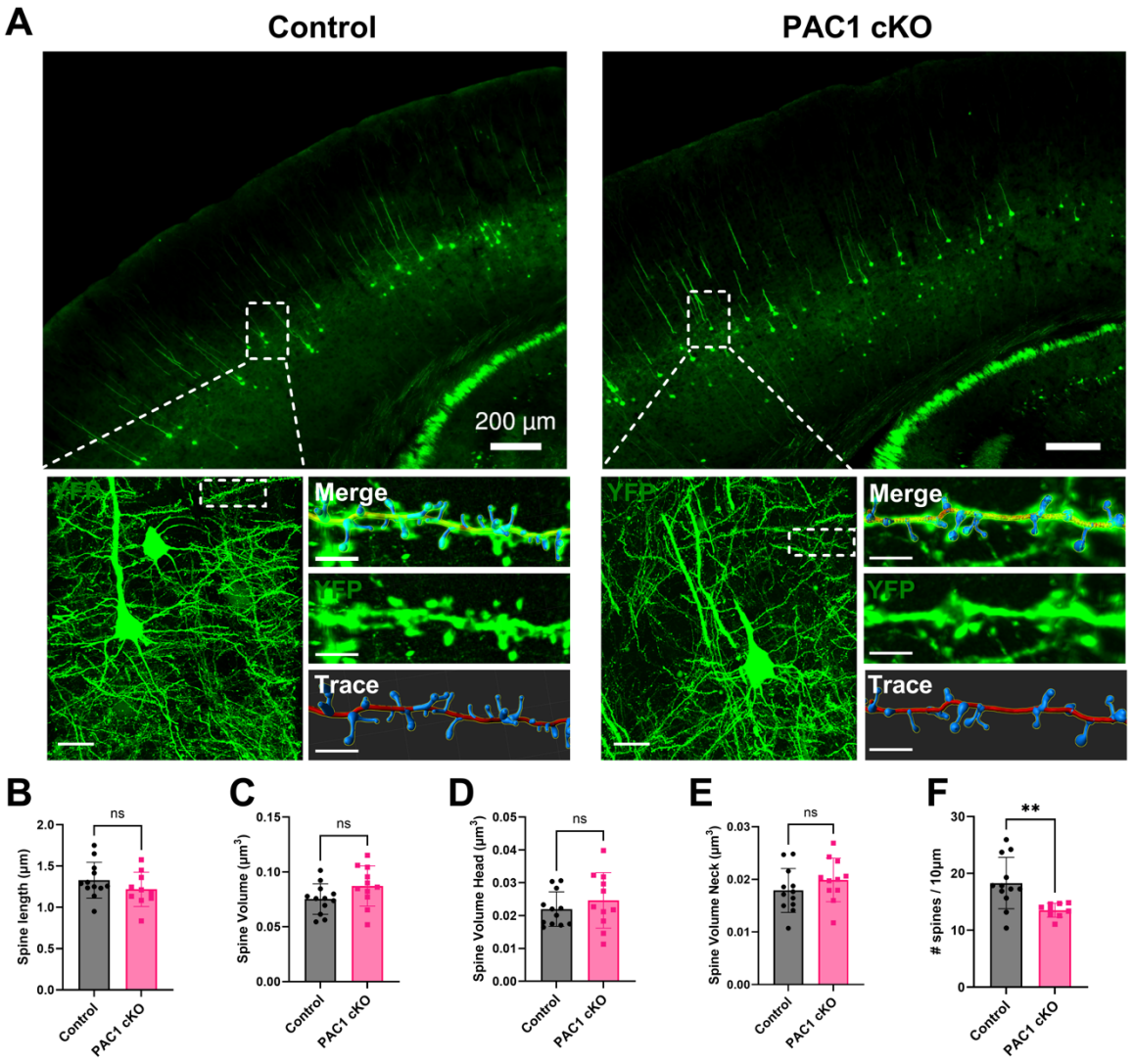
Supplementary Figure 2. YFP⁺ cell count in the hippocampus of wild-type and PAC1 conditional KO mice. *Quantification of Thy1-YFP⁺ cells in the (A) CA1, (B) CA2/3 and (C) Dentate gyrus of the hippocampus of control and PAC1 cKO mice under at baseline. n=4 mice per group. Data is plotted as mean ± SD and analysed using an unpaired t-test.*

PAC1 cKO = PAC1 conditional knockout



Supplementary Figure 3. Thy1-YFP⁺ neurons in layer V of the somatosensory cortex of control and PAC1 cKO mice. *Data shown is the average cell count ± SD. n=5 per genotype. Data was analysed using an unpaired t-test.*

ns = not significant, Thy1 = Thymus cell antigen 1, YFP = yellow fluorescent protein



Supplementary Figure 4. Cortical dendritic spine analysis in PAC1 cKO mice reveals reduced spine density. *(A) Representative images of dendritic spine tracing in the cerebral cortex of control and PAC1 cKO mice. Cortex scale bar = 200µm, neuron scale bar = 15µm, dendrite scale bar = 2µm. Measurements for (B) spine length, (C) spine volume, (D) volume of head of the spine, (E) volume of neck of the spine and (F) spine density. n=9-12 filaments/3 independent repeats for IMARIS filament tracing. Data is plotted as mean ± SD, as determined using an unpaired t-test. **p<0.01 vs control.*

ns = not significant, PAC1 cKO = PAC1 conditional knockout, YFP = yellow fluorescence protein.

Bridging section: Neuronal PAC1 depletion under demyelinating conditions

Based on the results from the outlined manuscript, the PAC1 receptor plays an important role mediating spatial memory formation and hippocampal plasticity. The work from this paper solely explored the role of PAC1 depletion under healthy conditions, allowing us to delineate fundamental neuron-specific functions associated with the receptor. However, as outlined in my introduction and based on findings from **chapter 3**, PAC1 signalling is also strongly neuroprotective under diseased conditions. Therefore, to continue to build knowledge on the role of PAC1 signalling during demyelination, we next explore the effects of PAC1 depletion in Camk2a expressing neurons exposed to the CPZ model for demyelination, Examining both neuronal-related effects as well as in oligodendrocytes.

Chapter 5:

Neuronal PAC1 deletion reduces axonal integrity and exacerbates oligodendrocyte loss after cuprizone demyelination

Status: not published

Margo I. Jansen, Judith Brinkman, James A. Waschek, Alessandro Castorina

5.1 Abstract

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating neurodegenerative disease of the brain and spinal cord, characterized by the formation of lesions in the white and often extending to the grey matter. Given the limited available treatments for people living with MS, there is an urgent need to explore new molecular targets that hold the potential to be employed as future therapeutics. The pituitary adenylate-cyclase activating polypeptide (PACAP)-preferring receptor, called PAC1, has previously been shown to be a as viable candidate for MS therapy. However, many questions regarding its cell-type specific function in central nervous system (CNS) cells remain, especially during experimental demyelination. Therefore, the aim of this study was to utilise the Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$ genetic mouse model to study the effects of PAC1 ablation in Camk2a-expressing excitatory neurons following a demyelinating insult with cuprizone (CPZ). While PAC1 conditional knockout (PAC1 cKO) showed decreased locomotion activity in the control group, after a four-week CPZ diet, there was no additional locomotor impairment in these mice, as assessed using the open field and rotarod tasks. Using the Thy1-YFP transgene present in these mouse model, which allows for high resolution imaging of Camk2a-expressing pyramidal neurons thus visualising PAC1 depleted neurons, we found that conditional PAC1 ablation caused loss of axonal integrity in the corpus callosum of demyelinating mice. PAC1 cKO mice also exhibited a constitutive loss of mature oligodendrocytes, and this effect was exacerbated under demyelinating conditions. Taken together, these findings highlight the importance of PAC1 signalling in neuron-oligodendrocyte communication, which is vital for myelination. By addressing receptor-specific roles in endogenous PACAP signalling, this study fills a critical gap in the current understanding of PACAP-PAC1's contribution to neuronal and glial functions. Furthermore, these results underscore the importance of PAC1 in both neuroprotection and myelin repair, offering new avenues for investigating PAC1 as a therapeutic target in multiple sclerosis and/or other neurodegenerative and demyelinating diseases.

5.2 Introduction

In recent years, the number of people diagnosed with the chronic autoinflammatory, demyelinating disease multiple sclerosis (MS) has increased dramatically [3]. This concerning trend is further amplified by the lack of a cure and limited availability of disease modifying therapies, especially for progressive MS [55,57,507,668]. Additionally, available treatments all focus on suppressing the immune system, without directly tackling other aspects of MS disease development. Therefore, there is an urgent need for further and broader-targeting therapies that provide protection against all aspects of the disease.

Previous work of our group and others have strongly implicated the potential of the Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and Vasoactive Intestinal Peptide (VIP) neuropeptide system as a potential therapeutic target in MS, given its neuroprotective and immunomodulatory function in the CNS [191,597]. However, research exploring the PACAP/VIP system in the CNS commonly utilise either exogenous PACAP (or VIP) administration, or global KO models to study its role in brain functioning under healthy and diseased conditions [201,202,606,609]. While this provides important information on the overall function of the neuropeptide system, it fails to delineate cell-type specific contributions to its protective effects. To further understand the mechanism of the signalling pathways in the CNS, research into the cell-type specific role of the system would provide this crucial insight.

My work in **chapter 3** shows the clear potential of PACAP-preferring receptor PAC1 as a strong candidate to further explore as a therapeutic target in MS. However, many questions regarding the function of PAC1 in the central nervous system (CNS) remain. While selective ablation of PAC1 in excitatory neurons (expressing Camk2a) seems to impact proper neuronal functioning under baseline conditions, especially in the hippocampus (**chapter 4**), we currently do not know how mice lacking PAC1 in Camk2a+ neurons would respond to demyelinating conditions.

Therefore, in the current study, we aimed to identify the effects of selective ablation of the PAC1 gene in excitatory pyramidal neurons after experimental demyelination, with a specific focus on general locomotion, neuronal health and oligodendrocyte numbers in the white matter.

5.3 Methods

5.3.1 Animals and tamoxifen injections

Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$ mice carrying both Thy1-YFP and Thy1-mitoCFP transgenes, generated from the breeding program of **chapter 4** were kept under environmentally controlled conditions in a 12-h light/dark cycle with access to food and water *ad libitum*. These mice also carry the Thy1-YFP construct (see **chapter 4**), allowing for visualisations of a subpopulation of Camk2a^{+} neurons and their axonal projections. All experiments were carried out with the approval of the University of Technology Sydney (ETH21-6281) and conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

To induce PAC1 depletion in Camk2a expressing neurons, 4-week-old mice received 75 mg/kg tamoxifen (in corn oil; Sigma) intra-peritoneal injections over a period of four consecutive days as outlined in the schematic shown for each experimental section. Control Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$ mice received vehicle intraperitoneal injections over that same period. Since tamoxifen is an estrogen-receptor agonist, a three-week washout was implemented to make sure tamoxifen was no longer bioactive prior to commencing any treatment or behavioural assessment.

5.3.2 Cuprizone diet

To study the effect of a targeted PAC1 deletion in neurons during a demyelination challenge, control or tamoxifen-induced *Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$* mice were fed either a standard diet or a 0.2% Cuprizone (CPZ; Sigma-Aldrich) diet for 28 days (n=12/group) as outlined in [597]. Group size was based on results obtained in previous studies using the same demyelination model [669,670]. Mice were randomly assigned to each experimental group using the freely available random group generator Keamk (https://www.keamk.com/). On day 29, mice were sacrificed using CO_2 asphyxiation (EA-33000TS SMARTBOX® Prodigy, Total CO_2 => 3.01 L/min) and brains were collected. See *Tissue collection and brain dissection* for additional details.

5.3.3 Open Field test

The open field test was performed as outlined in [597]. Briefly, four weeks following CPZ-induced demyelination, mice were acclimatised to the behaviour room for 30 min prior to

performing the Open Field test in the dark. The Open Field arena consisted of a dark grey square Perspex box (40 × 40 × 50 cm), where video recordings of the mice general locomotor activity behaviour were taken for 5 min from the top using an infrared video camera (Sony FDRAX53 4K Full HD Handycam, 1/50fps) held by a Manfrotto 190X tripod. Location tracking of the footage was performed using ezTrack (3 min/mouse) and measurements of overall distance travelled (in cm) and time spent in the centre of the arena (in seconds) were measured [554].

5.3.4 Rotarod

To measure general locomotor coordination in our mice we utilised the Rotarod apparatus with accelerating from 4-40 RPMs over a total of 300 seconds (Ugo Basile, Milan, Italy)[618,671]. Latency to fall, meaning the average time in seconds that each mouse spent on the rotating rod, was measured. A drop or full rotation of the mouse counts as the end of the trial.

5.3.5 Brain dissections and tissue collection

Following 4 weeks of CPZ exposure, brain tissues were collected for further molecular or histological analyses. For real-time quantitative PCR (RT-qPCR) or Western blot, fresh brain tissues were extracted under RNAse free conditions and snap-frozen using liquid nitrogen. White matter samples were micro-dissected as described in [597].

For immunofluorescence, mice were sacrificed by CO_2 asphyxiation, followed by a cardiac perfusion using cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS; pH 7.5). Afterwards, brains were collected and exposed to an overnight post-fixation step (in 4% PFA) at 4°C. Brains were subsequently embedded in Tissue Plus OCT Compound (ProSciTech) and 25µm thick coronal sections were cut using a cryostat (Cryostar NX70, Thermo Scientific), with the temperature set at -20°C. Sections were collected in a cryoprotectant solution (30% Ethyl Glycol, 20% Glycerol in 0.05M PB) and stored at -20°C until use.

5.3.6 RNA extraction, cDNA synthesis and Real-time quantitative PCR

RNA from the dissected brain samples was isolated using TRI-Reagent (Sigma Aldrich) and precipitated using 2-propanol as described in [220,597]. Complementary DNA was generated using the Tetro cDNA synthesis kit (BioLine) as per manufacturer's instructions using 1000µg of

RNA. RT-qPCR was performed and analysed as described in previous studies using the ΔΔCt method [261,597]. Primers sets used for gene expression studies are shown in Table 5.1

Table 5.1 Primer sets used in RT-qPCR. Forward and reverse primers were selected from the 5' and 3' region of each gene mRNA. The accession number reflects the mRNA sequence used for primer design. The expected length of each amplicon is indicated in the right column.

Accession #	Gene name	Primer sequences (5'-3-')	Product length (bp)
NM_001025251.2	MBP	Fwd:TATAAATCGGCTCACAAGGGATT Rev: TGTCTCTTCCTCCCAGCTTA	85
NM_011123.4	PLP1	Fwd: ATGCCAGAATGTATGGTGTTCT Rev:TTTAAGGACGGCGAAGTTGTAAG	200
NM_016967.2	OLIG2	Fwd:AAAGACAAGAAGCAGATGACTGA Rev: AGCATGAGGATGTAGTTTCGC	200
NM_011296.2	S18	Fwd: CCCTGAGAAGTTCCAGCACA Rev: GGTGAGGTCGATGTCTGCTT	145

5.3.7 Immunofluorescence

Selected brain tissue sections were washed in 1× PBS (3×, 10 min each) to remove the cryoprotectant solution and permeabilised for 30 min in 1× PBS + 0.1% Triton-X100 (0.1% PBST). Sections were blocked in 3% BSA (in 0.1% PBST, Bovogen) for 1 h followed by an overnight incubation with the primary antibody (1:250 in 3% BSA). The next day, sections were washed 3× in 0.1% PBST and incubated for 3 h in the appropriate secondary antibody. To counterstain nuclei, sections were incubated in DAPI stain (1µg/ml; Sigma-Aldrich) for 30 min followed by a final washing step in PBST. Stained sections were mounted using the VECTASHIELD Vibrance® Antifade Mounting Medium. Antibodies used in immunofluorescent staining were: rabbit-anti-NEFL (1:250; Thermo Fisher Scientific Cat# MA5-14981, RRID:AB_10984147), rabbit-anti-ASPA (1:250; Abcam Cat#ab223269, RRID: AB_3662876), Donkey Anti-Rabbit IgG H&L (Alexa Fluor 647; Abcam Cat# ab150063, RRID:AB_2687541).

5.3.8 Image acquisition & analyses

Twenty microns-thick Z-stacks of stained brain tissue sections were imaged using a Zeiss Axioscan Slide scanner (20× magnification) and analysed with 'extended depth of focus' images generated through the contrast algorithm from the Zeiss Zen Software (version 2.6). Mean grey intensity and % Area of the acquired 20× images were analysed using ImageJ software (v2.9.0).

5.3.9 Statistical analysis

Data is presented as mean ± standard deviation (SD). All statistical analyses and figure generation was performed using GraphPad Prism 9.3.1. Representative images of fluorescence staining were generated using OMERO Figure. To determine if data was normally distributed, quantile-quantile (Q-Q) plots were generated to compare the quantiles of the dataset to those of a theoretical normal distribution. Normality was evaluated based on the degree of alignment between the data points and the 45-degree reference line, which represents the expected distribution under normality. Once normal distribution was confirmed, pairwise comparisons were analysed using an unpaired two-tailed *t*-test. *p*-values ≤ 0.05 were considered statistically significant.

5.4 Results

5.4.1 Depletion of PAC1 in Camk2a$^+$ neurons does not exacerbate locomotor deficits under demyelinating conditions.

Work from our laboratory and several others have shown the importance of the endogenous PACAP/VIP signalling in maintaining CNS homeostasis and the protective effects of PACAP during a demyelinating challenge [193,201,202,597,600]. Therefore, our next objective was to investigate the effects of PAC1 depletion from Camk2a$^+$ neurons in the Cuprizone (CPZ) model of demyelination (Figure 1A).

Both control and PAC1 cKO mice underwent two different dietary regimes for 4 weeks: (1) standard diet or (2) diet supplemented with 0.2% CPZ. At the end of the 4-weeks, both genotypes were tested for changes in locomotor performance. Using the Rotarod test (Figure 1B), we report a minor reduction of the latency in PAC1 cKO mice, albeit not statistically significant (Figure 1C; t_{22}=1.732, p=0.0972). The CPZ diet did not further reduce the latency to fall in either of the two genotypes (Figure 1C). In the Open Field test, we observed a significant reduction in the total distance travelled by PAC1 cKO versus control mice when these were on a regular diet (t_{22}=2.147, *p=0.0431), which was not further aggravated when mice were fed a CPZ diet (Figure 1E). Likewise, no changes in exploration of the centre part of the Open Field arena could be identified (Figure 1F).

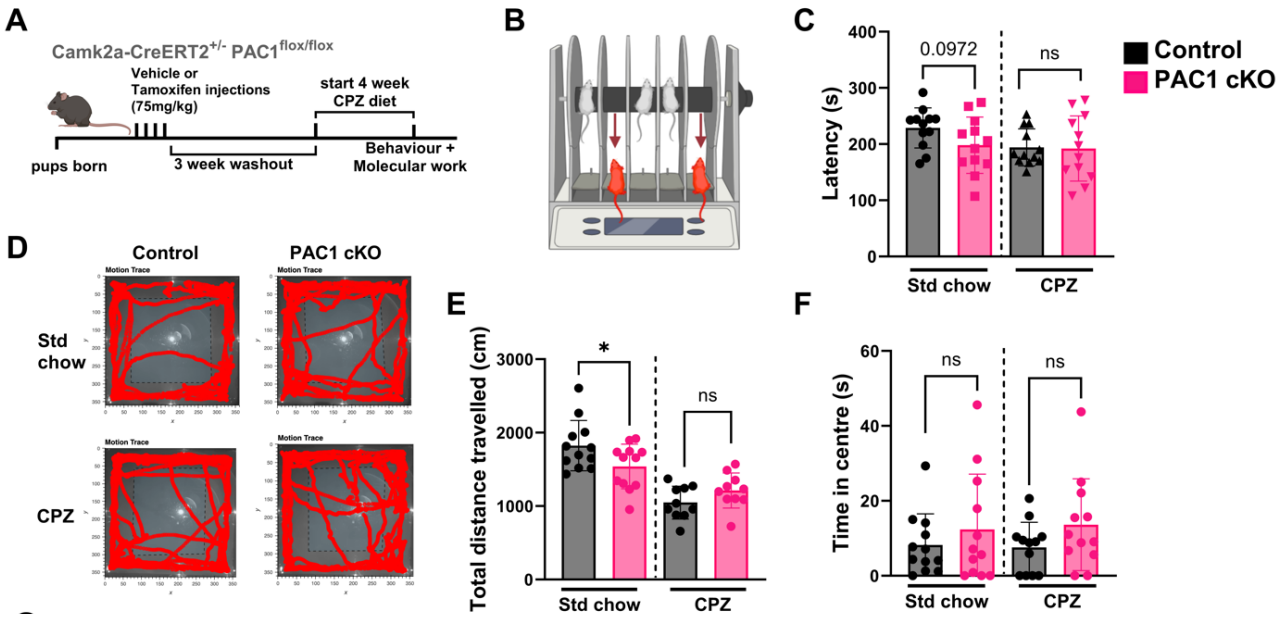


Figure 5.1. Locomotion analysis of PAC1 cKO mice after 4 weeks of demyelination. *(A) Timeline of the experimental paradigm. (B) Pictorial image of the Rotarod apparatus and (C) latency to fall (in sec) measured in control and PAC1 cKO mice after 4 weeks of standard or 0.2% CPZ diet. (D) Representative line tracks showing the trajectory of mice while exploring the open field apparatus. (E) Total distance travelled in cm and (F) Time spent in the centre in s. N=12 mice per group. Data plotted shows the mean ± SD and was analysed using an unpaired t-test. **p<0.01 vs control.*

CPZ = cuprizone, PAC1 cKO = PAC1 conditional knockout, Std chow = standard chow

5.4.2 PAC1 ablation in Camk2a expressing neurons reduce axonal projections in the corpus callosum of demyelinated mice

Neuronal damage is one of the major causes of clinical disability in MS [566,567]. Since a similar type of damage has been observed in the CPZ demyelination model, we next sought to examine the effect of Camk2a^{+} neurons PAC1 depletion on axonal damage after an acute demyelinating challenge [569,597]. Using neurofilament-light chain (NEFL), an established marker for axonal damage [571-573], we analysed its expression in the corpus callosum of both control and PAC1 cKO mice (Figure 5A,B). No differences in NEFL levels were observed in CPZ-fed mice, irrespective of the genotype (Figure 5B). However, semi-quantitative analyses of YFP fluorescent signals arising from axonal projections of Thy1^{+} neurons revealed a significant reduction of YFP intensity in the corpus callosum, suggesting a PAC1-dependent loss of axons in the white matter of CPZ intoxicated mice (t_8=3.826, **p=0.005; Figure 5C,D).

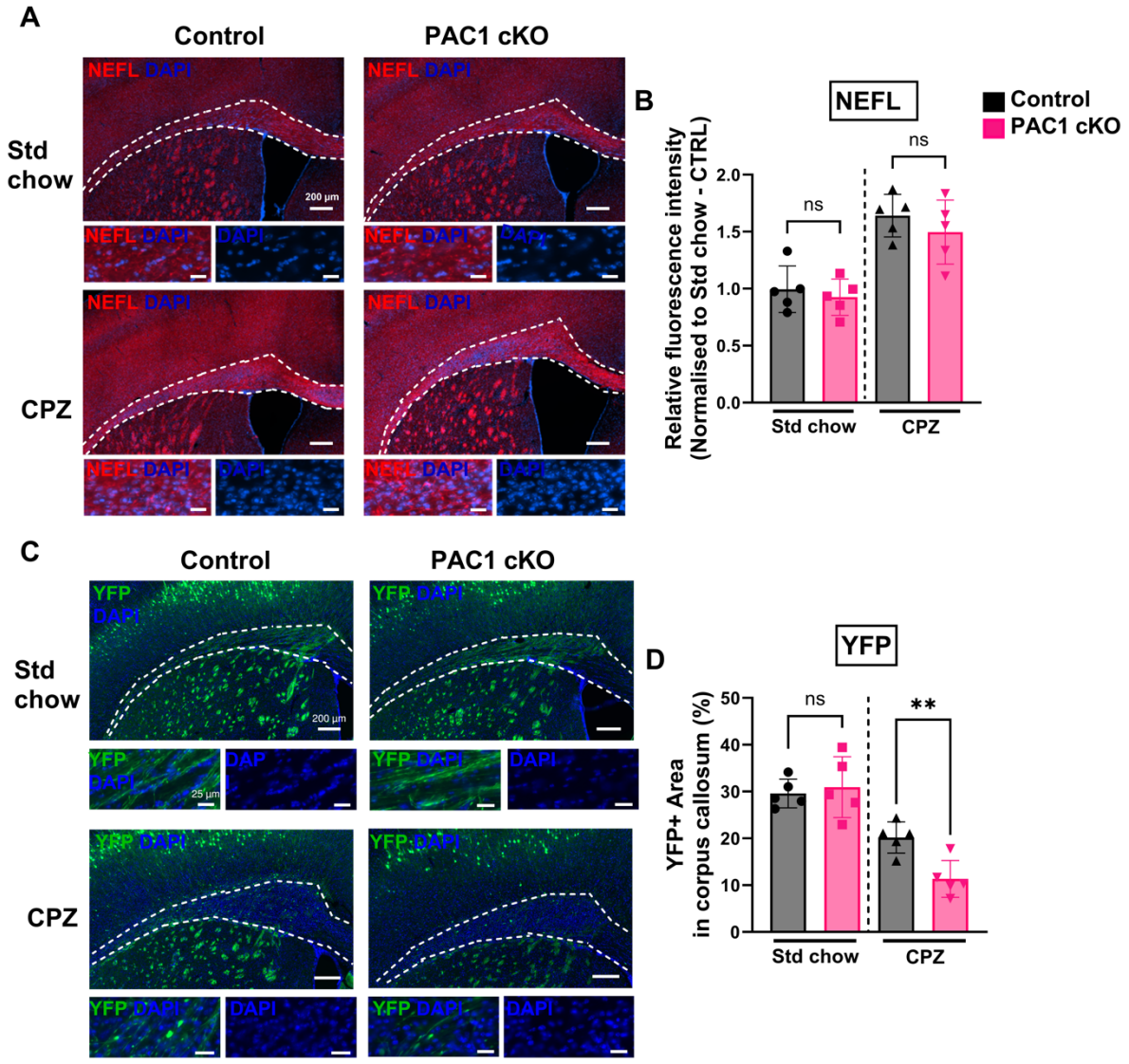


Figure 5.2 Reduced axonal integrity of projections from Thy1^{+} neurons in the corpus callosum of conditional PAC1 KO mice during cuprizone-induced demyelination. *(A) Representative images of NEFL staining in the corpus callosum of Control and PAC1 cKO mice under healthy as well as demyelinating conditions. (B) NEFL staining in the corpus callosum showed no change upon PAC1 depletion in neurons under demyelinating conditions. (C) Representative images of Thy-YFP signal in the corpus callosum of the control and PAC1 cKO mice. (D) Thy1-YFP^{+} axonal projections are decreased in the corpus callosum of demyelinating PAC1 cKO mice. N=3-4 mice per group for immunofluorescence studies. Data plotted shows the mean ± SD and was analysed using an unpaired t-test. **p<0.01 vs control.*

CPZ = cuprizone, NEFL= Neurofilament-light chain, PAC1 cKO = PAC1 conditional knockout, Std chow = standard chow, YFP= yellow fluorescent protein.

5.4.3 *Reduced maturation of oligodendrocytes in the brain of mice with neuronal depletion of the PAC1 gene*

Axonal integrity is known to be dependent on oligodendrocyte function and myelination [672,673]. On the other hand, there is evidence suggesting that diffuse axonal damage can interfere with oligodendrocyte health and myelin production [674,675], suggesting a bidirectional relationship between the two. Here, to further investigate whether impaired oligodendrocyte maturation contributes to the observed axonal loss in the corpus callosum of CPZ-fed PAC1 cKO mice, we conducted additional experiments to determine the expression of myelin genes and performed stereological measurements of mature (Aspartoacylase, $ASPA^+$) oligodendrocytes [676] in the corpus callosum of both control and PAC1 cKO mice after exposure to either a regular diet or a demyelinating diet with CPZ for 4 weeks. We observe that loss of PAC1 in $Camk2a^+$ neurons contribute to further reducing the expression of myelin genes under demyelinating conditions. Figure 6A shows the approximate topographical location of the regions of interests used for stereological assessments of mature oligodendrocytes.

After 4 weeks of CPZ diet, transcript levels of the myelin basic protein (*MBP)* gene show additional reduction in CPZ-fed PAC1 cKO mice compared to CPZ-fed control mice (t_8=2.749, *p=0.0251; Figure 6B). This is not seen when interrogating proteolipid protein 1 (*PLP1*) gene expression (p>0.05; Figure 6C). Measurements of the relative mRNA expression of *Olig2* – which encodes for a transcription factor crucial for the expression of myelin-related genes [677] – were consistent with MBP transcripts, demonstrating exacerbated reduction of gene expression in PAC1 cKO under a demyelinating challenge, although the result was not significant (t_8=2.075, p=0.0717; Figure 6D).

Stereological assessment of $ASPA^+$ oligodendrocytes demonstrated an unexpected reduction in the number of mature oligodendrocytes in the corpus callosum of PAC1 cKO mice fed a regular diet (t_6=4.793, **p=0.0030; Figure 5K). The 4-week dietary regime with CPZ further aggravated oligodendrocyte loss in cKO mice (t_6=2.699, *p=0.0356), suggesting a role of neuronal PAC1 in regulating oligodendrocyte maturation in the brain.

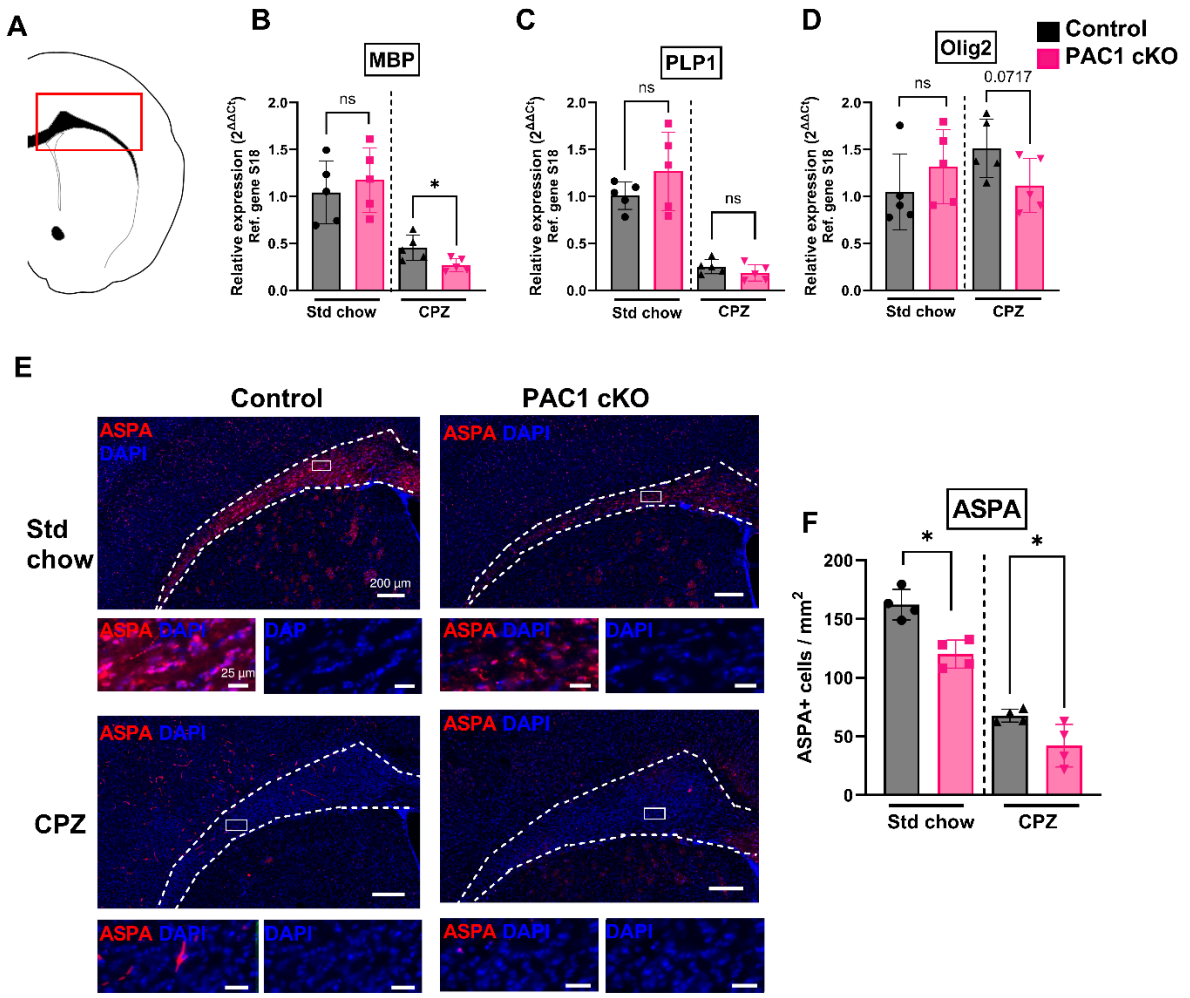


Figure 5.3. Loss of neuronal PAC1 impacts oligodendrocyte function in the corpus callosum of demyelinating mice. *(A) Schematic overview of the region of interest in this experimental set-up. RT-qPCR results for white matter markers (B) MBP, (C) PLP1 and (D) Olig2. (E) Representative images of ASPA staining in the white matter of control and PAC1 cKO mice under healthy and demyelinating conditions. (F) Quantification of ASPA+ cells in the corpus callosum of control and PAC1 cKO mice under baseline and demyelinating conditions. N=5 for RT-qPCR studies and n=4 mice for the immunofluorescence. Data is plotted as mean ± SD and analysed using an unpaired t-test. *p<0.05.*

ASPA = Aspartoacylase, CPZ = cuprizone, MBP = myelin basic protein, Olig2 = oligodendrocyte transcription factor 2, PLP1=proteolipid protein 1, PAC1 cKO = PAC1 conditional knockout, Std chow = standard chow.

5.5 Discussion

The critical role of glial cells, particularly oligodendrocytes, in supporting neuronal health and facilitating memory formation has gained significant recognition in recent years [678,679]. Bulk evidence indicates that PAC1 mediates protective roles both in neurons and myelin-producing cells in experimental models of CNS injury [196,204,302,305,600]. In this chapter, we investigated whether neuronal PAC1 contributes to neuronal damage, oligodendrocyte health and recovery both at baseline and after an acute demyelinating challenge with cuprizone. We observe that neuronal PAC1 depletion causes additional loss of axonal projections in the white matter of demyelinating mice, suggesting a neuroprotective function of PAC1. Previous work has already implicated that PAC1 depletion causes axonopathy in retinal neurons using a different model for MS, with evidence in our study corroborating these findings [204].

Neuronal-oligodendrocyte communication is essential for the maintenance of both neuronal and oligodendrocyte function [680,681]. Studies proposing a relationship between oligodendrocyte survival and axonal integrity already emerged in 1993; Barres and colleagues demonstrated that axonal degeneration leads to increased oligodendrocyte apoptosis [682]. This interaction is mediated, at least in part, by the bidirectional release of signalling factors that support both myelination and neuronal activity [683-686]. In our investigation of the effects of demyelination on neuronal function in the PAC1 cKO model, we also examined the impact of neuronal PAC1 deletion on oligodendrocytes. Notably, neuronal PAC1 deficiency under healthy conditions led to a significant reduction in the number of mature oligodendrocytes within the corpus callosum. This effect was exacerbated under demyelinating conditions, where further loss of mature oligodendrocytes was observed. However, future research would have to show if total numbers of oligodendrocytes are lost, or if oligodendrocytes simply lose their mature phenotype upon neuronal PAC1 deficiency. These findings suggest that neuronal PAC1 depletion may cause baseline deficits in axonal health, potentially disrupting axon-oligodendrocyte crosstalk and leading to oligodendrocyte loss [684,687].

Future research is needed to further decipher the role of the PAC1 receptor in neuron-oligodendrocyte crosstalk under healthy and demyelinating conditions. Since impaired neuron-oligodendrocyte interactions have now been recognised as early events in MS pathology, and PAC1 mediates protective responses in both neurons and oligodendrocytes [188,209,302,332,597,599,688], understanding the significance of this bi-directional communication

regulated through PACAP-PAC1 signalling could provide valuable insights into new ways to prevent and/or target early white matter damage in MS. Additionally, utilising the reversibility of the CPZ model, which allows to study remyelination, could provide important insights into the effects of PAC1 signalling during spontaneous myelin repair. It was recently shown that neurons are protected from damage and apoptosis during remyelination [689]. Since activation of the PACAP-PAC1 axis is neuroprotective, the effects of neuronal PAC1 ablation during spontaneous remyelination following a 4-week demyelinating insult could provide insights into the contribution of PAC1-driven neuroprotection in myelin recovery [523,599,690]. To further explore the role of PAC1 in neuroprotection within the context of MS, a potential future experiment should implement the experimental autoimmune encephalomyelitis (EAE) model in PAC1 cKO mice. Using this model of the disease, which better captures the pathogenic features of aberrant immune system activation and axonal dysfunction, as seen in people with MS, we could explore how neuronal PAC1 ablation interferes with axonal function from the perspective of inflammation-driven demyelination [691].

To summarise, this study shows how neuronal PAC1 expression is critical for the maintenance of axonal projections and oligodendrocyte maturation under demyelinating conditions. This suggests an important role for PACAP-PAC1 signalling in the bi-directional communication between neurons and oligodendrocytes in the effort to maintain homeostasis during demyelination.

Bridging section: Oligodendrocyte-specific PAC1 depletion

Given the finding that mice lacking neuronal PAC1 exhibit not only increased axonal damage but also exacerbated oligodendrocyte loss in the CPZ model for demyelination, I reasoned that there may be a disruption in neuronal-oligodendrocyte crosstalk upon ablation of the PAC1 gene from neurons. As such, my final aim was to generate an oligodendrocyte-specific inducible PAC1 cKO model. This approach would provide the opportunity to investigate if any disruption caused by PAC1 deletion is uni- or bi-directional (neurons to oligodendrocytes and vice versa). In the following and final chapter of my thesis, I outline the process of generating a tamoxifen-inducible, oligodendrocyte-specific PAC1 KO genetic mouse model.

Chapter 6:

Generation of an oligodendrocyte-specific inducible PAC1 knockout mouse model

Status: not published

Margo I. Jansen, James A. Waschek, Alessandro Castorina

6.1 Abstract

Oligodendrocytes, the myelin producing cells of the central nervous system (CNS), play a crucial role in maintaining healthy brain function and ensuring proper nerve conduction. In various neurological diseases, including multiple sclerosis, oligodendrocyte damage is a key driver of disease onset and progression, often resulting in downstream axonal damage and concurrent neuroinflammation.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide consisting of 27 or 38 amino acids that preferential binds through the PAC1 G-protein-coupled receptor (GPCR). PAC1-mediated signalling is associated with neuroprotective and oligoprotective effects within the CNS, particularly under demyelinating conditions. Additionally, research has demonstrated that neuronal PAC1 loss can directly affect the number of myelinating oligodendrocytes. These findings highlight the critical role of the PAC1 receptor in oligodendrocyte function. However, investigations into the specific role of PAC1 in the oligodendrocytes has been limited due to the lack of appropriate experimental tools. The aim of this work was to generate an oligodendrocyte-specific conditional PAC1 knockout mouse model using the CreERT-LoxP system to enable future studies on the role of PAC1 in oligodendrocytes. After two breeding rounds of PLP1-CreERT$^{+/-}$ C57BL/6 mice crossed with PAC1$^{flox/flox}$ mice, we successfully generated PLP1-CreERT$^{+/-}$-PAC1$^{flox/flox}$ mice. These mice exhibited significantly reduced PAC1 expression in the white matter one week and three weeks following tamoxifen induction. In conclusion, we have generated a transgenic mouse that will facilitate future *in vivo* on the oligodendrocyte-specific functions of PAC1.

6.2 Introduction

The pituitary adenylate cyclase-activating polypeptide (PACAP) and the vasoactive intestinal peptide (VIP) neuropeptides are endogenously produced molecules widely distributed throughout the CNS. These two neuropeptides bind with different affinities to the PAC1, VPAC1 and VPAC2 receptors, and are known to have neuroprotective and immunomodulatory signalling properties (for a detailed overview of the VIP/PACAP system see **1.1.3** and **1.1.4**). PACAP preferentially binds to the PAC1 receptor whereas VIP has equal binding affinity for all three receptors.

My previous work outlined in this thesis has shown a strong co-localisation of PAC1 with oligodendrocytes in the NAWM of MS patients (**Chapter 2**) and robust beneficial effects of targeting the PAC1 receptor specifically to preserve white matter under demyelinating conditions (**Chapter 3**). Additionally, PACAP treatment has been also found to stimulate oligodendrocyte proliferation but not differentiation *in vitro* [265]. Moreover, unexpected findings shown in the previous chapter (**Chapter 5**) highlighted how neuronal PAC1 loss triggers the loss of mature oligodendrocytes, as well as increase axonal damage under demyelinating conditions.

While this data points towards a crucial role for PAC1 signalling in oligodendrocyte functioning, much remains unknown about the specific function of PAC1 in this cell type under both healthy and diseased conditions. Since oligodendrocyte dysfunction drives MS lesion development, further unravelling of the role of this receptor under demyelinating conditions could provide essential insights into the molecular pathways driving PAC1-mediated oligodendrocyte protection. Once understood, these notions could serve the purpose of providing insights on the therapeutic potential of PAC1 in oligodendrocytes, and how to target the receptor specifically within the context of MS.

The lack of appropriate tools to study cell-type specific effects of PAC1 in the CNS has hindered our ability to study understand the differential roles of this receptor in the brain. While treating mice with the PAC1 agonist Maxadilan showed promising results regarding myelination and oligodendrocyte functioning, all PAC1-expressing cell types of the CNS are responding to the treatment. While Camk2a^{+} PAC1 depletion can provide important

information on the role of PAC1 in neuronal functioning, we currently lack the ability to study PAC1 in oligodendrocytes *in vivo*.

Therefore, the aim of this project was to generate a tamoxifen inducible oligodendrocyte-specific PAC1 knock-out genetic mouse model using the CreERT-LoxP system, creating the opportunity to study the role of PAC1 in mature oligodendrocyte functioning specifically. This unique model, utilising *PLP1* as a driver of our Cre expression, can be used to study the role of oligodendrocyte PAC1 in white matter homeostasis. Specifically, the field would benefit from CPZ-induced de- and remyelination studies in control versus PLP^{+} PAC1 cKO mice, to complement the neuronal PAC1 depletion findings during demyelination. Additionally, the newly generated an inducible oligodendrocyte-specific PAC1 knock-out genetic mouse model can be used for studies into the effects of neuroinflammation on PAC1-depleted oligodendrocytes, shining light on the immune-mediated effects on mature oligodendrocytes lacking PAC1.

6.3 Methods

6.3.1 Animals

Camk2a-CreERT2$^{-/-}$ PAC1$^{flox/flox}$ C57BL6/J mice obtained from our Camk2a-CreERT2 PAC1$^{flox/flox}$ breeding program (ETH21-6004) were crossed with PLP1-CreERT$^{+/-}$ mice obtained from the Jackson Laboratory (B6.Cg-Tg(Plp1-cre/ERT)3Pop/J, Strain #:005975) to generate our desired genotype (see 6.4.1). Mice were housed in individually ventilated cages (4 mice/cage) under a 12 h light/dark cycle, with access to food and water *ad libitum*. All experiments were carried out with the approval of the University of Technology Sydney (ETH21-6281) and conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

6.3.2 DNA extraction & genotyping polymerase chain reaction

At week 4 of age, mice were ear-notched and DNA was extracted using the KAPA Express Extract kit (KK7302, Roche) according to manufacturer's instructions. Briefly, DNA was extracted from tissue samples using 10µL 10X KAPA Express Extract Buffer, 2µL 1 U/µL KAPA Express Extract Enzyme in a total volume of 100µL by incubating the samples for 15 min at 75°C followed by 5 min at 95°C. After DNA extraction from the ear-notches,

polymerase chain reactions (PCR) were set up to (1) determine the allelic presence of the PAC1 flox construct and (2) resolve the presence of the PLP1-CreERT construct in our mice. Each reaction (final volume of 25µL) was set up as follows: 12.5µL 2X KAPA2G Fast (HotStart) Genotyping Mix with dye2, 1.25µL of forward and reverse primer (10µM), 1µL template DNA from the DNA extraction and topped up with 9µL ddH_2O. Primer sequences used for genotyping the PAC1 flox gene are: Forward: 5'-ACGTGCTCTCTTGGACCTTAGTAGC-3' and reverse: 5'-ATGGAGAGTAGGTGAATGAGCGACC-3'. Primer sequences to resolve the presence of the PLP1-CreERT construct in our mice are: Forward: 5'-CTTGGTAACATGGGCTGCTT-3' and reverse: 5'-CGGTTATTCAACTTGCACCA-3'. PCR reactions were run as outlined in Table 6.1 and resolved on a 2% agarose gel (Sigma-Aldrich in 1x Tris/Borate/EDTA buffer; 1x SYBR Safe DNA stain; ThermoFisher Scientific). Images were taken using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad).

Table 6.1. Genotyping PCR set-up

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	95ºC	3 min	1
Denaturation	95ºC	15 sec	35–40
Annealing	60ºC	15 sec	
Extension	72ºC	15 sec/kb	
Final extension	72ºC	1 min/kb	1

6.3.3 Induction of PAC1 Knockout

Tamoxifen injections were given according to the protocol outlined in **chapter 4** & **chapter 5**. Briefly, tamoxifen (Sigma-Aldrich) was dissolved in sterile corn oil (Sigma-Aldrich) to a final concentration of 20mg/mL. For four consecutive days, PLP1-CreERT$^{+/-}$ PAC1$^{flox/flox}$ or PLP1-CreERT$^{-/-}$PAC1$^{flox/flox}$ mice were injected with 75mg/kg tamoxifen solution and monitored for adverse symptoms. Brains were collected one week post final injection and three weeks post final injection to measure the effect on PAC1 levels.

6.3.4 Western blot

Brains were collected either one week or three weeks post tamoxifen induction and snap-frozen in liquid nitrogen. Following a microdissection of the white matter as described in [597], protein samples were extracted in radioimmunoprecipitation assay (RIPA) buffer (Sigma- Aldrich,

Castle Hill, NSW, Australia) containing 1x Protease Inhibitor cocktail (cOmpleteTM, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich, Castle Hill, NSW, Australia). Protein concentration was determined using the Bicinchoninic-Acid (BCA) Assay Protein Assay Kit (ThermoFisher Scientific) according to manufacturer's protocol. Absorbance was measured using the Tecan plate reader M1000 at 562nm. Protein samples (30µg) for Western blot were prepared using 3.75 µL of Laemmli Buffer (Bio-Rad) containing β-mercaptoethanol (Sigma-Aldrich; ratio 1:9 vol/vol) in a final volume of 15 µL. Following a protein denaturing step (10min, 70°C), samples were separated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% mini gels (Bio-Rad, Criterion 15-well Mini-Protean SFX). 5µL of the molecular weight ladder (Bio-Rad Pre-stained HyperLadder Precision Plus ProteinTM) was used as a reference for protein size. Proteins were transferred onto a 0.2µm PVDF membrane (Biorad) using the Trans-Blot Turbo instrument (Bio-Rad). After a blocking step (1h, 5% milk in TBS-T), membranes were incubated overnight with the appropriate diluted primary antibody in blocking buffer. Primary antibodies used in this experiment were: Rabbit-anti-PACAP receptor (1:500; GeneTex Cat# GTX30026, RRID:AB_3097721) and Rabbit-anti-GAPDH (1:1000; Bio-Rad Cat# VPA00187, RRID:AB_3431937). The next day, the membrane was washed in TBS-T (3x, 5min) and incubated in secondary antibody solution (1:10000, Abcam Cat# ab6721, RRID:AB_955447; 5% milk in TBS-T). The membrane was washed and subsequently imaged using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad) after a clarity step was performed to visualise bands (Clarity Western ECL Blotting Substrate (Bio-Rad)). Bands were analysed using ImageJ2 (v2.9.0/1.53t) as described in [692] using GAPDH as a reference protein.

6.4 Results

6.4.1 Breeding strategy for the generation of PLP-CreERT2$^{+/-}$ PAC1 $^{flox/flox}$ mice

Our aim for this final chapter was to generate an inducible oligodendrocyte-specific PAC1 KO genetic mouse model using the Cre-LoxP system. To selectively remove the PAC1 gene from oligodendrocytes, we use the highly selective expression of the *proteolipid protein 1* (PLP1) gene as a driver of our CreERT expression to express Cre recombinase only in myelinating cells [693,694].

To obtain our desired genotype of PLP1-CreERT$^{+/-}$ PAC1$^{flox/flox}$, a multi-step breeding protocol was employed. First, PLP1-CreERT$^{+/-}$ mice (B6.Cg-Tg(Plp1-cre/ERT)3Pop/J, Strain #:005975) were crossed with Cre$^{-/-}$ PAC1$^{flox/flox}$ mice obtained from the ETH21-6004 protocol (Figure 6.1A). As outlined in Figure 6.1A, the first generation off-spring is predicted to be 50% PLP1-CreERT$^{+/-}$ PAC1$^{flox/-}$ and 50% PLP1-CreERT$^{-/-}$ PAC1$^{flox/-}$. For example, off-spring number 1.1-1.6 and 1.9-1.10 are heterozygous for PLP1-CreERT, whereas off-spring 1.7 and 1.8 are PLP1-CreERT$^{-/-}$ (Figure 6.1B). As predicted, all off-spring from this first round of genotyping is PAC1$^{flox/-}$ and can be detected using PCR (Figure 6.1C).

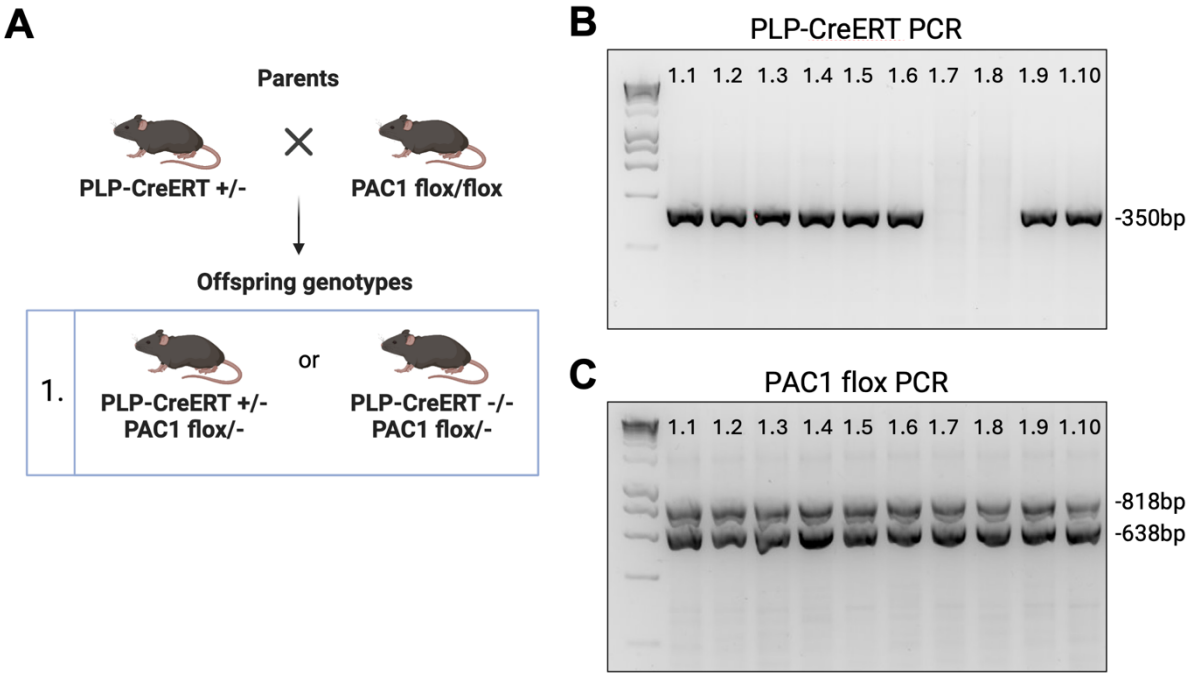


Figure 6.1 Overview of first round of breeding for the generation of PLP1-CreERT+/- PAC1flox/- mice. *(A) Schematic overview of the breeding strategy for the first round of generation of PLP-CreERT+/- PAC1flox/flox mice. (B) Example of genotyping results from the first round of offspring assessed for the*

heterozygous presence of the PLP1-CreERT construct. (C) Example of genotyping results for the PAC1 flox gene. As predicted all mice are heterozygous for PAC1 flox gene.

PLP = proteolipid protein , PAC1 = pituitary adenylate cyclase activating polypeptide receptor 1, PCR = polymerase chain reaction

Since both alleles of the PAC1 gene need to be floxed to induce a full PAC1 KO in PLP expressing cells, our next step was to backcross the first generation PLP1-CreERT$^{+/-}$ PAC1$^{flox/-}$ mice with the parental Cre$^{-/-}$ PAC1$^{flox/flox}$ mice to optimise the incidence of PLP-CreERT$^{+/-}$ PAC1$^{flox/flox}$ mice (Figure 6.2A). As can be seen in Figure 6.2B, we again generate PLP-CreERT heterozygous offspring in approximately 50% of our pups. Additionally, a selection of the offspring is homozygous for PAC1$^{flox/flox}$ and part is heterozygous for the PAC1 flox allele (Figure 6.2C). Thus, after two breeding rounds we have obtained our desired genotype of PLP-CreERT$^{+/-}$ PAC1$^{flox/flox.}$ Colony maintenance is set-up by crossing PLP-CreERT$^{+/-}$ PAC1$^{flox/flox}$ mice with PLP-CreERT$^{-/-}$ PAC1$^{flox/flox}$ mice.

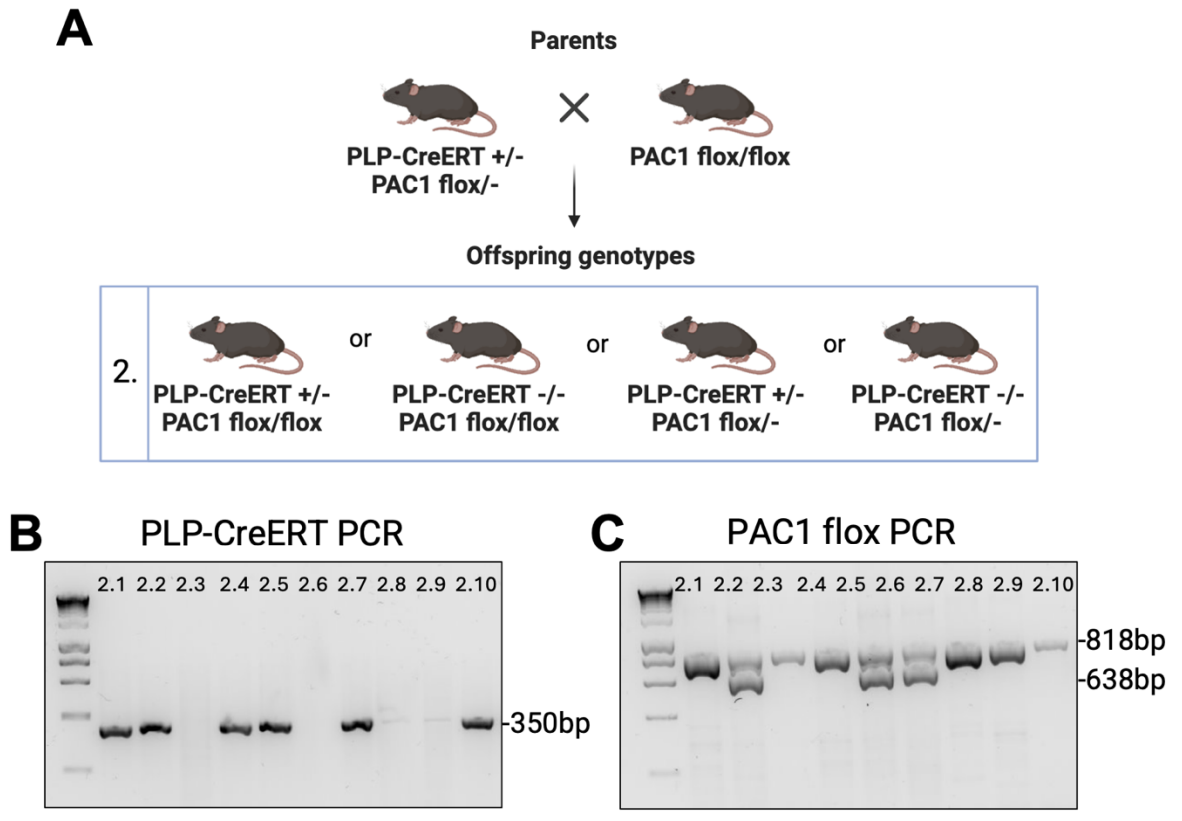


Figure 6.2 Overview of the second round of breeding to obtain our oligodendrocyte-inducible PAC1 conditional knockout mouse model*. (A) Schematic overview of the second round of crossings with predicted offspring genotypes. (B) Example of the PLP1-CreERT genotyping result for the second round of offspring. (C) Example of the PAC1 flox genotyping for the second round of off-spring.*

PLP = proteolipid protein , PAC1 = pituitary adenylate cyclase activating polypeptide receptor 1, PCR = polymerase chain reaction

6.4.2 Validation of PAC1 reduction in the CNS white matter

To confirm that tamoxifen induction successfully activated nuclear Cre translocations and the consequent depletion of the PAC1 receptor gene in PLP1-CreERT$^{+/-}$ PAC1$^{flox/flox}$ mice, we conducted a time course experiment to monitor PAC1 expression over time. At four weeks of age, PLP1-CreERT$^{+/-}$ PAC1$^{flox/flox}$ and PLP-CreERT$^{-/-}$ PAC1$^{flox/flox}$ were injected with tamoxifen, as outlined in schematic overview, and monitored for either one week or three weeks post-induction (Figure 6.3A&B). At these endpoints, brains were collected, and a Western blot was completed on the callosal white matter collected from these mice, with the idea of capturing the region with the highest PLP1 expression. In figure 6.3C, representative Western blot results can be seen for PAC1 and reference protein GAPDH. Upon quantification, a significant drop in PAC1 expression can already be appreciated at one week post final tamoxifen injection (*p=0.0392; Figure 1D). This drop in PAC1 levels was slightly more prominent at 3 weeks post final tamoxifen injection (**p=0.0061).

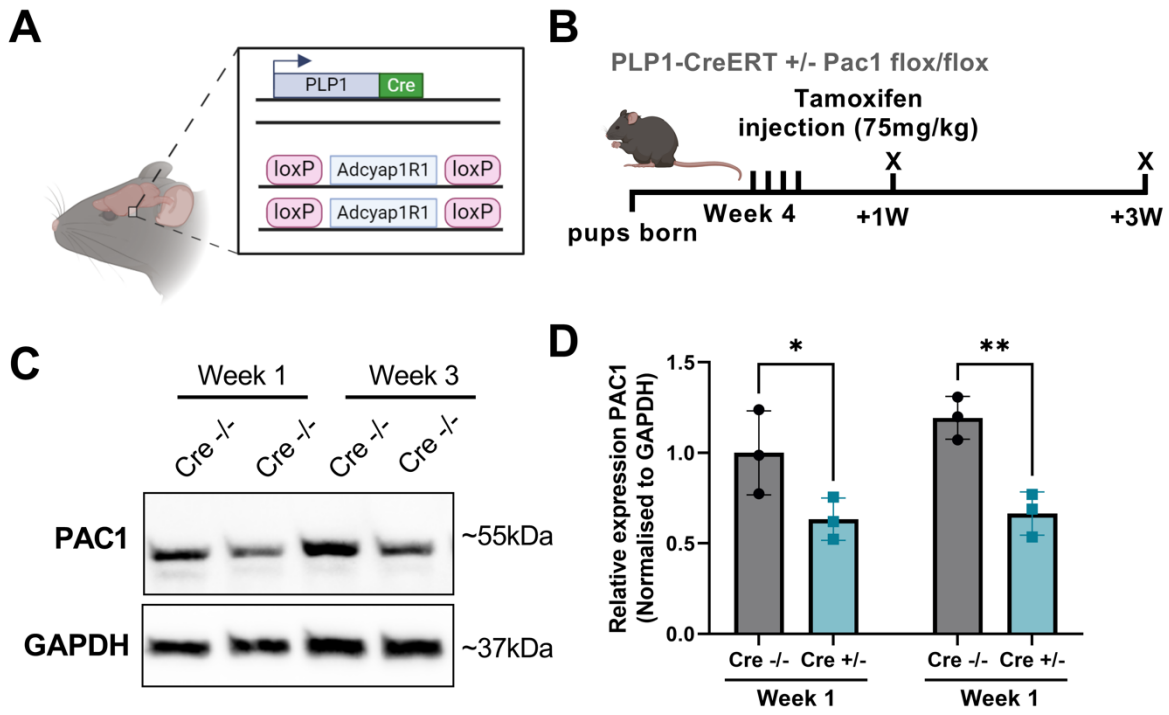


Figure 6.3 Validation of PLP1-driven PAC1 cKO depletion in the white matter of generated PLP1-CreERT$^{+/-}$ PAC1$^{flox/flox}$ mice. *(A) Schematic overview of the final genotype generated through our breeding*

*strategy. (B) Visualisation of the experimental timeline, where 4-week-old mice (PLP1-CreERT$^{+/-}$ PAC1$^{flox/flox}$ or PLP1-CreERT$^{-/-}$ PAC1$^{flox/flox}$ received four tamoxifen injections (75mg/kg) to induce PAC1 KO. The brain was collected and processed for Western blot analysis one week and three weeks post final injection. (C) Representative blots of PAC1 and reference protein GAPDH. (D) Quantification shows significant depletion of PAC1 in PLP1-CreERT$^{+/-}$ mice one-week post injection (*p<0.05), with the effect persisting 3 weeks post induction (**p<0.01).*

PLP1 = proteolipid protein 1, PAC1 = pituitary adenylate cyclase activating polypeptide receptor 1, cKO = conditional knock out. +1W = 1 week, +3 week = 3 weeks

6.5 Discussion

Studies on PACAP-PAC1 signalling in the white matter *in vivo* have been limited by the inability for researchers to study the role of PAC1 exclusively in mature oligodendrocytes. Therefore, we have generated a world's first oligodendrocyte-specific PAC1 conditional KO model using the PLP1 promoter as the driver for Cre recombination. This unique model can be used to study the role of PAC1 in oligodendrocyte functions in the brain under healthy and diseased conditions.

Although further validation experiments of this new model are needed, we show that PAC1 can be depleted from the white matter of mice in a temporally controlled manner. Since PLP1 expression peaks in mature oligodendrocytes at post-natal day 28 (P28) and remains stable afterwards, inducing PAC1 depletion after this day allows for precise loss of PAC1 in mature oligodendrocytes only [695,696]. Inducing the cKO prior to P28 could result in more widespread Cre activation outside of oligodendrocyte lineage cells, making it important for future research to closely consider the timepoint of induction [695]. A critical step in the validation of a newly generated Cre recombinase mouse models is through cross-breeding the mice with a *reporter* mouse line [697]. Although not possible within the timeline of my PhD, future work ought to validate the spatial location of our Cre activity in mature oligodendrocytes with the help of a reporter mouse [698]. This is important because PLP1-CreERT, albeit minimally, could also target Schwann cells, which could impact any interpretation of behavioural characterisation of these mice [694].

To be able to validate the spatial location of the Cre activity in our PLP1-CreERT$^{+/-}$-PAC1$^{flox/flox}$ mice, animals should be crossed with a *reporter* mouse strain to trace Cre activity in the CNS

and PNS. For example, a common and well-established *reporter* line to trace this activity is to utilise the ROSA$^{nT\text{-}nG}$ (B6N.129S6-Gt(ROSA)26Sortm1(CAG-tdTomato*,-EGFP*)Ees/J) *reporter* mouse line [699]. By crossing our PLP1-CreERT$^{+/-}$-PAC1$^{flox/flox}$ with ROSA$^{nT\text{-}nG}$ mice, there will be ubiquitous tdTomato expression throughout all cells in Cre naïve mice which, upon Cre activation, will switch to eGFP expression. Thus, we expect to observe green fluorescence only in cells expressing PLP, whereas the other cells in the CNS will remain red fluorescent (tdTomato+). This prediction could subsequently easily be confirmed with the help of immunofluorescence.

Another key point to keep in mind for the maintenance of this colony is to only crossbreed PLP1-CreERT$^{+/-}$-PAC1$^{flox/flox}$ with CreERT2$^{-/-}$ PAC1$^{flox/flox}$ mice, thereby maintaining homozygosity of the PAC1 flox gene in the progeny. Additionally, this would provide the certainty that the PLP-CreERT construct can only be present as heterozygous. By keeping Cre heterozygosity, the chances of off-target activity of Cre can be considerably reduced, thereby preventing unwanted recombination events as well as avoid cellular toxicity induced by high Cre expression [700-702].

The experimental opportunities with these mice are vast. A potential ideal future experiment would be to expose these mice to the same demyelinating conditions similar to the Camk2a-CreERT2$^{+/-}$ PAC1$^{flox/flox}$ mice from my two previous chapters and compare responses (see **Chapter 4&5**). After inducing the cKO at P28 and the standard 3-week washout to clear any residual tamoxifen, first the baseline effects of PLP-driven PAC1 depletion can be measured using the Open Field and Rotarod. Following a 4-week CPZ-induced demyelination paradigm, locomotion should be reassessed, and the brain studied for molecular effects. Firstly, oligodendrocyte numbers and health could be measured using *Olig2*, Quaking7 (CC1) and ASPA staining. Since Camk2a-driven PAC1 depletion caused loss of mature oligodendrocytes, it would be interesting to study the finding in the opposite direction. Namely, does loss of oligodendrocyte-specific PAC1 impact neuronal health? The answer to this question could provide a starting point for a plethora of studies examining neuron-oligodendrocyte communication through PAC1 signalling.

To summarise, I have been able to successfully generate a PLP1-driven PAC1 cKO mouse model using the Cre-LoxP system. Inducing the cKO with tamoxifen was able to deplete PAC1

levels in the white matter of PLP1-CreERT2$^{+/-}$ PAC1$^{flox/flox}$ mice paving the way for future research examining the role of the PAC1 signalling axis in mature oligodendrocyte functions.

Chapter 7:
General Discussion

7.1 Summary of main findings

The limited treatment options available for individuals with MS highlight the urgent need for further research into alternative therapeutic strategies to combat this chronic disease. As outlined in my introduction, the VIP/PACAP neuropeptide system has been proposed as a promising target to address the underlying pathology of MS. Especially since research has shown a beneficial role for this neuropeptide system in both MS-associated molecular pathology (**reviewed in *1.1.3)*** as well as targeting the comorbidities of the disease (**reviewed in *1.1.4*).** However, research so far has mostly focussed on the role of this neuropeptide system and the peripheral immune system in MS. We currently lack knowledge about the role of these neuropeptides and their respective receptors during the demyelination process of the disease, as well as their effect on neuronal function and neuroinflammation. Therefore, the aim of my thesis was to further dissect the role of the VIP/PACAP system in MS, specifically focussing on the role of the neuropeptide system in the CNS.

An overview of all findings outlined in this thesis can be found in Figure 7.1. First, we studied the expression pattern of the VIP/PACAP system in the NAWM of human MS donors and found a distinct difference in the expression levels of PAC1, VPAC1 and VPAC2 receptors in the white matter of progressive MS patients (**chapter 2**). Specifically, the unique pattern of decreased PAC1 expression in progressive MS struck our interest and made us want to dissect the contribution of this receptor specifically during demyelination (**chapter 3**). Targeting the PAC1 receptor during cuprizone-induced demyelination prevented demyelination, reduced neuroinflammation and exerted neuroprotective effects. Since neurodegeneration is correlated with worsened disease progression in MS [503,566,703], we further explored the role of PAC1 in neurons using an inducible/conditional KO mouse model (**chapter 4**). Through these studies, we found that the PAC1 receptor plays an essential role in controlling dendritic spine formation in Camk2a^{+} neurons of the cortex and hippocampus. Additionally, cuprizone-induced demyelination caused exacerbated axonal loss in PAC1 cKO mice and aggravated the loss of mature oligodendrocytes. These results imply the importance of PAC1 signalling in the crosstalk between neurons and oligodendrocytes (**chapter 5**). To further explore the role of the PAC1 receptor in oligodendrocytes, we generated an oligodendrocyte-specific conditional KO mouse model using the advanced CreERT-LoxP system (**chapter 6**). This novel transgenic mouse model can be utilised in future studies to examine *in vivo* the specific role of PAC1 in oligodendrocytes.

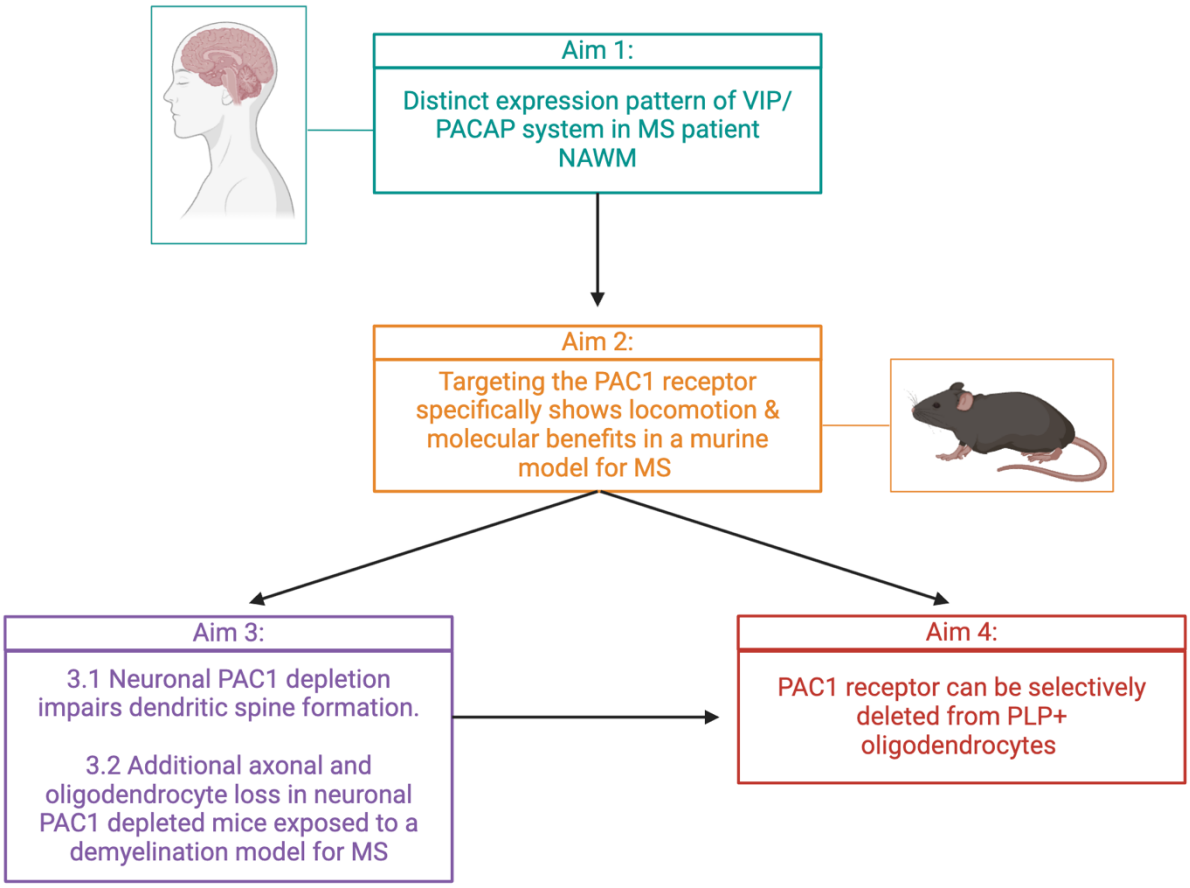


Figure 7.1 Summary of the main findings reported in the thesis.

7.2 Potential of PAC1 agonists as therapeutic targets in MS and other demyelinating diseases

As highlighted above, the overarching consensus of the work presented in my thesis demonstrates the potential of PAC1 as a therapeutic target to prevent demyelination. While previous studies have identified a plethora of beneficial effects of the VIP/PACAP system that are linked to the peripheral activity in MS [187,191,193], my work finds strong and positive effects the receptor in the CNS.

Current therapeutic strategies to combat MS progression focus on supressing the immune system to prevent disease progression [55,507]. While these treatments help reduce relapse rates and slow disease progression, they do not directly address the primary issue in MS development: the loss of oligodendrocytes. PAC1 activation seems to prevent the loss of oligodendrocytes during cuprizone-induced demyelination (**chapter 3**). In line with the loss of PAC1 seen in the NAWM of progressive MS cases (**chapter 2**), early administration of a PAC1

agonist – which restores PAC1 expression to healthy levels, may potentially prevent the myelin deterioration and oligodendrocyte loss in the NAWM of people afflicted with MS.

Targeting PAC1 activation could also promote remyelination in people with MS. This is especially true considering how PACAP treatment has been shown to promote remyelination following nerve injury [211]. The anti-inflammatory effects of PAC1 signalling could also promote oligodendrocyte proliferation and support myelin formation since inflammation, although indirectly, can hinder the maturation of oligodendrocyte progenitor cells, preventing proper generation of newly differentiated oligodendrocytes that can remyelinate the CNS [704].

However, to my knowledge, there are currently no published studies investigating the role of PAC1 signalling during remyelination. Therefore, a critical and much needed set of investigations is to harness the full potential of the CPZ model to explore how PAC1 affects the remyelination process. One key strength of the CPZ model is that, following a demyelination insult of 4 weeks, mice that are returned to a normal diet spontaneously remyelinate almost completely in a period ranging from 2 to 4 weeks [206,542,705]. To build upon my findings from **chapter 3**, it would be interesting to examine the remyelinating properties of PAC1 activation using the remyelination properties of the CPZ model. This could expand the therapeutic significance and our knowledge of the positive effects of targeting PAC1 receptors to promote myelin repair in MS.

It is worth noting that the impact of the findings shown in my thesis may extend beyond PAC1 therapeutic potential in MS. There are several other white matter diseases where targeting PAC1 could elicit beneficial effects. For example, in amyotrophic lateral sclerosis (ALS), a fatal disease characterised by the degeneration of motor neurons with associated white matter abnormalities [706,707], patients could potentially benefit from a PAC1-targetting therapy. In ALS patients, similarly to MS, disruptions in oligodendrocyte functions have been observed prior to axonal loss or neurodegeneration [708]. In parallel, similar mechanisms underlying WM damage in MS and ALS have been proposed [707]. Therefore, it is not improbable that targeting PAC1 could be beneficial in ALS, especially since ALS is also characterized by neuroinflammation and disease progression is driven by neuronal degeneration [706,709]. Therefore, targeting the PAC1 receptor could have similar broad protective effects in the nervous system of ALS patients similarly to what we see in MS. Although the role of the VIP/PACAP system in ALS is not well-understood, an interesting paper showed how in an *in-vitro* model of human induced

Pluripotent Stem Cells (iPSC)-derived motor neurons undergoing degeneration, exogenous PACAP treatment prevents apoptosis [710]. Further research on the role of PAC1 signalling in preclinical models of ALS is warranted to provide the critical proof-of-concept data needed to establish if targeting the PAC1 receptor could be protective in the CNS of ALS patients.

There are also several demyelinating diseases where the role of PAC1-mediated signalling should be better understood. For example, in Neuromyelitis Optica Spectrum Disorders (NMOSD), targeting the PAC1 receptor could provide beneficial effects in the CNS. These patients suffer from an inflammatory autoimmune disease of the CNS, associated with astrocyte dysfunction, demyelination and neurodegeneration in the spinal cord and optic nerve [711]. In these people, there is an immune-attack against Aquaporin-4 (AQP-4), a water channel highly expressed in astrocytes, which leads to a disruption in cellular homeostasis causing astrogliosis, oligodendrocyte and neuronal damage [712]. Since our anti-inflammatory effects triggered by PAC1 activation in the CNS were mostly mediated by astrocytes and could also prevent neuronal damage and oligodendrocyte loss (**chapter 3**), it is conceivable that targeting PAC1 activation could be beneficial in preventing demyelination in NMOSD as well as other early onset demyelinating diseases with strong underlying inflammatory and neurodegenerative components such as Acute Disseminated Encephalomyelitis or even acute transverse myelitis [713,714].

7.3 Neuronal function and the PAC1 receptor

In **chapter 4**, my work demonstrates that loss of PAC1 in Camk2a expressing neurons significantly reduces dendritic spine density in the hippocampal CA1 region. Dendritic spine density is a measurement that reflects excitatory synaptic connections in neurons, underlying memory formation in the hippocampus [715]. Thus, PAC1 signalling is critical for proper neuronal functioning and the maintenance of established synaptic connections within the hippocampus and cortex.

The dynamic mechanism underlying the modulation of synaptic connections is named synaptic plasticity [716]. This process drives important functions allowing us to adapt and learn from experiences by modulating the brains neuronal circuit. Since my work showcases the structural/static synaptic changes caused by PAC1 depletion *in vivo,* an important future experiment would be to examine the dynamic/functional changes in synapse loss/formation in

these mice using live-cell imaging. Live-cell imaging is a high-end, well-established tool that allows for the continuous capturing of mainly *in-vitro* cultured cells [717]. Therefore, an ideal future experiment to complement my findings on synapse morphology and numbers from **chapter 4** would be to study the effect of induced PAC1 depletion *in vitro* using hippocampal neuronal cultures (Figure 7.2). By culturing Camk2a+ neurons isolated from the Camk2a-CreERT2$^{+/-}$Pac1$^{flox/flox}$ mice and inducing PAC1 depletion using tamoxifen at different time-points, we can study the effect of this on synapse development in real-time under healthy as well as degenerating conditions. Utilising IMARIS live-cell analysis tools, we in turn can measure and quantify these dynamic changes in synaptic morphology in detail.

In the same line, live-cell imaging could also be used to trace mitochondria in these same cultured hippocampal neurons, since one of my other main findings in **chapter 4** was reduced mitochondrial density in the dendrites of PAC1 depleted neurons *in vivo*. Mitochondria are crucial for proper neuronal morphometric functioning [661,718]. Therefore, an equally important follow-up experiment would be to assess mitochondrial motility in these neurons as well and study the effect of PAC1 on this process. By utilising the build-in expression of Thy1-YFP and Thy1-mitoCFP of the Camk2a-CreERT2$^{+/-}$Pac1$^{flox/flox}$ mice, both questions regarding synapse morphology and mitochondria motility alterations under healthy and degenerating conditions can be answered in detail by culturing these cells *in-vitro* combined with live-cell imaging.

The functional implications of PAC1 depletion on neuronal connectivity remain unknown. Functional synaptic connectivity is often studied within the context of -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and N-methyl-d-aspartate (NMDA) mediated long-term potentiation (LTP) [719]. Long-term potentiation (LTP) is defined as the strengthening of synaptic connections upon prolonged signal transduction [720]. The change in LTP is measured using electrophysiological tools, quantifying current changes in neurons in the pre- and post-synapse [721,722]. To complement my morphological findings on dendritic spine plasticity in PAC1 depleted Camk2a+ neurons, an important follow-up experiment would be to perform *in vitro* and *ex vivo* patch-clamp recordings on our Camk2a-CreERT2$^{+/-}$Pac1$^{flox/flox}$ mice. With this approach, we can determine the effects of PAC1 depletion on the electrical current in the post-synapse of hippocampal Camk2a+ neurons. Since PACAP treatment enhances AMPA and NMDA-mediated excitatory postsynaptic potential (EPSP) [723-725], it is possible that the removal of PAC1 could have detrimental effects on LTP formation in hippocampal neurons. Thus, any live-cell and electrophysiological studies would complement

the morphological findings on the role of PAC1 and synapse density in the hippocampus within the complex environment of the brain.

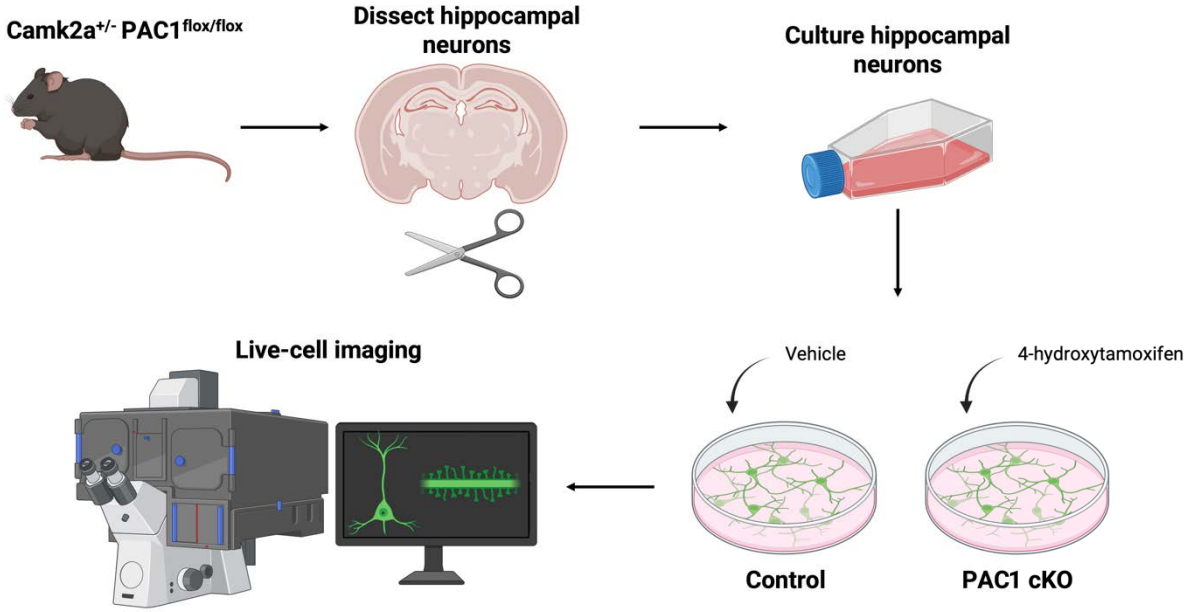


Figure 7.2. Experimental outline to study synaptogenesis in PAC1 cKO neurons in vitro using live-cell imaging.

7.4 The emerging role of PAC1 in neuron-oligodendrocyte crosstalk

In recent years, it has become clear that bidirectional communication between neurons and oligodendrocytes is crucial for oligodendrocyte differentiation and maturation, as well as maintaining axon integrity [726,727]. Neuronal activity and motor learning regulate myelination [728-730]. Additionally, oligodendrocytes express several molecules that establish synapse-like connections between axons such as NMDA receptors, potassium ion channels and cell-adhesion molecules, that influence myelin sheath formation [731-734]. Neuronal activity can also stimulate the release of exosomes from oligodendrocytes that are, in turn, taken up by neurons to induce protective effects by preventing oxidative stress and starvation [735]. In the NAWM of MS patients, impaired adhesion has been seen in sites of axon-myelin blistering, linking compromised neuron-oligodendrocyte communication to early phases of CNS damage [688].

My work shows that loss of neuronal PAC1 can reduce oligodendrocyte numbers in the brain, providing further evidence of the importance of proper communication between the two cell types (**chapter 4**). Oligodendrocytes express and release PACAP, which could interact with neuronally expressed PAC1 [736,737]. Vice versa, oligodendrocytes express PAC1 and thus could respond to neuronally released PACAP (**chapter 2 & 5**) [265]. Therefore, with the current availability of Camk2a-CreERT2$^{+/-}$-PAC1$^{flox/flox}$ and PLP1-CreERT2$^{+/-}$-PAC1$^{flox/flox}$ transgenic mouse lines, there are several exciting future experiments that could be designed to establish the role of the PAC1 receptor in the bi-directional communication between neurons and oligodendrocytes.

Firstly, any microscopic impact on white matter in mice lacking PAC1 in oligodendrocytes and neurons ought to be investigated. Using electron microscopy, ultrastructural changes in myelin conformation may be detected in myelinated axons of transgenic mice both at baseline and after a demyelinating challenge. Specifically, the 'g-ratio', which reflects the ratio of the inner and outer diameter of the myelin sheath, is a reliable measurement of the degree of myelination of axons that warrants additional investigations [738-740].

Secondly, we could examine the electrophysiological effects of neuronal PAC1 depletion in oligodendrocytes. Vice versa, we can study the effect of oligodendrocyte PAC1 depletion on neuronal electrophysiological properties. Both approaches provide valuable insights into the role of PAC1 in both cell types and their respective role on maintaining cellular conductivity and homeostasis. This can be further achieved using *ex vivo* patch clamping experiments. Oligodendrocytes and/or neurons could be patched in both neuronal PAC1 cKO brain slices and oligodendrocyte-targeted PAC1 cKO brain slices where the effects on calcium (Ca^{2+}) currents can be measured. Changes in oligodendrocyte Ca^{2+} dynamics play a role in proliferation, differentiation and myelination of these cells [741,742]. Whereas neuronal Ca^{2+} spikes provide insights into synaptic plasticity and signal integration [743]. Performing this experiment would provide a comprehensive electrophysiological evaluation of the bi-directional effects driven by the PAC1 receptor in oligodendrocytes and neurons.

7.5 Limitations of current studies

Currently, the prevention of disability progression in MS is a main focus in therapy development [93,95,541]. Disease progression is strongly associated with axonal damage and

neuronal loss due to prolonged demyelination and limited remyelination abilities of oligodendrocytes[503]. Therefore, large efforts are now being made to find druggable targets promoting remyelination in the CNS.

Although my work shows the key protective functions of PAC1 during demyelination, these findings do not delineate the contribution of the receptor in remyelination. This would be the main limitation of the work outlined in my thesis. As highlighted in section 7.2, the CPZ model provides the unique opportunity to study active demyelination during the first 4-5 weeks of CPZ diet, followed by spontaneous remyelination in the 2-4 weeks following CPZ-diet cessation. Since my work shows protective effects of PAC1 signalling during demyelination, it would be important to determine whether these effects translate into accelerated remyelination during the spontaneous recovery phase. To test this hypothesis, mice should receive the CPZ diet for four weeks to induce demyelination. Afterwards, mice would be placed back on a standard diet and receive injections of PACAP, VIP or Maxadilan every other day. Receiving neuropeptide injections after a prolonged demyelinating insult and the start of spontaneous remyelination will help investigate whether the PACAP/VIP system could be targeted to promote remyelination and oligodendrocyte proliferation *in vivo*. Using the previously used Open Field and Rotarod tests, we could assess any functional outcomes of improvements by any enhanced remyelination. This provides an indication whether this treatment could lead to clinical improvement in people with MS. Additionally, studies on oligodendrocyte number and myelin production, as well as any neuroprotective effect should be measured using immunohistochemistry and electron microscopy as it is crucial to see what molecular effects the treatment has on CNS cells during remyelination.

Lastly, examining transcriptomic and/or proteomic changes after complete demyelination and during different remyelination stages in these mice could provide critical information on the downstream intracellular pathways targeted by PAC1 activation during demyelination and at different stages of spontaneous remyelination. These data would offer us a clearer understanding of the changes in the molecular landscape that occur in the presence or absence of the PAC1 receptor in oligodendrocytes and unveil the pathways that mediate its protective effects *in vivo*.

7.6 Future perspectives

Ideally, future research should focus on the role and efficacy of PAC1 specific agonists in demyelinating and neurodegenerative diseases. My work, and those of others, have unveiled some promising results regarding to PAC1 activation in the CNS in the context of several neurological diseases, since its activation reduces clinical signs of disease, axonal damage and diminishes inflammation [187,298,302,597]. However, many questions regarding the fundamental functions of PAC1 signalling and the translatability of PAC1 agonists in clinic remain to be answered. Expanding on the broad protective functions of PAC1-signalling outside demyelinating diseases, PAC1 agonists should also be further explored in other neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease or ALS [190,744].

An important point to consider for future research into the role of PAC1 agonists in demyelinating and neurodegenerative diseases is the delivery mode. To avoid off-target effects, systemic breakdown of drugs by the body, and circumvent any limitation imposed by biological barriers such as blood-brain barrier (BBB) permeability, the establishment of an optimal drug-delivery route is a critical factor to consider if/when implementing PAC1 agonists for the treatment of neurological diseases. The BBB prevents around 98% of small organic compounds from entering the CNS and is a major hurdle in drug development [745,746]. While PACAP and VIP are able to cross the BBB, a large percentage of infused peptides gets metabolised before it reaches the brain [588,747]. With the help of recent sophisticated developments in precise nanoparticles for drug delivery, PAC1 agonist could be delivered exactly at the location in the brain where it is needed [748]. This is especially important since PAC1 antagonists have proven beneficial effects in a different set of neurological conditions, such as for neuropathic pain conditions and combatting migraines [455,459]. Giving systemic PAC1 agonists could thus lead to potential unwelcome side-effects in the nervous system. Therefore, precise PAC1 agonists and specific drug delivery modes are to be trialled to minimise any off-target effects in patients.

Additionally, the field has been limited in their ability to study the role of PAC1 in oligodendrocytes due to the lack of available tools. Now, with the development of PLP-CreERT$^{+/-}$PAC1$^{flox/flox}$ mice, research into the oligodendrocyte-specific functions of PAC1-mediated signalling can be studied. This high-end tool can help us unravel if the absence of PAC1 alters oligodendrocyte homeostasis as well as any downstream effects on neuronal functioning.

7.7 Concluding remarks

In summary, we demonstrate, using human MS patient brain tissue and animal models of MS, the involvement of the PAC1 receptor in MS CNS pathology. PAC1 expression is dysregulated in the NAWM of MS patients, while activating the PAC1 receptor shows broad protective effects in the CNS of demyelinating mice. We show that targeting PAC1 prevents oligodendrocyte loss, attenuates neuroinflammation and limits axonal damage. Additionally, using an inducible/conditional knockout model targeting neuronal PAC1 expression, we provide strong evidence for the importance of the PAC1 receptor as a modulator of synaptic plasticity in hippocampal and cortical neurons. Under demyelinating conditions, my work shows how neuronal PAC1 impacts the number of mature oligodendrocytes in the corpus callosum. Moreover, to facilitate studies addressing the role of PAC1 in oligodendrocytes, we generated a PLP-driven conditional PAC1 knockout genetic mouse model. This unicum will allow future research to truly dive into the role of PAC1 in oligodendrocyte health and function as well as, in combination with the Camk2a-CreERT2$^{+/-}$ PAC1$^{flox/flox}$ model, allow for the ability to perform sophisticated studies on the bi-directional role of PAC1 in the maintenance of healthy neuron-oligodendrocyte interactions.

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