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MITIGATING NEUROINFLAMMATION: NEW POTENTIAL THERAPEUTICS AND TARGETS

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Mitigating Neuroinflammation: New Potential Therapeutics and Targets

Thesis for Doctoral Degree (PhD)

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For Cara and Jude

POPULAR SCIENCE SUMMARY OF THESIS

With larger proportions of populations around the world aging, the burden of chronic neurodegenerative disorders such Alzheimer's disease (AD) and Parkinson's disease (PD) is also increasing. The lack of treatments for neurodegenerative disorders makes the repurposing of pharmaceuticals already in use a potentially faster route to bringing drugs to market for those who need them most. Glucagon like peptide-1 (GLP-1) based receptor agonists (incretin mimetics), commonly used to treat type-II diabetes mellitus (T2DM) and recently for weight loss, and Rho-associated coiled-coil containing kinase (ROCK) inhibitors, used for glaucoma treatment, are two such drug classes. Incretin mimetics are much further along in the process for repurposing, but both present great promise as potential neurodegenerative therapies.

The incretins, including GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), are normally produced postprandially to regulate blood sugar, but in T2DM, their function is often impaired. Glucagon (Gcg), another peptide involved in blood sugar regulation, is also dysregulated in T2DM. Interestingly, diabetes is one of the greatest risk factors for developing a chronic neurogenerative disorder, leading scientists to believe there is a clear connection between metabolic disease and brain health. Early studies with Exenatide, the first US Food and Drug Administration (FDA) approved incretin mimetic, proved this drug class had a protective function in the brain and accelerated research into their potential utility in treating nervous system dysfunction. Whereas incretins act through many different cellular pathways, ROCK inhibitors target the two isoforms of the ROCK protein, ROCK1 and ROCK2. ROCK is elevated in chronic neurodegenerative diseases and its activation within the brain prevents neuron repair. Endogenous ROCK activation occurs not exclusively, but prominently, through the Nogo signaling system, which in a healthy brain, helps maintain stable neuronal connections.

Although neurons are typically the cells of the nervous system that receive the most attention, microglia, once thought to just be the "glue" of the brain, are increasingly being found to play very important roles in health and disease. In a healthy brain, microglia are evenly tiled throughout the tissue and make up around 10% of total cells. As the resident immune cells of the brain, they perform routine homeostatic functions such as clearing waste, providing metabolic support to other cells, and shaping neuronal connections. However, during pathological conditions, microglia respond by performing a suite of other roles to contain damage, including the initiation of the inflammatory response and clearing of damaged tissue. Often, this process results in damage to healthy tissue that can further exacerbate a condition or disease. Therefore, balancing the benefits of microglial activation to the potential damaging effects is essential for the most positive prognosis.

One common symptom and potential cause of neurodegenerative disorders is microglial induced neuroinflammation, making this an ideal target for any drug therapy, including incretin mimetics and ROCK inhibitors. Incretin mimetics are now undergoing human clinical trials for PD, AD, and other neurological disorders, while ROCK inhibitors have entered clinical trials for treating amyotrophic lateral sclerosis (ALS), a disease that affects neurons controlling motor function. ROCK inhibitors have yet to enter trials for treating other more common neurodegenerative diseases such as AD and PD, although these current human trials highlight growing interest in both drug classes. While these studies are ongoing, preclinical science, including both cellular and animal models such as those performed in the current thesis, is essential to understand 1) which formulations of these and other drugs have the highest brain uptake, crossing the blood brain barrier (BBB); 2) mechanisms of action, including

activation or inhibition of key cellular pathways; and 3) overarching phenotypes, both behavioral and pathological, resulting from the drug treatment or genetic manipulation.

In the current thesis, I utilized cellular models of microglia and rodent models of brain injury to understand pharmacologic and genetic underpinnings of neuroinflammation. The injury model used throughout my projects involves the induction of traumatic brain injury (TBI) in mice. TBI is an excellent model of neurodegeneration because it can be performed rapidly and replicates many features of neurodegenerative disease, including neuronal cell death and activation of microglia. In addition, TBI remains one of the most prevalent causes of injury and death throughout the world, making it an important public health issue to study in parallel to chronic neurodegenerative conditions. Each constituent paper of my thesis incorporates microglial responses to either drug treatment or genetic manipulation. In two studies, we investigate new incretin mimetic formulations, including a slow release formulation of Exenatide, a common diabetes medication, and a unimolecular triagonist that targets GLP-1, GIP, and Gcg receptors. In another study, I sought to understand if the circulating metabolite of GLP-1 had any nervous system function, adding to the current understanding of how the properly functioning incretin signaling system may affect brain health. In the last two studies, I investigated how ROCK inhibition affects microglial activation in cell culture and identified for the first time roles of microglial Nogo, an endogenous ROCK activator, in delaying recovery from brain injury.

Overall, my thesis advances understanding and potential utility of using incretin mimetics and ROCK inhibitors to blunt neuroinflammation. In addition, I show a novel role of the Nogo signaling system in microglia. Across all model systems used, I show anti-inflammatory effects of drug and genetic manipulation. Lastly, I have advanced the use of a new immortalized microglial cell line (IMG cells), that can potentially be used as a platform for screening new drugs that target microglia, without the use of live animals.

ABSTRACT

Neurodegenerative disorders, from chronic diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) to more acute injuries such traumatic brain injury (TBI), present a particular challenge to public health as they lack treatments and have devastating consequences. Across the variety of central nervous system (CNS) diseases and injuries, inflammation is a hallmark and potential target for effective therapies. Microglia, the immune cells of the brain, normally perform homeostatic maintenance roles in the brain; however, in cases of injury and disease, they are often dysfunctional and produce excessive quantities of pro-inflammatory proteins that exacerbate conditions. This makes microglia a prime target for intervention. As there is a current lack of drugs available to treat brain injuries and diseases, repurposing clinically approved medications may be the fastest route for new drugs to reach patients. Understanding cellular mechanisms and proteins that mediate inflammation are valuable for the development of new therapies. Overall, this thesis aims to evaluate several drug classes, including incretins and Rho-associated coiled-coil containing kinase (ROCK) inhibitors, which have potential for repurposing from their approved uses to treating neurodegenerative disorders. Herein, novel cellular mechanism and proteins that induce neuroinflammation are also investigated.

In this thesis, the incretins, which are endogenous insulin regulating hormones currently used to treat diabetes, and ROCK inhibitors, clinically used for glaucoma, are evaluated in different injury and disease models to understand their ability to affect brain health. With these studies, we evaluate microglia as a primary source of inflammation and utilize both cellular and rodent models of neuroinflammation. Our cellular studies leverage the potent inflammatory properties of lipopolysaccharide (LPS) to mimic *in vivo* neuroinflammation observed in our rodent models of mild-moderate traumatic brain injury (mTBI), which are also used throughout this thesis. We apply pharmacologic or genetic manipulations to understand how pathology progresses with the intervention, assessing proinflammatory protein production and microglial activation via enzyme-linked immunosorbent assays (ELISA), electrochemiluminescence, and immunochemistry, among other methods.

Incretin effects on the brain are the focus of **Papers I-III**. In **Paper I**, we investigate the pharmacokinetics and efficacy of PT302, a new slow-release formulation of the GLP-1 mimetic, Exenatide. Exenatide was initially approved for clinical use as a twice daily subcutaneously administered drug. We demonstrate steady-state plasma levels of Exenatide formulated as PT320 for up to three weeks after a single subcutaneous injection, with brain penetration reaching up to 2 to 3% of peak plasma concentrations, which is reasonable for a peptide-based drug and appears to be within its pharmacologically relevant range. Notably, PT302 administered at a dose that is translatable to human use is anti-inflammatory in the mouse brain, as we demonstrated the drug's ability to decrease microglial activation following a mTBI injury. Behavioral deficits were also mitigated by PT302. For the next study (**Paper II**), we also utilized the mTBI model to investigate efficacy of a novel monomeric incretin-based triagonist, which showed similar effects as PT302 at mitigating behavioral deficits and inducing cellular signaling pathways that are neuroprotective. Additionally, I introduce a primary microglia (PMg) cell model for neuroinflammation and show potent anti-inflammatory potential for the triagonist. The last of the incretin studies (**Paper III**) utilizes cellular models of neurodegeneration and neuroinflammation to understand the physiological roles in the nervous system of the GLP-1 metabolite, GLP-1(9-36). This work has relevance as GLP-1(9-36) circulates in the bloodstream long after its insulinotropic parent peptide is inactivated. Our work indicates this metabolite, indeed, has physiologic

roles in nervous system cells, including microglia. In this study, I utilized the relatively new immortalized (IMG) mouse microglial cell line as a model system for PMg.

The Nogo-signaling system, known for its neuronal plasticity restricting properties via RhoA and ROCK1 and ROCK2 activation, has recently been implicated in microglial inflammation processes. In **Paper IV**, we sought to elucidate the roles that RhoA and ROCK play in influencing the inflammatory process. We show that ROCK inhibitors potently inhibit LPS induced inflammation via a variety of mechanisms in both primary and IMG microglia. In parallel to the inquiry of ROCK influence on inflammation, this study serves as a "proof of concept" for the utility of using IMG cells in preclinical drug development and screening.

Lastly, in **Paper V,** we investigated the effects of microglial conditional deletion of a potent endogenous activator of RhoA/ROCK signaling in nervous system cells, Nogo. I developed a novel mouse model to perform this genetic manipulation and applied a moderate TBI injury, via a controlled cortical impact (CCI) injury to exposed brain tissue. Mice with conditional deletion of microglial Nogo (MinoKO mice) show signs of decreased microglial and astrocytic activation following CCI. CCI injured mice with microglial specific Nogo deletion exhibited hyperactivity among other phenotypes post-injury. MinoKO mice did not exhibit asymmetric motor function one week post-CCI as their control cohorts did, thereby providing further evidence of microglial Nogo negatively influencing recovery.

Overall, the studies within this thesis provide strong evidence for the utility of using incretins to treat neurodegenerative conditions. In addition, the current studies elucidating novel roles of RhoA, ROCK, and Nogo in microglial-induced inflammation add to the growing list of potential drug targets that may mitigate disease pathology.

SCIENTIFIC PAPERS INCLUDED IN THESIS

I. Bader, M., Choi, H.-I., Rubovitch, V., Li, Y., Kim, H.K., **Glotfelty, E**., Pick, C.G., Lecca, D., Hoffer, B.J., Kim, D.S., Tweedie, D., Greig, N.H., Rachmany, L. (2018). Pharmacokinetics and efficacy of PT302, a sustained-release Exenatide formulation, in a murine model of mild traumatic brain injury. *Neurobiology of Disease*. 124, 439–453.

II. Li, Y., **Glotfelty, E.J**., Namdar, I., Tweedie, D., Olson, L., Hoffer, B.J., DiMarchi, R.D., Pick, C.G., Greig, N.H. (2020). Neurotrophic and neuroprotective effects of a monomeric GLP-1/GIP/Gcg receptor triagonist in cellular and rodent models of mild traumatic brain injury. *Experimental Neurology*. 324, 113113.

III. Li, Y., **Glotfelty, E.J**., Karlsson, T., Fortuno, L. V., Harvey, B.K., Greig, N.H. (2021). The metabolite GLP-1 (9-36) is neuroprotective and anti-inflammatory in cellular models of neurodegeneration. *Journal of Neurochemistry*. 159, 867–886

IV. **Glotfelty, E.J**., Tovar-Y-Romo, L.B., Hsueh, S., Tweedie, D., Li, Y., Harvey, B.K., Hoffer, B.J., Karlsson, T., Olson, L., Greig, N.H. (2023). The RhoA-ROCK1/ROCK2 pathway exacerbates inflammatory signaling in immortalized and primary cells microglia. *Cells*. 12, 1367

V. **Glotfelty, E.J.**, Hsueh, S., Bedolla, A., Karlsson, T., McGee, A., Luo, Y., Greig, N.H., Olson, L. (2023). Microglial Nogo delays recovery following traumatic brain injury in mice. *Manuscript*

Related Reviews and Research Not Included in Thesis

I. Chen, S.; Yu, S.-J.; Li, Y.; Lecca, D.; **Glotfelty, E.J**.; Kim, H. K.; Choi, H.-I.; Hoffer, B. J.; Greig, N. H.; Kim, D.-S., Wang, Y. (2018). Post-Treatment with PT302, a Long-Acting Exendin-4 Sustained Release Formulation, Reduces Dopaminergic Neurodegeneration in a 6-Hydroxydopamine Rat Model of Parkinson's Disease. Sci. Rep. 8 (1), 10722
.

II. **Glotfelty, E.J**., Delgado, T.E., Tovar-y-Romo, L.B., Luo, Y., Hoffer, B.J., Olson, L., Karlsson, T.E., Mattson, M.P., Harvey, B.K., Tweedie, D., Li, Y., Greig, N.H. (2019). Incretin Mimetics as Rational Candidates for the Treatment of Traumatic Brain Injury. *ACS Pharmacol. Transl. Sci*. 2, 66–91

III. .**Glotfelty, E.J**., Olson, L., Karlsson, T.E., Li, Y., Greig, N.H. Glucagon-like Peptide-1 (GLP-1)-Based Receptor Agonists as a Treatment for Parkinson's Disease (2020). *Expert Opin. Investig. Drugs*, 29, 595–602

IV. Yu, S.-J., Chen, S., Yang, Y.-Y., **Glotfelty, E.J.**, Jung, J., Kim, H.K., Choi, H.-I., Choi, D.-S., Hoffer, B.J., Greig, N.H., Wang, Y. (2020) PT320, sustained release Exendin-4, mitigates L-DOPA-induced dyskinesia in a rat 6-hydroxydopamine model of Parkinson's disease. *Front. Neurosci*. 14, 785.

V. Kopp, K.O., **Glotfelty, E.J**., Li, Y., Greig, N.H. (2022). Glucagon-like peptide-1 (GLP-1) receptor agonists and neuroinflammation: Implications for neurodegenerative disease treatment. *Pharmacol. Res.* 186, 106550.

Scientific Papers Not Included in Thesis

I. Greig, N.H., Lecca, D., Hsueh, S.C., Nogueras-Ortiz, C., Kapogiannis, D., Tweedie, D., **Glotfelty, E.J**., Becker, R.E., Chiang, Y.H., Hoffer, B.J. (2020). (−)-Phenserine tartrate (PhenT) as a treatment for traumatic brain injury. *CNS Neurosci. Ther*. 26, 636-649.

II. Mcnutt, P. M., Vazquez-Cintron, E. J., Tenezaca, L., Ondeck, C. A., Kelly, K. E., Mangkhalakhili, M., Machamer, J. B., Angeles, C. A., **Glotfelty, E. J.**, Cika, J., Benjumea, C. H., Whitfield, J. T., Band, P. A., Shoemaker, C. B., & Ichtchenko, K. Neuronal delivery of antibodies has therapeutic effects in animal models of botulism. *Sci. Transl. Med.* 13(575), eabd7789.

III. Lecca, D., Jung, Y. J., Scerba, M. T., Hwang, I., Kim, Y. K., Kim, S., Modrow, S., Tweedie, D., Hsueh, S. C., Liu, D., Luo, W., **Glotfelty, E. J**., Li, Y., Wang, J. Y., Luo, Y., Hoffer, B. J., Kim, D. S., McDevitt, R. A., & Greig, N. H. (2022). Role of chronic neuroinflammation in neuroplasticity and cognitive function: A hypothesis. *Alzheimers Dement*. 18, 2327–2340.

IV. Kopp, K. O., Greer, M. E., **Glotfelty, E. J**., Hsueh, S.-C., Tweedie, D., Kim, D. S., Reale, M., Vargesson, N., & Greig, N. H. (2023). A New Generation of IMiDs as Treatments for Neuroinflammatory and Neurodegenerative Disorders. *Biomolecules.* 13(5), 747.

TABLE OF CONTENTS

List of abbreviations

AD	Alzheimer's Disease
PD	Parkinson's Disease
GLP-1	Glucagon-like peptide-1
T2DM	Type-II diabetes mellitus
ROCK	Rho-associated coiled-coil containing kinase
GIP	glucose-dependent insulinotropic polypeptide
Gcg	glucagon
FDA	Food and Drug Administration
ALS	amyotrophic lateral sclerosis
BBB	Blood-brain barrier
IMG	immortalized microglia
CNS	Central nervous system
LPS	lipopolysaccharide
ELISA	enzyme-linked immunosorbent assay
RhoA	ras homolog gene family member A
CCI	controlled cortical impact
GWAS	genome-wide association study
US	United States
GTP	guanosine triphosphate
PRR	pattern recognition receptor
TLR	toll-like receptor
DAMPs	damage associated molecular patterns
HMGB1	high mobility group box 1
NF-κB	nuclear factor kappa-B
IκB	Inhibitor of kappa-B
TNF-α	tumor necrosis factor- alpha
KC/GRO	keratinocyte chemoattractant/human growth regulated oncogene
IFNγ	Interferon gamma
CCL5	chemokine (C-C motif) ligand 5
IL	interleukin

RNAseq	RNA sequencing
Iba1	ionized calcium-binding adaptor molecule 1
ICC	immunocytochemistry
P2Ry12	P2Y purinoceptor 12
TMEM119	transmembrane protien 119
CX3CR1	CXC chemokine receptor 1
ATP	adenosine triphosphate
C1q	complement component 1q
GPCR	G-protein coupled receptor
DPP-IV	dipeptidyl peptidase-4
Ex-4	Exendin-4
NASH	Nonalcholic steatohepatitis
NAFLD	Nonalcoholic fatty liver disease
GLP-1R	glucagon-like peptide-1 receptor
GIPR	glucose-dependent insulinotropic polypeptide receptor
GcgR	glucagon receptor
cAMP	Cyclic adenosine monophosphate
LID	levodopa-induced dyskinesia
AMPK	adenosine monophosphate-activated protein kinase
LTP	long-term potentiation
MAI	myelin-associated inhibitor
MAG	myelin-associated glycoprotein
OMgp	oligodendrocyte myelin glycoprotein
RTN4	reticulon 4
Sdc3	syndecan 3
Sdc4	syndecan 4
S1PR2	sphingosine-1-phosphate receptor 2
NgR3	Nogo receptor 3
NgR1	Nogo receptor 1
Aβ	Amyloid-beta
Tg	transgenic

BDNF	brain-derived neurotrophic factor
CREB	Cyclic adenosine monophosphate response element binding protein
KO	knockout
KD	knockdown
SCI	spinal cord injury
IP	intraperitoneal
POLY (I:C)	polyinosinic: polycytidylic acid
LIMK	LIM kinase
SHH	slingshot
WD	weight drop
FPI	fluid percussion injury
ER	estrogen receptor
TAM	tamoxifen
HSP90	heat shock protein 90
MinoKO	Microglial Nogo knock-out
mTBI	mild traumatic brain injury
CSF	Cerebrospinal fluid
s.c.	subcutaneuous
PMg	primary microglia
HBSS	Hank’s Balanced Salt Solution
PFA	paraformaldehyde
OCT	optimal cutting temperature
NOR	novel object recognition
RT	room temperature
GFAP	glial fibrillary acidic protein
SR	slow release
ROS	reactive oxygen species
PKA	protein kinase A
α-syn	Alpha-synuclein
PKC	protein kinase C
MLCK	myosin light chain kinase

iNOS	inducible nitric oxide synthase
NOD1	Neucleotide-binding oligomerization domain-containing protein 1
Narc	Narciclasine
MGnD	Neurodegenerative Microglia
OFT	open field test
EPM	elevated plus maze
MWM	Morris water maze
EBST	elevated body swing test

1 LITERATURE REVIEW

1.1 Introduction

Chronic neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) to more acute injuries such traumatic brain injury (TBI), present a particular challenge to public health as they lack treatments and have devastating consequences. Across the variety of central nervous system (CNS) diseases and injuries, inflammation is a hallmark and potential target for effective therapies. Microglia, the immune cells of the brain, normally perform homeostatic maintenance roles in the nervous system; however, in cases of injury and disease, they are often dysfunctional and excessively produce pro-inflammatory proteins that exacerbate conditions. This makes microglia a prime target for intervention.

Notably, a recent genome-wide association study (GWAS) found that AD and related dementias may be driven by elevation of inflammation-related pathways as a risk for these chronic conditions.[1] Increasing evidence points to the use of incretin mimetics, many of which are approved by the United States (US) Food and Drug Administration (FDA) and parallel foreign governmental regulatory bodies for treating diabetes, as potential treatments for neurological diseases and injuries. Long-acting monomeric incretin mimetics have strong anti-inflammatory and neuroprotective properties[2,3] through the utilization of single or combined active regions of the endogenous incretins glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and glucagon (Gcg). Although neuroinflammation is widely understood as a context-dependent microglial immune response to injury or disease, some mechanisms of the response are not entirely understood. The Nogo-signaling system, known for its neuronal plasticity restricting properties via GTPase ras homolog gene family member A (RhoA) and Rho-associated coiled-coil containing protein kinases 1 and 2 (ROCK1 and ROCK2) activation, has recently been implicated in microglial inflammation processes.[4,5] Further investigation into microglial specific Nogo-signaling and its downstream components, RhoA and ROCK 1 and 2, may provide a new understanding of neuroinflammation and novel targets for treatment. *In vitro* and *in vivo* models of neuroinflammation are key to evaluating new targets for neuroinflammation and preclinical drug efficacy.

1.2 Microglia: The Source of the Neuroinflammatory Response

Neuroinflammation arises from microglia, the highly motile innate immune cells of the brain. These cells, which comprise approximately 10% of total cells in the brain, also recruit peripheral immune cells that contribute to neuroinflammation and containment of CNS damage. Although neuroinflammation is most widely associated with pathological effects, especially with prolonged injuries or disorders, it is important for a variety of positive physiologic responses, including neurorepair.[6] In a healthy brain, microglia perform homeostatic functions and are highly ramified, possessing a complex network of processes emanating from the soma. Microglia are distributed evenly throughout the brain parenchyma to shape synapse formation and elimination and to survey surrounding areas for damage or indicators of abnormalities.[7] Microglia, and other glial cells such as astrocytes, possess families of pattern recognition receptors (PRRs) and others, including toll-like receptors (TLRs) and purinergic receptors, which are suited to detect damage associated molecular patterns (DAMPs). DAMPs include high mobility group box 1 (HMGB1) protein, other alarmins, and purines released by dying or damaged cells.[8–10] Detection of DAMPS initiates the inflammation response in microglia, which involves the coordinated production and release of a cascading milieu of proteins including both pro- and anti-inflammatory cytokines, chemokines, reactive oxygen species (ROS), and others.[11]

Microglia initiate the immune response almost uniformly by activating the transcription factor nuclear factor kappa-B (NF-κB), responsible for expression of a large family of inflammation-related genes.[12] During homeostatic conditions, NF-κB resides in the cell cytoplasm and is inhibited from performing transcription in the nucleus by inhibitor of kappa-B (IκB).[13] NF-κB is liberated from IκB via a variety of external stress signals including LPS, pathogens, or cytokines such as tumor necrosis factor-alpha (TNF-α), which bind to their respective cell receptors and initiate signaling cascades that results in the inhibitory phosphorylation and degradation of IκB.[12] This liberates NF-κB and allows it to translocate to the nucleus and bind to κB elements on gene promoters and influence the transcription/expression of over 400 different genes,[14] including expression of cytokines and chemokines such as TNF-α, keratinocyte chemoattractant/ human growth regulated oncogene (KC/GRO), interferon gamma (IFNγ), chemokine (C-C motif) ligand 5 (CCL5), and the interleukins 1 alpha (IL-1α), 1 beta (IL-1β), 2 (IL-2), 5 (IL-5), 6 (IL-6), 10 (IL-10), and 12 (IL-12). For full list see Boston University resource "NF-κB Target Genes": https://www.bu.edu/nf-kb/gene-resources/target-genes/. Expression patterns of all or some of these signals induced by mechanical injury (TBI) or TLR4 activation via LPS are explored in **Papers I-IV**. It is important to note that microglia also respond to anti-inflammatory stimuli/signals, such as IL-4, which can suppress NF-κB activation and pro-inflammatory gene production, and initiate an anti-inflammatory response.[15,16] The balance of pro- and anti-inflammatory signaling should be considered in any pathological context.

In microglia, external signals drive biochemical changes in the cells, such NF-κB nuclear translocation, marking a context-dependent starting point for what is broadly described as microglial "activation." Microglia produce proteins upon activation, which can function in an autocrine or paracrine fashion. Functional changes in microglia may include increased phagocytosis,[17] changes in migration ability,[18] altered proliferation patterns,[19] or even blood-brain barrier (BBB) protection.[20] The use of a dichotomous nomenclature of "M1" ("activated"/proinflammatory) or "M2" ("alternatively activated"/anti-inflammatory)[21] microglia describes potential differences in microglial activation states; albeit these terms also do not fully encompass the complexity of microglial function and diversity

(Figure 1).[22]

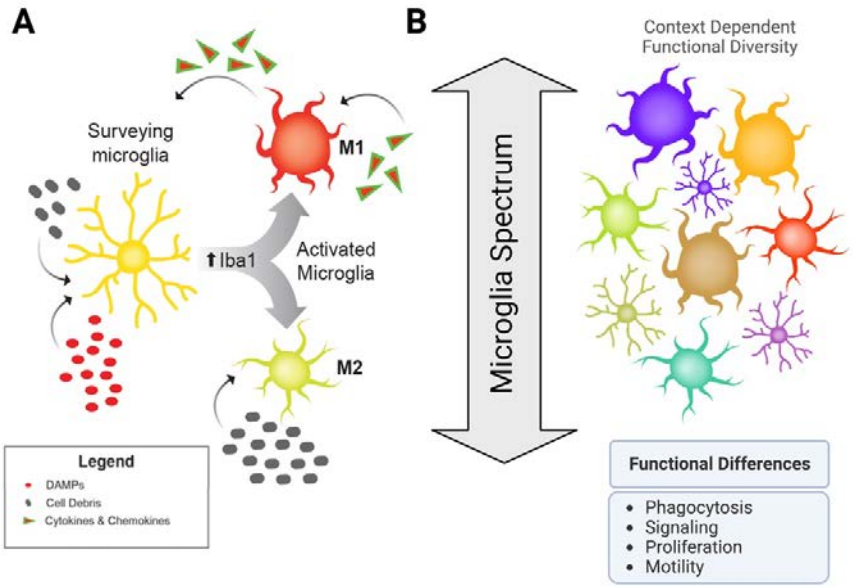

Figure 1. Microglial reactivity and diversity. ***(A)*** *In normal resting conditions, microglia remain in a homeostatic surveying state. Detection of DAMPs, cell debris, and other signaling proteins caused by disease, injury, or other aberrant processes in the CNS, cause microglia to become activated, formerly described as M1 (pro-inflammatory) or M2 (alternatively activated/anti-inflammatory). (B) Recent RNA sequencing experiments on microglial populations and single cells have revealed additional heterogeneity and distinct activation states during development, aging, and as a result of injury or disease. Furthermore, functional differences exist in healthy brains with genetically distinct regional populations. Figure adapted from Glotfelty et al. (2019).*[2]

Microglia can be described by a number of parameters, including morphology, transcriptome, and injury/disease state context. Indeed, it is apparent that some microglia interact with regenerative or reparative processes while others possess opposite functions.[23] Recent RNA sequencing (seq) and single cell RNAseq experiments have revealed regionally and developmentally distinct populations of microglia.[24–27] Furthermore, microglial heterogeneity occurs in response to injury[25,27] as well as in neurodegenerative diseases.[28,29] Single cell RNAseq provides evidence for several microglial phenotypes that could be targets for intervention and result in improved outcomes in injury or disease. Timing of interventions in acute brain injuries seems to be critically important, as weighted levels of microglial activation states may provide benefit in the short term.

Microglial activation is typically demonstrated experimentally via increased signal intensity of ionized calcium-binding adaptor molecule 1 (Iba-1) immunochemistry. Iba1 is intricately involved in actin fiber bundling, membrane ruffling, and phagocytic function, which accompany morphological changes during microglia activation (Figure 1).[30] An elevation in numbers of $Iba1^{+}$ cells indicates increased localization of microglia to a damaged area or possibly cellular proliferation. Iba1 immunochemistry is utilized in **Papers I**, **IV**, and **V**. Although Iba-1 is the most commonly used marker for microglia, others include P2Y purinoceptor 12 (P2Ry12) (used in **Papers III** and **V**), transmembrane protein 119 (TMEM119) (**Paper IV**), and CXC chemokine receptor 1 (CX3CR1) (**Paper V**). P2Ry12 is responsible

for sensing local changes in adenosine triphosphate (ATP) that can result from tissue damage[31] or even originating from neuronal firing.[32] There is currently no known functions of TMEM119 but it, along with P2Ry12 are exclusive markers of microglia, while their expression dramatically decreases in pathological contexts.[33] In the brain, CX3CR1's only ligand resides in neurons. A deficiency of CX3CR1 promotes an aging phenotype, leading to the hypothesis that its role is intricately involved in supporting neuron health.[34] Iba1, CX3CR1, and other markers of microglia are also expressed in peripheral macrophages, which is an important consideration, especially with the wide use of conditional Cre recombinase/loxP knockout (KO) models targeting microglia via the CX3CR1 promoter (used in **Paper V**).[35]

Though inflammation is a natural response to injury and thus biologically important to recovery,[36] aberrant and persistent inflammation can create a neurotoxic environment.[37,38] Although microglia initiate the neuroinflammatory response, there signaling produces secondary response in other cells, such as astrocytes. For example, a proinflammatory microglial state induces production and release of a broad range of inflammatory cytokines, including IL-1α, TNF-α, and complement component 1q (C1q), all of which have been implicated in the formation of a "powerfully neurotoxic" astrocyte subtype.[39] The neurotoxic components secreted from this astrocyte subtype are long chain saturated lipids contained in APOE and APOJ lipoparticles.[40] These astrocytes exist in normally aged mouse brains,[41] have been implicated in exacerbating neurodegeneration in models of PD,[3] and are present in the brains of AD, PD, Huntington's disease, ALS, and multiple sclerosis patients.[39] Astrocyte nomenclature was previously limited, describing reparative and harmful subtypes, but as with microglia, this simplified nomenclature has fallen out of favor. A broader descriptor, "reactive", reflects context-dependent phenotypes of astrocytes.[42] This "cycle of neuroinflammation" can result in an enduring loop of microglial and astrocyte activation common in chronic neurodegenerative conditions (Figure 2).[43]

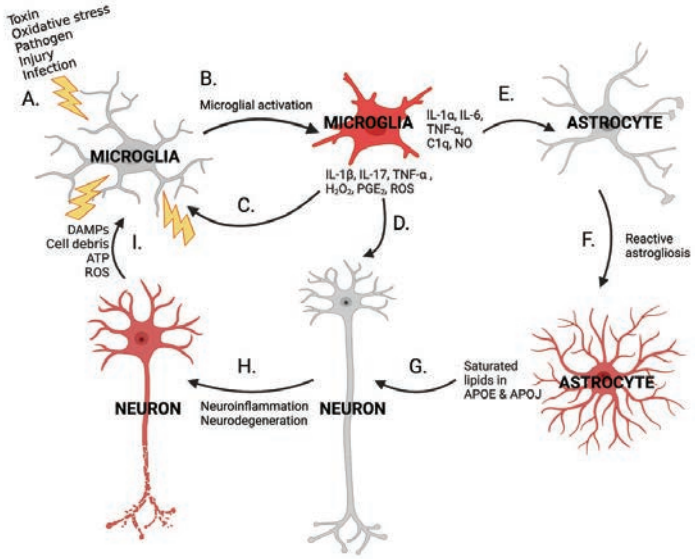

Figure 2. The cycle of neuroinflammation. *Microglial activation via a variety of stressors (A) initiates intracellular signaling pathways and activation of microglia (B) that lead to release of pro-inflammatory proteins that can affect neuronal health (D,H) and astrocyte reactivity (E, F). Subpopulations of reactive astrocytes secrete saturated lipids that are neurotoxic (G). Tissue degradation and cell death can chronically activate microglia via release of DAMPS, cell debris, ATP, and ROS, exacerbating the inflammatory response and preventing recovery (I). Figure from Kopp et al. (2022).*[43]

1.3 Incretins: From Treating Diabetes to Neurodegeneration

1.3.1 A Brief History

The endogenous incretin hormones GLP-1 and GIP are known for their postprandial insulinotropic properties. Produced in the L and K cells of the small intestine, respectively, these incretins account for approximately 70% of insulin produced in the islet β-cells of the pancreas where their corresponding G-protein coupled receptors (GPCRs) reside.[44] Gcg, produced in the pancreatic α-cells, has opposite effects of the incretins, increasing liver production of glucose during times of low blood sugar or fasting. Gcg receptor (R) agonists are used to treat severe hypoglycemia, often attributed to mismanagement of insulin regimens in diabetic patients.[45]

Within minutes of their release, GLP-1, GIP, and Gcg are all susceptible to cleavage and inactivation by dipeptidyl peptidase-4 (DPP-IV).[46,47] Along with their powerful glucoregulatory properties, the

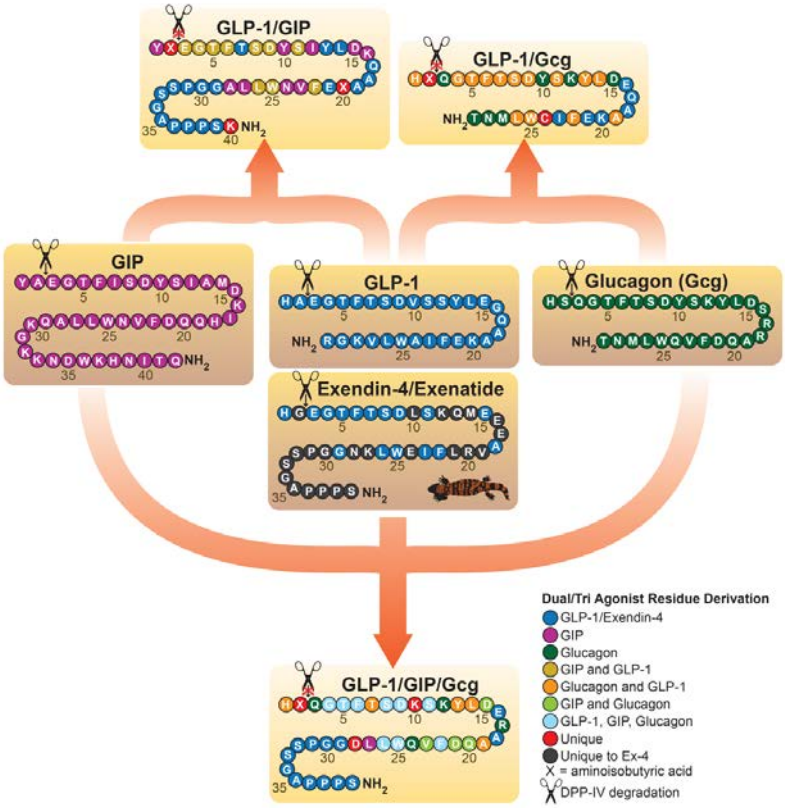

Figure 3. GLP-1 based incretin mimetics*. The endogenous peptides GLP-1, GIP, and Gcg, are all susceptible to cleavage by DPP-IV. The discovery of the DPP-IV resistant GLP-1 analogue, Exendin-4, in the venom of the Gila monster led to the development of a new class of US FDA approved diabetes medications. Pharma companies have since introduced monomeric dual and tri-receptor agonists, combining elements of single GLP-1, GIP, and Gcg. These new mimetics are being developed in clinical trials and may outperform single GLP-1R agonists. Figure from Glotfelty et al. (2019).*[2]

incretins and Gcg perform extrapancreatic functions, helping to control gastric emptying and satiety,[48] signaling of gastric distention,[49] and fluid homeostasis (thirst)[50] among others. The predominant active isoform, GLP-1(7-36)amide has been a primary target of research and drug development for treating type-II diabetes mellitus (T2DM), as deficits in incretin signaling are a hallmark of the disease.

The 1992 discovery of a naturally occurring DPP-IV resistant analogue to human GLP-1, Exendin-4 (Ex-4), in the venom of the Gila Monster *(Heloderma suspectum)*[51] paved the way for the 2005 US FDA approval of a synthetic version of Ex-4, Exenatide. Since then, potentially more efficacious and amenable monomeric dual and tri-agonists that include combinations of active regions of GLP-1 with GIP and/or Gcg have recently entered into clinical trials for a variety of metabolic disorders, including T2DM, non-alcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease (NAFLD) and obesity.[2,44,52] Amino acid sequences for the endogenous peptides (GLP-1/Exendin-4, GIP, and Gcg) and their monomeric dual and tri-agonist mimetics are shown in Figure 3.

In parallel to Exenatide's clinical development for treating T2DM, GLP-1Rs were discovered in the brains of both rodents[53,54] and humans,[55,56] though their function was not known. Mechanistic studies uncovered novel neurotrophic and neuroprotective properties of GLP-1[57–60] and sparked an interest for utilizing incretins as potential treatments for neurological disorders.[61,62] As insulin dysregulation is a risk factor for a variety of neurodegenerative disorders and stroke, repurposing incretin therapies for treating these diseases appeared to be a fruitful avenue of research.[63,64] Since the US FDA approval of Exenatide, characterization of receptor localization for all three secretins, GLP-1, GIP, and Gcg, indicate distribution in a wide variety of tissues,[65] including many brain structures and cell types in the brain (Figure 4).

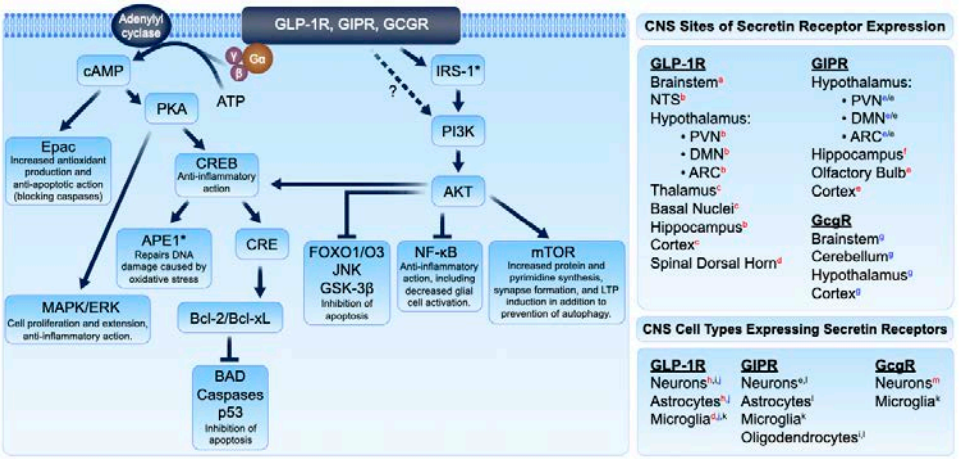

Figure 4. GLP-1, GIP, Gcg receptor activation produces pleiotropic effects in a variety of brain regions. *Pathways activated are broadly anti-inflammatory and neuroprotective. Secretin receptor expression in rats (red superscript), mice (blue superscript), and humans (black superscript) are shown in the right panel. (a) ref[66]; (b)ref[67]; (c) ref[68]; (d) ref[69]; (e) ref[70]; (f) ref[71]; (g) ref[72]; (h) ref[73]; (i) ref[74]; (j) ref[75]; (k.) ref[76] ; (l.) ref[77]; (m.) ref[78]. Figure from Kopp et al. 2022.*[43]

Activation of all three receptors, separately or together, produces pleiotropic signaling effects, a potential benefit over insulin therapy alone, which has not proved beneficial in treating neurodegenerative disorders . The neuroprotective and anti-inflammatory effects observed in the many

preclinical studies and human trials likely are working to restore disrupted insulin signaling and resistance that is not overcome with insulin delivery alone.

1.3.2 Incretins in Animal Models of Neurodegeneration

The pleiotropic properties of GLP-1, GIP, and Gcg make them interesting candidates for filling the pharmacologic void for treating neurological disorders, especially in their long-acting forms. A major reason for selecting GLP-1R agonists for drug repositioning towards PD was their ability to activate multiple, potentially beneficial, downstream pathways consequent to their potent secondary messenger cyclic adenosine monophosphate (cAMP) action, following their selective GPCR engagement (Figure 4).[79] While many drugs considered for repositioning act on single pathways, GLP-1R agonists, along with GIPR and GcgR agonists, initiate anti-oxidant, anti-apoptotic, trophic, and potent anti-inflammatory actions (summarized in Figure 4), which are potentially valuable across a variety of neurological conditions including TBI,[2] PD,[80–82] AD,[63] and stroke.[83]

1.3.3 Incretins in Human Clinical Trials for Neurologic Conditions

Human clinical trials are currently underway using GLP-1R agonists to treat PD,[82] with several already completed indicating positive benefits for patients treated with the drugs.[84,85] A full list of human clinical trials related to neurological disorders can be found in Table 1.[43] Preclinical experiments show that incretin mimetics may provide respite to levodopa-induced dyskinesia (LID), a condition caused by prolonged exposure to *Levodopa*, the primary treatment for PD symptoms.[86] A recent Phase II randomized, placebo controlled, double blind clinical trial using the PT320 Exenatide formulation (featured in **Paper I**) met primary outcomes in reducing head pressure from idiopathic intracranial hypertension.[87] Furthermore, meta-analyses of diabetes patients treated with DPP-IV inhibitors and/or GLP-1R agonists showed significant reductions in PD[88] and stroke[89,90] morbidity. These datasets provide strong evidence for a prophylactic incretin effect on chronic neurodegenerative disorder development and further highlight connections between insulin resistance and CNS disease.

Although GLP-1R agonists have been utilized in human clinical trials for neurological disorders, human clinical trials of the monomeric dual and triagonist mimetics have not been tested for these same conditions. A polypharmacologic approach may provide even more robust outcomes than GLP-1R agonism alone. Importantly, the dual agonist compounds (Figure 3) have been safely tested in human clinical trials for metabolic disorders[91–94] and show a tolerability similar to GLP-1R agonists alone. Recent *in vitro* and *in vivo* research is establishing the triagonist compound as a potentially even more potent and balanced treatment for neurological conditions.[95–97] Tirzepatide, a dual GLP-1R/GIPR agonist, was recently shown to be potentially even more effective than the GLP-1R agonist semaglutide as a weight loss drug in humans (SURMOUNT-II, clinical trial #NCT04657003), though head to head comparisons have yet to be performed. Still, these studies highlights that these drugs are safe in humans and potentially more efficacious, including for the treatment of neurodegenerative disorders. Activation of multiple GPCRs simultaneously through the dual and triple incretin-based agonists seems a promising strategy to study for future neurological disorder/injury therapies. Optimizing which of these drugs best enters the brain and which patients will best respond will be important steps to moving these compounds into the clinic.

Company	Drug Name		Use	Clinical Trial	Phase	Status	Outcomes
AstraZeneca	Exenatide	Bydureon®	PD	#NCT03456687	1	Ongoing	N/A
				#NCT01971242	2	Completed (2020)	Treatment improved off-medication motor scores. See (a.) *Athauda et al., 2017.*
				#NCT04305002	2	Recruiting	N/A
				*EUCTR2019-000732-26-SE	2	Ongoing	N/A
				#NCT04232969	3	Ongoing	N/A
				*ISRCTN14552789	3	Ongoing	N/A
				*ACTRN12620000627954	4	Recruiting	N/A
		Byetta®	AD	#NCT01255163	2	Completed (2016)	Treatment reduced amyloid-β-42 concentration in extracellular vesicles. See (b.) *Mullins et al., 2019.*
			PD	#NCT01174810	2	Completed (2013)	Treatment improved motor abilities and cognition. See (c.) *Aviles-Olmos et al., 2013.*
			FRDA	*EUCTR2014-003598-41-BE	Pilot	Completed (2019)	Treatment improved frataxin levels in FRDA patients. See (d.) *Igoillo-Esteve et al., 2020.*
Novo Nordisk	Liraglutide	Victoza®	AD	#NCT01469351	2	Completed (2013)	Treatment maintained healthy brain glucose metabolism. See (e.) *Gejl et al., 2016.*
				#NCT01843075	2	Ongoing	N/A
			PD	#NCT02953665	2	Completed (2022)	Treatment improved non-motor symptoms, quality of life, and mobility. See (f.) *Hogg et al., 2022.*
	Semaglutide	Ozempic®	PD	#NCT03659682	2	Not yet recruiting	N/A
		Rybelsus®	AD	*ISRCTN71283871	2	Recruiting	N/A
				#NCT04777409	3	Recruiting	N/A
				#NCT04777396	3	Recruiting	N/A
Neuraly Inc.	Exenatide	NLY01	PD	#NCT03672604	1	Ongoing	N/A
				#NCT04154072	2	Ongoing	N/A
Peptron	Exenatide	PT320	PD	#NCT04269642	2	Ongoing	N/A
Sanofi-Aventis	Lixisenatide	Adlyxin® / Lyxumia®	PD	#NCT03439943	2	Ongoing	N/A
Various	Any GLP-1R agonist approved for metabolic disease treatment	Various	Glaucoma	N/A	N/A	Completed (2021)	GLP-1R agonist treatment cohort had reduced risk of developing glaucoma. See (g.) *Sterling et al., 2021.*

Table 1. Incretin repurposing: clinical trials to treat neurological diseases. *Drugs that are red and green are FDA approved in the USA or approved internationally, respectively, to treat metabolic diseases. Drugs in blue are in clinical trials but are not clinically approved for any treatment. Bydureon®, Byetta®, and Victoza® have completed clinical trials and demonstrated efficacy for treating a nervous system disease. #: https://clinicaltrials.gov (USA clinical trials); *: https://trialsearch.who.int for trials outside the USA. FRDA = Friedreich ataxia. Sources: (a)[84], (b)[98], (c)[99], (d)[100], (e)[101], (f)[102], (g)[103]. Table from Kopp et al. (2022).*[43]

1.3.4 The GLP-1(7-36)amide metabolite GLP-1(9-36): A biological role in the CNS?

GLP-1 is derived from the posttranslational processing of the proglucagon gene, as is GIP and Gcg, and exists in several bioactive forms, most notably its insulinotropic, N-terminally truncated GLP-1 (7-37) (glycine extended) and the more abundant and widely studied amidated GLP-1 (7-36)amide.[104,105] These two forms are equipotent,[106] while GLP-1's full length form, GLP-1 (1-37), shows little activity.[107,108] Following cleavage of the highly insulinotropic and active GLP-1 (7-36)amide by DPP-IV, its metabolite GLP-1(9-36) is formed (Figure 5).[109]

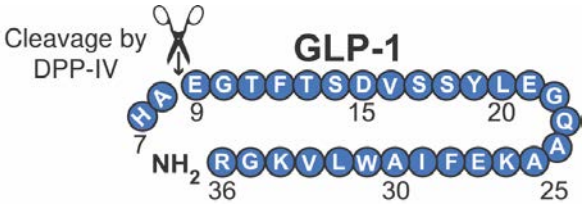

Figure 5. *The metabolite of GLP-1(7-36), GLP-1(9-36), is formed via DPP-IV cleavage. Amino acids at each position are indicated by their single letter abbreviation. Graphic from Li et al. (2021).*[96]

GLP-1 (9-36) remains in the bloodstream at circulating levels 10-fold that of its precursor.[110] Interestingly, despite lacking insulinotropic properties,[111] GLP-1(9-36) does suppress glucose production via an insulin-independent mechanism.[112] This effect was observed in pigs[113] and in humans[114] but not mice.[115] Previous research has shown GLP-1(9-36) does not bind to the GLP-1 receptor (GLP-1R) with high affinity,[110] which supports a potential novel or unknown receptor via which it confers activity.

Some incretin therapies are susceptible to DPP-IV cleavage and may also result in circulating levels of the GLP-1(9-36) metabolite. Liraglutide, a GLP-1R agonist, for example, was shown to confer much of its positive actions in a mouse model of intracerebral hemorrhage via the GLP-1(9-36) metabolite. The positive effects included reduced brain edema, neurological effects, and neuroinflammation. Liraglutide co-administered with a GLP-1R antagonist did not block these observations, but co-administration with a DPP-IV inhibitor did. Hou et al. (2012)[116] later demonstrated in this same study that the effect was mediated by a GLP-1R independent activation of 5'-adenosine monophosphate-activated protein kinase (AMPK). Indeed, recent research (preprint) has provided evidence for GLP-1(9-36) activating the Gcg receptor (GcgR) in the gut.[117]

Further evidence of a role for GLP-1(9-36) in the brain comes from previous research showing the metabolite can rescue synaptic plasticity and memory deficits in an AD mouse model.[118] GLP-1(9-36) was also shown to enhance hippocampal long term potentiation (LTP)[119]; however, other studies have demonstrated no enhancement of LTP.[120] Clearly, much is not understood about the mechanisms via which the GLP-1(9-36) fragment engages the nervous system, including target receptors and biological function. **Paper III** attempts to answer some of these key questions using *in vitro* models of neurodegeneration.

1.4 The Nogo Signaling System

1.4.1 Function and Proteins Involved

Brain function is critically reliant on the precise assembly, maintenance, and plasticity of the intricate synaptic network. During development, an initial scaffold of neural circuits is laid down and then shaped by experience-driven neural plasticity. This declines after an early postnatal critical period so that

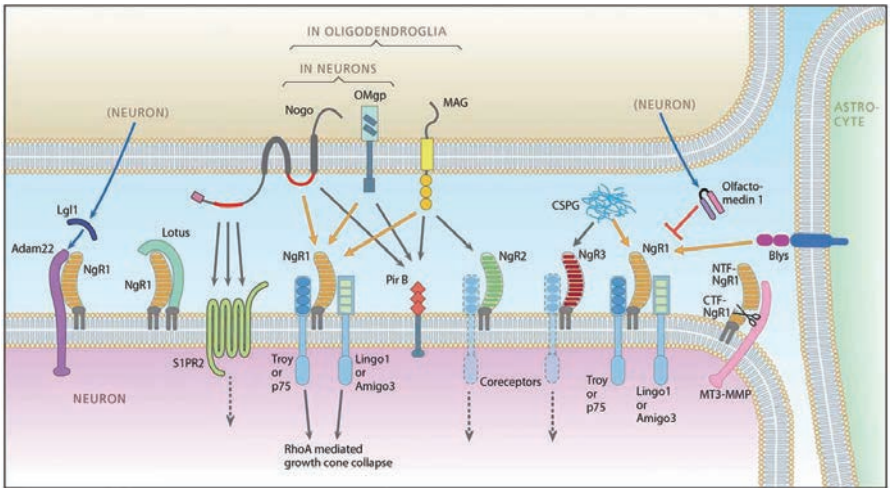

Figure 6. The Nogo signaling system ligands, receptors, co-receptors, and endogenous inhibitors. *Nogo signaling broadly results in RhoA mediated growth cone collapse. Figure from Karlsson et al (2017)[129] with permission. Illustration by Annica Röhl.*

limited, but crucial, neural network plasticity remains to optimize microcircuits within the mature brain to changing conditions, such as allowing the formation of lasting memories.[121–125] This restricted plasticity stabilizes the neuronal architecture but can limit functional recovery following a CNS insult, such as a stroke or TBI, as well as aggravate neurodegenerative disorders such as AD. A diverse group of molecules are known to limit adult structural synaptic plasticity. Particularly important is the Nogo-like signaling system and the myelin- associated inhibitors (MAIs) including Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp).[126] Nogo originates from the reticulon 4 (RTN4) gene and is expressed as either Nogo-A, -B, or -C, all three of which share a highly conserved C-terminus and a 66-amino acid signaling domain known as Nogo-66. All three isoforms are highly expressed on cell surfaces and in the endoplasmic reticulum.[127] Nogo-66, MAG, and OMgp signaling is most prominently mediated by the glycosyl-phosphatidylinositol-anchored Nogo receptor 1 (NgR1), with several coreceptors and modulators also participating.[128] The total Nogo-signaling system comprises upwards of 20 different proteins, including ligands, receptors, co-receptors, and endogenous inhibitors (Figure 6).[129] Nogo-A has an additional signaling domain known as Δ-20, or amino-Nogo, that activates sphingosine 1-phosphate receptor 2 (S1PR2).[130] Despite differences in ligands, receptors, and co-receptors, overall Nogo-signaling converges on a similar intracellular pathway, activating RhoA and its downstream target, ROCK.[131]

1.4.2 Modulating Nogo Ligand and Receptor Expression

Blockade of Nogo-A, NgR1, and other related proteins, has been shown to augment synaptic plasticity and translates into behavioral improvements across a broad range of CNS injury models. This has been demonstrated by the use of neutralizing antibodies,[132,133] small interfering RNAs,[134] soluble receptor fragments,[135] peptide antagonists,[136] gene KO or knockdown (KD),[137] as well as blockade of the RhoA or ROCK signaling cascades.[138] All of these approaches enhance regenerative sprouting and growth of lesioned fibers as well as compensatory collateral sprouting of intact fibers in models of spinal cord or brain injury. Proof of concept studies lowering Nogo activity have been undertaken in nonhuman primates, and clinical studies have been initiated with Nogo-A and Lingo-1 antibodies.[139–141]

Patients with temporal lobe epilepsy,[142] multiple sclerosis,[143] schizophrenia,[144] AD,[145] and PD[146] show elevated levels of Nogo-A in nervous system tissue. Nogo-A is upregulated in AD hippocampus and is localized to amyloid plaque deposits and reactive glial cells.[145] Likewise, NgR1 expression appears to be elevated in the CA1 and CA2 regions of AD hippocampus.[147] NgR1 is considered to have a role in amyloid-β peptide (Aβ) generation, with over-expression resulting in lowered Aβ[148]; mice lacking NgR1 demonstrate elevated Aβ accumulation,[148] but a reduction (deletion) of Nogo-A results in improved performance and less neuropathology in AD transgenic (Tg) mice.[137] Conversely, overexpression of NgR1 in forebrain neurons in AD Tg mice impairs spatial cognition without influencing amyloid plaque formation.[149] Increasing research suggests that there is extensive crosstalk between pro- and anti-plasticity signaling pathways, such as those involving BDNF that can attenuate myelin-associated inhibitors of synaptic plasticity in a phospho (p)-cAMP element binding protein (CREB)-dependent manner,[128] whereas Nogo-A decreases CREB activation leading to down-regulation of neuronal growth/plasticity programs.[150] Pharmacologically regulating this balance may provide valuable treatment strategies for neurological disorders.

Interestingly, results have been mixed with Nogo ligand and receptor KO studies versus antibody treatments.[151] Furthermore, there were mouse strain-specific differences in axonal recovery from spinal cord injury (SCI) observed in Nogo-A KO studies,[152] although this has been disputed[153] As most KO

studies target all cells in the body, it is important to consider cell type specific contributions to observed phenotypes. As the Nogo-signaling system involves many proteins, understanding cellular localization of each may be beneficial in focusing targeted KO studies.

1.4.3 Cell Type Specific Nogo-like Signaling: A Focus on Microglia

Nogo related protein distribution throughout subpopulations of cells within the brain may contextualize understanding of the diverse roles that these receptors and ligands undertake in both chronic neurodegenerative disorders and acute injuries such as SCI, TBI, or stroke. Many studies have used germline KO of Nogo ligands and receptors,[137,154,155] which provide insights to total expression contributions of these proteins to phenotypes; however, outcomes of injury models are likely more

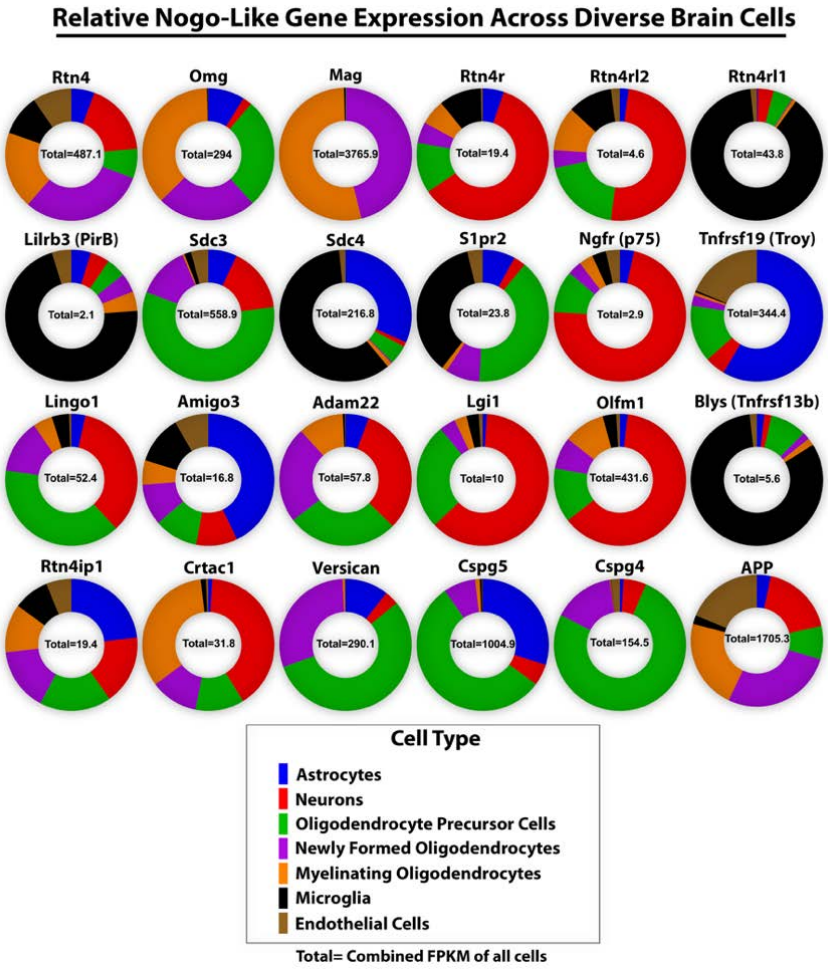

Figure 7. Nogo-like gene expression in a variety of brain cells. *Data retrieved from Zhang et al. (2014)[75] shows a wide variety of cell-type specific expression of Nogo-related genes. The "total" FPKM contribution of all cell types is shown in the center of each donut graph with the fraction of each cell-specific expression represented proportionately as a shaded region of each ring. If differing, gene abbreviation-protein abbreviation: Rtn4-Nogo; Omg-OMgp; Rtn4r-NgR1; Rtn4rl2-NgR2 (Nogo receptor 2); Rtn4rl1-NgR3 (Nogo receptor 3); Lilrb3-PirB; Ngfr-p75; Tnfrsf19-Troy; Tnfrsf13b-Blys; Rtn4ip1-Reticulon 4 interacting protein 1.*

nuanced when KO/ knock down (KD) or overexpression are undertaken in specific cell types. Preliminary mouse studies of Nogo-A KO in oligodendrocytes and neurons affect dendritic arborization and spine density differentially,[156] with oligodendrocyte KO more beneficial to SCI recovery than neuronal KO.[157] Until this present thesis, genetic manipulation of Nogo-related proteins in microglia has not been investigated.

As shown in Figure 7, Nogo-related gene expression spans diverse cell types with varied levels of expression throughout.[75] From the Zhang et al. (2014)[75] dataset, Rtn4 (Nogo-A), Rtn4rl1 (NgR3), Sdc4 (Syndecan 4), and Tnfrsf13b (Blys) are likely candidates for microglial (shown in black) protein expression, with Rtn4r (NgR1) and Lingo potentially also showing expression to lesser extents. Evidence that microglia express NgR1 and respond to NgR1 ligands with impaired mobility/adhesion capabilities and increased inflammatory signaling has been increasing over the past decade.[4,158,159]

NgR1's involvement in Aβ clearance may have particular relevance for microglia as they are the primary phagocytes of the brain and are directly implicated in the seeding/clearance of amyloid plaques in AD.[160] In a moderate, mouse TBI injury model, mice treated with the NgR1 antagonist, NEP1-40, showed shifts in activation states of microglia to a more reparative rod and macrophage type morphology, directly implicating Nogo signaling in microglia function.[161] An overexpression of NgR1 in forebrain neurons of mice subjected to mild-TBI impaired their recovery.[162]

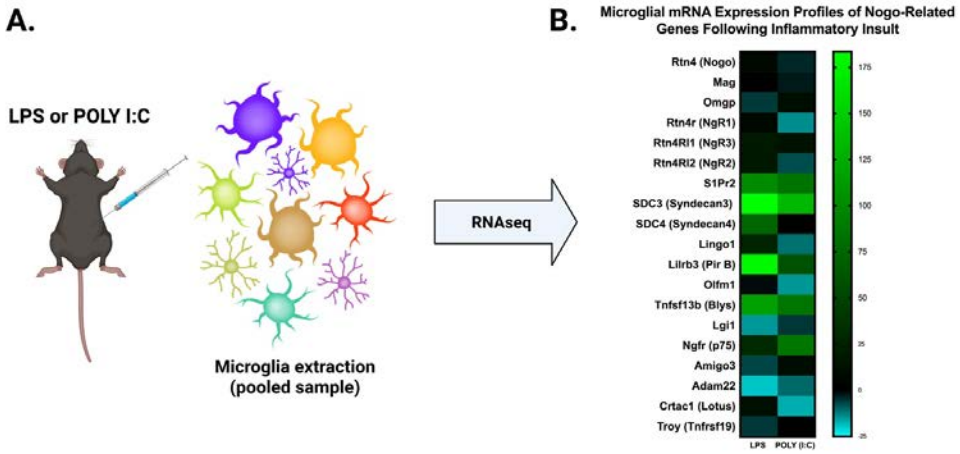

Figure 8. Evidence of Nogo-like signaling regulation in microglia during inflammatory challenges. *Data retrieved from Kang et al. (2018)[164] (A) RNAseq was performed on total microglia population extracted from mouse brains 24 h after IP injected LPS of POLY (I:C). (B) Nogo-like signaling gene patterns in relation to sham injected animals are show; n=3-4/group. Figure partially created using BioRender.com. .*

Publicly available data of RNAseq datasets (as shown above) are widely available and provide a generalized overview of microglial gene expression; however, the diverse phenotypes that microglia are known to express may not be fully represented in the RNA expression patterns observed with pooled cell data (Figure 8A). Averaging transcript counts and consequent dilution of rare gene transcripts does not allow for interrogation of cell types of interest for a certain biological effect (*e.g.,* pro-inflammatory, anti-inflammatory, or phagocytic subtypes). Microglia are no exception. Common *in vivo* assays for neuroinflammation use intraperitoneal (IP) delivered LPS or *Polyinosinic: polycytidylic acid (POLY I:C)* that initiate an inflammation response via their respective activations of TLR4 or TLR3 and the

downstream transcriptional regulator NF-κB.[163] We utilized LPS *in* vitro in **Papers II**, **III**, and extensively in **Paper IV**. Kang et al. (2018)[164] utilized these models to assess transcriptional changes in microglia following a systemically induced inflammatory insult. Though applied to activate microglia globally, multiple activation states of these cells are not reflected in the RNAseq results. It can be observed, however, that S1Pr2, Sdc3, Sdc4, PirB, and Blys seem to be consistently upregulated during the inflammatory insults, whereas downregulation of Adam22, Crtac1, Lgi1, and, to a smaller extent, NgR1 are elicited (Figure 8B). To understand whether or not activation state determines Nogo-signaling gene expression, single cell sequencing of microglia must be performed and analyzed for such comparisons. This technology is still in its infancy; however, its growing development and use makes such comparisons possible.

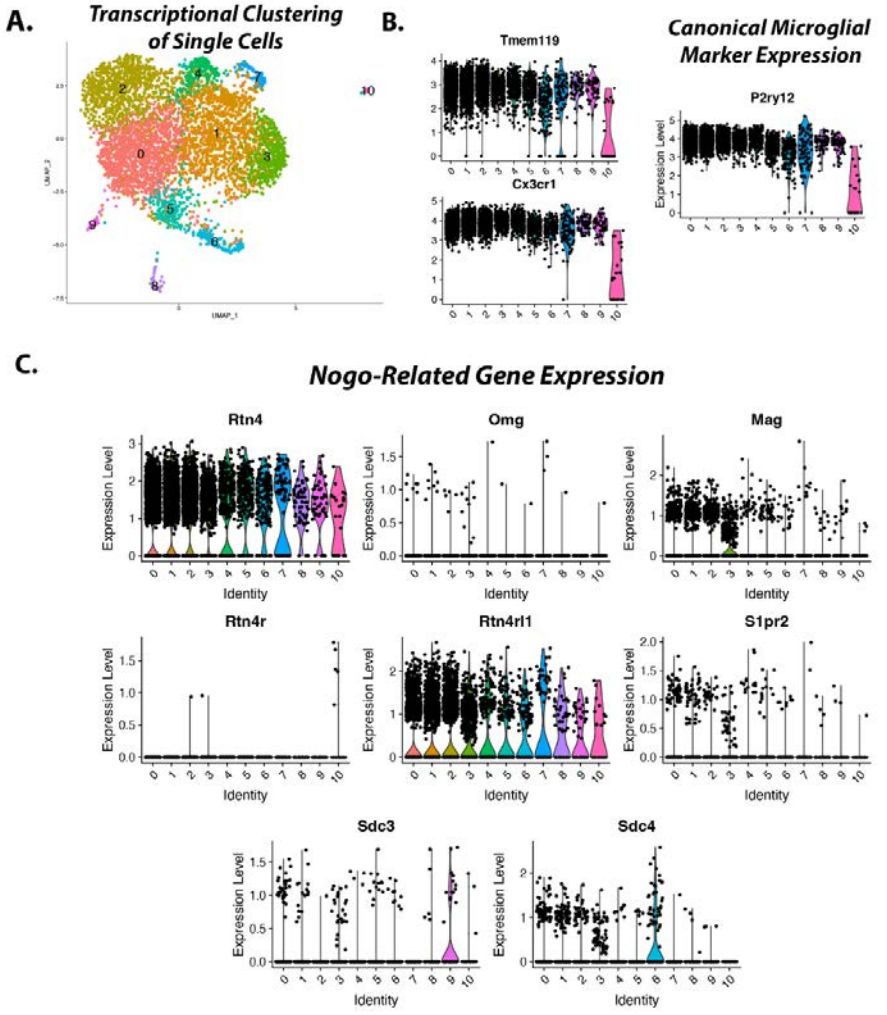

Figure 9. Single cell sequencing reveals Nogo genes expressed in subsets of microglia. *In an experiment of single cell sequencing of mouse microglia following a mTBI injury, we identified 11 transcriptionally related clusters of cells (A). Clusters 0-9 express high levels of canonical microglia markers, while cluster 10 contains a group of non-microglia contaminating cells (B). Nogo- related gene expression was probed in each cluster (C). Other non-expressed Nogo-related genes are not shown.*

For example, we have performed single cell sequencing of cortical microglia following an mTBI (unpublished results). We have found 10 separate transcriptionally related clusters of microglia from the samples assessed, all of which exist in the injured and uninjured brain (Figure 9A). Our purification technique was verified as each cluster of cells (0-9) express canonical markers of microglia, including TMEM119, Cx3cr1, and P2ry12 (cluster 10 includes contaminating cells) (Figure 9B). Using this single cell sequencing, we can screen each cluster of cells for expression of any gene. As is evident, Rtn4 (Nogo) and Rtn4rl1 (Nogo receptor 3) are the most highly expressed Nogo-related genes across most microglial subpopulations. MAG is expressed in many, but not a majority of microglia, with Rtn4r (NgR1), S1pr2, and the syndecans (Sdc3 and Sdc4) minimally expressed in microglia (Figure 9C). Other non-expressed Nogo-related genes are not shown. Several groups have described microglial NgR1 expression,[158,159,165] though our single cell sequencing data indicates little if any expression in cortical microglia. Since a majority of cortical microglia express Nogo, we used this as justification for generating the conditional Nogo-KO mouse model found in **Paper V**. Recent interest in Nogo-related regulation of inflammation[5] also necessitated the creation of this KO mouse.

1.4.4 Nogo and Inflammation

Our focus targeting microglial Nogo as a potential mediator of neuroinflammation is supported by a variety of recent studies. Antibody treatment targeting Nogo-A reduced TNF-α expression in a retinal injury model.[166] In macrophage models of inflammation, Nogo-A was shown to play a critical role in the regulation of pro-inflammatory genes,[167] with Nogo-B similarly facilitating LPS induced increases in pro-inflammatory cytokine production.[168] In a separate *in vitro* LPS model using PC12 cells, Nogo-A silencing via RNA interference downregulated TNF-α and IL-6 protein release and additionally blocked reductions in tyrosine hydroxylase, a common feature of PD.[169] Earlier studies demonstrate a role for Nogo-B increasing recruitment of macrophages to injury sites, with macrophages lacking the protein showing a reduced ability to spread and migrate.[170] Nogo mediated inhibition of adhesion and migration of microglia was later showed to be a RhoA/ROCK mediated response.[158] Although our single cell data above shows no NgR1 expression in cortical microglia, others have demonstrated NgR1 mediated inflammatory responses in microglia.[4] It should be noted that our work in **Paper IV** did not replicate this response. Nonetheless there is strong evidence of RhoA/ROCK influence over the production of inflammatory proteins in microglia.

1.4.5 RhoA/ROCK Signaling

The LPS signaling cascade that leads to the rapid production of inflammatory proteins can be blocked by prophylactic administration of inhibitors of the RhoA/ROCK signaling pathway,[171] a signaling pathway that also occurs following the activation of NgR1 and is responsible for the neuronal plasticity restricting properties of the Nogo-related proteins. Fasudil, a ROCK inhibitor, reduces microglial production of cytokines following LPS challenge, while at the same time altering global activation of microglia populations.[172] Microglia ability to perform important functions, such as migration and phagocytosis during the inflammation response, depends on the ability of the actin cytoskeleton to rearrange.[173] Microglial phenotypes are reflective of these rearrangements, and indeed, these processes area affected by RhoA/ROCK signaling.[172] Clearly, the key activity mechanism for Nogo-signaling, RhoA/ROCK, is implicated in the inflammation response,[4,174] but further research is needed to understand the influence that Nogo-related proteins in microglia have in pathological conditions such as chronic neurodegeneration or TBI.

ROCK1 and ROCK2 exhibit homology in the kinase regions, but these proteins have previously been shown to have differing roles and act in an independent manner, including in CNS tissue.[175–178] Although ROCK2 is more highly expressed overall in the brain,[179] ROCK1 is more highly expressed in microglial cells than ROCK2.[74] Independent characterization of the roles of ROCK1 and ROCK2 in the brain, and in microglia specifically, are currently lacking and necessitate study.

1.4.6 Cofilin

Cofilin is a terminal effector molecule in the Nogo-signaling pathway and becomes phosphorylated (inactivated) following NgR1 activity, with final activation status ultimately determined by LIM kinase (LIMK) which phosphorylates and inactivates, or phosphatases such as Slingshot (SHH), that dephosphorylate and activate the protein.[180] This results in the termination of actin cytoskeleton assembly,[181,182] which is detrimental to neuronal plasticity, outgrowth, and repair. Interestingly, after a brief period, deactivated cofilin is switched back to an active state, and neurite outgrowth restriction is maintained.[181] Clearly, cofilin phosphorylation is key to restricting neurite outgrowth, but much is still unknown about these changes locally (*e.g.,* in an individual synapse) or in other cell types (*e.g.,* microglia). Active and inactive forms of cofilin are also involved in microglial actin dynamics. While cofilin is important for microglial mobility, morphology, and activation profile, unregulated levels of active cofilin during an inflammatory challenge leads to the formation of neurotoxic proinflammatory microglia.[183] Alhadidi et al. (2018)[183] demonstrated cofilin's ability to mediate LPS induced microglial pro-inflammatory activation and subsequent neurotoxicity, with inactive forms of the protein being drastically reduced during LPS challenge and oxygen glucose deprivation. The group later performed a study demonstrating that cofilin KD effectively reduces microglial activation and oxidative stress in a mouse model of intracerebral hemorrhage.[184] Targeting this endpoint of the canonical Nogo-signaling pathway may be of particular importance for a treatment strategy for inflammation in the CNS. Indeed, treating cells with Ex-4 increases the phosphorylation (inactivation) of cofilin,[185] which may have anti-inflammatory effects in microglia. More research is needed to understand the roles that incretins may have in influencing the complex Nogo-signaling system.

1.5 Traumatic Brain Injury (TBI)

1.5.1 Rodent Models of TBI

	Microglial Activation	*Astrocyte Activation*	*Cytokine Elevation*	*Cognitive Deficits*	*Cell Death*	*Neuronal Dysfunction*	*BBB Permeable*	*Oxidative Stress*
WD	186–189	190–192	193–195	190,193,196	190,197,198	199,200	201,202	203,204
Blast	202,205	206–210	211,212	211,213–215	205,211,215	214,216,217	218,219	211,218,219
FPI	220–224	224,225	220,226,227	220,227,228	224,225,229	230–232	233–235	221,232,236
CCI	237–241	242–248	244,247,249	246,249,250	249,251,252	251–253	254,255	253,256,257

Table 2. Observed effects following animal models of TBI. *The WD, blast, fluid percussion injury (FPI), and closed cortical impact (CCI) models of TBI effectively model common features of human TBI, which are included in each column heading. Each model varies in reproducibility, time requirements, and intensity, which should be assessed before experimental design. All models present unique caveats. BBB= Blood-brain barrier. Table reproduced from Glotfelty et al. (2019).*[2]

Our group has experience and publications with a variety of techniques used to generate concussive TBI in mice and rats, including mild injury with weight drop (WD) and moderate to severe injury within fluid percussion injury (FPI) or controlled cortical impact (CCI). An additional model of TBI involves blast injury, which we have previously used[215] either in the open field with explosives, or simulated explosions within a laboratory setting transferred via a shock tube. We used mice in all TBI models, utilizing WD in **Papers I and II** (Figure 10A) and CCI in **Paper V** (Figure 10B). Although some of our previous work has also been performed in rats, there is extensive literature showing that these TBI instruments, also easily work on mice with very positive effects. WD and CCI allow a range of injury severity with little variability. We model moderate TBI with CCI rather than compare different levels of intensity since our data indicates this moderate level of injury is most relevant for our work in understanding and treating pathology associated with neurodegenerative disease. As we previously reviewed,[2] all four of the mouse TBI models produce a wide range of pathologies, all of which are also characteristics of human TBI injury (detailed in Table 2).

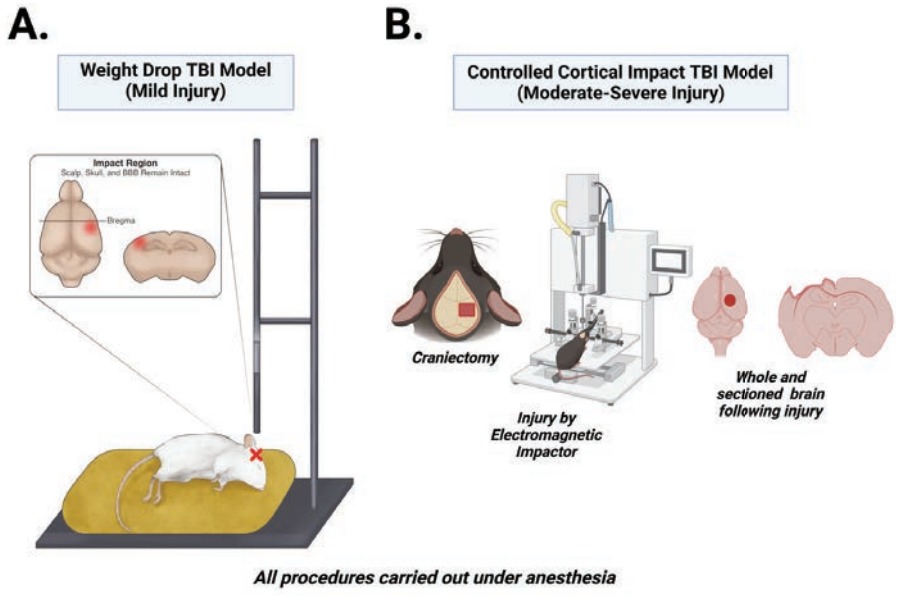

Figure 10. Models of TBI used in current thesis. *We utilize the WD (A) and CCI (B) models to induce TBI in mice. The WD model involves the dropping of a dense 30g metal cylinder on the temporal region of the mouse head. This is a closed head injury that can be performed rapidly. The brain remains intact with noticeable pathology evident using immunochemistry. The CCI model is a more complex and time intensive injury involving the removal of a small region of skull and directly impacting the brain at a programmed depth using an impactor. Injury intensity can be increased or decreased depending on depth of impact. Thereafter, the surgery wound is closed using sutures and the mouse allowed to recover. Tissue is generally compromised upon brain extraction as shown. (A) Illustration by Lauren Brick. (B) Created using Biorender.com.*

1.5.2 *Male and Female differences in TBI*

Males are more likely to suffer from a TBI than women across nearly all age groups,[258] although similar levels of TBI are experienced in the elderly of both sexes.[259] Men and boys are more likely to engage in risky behavior than girls and women, including reduced wearing of head protection.[259,260] Although much of the increases in public attention and general knowledge of TBI has come from media coverage of sports-related head injuries, the elderly account for the largest increases in TBI-related emergency

room visits.[261] Additionally, TBI occurring in old age causes higher mortality and worse future prognosis than the same injuries occurring in younger individuals.[262,263]

TBI is a heterogenous injury. Meta analyses of TBI outcomes in men and women show a majority of studies looking at sex differences show there are worse outcomes in women than men (47%) following alike injuries, with another 27% of these studies showing that men have worse outcomes than women.[264] Clearly, it is difficult to directly compare one head injury to another, but the results of this human meta analyses highlight needs to include both sexes in TBI studies. Many studies, including ours (**Papers I** and **II**), use only male mice in TBI models to evade possible differences in how different sexes respond at a cellular and molecular level to injury, including sex hormones such as estrogen, which is neuroprotective and can potentially effect outcomes, especially in the mTBI model.[265] In general, in preclinical TBI studies, female rodents have better outcomes compared to males, following alike injury,[264] which is in contrast to humans. Additionally, there are pharmacokinetic and pharmacodynamic differences related to drug processing between the sexes that may affect initial preclinical assessments.[266] Although we did not include both sexes in our pharmacological studies, we did include both sexes in our moderate TBI studies involving genetic manipulation of Nogo protein in microglia **(Paper V)**.

1.6 Conclusion

Microglial influence in shaping neuronal circuits, monitoring homeostasis, and responding to injury or disease has become increasingly evident in the past ten years. The many functions that microglia simultaneously perform in the brain are starting to be understood as more advanced techniques to monitor them become available. Profiling activation states of microglia is paramount to understanding their many functions, with single cell sequencing leading the way for these discoveries. With these technologies, exploration of novel signaling paradigms are also possible, including the Nogo-signaling system that has previously been neglected in microglia. Our group has used several models (*in vivo* and *in vitro*) of neurodegeneration and neuroinflammation, including mild/moderate TBI in mice and microglial cell cultures, to understand activation states of microglia and to modulate their function pharmacologically or genetically. In *in vivo* mTBI models and PMg cell culture, we have effectively mitigated behavioral deficits (**Papers I-II**) and microglial proinflammatory states associated with these models (**Papers I-III**) using various formulations of incretins or common metabolites. These include a slow release form of Exenatide called PT302 (**Paper I**), a monomeric GLP-1/GIP/Gcg receptor triagonist (**Paper II**), and the common metabolite of GLP-1 (**Paper III**). We additionally profiled the pharmacokinetics of a new sustained release form of the GLP-1R agonist Exendin-4, PT302 (**Paper I**) which has recently completed a Phase 2 clinical trial, successfully treating symptoms of intracranial hypertension.[87] This importantly links our preclinical studies to human ones, by ensuring that the dose used in the former are clinically translatable to human use. Our studies have clarified possible functional roles in the nervous system of the ubiquitously circulating metabolite of insulinotropic GLP-1(7-36)amide, including neurotrophic and anti-inflammatory properties (**Paper III**). Identification of novel mechanisms of neuroinflammation is key to drug development focused on brain injury and diseases. The Nogo signaling system and the canonical RhoA/ROCK pathway it induces sheds light on new drug targets for treating brain injury and disease. Using a relatively new immortalized microglial cell line (IMG cells), we effectively demonstrate mechanisms by which RhoA/ROCK influence inflammatory pathways (**Paper IV**). Lastly, we have for the first time demonstrated a pathological effect of microglial Nogo protein following injury using a conditional KO model (**Paper V**). This research thereby bridges

basic and translational science, aiding bench to bedside clinical drug development and defining drug mechanisms underpinning clinical responses to potentially optimize future drug treatments.

2 RESEARCH AIMS

1- To describe rodent brain penetration of a slow release Exenatide, PT302. Additionally, to assess the antifinflammatory potential of this drug in a model of mild traumatic brain injury at clinically achievable dosing.

2- To apply lipopolysaccharide induced neuroinflammation in a primary microglia model to demonstrate the anti-inflammatory capacity of a novel, monomeric GLP-1, GIP, Gcg receptor triagonist.

3- To determine if the non-insulinotropic metabolite of GLP-1(7-36)amide, GLP-1(9-36), has any central nervous system disease modifying effects. This includes exploration of neutrophic and anti-inflammatory properties of this important metabollite.

4- To apply an imortalized microglial cell line (IMG cells) in parallel to primary microglia to explore the roles of RhoA/ROCK signaling in neuroinflammation and define its utility in neurological drug development.

5- To identify roles of microglial-Nogo proteins in both healthy and injured adult mouse brain.

3 MATERIALS and METHODS

Materials and methods specific to this thesis are included in detail below. Common experimental procedures (Western blot, ELISA, immunochemistry) are included briefly with more detailed descriptions within the constituent papers.

3.1 Ethical Considerations and Permissions

All animal experiments in this thesis were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and followed current European Union regulations (Directive 2010 / 63 / EU) agreement number A1301310. Experimental animal protocols were approved by the Animal Care and Use Committee of the Intramural Research Program, National Institute on Aging (438-TGB, 331-TGB, 497-TGB, 488-TGB) and followed the guidelines for animal experimentation of the National Research Council (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) and the National Institutes of Health (DHEW publication 85-23, revised, 1995). Use of animals was also approved at the Karolinska Institutet (Permit#: 17038-2020). Of particular importance throughout our studies was applying the 3R principle (reduce, refine, and replace) regarding use of animals. Throughout my thesis, I have aimed to minimize the number of animals used in studies, while also maintaining the highest levels of care to minimize pain, suffering, and stress incurred on animals. Animal numbers were chosen based on previous experiments in our group. One major objective of my thesis was the further characterization and use of the IMG cell line to replace PMg cell cultures for inflammation related drug screening. These cells express canonical markers of microglia and respond similarly to challenges such as LPS as do primary microglia, which require the use of many animals per cell culture. **Papers III and IV** utilized the IMG cell line which, according to our studies, is a very robust model for PMg and will be utilized in further studies carried out by my laboratories at the NIH and Karolinska Institutet to replace PMg cell cultures.

3.2 Animals used throughout studies

Animals throughout all studies were housed with *ad libitum* food and water in normal 12 h dark- 12 h light cycles. **Papers I and II** utilized all male cohorts of adult CD1/ICR (Charles River Labs) outbred mice weighing approximately 30-40 grams, housed five animals per cage. In **Paper V** mice were singly housed to allow for individual activity tracking, which required a special amendment to our study protocol (488-TGB-2022). Male Sprague Dawley rats (Charles River Labs) were used to assess brain penetration of Exenatide in **Paper I**. PMg cultures (**Paper III** and **Paper IV**) utilized young male CD1 mice for microglia extraction. Studies in **Paper V** utilized both male and female mice (described below).

3.2.1 Developing the MinoKO mouse

In **Paper V** we developed a conditional, microglial specific Nogo KO mouse that utilizes Cre-Lox recombination technology. Cell type specific Cre-Lox recombination works by breeding one mouse that has a gene of interest flanked by two loxP sites (floxed gene) with another mouse that expresses Cre recombinase (Cre) enzyme under the control of a cell type specific promoter. Cre recognizes the floxed sites and excises the gene, resulting in zero or disrupted downstream protein expression. To make this system inducible (i.e., turn on at specific time), Cre is fused with an estrogen receptor (ER) that does not bind to the endogenous hormone but has high affinity for tamoxifen (TAM). CreER resides in the cell cytoplasm where it fuses to another protein, heat shock protein-90 (HSP90), that inhibits Cre from entering the nucleus and performing recombination. When animals are introduced to tamoxifen,

typically injected over several days, CreER is activated and dissociates from HSP90 to move into the nucleus where it recognizes the loxP sites flanking the gene of interest.

To develop the microglial specific Nogo-KO mouse, we used *Cx3cr1*$^{CreER\text{-}YFP+/+}$ mice obtained from Jackson Labs (Stock# 021160) and bred with Rtn4$^{loxP/loxP}$ mice,[157] gifted to our group from Binhai Zheng (available at Jackson Labs as Stock# 033364). Both of these mice have the inbred C57BL/6J background. The *Cx3cr1*$^{CreER\text{-}YFP+/+}$ have inducible CreER and enhanced yellow fluorescent protein (EYFP) knocked into the Cx3cr1 locus, a gene specific to microglial cells of the brain and peripheral macrophages. This allows for constitutive expression of CreER and YFP in microglia under the control of the Cx3Cr1 promoter. The RTN4$^{loxP/loxP}$ mice have loxP sites inserted to flank the first and second common exons of the three isoforms of Nogo: Nogo-A, Nogo-B, and Nogo-C, (Exons-4 and -5 specifically).[157] Through multiple stages of breeding, we generated a conditional **Mi**croglial **No**go **K**nock-**O**ut (MinoKO) mouse with confirmed genotypes heterozygous for CreER-YFP and homozygous for the floxed RTN4 gene: Cx3Cr1$^{CreEr\ YFP\ +/-}$RTN4$^{loxP/loxP}$. This floxed region targeted is commonly referred to as Nogo-66 and is one of the most prominent ligands of the Nogo signaling system. To induce the KO of Nogo in microglia, we administered TAM (100 mg/kg) daily via IP injection over five days, alternating injection sites. Before any experimentation, we allowed peripheral macrophage populations expressing Cx3Cr1 to turnover (approximately six weeks),[267] ensuring only the effect of microglial recombination is observed. A full summary of MinoKO mouse design is shown in Fig. 11A**.** The Cx3Cr1 promoter is one of the most widely used to specifically target microglial specific Cre expression. Importantly, Cre does not drive any adverse phenotypes or activation of microglia in adult mice.[268] We used three different genotypes of mice in the **Paper V** experiments; two control animals, 1) which expressed CreER in microglia (Cre Control) and 2) another with no CreER but floxed RTN4 sites (Floxed Control), and 3) the MinoKO mouse which expresses CreER in microglia and also has a floxed RTN4 gene (Fig. 11B).

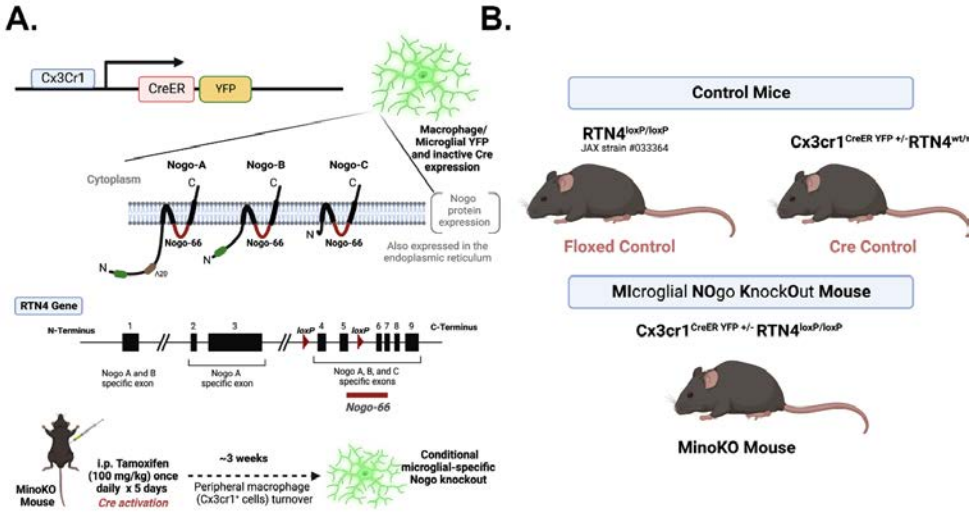

Figure 11. Design of MinoKO mouse and other mice used in Paper V. *(A) The MinoKO mouse utilizes CreER-Lox recombination to selectively delete the Nogo-66 region common to Nogo-A, -B, and -C. (B) Three genotypes of mice are used in Paper V.*

3.3 Injury Models of TBI: WD and CCI

Our studies utilize rodent TBI to model human head injury but also to quickly induce pathology that is common in neurodegenerative disorders, most notably, neuroinflammation. This allows our group to screen drugs *in vivo* that have already demonstrated *in vitro* efficacy. Animal models of neurodegeneration often involve expensive Tg mice that are resource intensive, including long periods of aging for up to years at a time. Evaluation of TBI saves time and resources and helps pinpoint the most promising drugs to advance into more complex aging or neurodegenerative disease models. We utilized the WD method to induce a mTBI (**Paper I** and **Paper II)** and CCI (**Paper V)** to induce a moderate TBI. Schematic diagrams of the TBI models used herein are shown in Fig. 9. Briefly, WD procedures were performed while mice were anesthetized with isoflurane (5%) and placed on a sponge with the temporal region of the head directly under the WD tube. A 30 g cylindrical weight (approximately the same weight as the mouse) was then dropped on the closed head to induce the mild injury. Mice were then placed back in cages for observation. This WD mTBI model has parallels to concussive head injury in humans, in which a person falls under their own weight and strikes their head or clashes heads with a person of approximately their same weight. For CCI injuries, mice were placed under deep aesthesia using 2.5% tribromoethanol (Avertin: 250 mg/kg; Sigma, St. Louis, MO, USA) and placed in the stereotaxic frame. A craniectomy was then performed, removing a small portion of the skull near the right motor cortex. The CCI device, Impact One (Leica Biosystems Inc., Buffalo Grove, IL, USA) allowed for adjustable impact depth. We used a 1 mm depth based on our prior experience inducing head injuries that impact motor function.

3.4 Pharmacokinetic Studies

In **Paper I**, we assessed blood plasma pharmacokinetics of a slow release Exenatide formulation (PT302, *Peptron*) in mice, while CNS assessment was performed in Sprague-Dawley rats. For the mouse studies, dose range studies were initially performed in mice without mTBI (PT302 dose evaluation range 0.1 to 2.0 mg/kg; blood sampling 7 days later) to assess dose-dependent linearity of plasma Exenatide levels. We also assessed blood plasma Exenatide levels in mTBI challenged mice, using a single PT302 dose (0.6 mg/kg) administered 1 h post-TBI and blood samples withdrawn at 12, 24 and 72 h. This allowed us to understand whether mTBI affected metabolism or brain uptake of the Exenatide, thus reducing circulating levels. Three doses of PT302 were chosen based on these initial studies (low: 0.024 mg/kg, medium: 0.12 mg/kg and high: 0.6 mg/kg) for the 7 day time-dependent release, with samples collected before dosing and at 1, 24, 168, 336, and 504 h after dosing. One or more time-dependent blood samples (approximately 0.25 mL) were obtained from the sub- mandibular vein[269] to assess blood Exenatide levels. This allowed us to assess the steady-state plasma levels of PT302 maintained over three weeks. These studies allowed evaluation of steady-state plasma Exenatide concentrations that were then matched to human studies to allow our selection of drug doses in our rodent studies that provided alike plasma drug levels in humans. In this manner (and in accord with US FDA guidelines) or our studies in rodents are clinically translatable to humans.

Although most of our studies are carried out in mice, we use rats to test the ability of the various formulations of Exenatide to penetrate the BBB, as their size allowed the sampling of a sufficient volume of cerebrospinal fluid (CSF) (approximately 100 µL) for quantification of the drug. We previously performed pilot studies in mice that showed excessive background in samples did not allow for accurate Exenatide readings below 100 pg/mL. Other studies have established BBB permeability to

be roughly equivalent in mice and rats across a wide range of drugs.[270] We therefore evaluated Exenatide uptake into CSF as a measure comparable to human studies using the rat as an animal model.

As prior studies have demonstrated that there is no change in the integrity of the BBB following a 30 g WD mTBI challenge in mice [271], our studies to compare Exenatide CSF and plasma levels did not involve a TBI challenge. In separate groups of animals (N=4 to 5 per group), Exenatide was administered s.c. in the form of either (i) PT302 containing 0.46mg or 0.92mg Exenatide, (ii) via mini pump (Alzet model 2ML2, Cupertino, CA) delivering 7.0 pM/kg/min or 15.0 pM/kg/min as an alternative means to maintain steady-state plasma levels, or (iii) twice daily immediate release Exenatide (4.6 or 10 µg/kg daily). Pumps were implanted aseptically via a small incision in the back of the neck under isoflurane anesthesia. Plasma and CSF (from cisterna magna) samples were obtained from all animals on day 14 after the initiation of Exenatide administration. For the immediate release Exenatide, plasma and CSF were collected 90 min following the final subcutaneous (s.c.) dose.

Across our pharmacokinetic studies, blood samples were collected into heparinized tubes that contained aprotinin; blood was centrifuged (10,000 g× 4 min at 4°C) and plasma collected and immediately stored at -80 °C. Plasma Exenatide concentrations were quantified with an Exenatide fluorescent immunoassay kit (Phoenix Pharmaceuticals Inc., Burlingame, CA). An organic solvent extract was obtained by adding methanol to the plasma that contained either the Exenatide standards or the *in vivo* obtained test samples. Our calibration curves ranged from 20 to 5000 pg/mL, and quality control samples were prepared at the concentrations of 40, 150, and 300 pg/ mL. CSF samples were withdrawn from animals and immediately frozen at -80°C. Calibration curves were prepared from 100 to 1000 pg/mL. All samples, including the Exenatide standards, the quality control samples, and the test samples, were assayed in duplicate. Fluorescence was detected with a Tecan GENios (Grödig, Austria) microplate reader at an excitation wavelength of 320 nm and an emission wavelength of 420 nm.

3.5 Cell Culture

All cell cultures were maintained in a 37°C incubator (5% CO_2 and 95% air), with media replacement every other day or as indicated.

3.5.1 HMC3 and IMG Cell Culture

The IMG cell line[272] (Sigma-Aldrich, cat# SCC-134) were used in **Papers III and IV.** Cells were cultured in High Glucose DMEM (Sigma Cat. No. D6546) with 10% fetal bovine serum (Gibco™ cat#10082147), 1x L-Glutamine (Sigma, cat# TMS-002-C) and 100 U/mL penicillin/streptomycin (Gibco™, cat#15140148). For subculture, Accutase® detachment media (Sigma, cat#A6964) was used. Cells were grown in 24- or 48-well tissue culture treated (TCT) plates and plated at a density of 40,000 or 20,000 cells/well respectively in 500 mL of media for experiments. Fresh media was added on day 1 after passaging and changed every two days as needed. For Western blot studies and protein extraction, IMG cells were grown in 6 cm TCT dishes at a density of $1x10^6$ cells/dish.

The HMC3 transformed human microglial cell line (cat# CRL-3304, ATCC), was utilized in **Paper III.** HMC3 cells were cultured similarly to IMG cells but with EMEM media (ATCC). We use 0.25% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen, cat#AM9912) for subculture. A maximum of 10 passages were used for the IMG and HMC3 cells.

3.5.2 Primary Microglia (PMg) Isolation and Cell Culture

PMg cultures were utilized in **Papers II and IV**. Microglia were isolated from one-month old adult CD1 mice as previously described (Hammond et al., 2019 and Lee & Tansey, 2013) with minor changes. Briefly, animals were anesthetized with isoflurane and transcardially perfused with ice cold Hank's Balanced Salt Solution (HBSS) (Gibco cat#14175095). The brains were rapidly removed and placed in HBSS on ice, minced, and mechanically processed with a 15 mL Dounce homogenizer (Wheaton) using the loose followed by the tight-fitting pestle (8 strokes with rotation from each pestle). Cell suspensions were filtered on a sterile, pre-wet (HBSS), 100 µm filter (Corning™ cat# 431751) and centrifuged (600×g, 8 min, 4°C). Using a percoll gradient (37% on top of 70% isotonic Percoll) with centrifugation (700 × g, 18 °C, no brake) we were able to isolate immune cells from myelin and other cell debris. These cells were washed and further purfied using MACS CD11b staining and separation (Miltenyi Biotec) according to the manufacturer protocol. CD11b positive cells were plated on Poly-D-Lysine (PDL) coated 96-well plates (Corning Biocoat Poly-D-Lysine cellware cat# 354461) (density of 10,000 cells/well) and grown for 12–14 days in 150 µL complete microglia medium (MM, ScienCell Research Laboratories cat# 1901) with half media changes every other day.

3.6 Drug Treatments and Cellular Challenges

3.6.1 Triagonist, GLP-1(9-36), Nogo-P4, Narciclasine, RKI1447, Y27632

Cells were pretreated with any of the various pharmacologic interventions prior to any challenge; 24 hours for GLP-1(9-36) (**Paper III**) and 1 h for other pharmacological interventions (**Paper IV**). This allows drug treatments to activate or inhibit pathways of interest and provides opportunity to understand how these pathways affect the inflammation response. Incubation of drugs or other pharmacologic intervention continued in parallel with the LPS challenges. We determined dose ranges of Triagonist, GLP-1(9-36), Nogo-P4, Narciclasine (Narc), RKI1447, and Y27632 via pilot experiments, including viability and ELISA cytokine assessments, along with previously published studies.

3.6.2 Lipopolysaccharide (LPS) Cell Culture Challenges

LPS was used as an inflammatory challenge in **Papers II, III, and IV**. PMg and IMG were challenged with LPS (from *E. coli* O55:B5) (Sigma, cat# L2880) at varying doses to optimize challenge conditions. In **Paper III**, cells were incubated with GLP-1(9-36) at two doses (100 nM and 1 µM) for 24 h prior to LPS challenge. For other studies, LPS challenges were carried out 1 h after any pharmacologic intervention. A submaximal LPS dose of 1-10 ng/mL was used throughout these studies. Importantly, these dosages are useful for assessing whether the addition of an experimental drug/compound can either lower or further elevate cytokine levels. For experiments visualizing NF-κB nuclear translocation in **Paper IV**, the LPS challenge was only for 15 minutes which allowed for peak activity of NF-κB to be visualized.

3.7 Brain Fixation and Processing

All brains used for histological or immunochemical staining were removed from mice that underwent transcardial perfusion, first with PBS and then followed by 4% paraformaldehyde (PFA) for fixation. After brain dissection, they were immersed for another 24 h in PFA. We then performed glucose saturation, first with a 20% glucose solution for 24 h and then 30% glucose for 24 h. To cut the tissue, brains were mounted on cryosectioning chucks with optimal cutting temperature (OCT) compound.

Brains were then sliced in 20 μm sections and stored in cryoprotectant for later use. (**Papers I, II, IV, V**).

3.8 Immunocytochemistry (ICC) and Immunohistochemistry (IHC)

Briefly, tissue or cells underwent a blocking/permeabilization step for 1 h in a PBS solution consisting of 3% BSA with either 0.1% saponin (for cell culture) or 0.1-0.2% Triton (for free floating brains). Primary antibody was then applied for either 1 h at room temperature (RT) or overnight at 4°C and followed by three washes. Primary antibodies used are shown in Table 3. Corresponding secondary antibodies were then applied for 1 h at RT followed by washing. Cells or brain slices were often counterstained with mounting media containing DAPI for nucleus visualization. All immunochemistry experiments were imaged using a Zeiss LSM 880 confocal microscope. (**Papers I-V**)

3.9 Giemsa Staining

Quantification of brain lesion and lateral ventricle size in TBI animals was performed using FIJI from images gathered from Giemsa stained serial 2-weeks post-CCI brain sections (**Paper V**). Serial brain slices from 2-week post-TBI or sham (20 μm) were mounted on slides, air dried, rehydrated in 1/15 M KH_2PO_4 (9.07 g/ L; Sigma, cat# P0662) buffer for 10 min followed by 30 min saturation in 10% Giemsa (Sigma, cat #12-0440) KH_2PO_4 buffered solution (pH 4.5) at 40°C. Following a wash in KH_2PO_4 solution, the slides were de-saturated and dehydrated in a series of absolute ethanol washes (1 min × 3). Xylene (Sigma, cat# 534056) was then used to clear the tissue cover-slipped (Epredia™ Cytoseal™ 60, FisherScientific, cat#23-244257). Slides were imaged using the BZ-X710 (Keyence Corporation of America, Itasca, IL, USA) microscope, and brain image areas were quantified using ImageJ 1.52q software (National Institutes of Health, Bethesda, MD, USA).

3.10 NF-κB signal quantification

IMG single cell nuclear localization of NF-κB (**Paper IV**) was performed using FIJI[274] in images stained with NF-κB and DAPI. We used the following protocol: Separate Channels>Manually threshold DAPI>Fill Holes (DAPI)>Watershed (DAPI)>Analyze Particles (Size: 50-infinity, Circularity: 0.20-1.00, select: Add to Manager) (DAPI)> Select NF-κB Channel Image> Overlay from ROI Manager> Select "Measure" from ROI manager (NF-κB channel image). Mean gray value (MGV) [the sum of the gray values (Optical Density, OD) of all the pixels in each nucleus divided by the total number of pixels/nucleus] was recorded for each nucleus. Multiple fields of view were used for analysis in each group, with image averages making up a single biological replicate. Approximately 550 cell nuclear signals were averaged per n. Two separate cell cultures confirmed results for both ROCK inhibitor treatment and siRNA treatment.

3.11 Microglia Morphology Analysis

We used MotiQ, an automated analysis software developed for use in ImageJ (**https://github.com/hansenjn/MotiQ**) to assess $Iba1^+$ microglial morphological parameters (ramification index, spanned area per cell, branch end points, and total length of tree skeleton). Healthy microglia have highly complex branching to survey surrounding tissue. When microglia sense damage or other abnormal signaling, they retract their processes to respond, often activating phagocytic cellular programs and also secreting proinflammatory proteins. Although morphology isn't completely wholistic in identifying microglial function, it provides clues to the inflammatory state of the cells.[22]

Ramification index is a unit free metric calculated by comparing the ratio of the cell surface area to the surface area of a perfect sphere with the same volume as the analyzed cell. A ramification index of 1 corresponds to a perfectly round cell with completely retracted processes, with higher ramification indices seen in homeostatic microglial with increased branching complexity. Data shown are averages of all microglia measured from each brain region (20 microglia per brain region).

3.12 Western Blot

IMG cells lysates were isolated form 6 cm dishes with respective treatments [Vehicle, Y27632 (100 μM), RKI1447 (10 μM) ± LPS (10 ng/mL)]; n=3 or 4 plates/group. For each lane 40 μg of protein were resolved on precast 15-well Nu-PAGE™ (ThermoFisher) 4-12% (cat# NPO323BOX) or 10% (cat# NP0303BOX) Bis-Tris gels and transferred to PVDF membrane, 0.45 μm, (Immobilon, FL-Transfer Membrane, cat # IPFL00010). Primary antibodies (Table 3) were applied overnight at 4°C. Following three washes secondary antibodies were applied at RT. Membranes were then imaged using the LI-COR imaging system (Odyssey Clx Imager, RRID:SCR_014579, LI-COR Biosciences, 4647 Superior Street,

Table 3. *Antibodies used across all studies.*

Antibody	Company	Catalog #	Species	Paper	Dilution/Method
Iba1	Abcam	ab5076	Goat	I	1:500/IHC
Map-2	Synaptic Systems	188003	Rabbit	III	1:1000/ICC
P2Ry12	Biolegend	848002	Rat	III	1:200/ICC
GLP-1R	Invitrogen	PA5- 97789	Rabbit	III	1:100/ICC
Iba1	Synaptic Systems	234004	Guinea Pig	III, IV, V	1:500/IHC and ICC
TMEM119	Synaptic Systems	400001	Mouse	IV	1:200/IHC and ICC
NF-κB	Cell Signaling	6956S	Mouse	IV	1:500/ICC
Phalloidin	Invitrogen	A12379	n/a	IV	1:500/ICC
Cofilin	Invitrogen	MA5-27737	Mouse	IV	1:1000/ Western Blot
P-Cofilin (Ser3)	Cell Signaling	3313-S	Rabbit	IV	1:1000/Western Blot
GAPDH	Invitrogen	MA5-15738	Mouse	IV	1:5000/Western Blot
GAPDH	Cell Signaling	2118S	Rabbit	IV	1:1000/Western Blot
iNOS	Cell Signaling	13120S	Rabbit	IV	1:1000/ Western Blot
GFP/YFP	Abcam	ab290	Rabbit	V	1:500/IHC
Nogo	Santa Cruz	271878	Mouse	V	1:50/IHC
GFAP	Invitrogen	PA1-10004	Chicken	V	1:1000/IHC
CD11B-488	Invitrogen	53-0112-80	Rat	V	0.5 μg/100μL : FACS
P2Ry12-APC	Biolegend	848006	Rat	V	1 μg/ 100μL : FACS

Lincoln, Nebraska, USA.) Anti-inflammatory effect from drug treatment was confirmed before blotting with two experiments confirming results.

3.13 Cell Viability/Toxicity Assay

We examined cell viability/drug toxicity with the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (MTS) (Promega, cat#G3580) according to the manufacturer's protocol (**Papers III and IV**). The MTS assay works by analyzing formazan concentrations, a product produced in proportion to the viable cell populations (490 nm absorbance). Following experiments, media was collected and replaced and incubated with MTS reagent. After a 1 h incubation at 37°C, plates were read using the Tecan infinite M200 Pro plate reader. Results of the MTS assay were used to normalize subsequent cytokine readouts determined via ELISA or MSD assay.

3.14 Single plex mouse TNF-α and IL-6 ELISA

Single plex enzyme linked immunosorbent assays (ELISAs) were used to evaluate treatment effects on the inflammation related cytokines TNF-α and IL-6 (**Papers II, III, and IV**). BioLegend's Mouse TNF-α ELISA MAX™ Deluxe Set (cat#430904), Mouse IL-6 ELISA MAX™ Deluxe Set (cat#431304), or Human IL-6 ELISA MAX™ Deluxe Set (cat#430904) were used, according to the manufacturer's protocol. At least three separate cultures confirmed the effects of Y27632 and RKI1447 against an LPS challenge. Two separate experiments confirmed effects of Narc and LPS on cytokine production. All cytokine results were normalized to MTS assay readouts. Single plex ELISAs were read using the SpectraMax PLUS 384 plate reader with Softmax Pro v. 7.1 software.

3.15 Mesoscale Discovery (Electrochemiluminescence) Detection of Cytokines

Media samples obtained from LPS, Y27632 and RKI1447 treated primary and IMG cells (**Paper IV**) were subjected to multiplex analysis examining the levels of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α. We used the V-PLEX Mouse Proinflammatory Panel 1, MULTI-SPOT® 96-well, 10-spot plate (cat#N05048A-1, Meso Scale Discovery) according to the manufacturer's protocol. IL-4 and IFNγ were below the level of assay detection and hence are not included in graphs.

3.16 Behavioral Assessments

We employed a variety of behavioral assessments throughout **Papers I, II, and V**. In **Papers I and II** working and recognition memory were tested with the spontaneous alteration test (Y-maze) and novel object recognition (NOR) tests. A modified version of the spontaneous alteration test was also used in **Paper V**. Briefly, the spontaneous alteration test measures subjects' willingness to explore new areas, while the NOR measures the ability of subjects to recognize new objects. Mice with memory deficits will not explore the new areas or objects more than old or familiar spaces/objects. Anxiety was evaluated using the elevated plus maze (EPM) in **Papers I, II, and V** together with the open field test (OFT). Mice that have increased anxiety will explore open arms of the EPM less than mice with no anxiogenic phenotypes. In the OFT, mice that are anxious tend to not explore the center of the apparatus. The OFT was additionally used as a measure of locomotion, which was determined over 30 minutes of exploration allowed by each mouse. In **Paper V** we also used the Morris Water Maze (MWM) and conditioned fear

paradigms to assess memory before and after CCI, respectively. The MWM measures the ability of mice to remember where a rescue platform is located. Several trials of training allow mice to learn where the platform is. Mice with poor memory will spend longer times locating the platform. In the conditioned fear paradigm, mice learn to associate a shock with a sound and will typically exhibit freezing behavior in response to a sound and anticipation of shock as training in the test progresses. Those that do not associate the tone with the shock freeze less and typically are considered to have deficits in learning/memory. In **Paper V**, mice were singly housed with activity continuously tracked using the digital individually ventilated cage system (DVC™; Tecniplast). Animals were tracked for 42 days pre-CCI and 14 days post-CCI. The last test behavior evaluated in **Paper V** was asymmetrical motor function assessed via the elevated body swing test (EBST).[275] Briefly, the EBST measures the tendency for unilaterally brain injured mice to turn their body contralaterally when held by the base of the tail. Motor function disruptions on one side of the brain bias ability to turn towards the contralateral side. Normally, animals will turn roughly 50% to either side. Mice were assessed over 20 turns before CCI and 7 and 14 days post-CCI. Full descriptions of behavioral assessments are found in constituent papers.

3.17 siRNA Transfection

To assess ROCK1 and ROCK2 specific roles in inflammation, we utilized an siRNA approach in IMG cells (**Paper IV**). Silencer Select pre-designed siRNAs (ThermoFisher) for ROCK1 (cat#4390771), ROCK2 (cat#4390771), and negative control/scrambled moieties (cat#4390843) were used. Transfection was performed according to manufacturer's protocol (#MAN0007825 Rev1.0) with Lipofectamine™ RNAiMAX transfection reagent (ThermoFisher, cat#13778075) in OptiMEM™ reduced serum media (Gibco™, cat#31985070). We performed the transfections with ROCK1 and ROCK2 siRNAs both separately and combined to understand if there was an individual or combined contribution of ROCK inhibition responsible for decreased inflammatory cytokine production. Cells were treated with siRNAs for 24 h and then challenged with LPS for an additional 24 h. siRNA transfection continued in parallel to the LPS challenge. Media was collected and evaluated for TNF-α and IL-6 via ELISA.

3.18 FACS sorting

Microglia were extracted from MinoKO, Cre Control, and Floxed Control animals as described above for PMg cultures (**Paper V**). Cells were resuspended in 100 mL FACS staining buffer (HBSS without calcium or magnesium, 1mM EDTA, and .5% UltraPure BSA) and incubated in TruStain FcX™ Block (BioLegend) for 10 minutes followed by subsequent addition antibodies (P2Ry12 and CD11b) at manufacturer recommended dilutions. The Beckman Coulter Moflo XDP cell sorter was used with a 70 μm nozzle, 39psi sheath pressure, and a sample flow rate of 2000-3000 cells/second. Samples were collected on ice cold 100 mL FACS collection buffer (HBSS, 1mM EDTA, and 1% UltraPure BSA) in RNAse free 500 mL tubes). Live cells were gated from total isolated cells, followed by a doublet exclusion step and subsequent gating via antibody stains. Controls were used to establish gating boundaries for final sort. RNA was immediately extracted from samples using the Arcturus™ Pico-Pure (Cat#KIT0204) kit according to manufacturer's protocol.

3.19 RNA Quantification and Quality Assessment

For RNA isolated from FACS sorted microglia (**Paper V**), we employed the Agilent 2100 Bioanalyzer using the RNA 6000 Pico Kit (cat # 5061-1513) to quantify and assess quality of each sample. RNA samples with RNA integrity number of 7+ were used for downstream RT-qPCR to confirm

recombination in MinoKO mice. Quality and concentration of total RNA from IMG cells (**Paper IV**) were determined using the NanoDrop™ 2000 spectrophotometer (ThermoFisher, cat# ND-2000, RRID:SCR_018042) by measuring absorbance at 230, 260 and 280 nm wavelengths. Only RNA within acceptable ratios was used for subsequent analysis.

3.2O RT-qPCR for IMG Cells

RNA isolated from IMG cell treatments (**Paper IV**) was quantified using the RNAEasy kit (Qiagen, cat# 74136) according to manufacturer's protocol. Total RNA (1 µg) was converted to cDNA using the iScript™ cDNA Synthesis kit (20 µL reaction) according to manufacturer's protocol using a BioRad MJ Mini™ Personal Thermocycler. cDNA generated (10 ng/reaction) was used for downstream RT-qPCR using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, cat#1725121;10 µL reaction). Primer sequences used are shown in Table 4.

Table 4. *Primer sequences for primers used in the evaluation of mRNA transcript levels.*

Probe Name		5'-3' Sequence	Source
ROCK1	Fw	GCTCATCTCTGTGTGACTCT	NM_009071.2
	Rv	TACGGAAAGCAAGTCAGACC	
ROCK2	Fw	GGTCAATCAGCTCCAGAAAC	NM_009072.2
	Rv	GTTTGGAACTTTCTGCCTGG	
TNF-α	Fw	GGCAGGTCTACTTTGGAGTCATTG	276
	Rv	ACATTCGAGGCTCCAGTGAATTCGG	
iNOS	Fw	TACTCCATCAGCTCCTCCCA	NM_010927.4
	Rv	GTTCCTGATCCAAGTGCTGC	
IL-6	Fw	TTCTTGGGACTGATGTTGTTGAC	277
	Rv	AATTAAGCCTCCGACTTGTGAAG	
β-actin	Fw	TGAGAGGGAAATCGTGCGTGAC	NM_007393.4

4 RESULTS and DISCUSSION

4.1 Incretin Mimetics are Anti-Inflammatory in *in vitro* and *in vivo* mouse models of neuroinflammation (Papers I–III)

4.1.1 Paper I

In mice, PT302, a slow release Exenatide formulation, is anti-inflammatory in a model of mTBI and dose-dependently maintains blood plasma concentrations, entering the brain at therapeutic concentrations.

As there are many formulations of incretin mimetics already developed for metabolic diseases, repurposing these drugs towards treating neurodegenerative disorders requires understanding of their ability to enter the brain and confer reparative mechanisms.[43,82] Incretins are peptide drugs that have large molecular weights, making it a challenge to optimize formulations that readily cross the BBB. In **Paper I** we sought to characterize the pharmacokinetics and action of a new slow release formulation of Exenatide, PT302 (currently referred to PT320). Delivering the drug subcutaneously in mice at several doses (.1-2 mg/kg), we initially established dose-dependent plasma concentrations at 7 days post injection (Figure 12A). From this data we selected three doses that skirt plasma concentrations achievable in humans administered Exenatide, and then administered these to mice as a single injection of low (.024 mg/kg), medium (0.12 mg/kg), and high (0.6 mg/kg) doses of PT302 to evaluate their time-dependent concentrations over 21 days. Each dose maintained steady-state plasma levels of Exenatide over the 21 days, with peak concentrations occurring at 14 days (Figure 12B). These results are in line with steady-state plasma levels achieved with a common clinical dose (2 mg) of Exenatide (*Bydureon*™), which were found to provide therapeutic benefit for PD patients in a recent clinical trial.[84] Importantly, mTBI had no effect on plasma Exenatide concentrations, indicating no disruption in drug

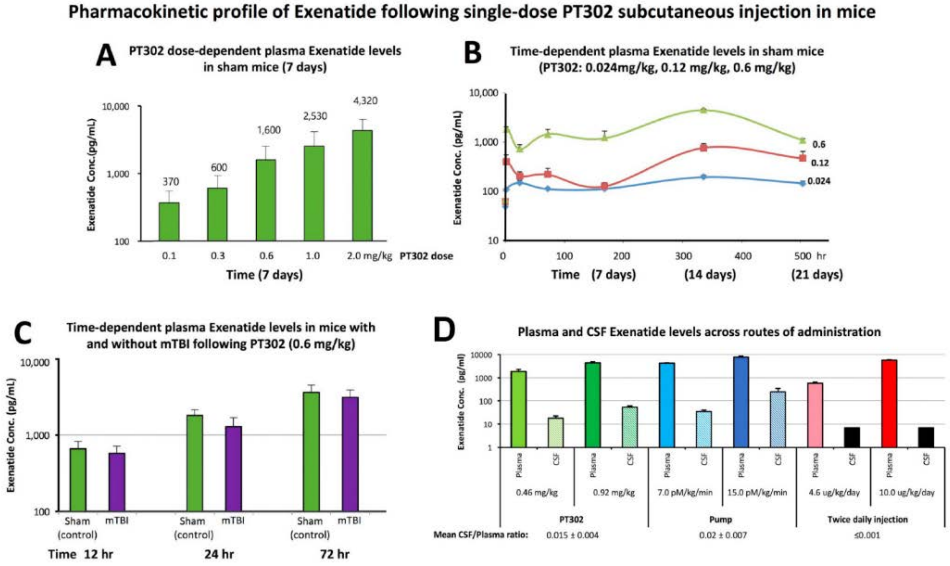

Figure 12. *Single subcutaneous injection of PT302 produces dose dependent steady state plasma concentrations of Exenatide that also enters the brain.*

metabolism/distribution from the injury (Figure 12C). Producing steady-state plasma levels is important in order for the peptide to enter the brain, as constantly maintained plasma drug levels act as a primary driving force to achieve brain entry.

As our study evaluating Exenatide concentrations in CSF shows, immediate release Exenatide did not achieve any detectable brain penetration, while PT302 (subcutaneous) and Exenatide delivered via mini-osmotic pump, did at levels 1.5%-2% of accompanying plasma levels (Figure 12D). The mini-osmotic pump was used in lieu of availability of the clinical grade, once-weekly delivered slow release form of Exenatide called *Bydureon*, and the immediate release formulation mimicked the clinically approved *Byetta*. Both of these drugs are commonly prescribed for diabetes patients. However, *Byetta* and *Bydureon* recently have been discontinued by Astrazeneca due to business rather than safety or efficacy reasons; thereby making the development of PT302 yet more important.

Along with the pharmacokinetic profile of PT302, we examined its effects in mice that were subjected to mTBI. Our group has previously established that the weight drop model of inducing mTBI produces behavioral deficits in mice, including spatial and recognition memory tests, the Y-maze and novel object recognition (NOR) tests, respectively.[198] We have also shown incretin mimetics to be effective at mitigating these deficits while conferring neuroprotection and anti-inflammatory action against the same TBI injury.[196,214,215,278,279] With all of this evidence, we hypothesized a therapeutic benefit would also be expected with PT302 administered at clinically translatable doses via the clinical route (subcutaneous). Indeed, cognitive impairment was reversed both 7 and 30 days post mTBI, via the Y-maze and NOR tests, with our studies confirming that PT302 reduced the amount of neuronal cell death induced by the injury. Neuroinflammation was also reduced, demonstrated via reduced microglial (Iba1) and astrocyte (glial fibrillary acidic protein-GFAP) immunostaining of affected brain regions, Iba1 and TNF-α colocalization, and microglial coverage post mTBI. An example of the effects of PT302 on microglial immunoreactivity 72 hours post mTBI is shown in Figure 13. mTBI increases Iba1 immunoreactivity, which is reduced by both a low and high dose of PT302.

Although direct comparison to *Bydureon*™ was not possible in this study, our group has since performed head to head pharmacokinetic studies between *Bydureon* and PT302 in non-human primates.[280] While total exposure to Exenatide was similar between PT302 and *Bydureon,* PT302 showed superiority in more rapidly reaching and maintaining steady-state blood plasma levels, while *Bydureon* dipped below therapeutic concentrations earlier and behaved in a biphasic manner.

Brain concentrations of Exenatide delivered in a slow release form reach similar levels in humans.[84] Interest in small molecule, non-peptide designer GLP-1R agonists including TTP273[281] and TT-OAD2[282] may provide a new option to explore GLP-1R agonists that confer higher potency in the CNS. Although these small molecules have potential for increasing brain penetration, they have yet to be proven more effective at crossing the BBB.[283] Regardless, we have shown that maintaining steady-state plasma levels of Exenatide is an important factor in increasing the amount of drug that enters the brain. Structurally equivalent rapid release Exenatide, did not enter the brain at detectable levels, highlighting the need for more studies to understand which clinically approved medications should move forward for repurposing towards treating neurodegenerative disorders.

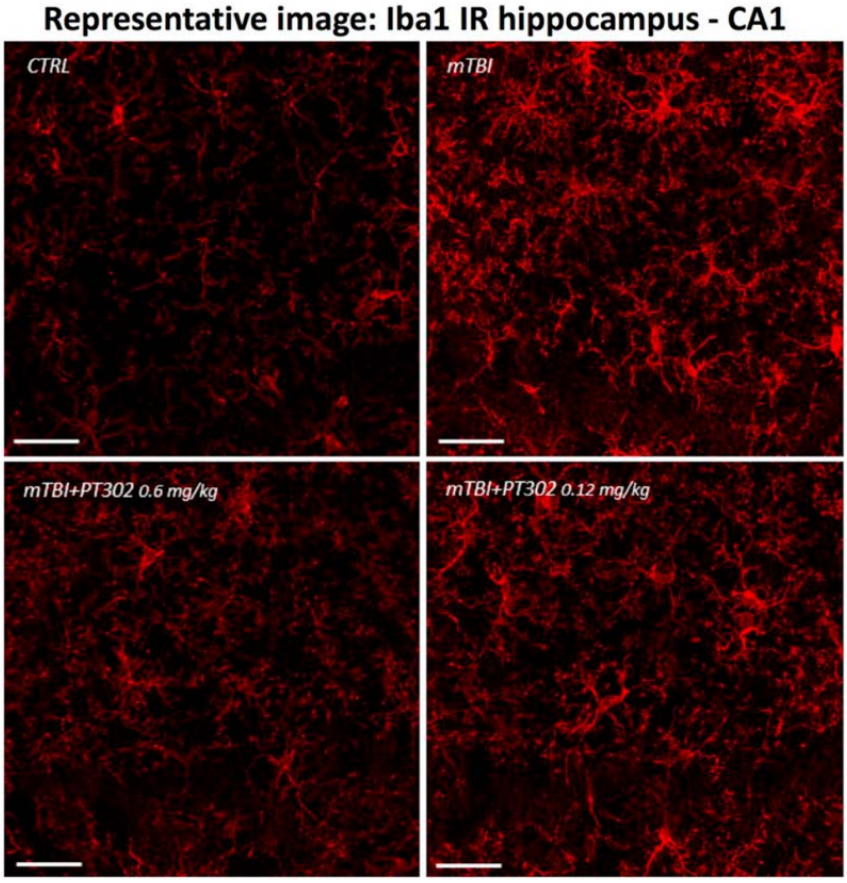

Figure 13. *mTBI induced increases in hippocampal microglia immunoreactivity is decreased with both low (.12 mg/kg) and high (.6 mg/kg) doses of PT302 (72 h post-TBI)..*

4.1.2 Paper II

A monomeric GLP-1/GIP/Gcg receptor triagonist induces neuroprotective cellular pathways, reduces TNF-α released from PMg cultures, and mitigates behavioral deficits associated with mTBI.

Joining a growing body of literature showing that incretins exert protective action on the brain, we showed in **Paper I** the neuroprotective and anti-inflammatory effects of a new slow release formulation of Exenatide, PT302. Though single GLP-1R agonists are very effective for treating diabetes clinically and mitigating pathology in models of brain injury/neurodegeneration as we and others have previously shown,[284–286] there is growing interest in delivering unimolecular multi-agonist incretin mimetics that may potentially provide even more robust effects.[52] Dual agonism of GLP-1/GIP receptors or GLP-1/Gcg receptors with monomeric compounds have been shown to provide enhanced effects over single receptor agonists in animal models of neurodegeneration[286–289] and in treating metabolic diseases in humans.[290–292] In a recent clinical trial, *Tirzepatide*, a dual GLP-1/GIP receptor agonist, produced enhanced weight loss compared to the single GLP-1R agonist, semaglutide.[293] Although in infant stages of development, unimolecular triple receptor agonists combining GLP-1, GIP, *and* Gcg have been recognized to be superior to dual receptor agonists for treating metabolic diseases in humans and in animal models,[294,295] producing more physiologically balanced signaling.[296] Triple receptor agonists were also beneficial to mitigating pathology and cognitive deficits associated with AD mouse models.[96,97,297]

As triple agonists are currently being developed for treating metabolic diseases and in the light of the growing evidence of synergistic benefits of activating all three receptors, we hypothesized in **Paper II** that a unimolecular incretin based triagonist would be beneficial in our models of neurodegeneration and neuroinflammation, including the WD model of TBI. In addition, we used a variety of cellular assays to assess signaling characteristics of the drug. We used the SH-SY5Y neuroblastoma cell line as a model system for neuronal cultures and assessed the levels of cAMP produced by the drug. cAMP is a primary upstream regulator of several cell signaling pathways that are neurotrophic, neuroprotective, anti-apoptotic and anti-inflammatory.[2,298] The triagonist dose dependently increased production of cAMP in SHSY-5Y cells, sustaining steady expression levels over one hour. Additionally, the triagonist produced significantly more cAMP in both SH-SY5Y cells and primary astrocytes than Exendin-4 (single GLP-1R agonist), in line with the previous studies mentioned above. We further showed the triagonist provided superior neuroprotection against glutamate induced toxicity than dual or single incretin receptor agonists. Using various combinations of GLP-1, GIP, and Gcg receptor antagonists, we showed that this particular formulation of triagonist does not exhibit biased agonism towards one receptor; rather, its neuroprotective benefit relies on all three receptors being activated.

For this study, I introduced the primary mouse microglial model of neuroinflammation and found that the triagonist exerts potent anti-inflammatory action against an LPS challenge, significantly reducing TNF-α production from the cells (Figure 14). This is the first application of the triple agonist performed on PMg cultures and is in line with the *in vivo* studies showing an incretin-based triagonist reduced microglial and astrocyte activation in an AD mouse model.[297] As we showed in **Paper I**, the mouse WD TBI model produces lasting cognitive deficits that were ameliorated by administration of a GLP-1R agonist. We therefore assessed whether the triagonist produced similar cognitive benefits. Triagonist was administered at a clinically translatable dose via its clinical route (subcutaneous) once daily for

seven days after the TBI and produced lasting effects, as the deficits shown in the Y-maze spontaneous alteration test and NOR test were reversed both 7 and 30 days post mTBI.

One disadvantage of the triagonist studied in **Paper II** versus PT302 of **Paper I** is the requirement for daily injections. Pain associated with injections can affect patient willingness to use a drug, and increased frequency of this delivery mechanism can reduce overall compliance.[299] As the benefits of triple receptor agonists become increasingly understood and applied to human conditions, technology advances made for delivering single GLP-1R agonists, such as PT302, will undoubtedly be applied to these peptides. Nevertheless, this current study cross-validates efficacy shown in our previous work with single and dual incretin receptor agonists in animal models of TBI[188,196,215,300,301] along with other assessments of triple incretin agonists in mouse AD models.[96,297] As studies of triple incretin receptor agonists in humans advance, parallel assessment of these compounds for neurological conditions is warranted.

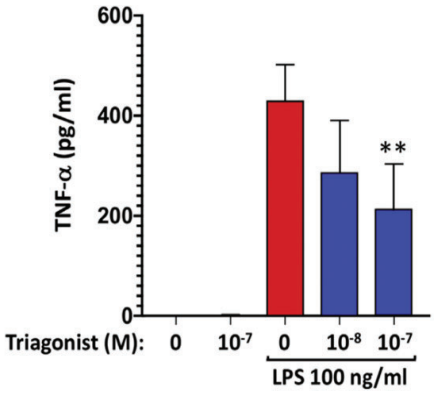

Figure 14. *Anti-inflammatory effects of Triagonist against on LPS challenge in PMg cultures. Cells were treated with triagonist 1 h before LPS challenge. TNF-α levels were measured after 24 h. **P < 0.01 vs. LPS alone (red bar) (n = 4 per group).*

4.1.3 Paper III

The insulinotropic GLP-1(7-36) metabolite, GLP-1(9-36), is neuroprotective and anti-inflammatory in models of neurodegeneration.

As we have previously described in **Papers I** and **II**, incretin mimetics are widely used for treating diabetes and are now being repurposed towards use in neurodegenerative orders. Individuals with diabetes have heightened risks for developing chronic neurodegenerative disorders, including AD[302] and PD,[303] with insulin resistance also a strong link to these diseases.[63] Interestingly, diabetes patients treated with incretin mimetics have lower risk for developing PD[88,304] or stroke,[89,90] further highlighting the connection of metabolic dysfunction with brain disease. Abnormal incretin and insulin signaling play roles in the eventual development of disease, making insulin a possible treatment for neurodegeneration; however, a clinical trial (NCT01767909) was performed testing the feasibility of using intranasal insulin to treat cognitive impairment and no benefit was observed over a 12-month treatment period.[305] This is in contrast to PD disease modifying effects observed from once weekly administration of a GLP-1R agonist in a human clinical trial (NCT01971242).[84] One reason for discrepancy and possible increased benefits from using incretin mimetics over insulin is that incretin mimetics restore insulin signaling pathways that insulin alone cannot repair.[306] Additionally, the pleiotropic action of incretins, compared to insulin, on many cell types throughout the brain and body via their respective GPCRs activate pathways particularly beneficial to reducing neurodegenerative pathology.[307]

While much focus has been put into understanding how incretin and insulin dysfunction play roles in neurodegeneration, very little attention has been paid to biological roles of long acting circulating metabolites of GLP-1(7-36), most notably GLP-1(9-36), which is produced via the rapid cleavage of the parent peptide by DPP-IV. GLP-1(9-36) remains in the blood at up to 10-fold higher levels than GLP-1(7-36)[111] and was long thought to be inert or confer no biological role. Although, it is not insulinotropic, it does confer glucoregulatory properties by potently inhibiting hepatic glucose production in humans.[111] With the evidence that the incretin system is impaired in T2DM patients,[308] and these patients also showing increased risk of developing neurodegenerative disease,[309] the circulating GLP-1(9-36) metabolite may play an active role in this prognosis.

With all of this in mind, the goal of **Paper III** was to determine if GLP-1(9-36) has biological function in nervous system cells. We utilized SH-SY5Y neuroblastoma cells as we did in **Paper II**, but also employed a GLP-1R overexpressing variety of SH-SY5Y cells we have previously used,[310] called #9 cells. Interestingly, the metabolite did produce a peak cAMP response, albeit much smaller, similar to GLP-1 at 5 minutes after administration to SH-SY5Y cells. However, the response quickly returned to baseline. When applied to the #9 cells, the metabolite produced and sustained a much higher cAMP response up to one hour after administration. Notably, a high dose of GLP-1(9-36) also increased cAMP production in primary cortical neurons up to one hour post administration, verifying the result observed in the SH-SY5Y neuronal cell models. GLP-1(9-36) also exhibited neurotrophic and neuroprotective action, increasing cell viabilities in the presence or absence of a toxic glutamate challenge. Glutamate is an endogenous excitatory neurotransmitter; however, overactivation of its receptors in neurons is toxic and is a feature observed in neurodegenerative disease and acute brain injury, including TBI and stroke.[311]

Several studies purport GLP-1(9-36) acts via a receptor independent from the GLP-1R.[116,117,312,313] However, we show in #9 cells that the metabolite exerts GLP-1R mediated neuroprotective action against a hydrogen peroxide challenge, a model of ROS toxicity. The metabolite's protective action was

abolished when applied in parallel with a selective GLP-1R antagonist. We also differentiated the SH-SY5Y cells and #9 cells to exhibit more neuronal-like features, including dendritic processes, and showed similar neuroprotective effects of the metabolite in the hydrogen peroxide challenge. To elucidate signaling cascades that may be involved in the neuroprotective and neurotrophic effects of GLP-1(9-36) that we observed in #9 cells, we inhibited the cAMP dependent signaling proteins protein kinase A (PKA) and AMPK, via H89 and Compound C respectively, in the presence or absence of GLP-1(9-36) and a toxic glutamate challenge. Against a glutamate challenge, both PKA and AMPK inhibition blocked the protective effects exerted by GLP-1(9-36).

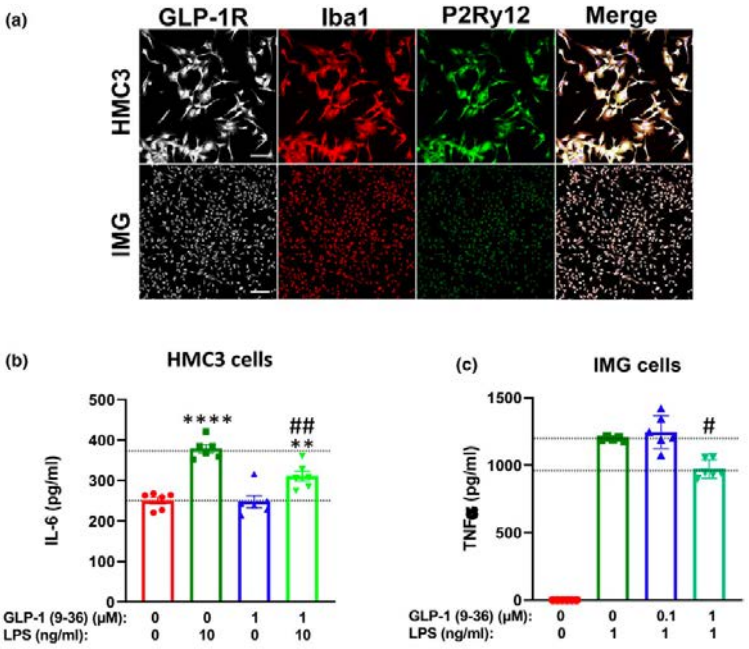

Figure 15*. Anti-inflammatory effects of GLP-1(9-36) in human HMC3 and mouse IMG microglial cells against a LPS challenge. (a) We establish these cell lines express microglial markers, Iba1 and P2Ry12, as well as the GLP-1R. Anti-inflammatory effects were observed in both (b) HMC3 and (c) IMG cells. One-way analysis of variance (ANOVA) tests were used for comparison of multiple samples, followed by post hoc Tukey's multiple tests. Statistical comparison vs. vehicle-treated control: **P < 0.01; ****P < 0.0001; comparison vs. LPS alone: #P < 0.05; ##P < 0.01) (scale bar= 100 μm)*

We next established GLP-1R, Iba1, and P2Ry12 protein expression in two microglial cell lines, human (HMC3) and mouse (IMG) immortalized microglia, via ICC (Figure 15a). Importantly, these cell lines express two of the canonical markers of microglia *and* a receptor that may confer biological action of GLP-1(9-36). A 24 hour pre-incubation of high doses of GLP-1(9-36) (1μM) reduces pro-inflammatory cytokines (IL-6 and TNF-α) observed in HMC3 and IMG cells challenged with low doses of LPS (Figure 15b and c). HMC3 cells are unable to produce TNF-α, which is one disadvantage of these cells as a model microglia system.[314] These cells also expressed high levels of baseline IL-6, which is not typical of unstimulated microglia. Confirming anti-inflammatory effects in both human and mouse microglial cell lines cross-validates anti-inflammatory effects of other GLP-1R activating incretin mimetics described in **Papers I** and **II**.

We lastly applied the metabolite to cellular models of PD and AD using mouse primary cortical and hippocampal mixed cultures. For the PD model, alpha-synuclein (α-syn) was used to mimic pathological proteins present in the dopaminergic-rich substantia nigra in the human condition. The metabolite was minimally effective at counteracting the toxic effects of α-syn, though neurite length was significantly rescued in addition to microglial activation reduction. Aβ challenges were used in primary cortical cell cultures to model pathological proteins common throughout the human AD brain. A high dose of GLP-1(9-36) increased surviving neurons in this model.

Previous studies have demonstrated enhanced cAMP response from GLP-1(9-36) when the GLP-1R is allosterically modulated[315] and others have shown allosteric modulation of the metabolite can also have similar effects.[316] These indicate possible modifications of endogenous circulating metabolites may be an avenue to produce therapeutic products.[317,318] Although we did not modulate receptor or peptide shape, we did show increasing receptor density, as in the #9 cells, dramatically affected the level of response that GLP-1(9-36) could confer. In human PD patients' postmortem substantia nigra samples, GLP-1R mRNA was shown to be 10-fold higher than that of the healthy brains,[3] just one example of a pathological context where increased GLP-1R content may be a very active target to engage for treatment of neurodegeneration. While our studies do indicate GLP-1R activation by the metabolite, all GLP-1Rs in the brain likely are not engaged by the peptide. In murine brain tissue, for example, GLP-1(9- 36) that has been radiolabeled binds hippocampal tissue at similar levels of GLP- 1(7- 36), although GLP-1(7-36) labeled other areas of the brain at much higher levels.[319]

Despite this, there is body of growing research, including studies found in **Paper III**, that suggest a neuroprotective and likely homeostatic role of GLP-1(9-36) in the brain. Incretin deficits, including decreased baseline levels of circulating GLP-1(9-36), may potentiate or exacerbate neurodegenerative conditions, which our studies provide evidence for. A full summary of findings in **Paper III** are shown in Figure 16.

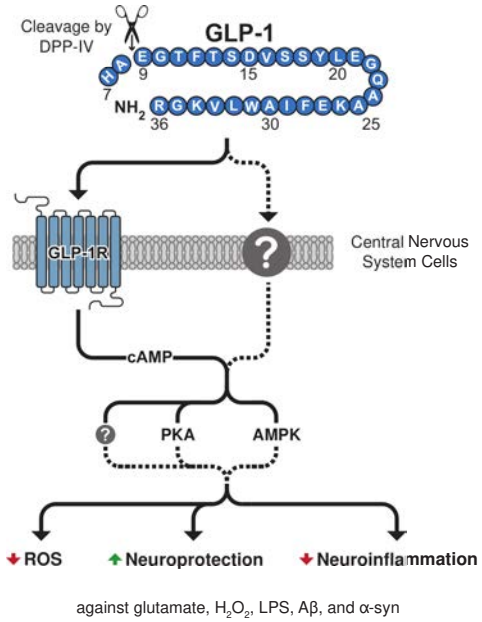

Figure 16. Summary of results in Paper III. *Dotted lines and "?" acknowledge literature indicating possible other GLP-1(9-36) mechanisms of action, with solid lines showing evaluations performed in Paper III.*

4.2 RhoA/ROCK and the Nogo signaling system in microglia (Papers IV-V)

4.2.1 Paper IV

RhoA-ROCK1/ROCK2 mediate signaling exacerbates LPS induced inflammatory signaling in cellular models of microglia

As we have already investigated phenotypic microglial responses to incretin treatment in **Papers I-III**, we sought to explore more novel mechanisms of neuroinflammation including the influence of ROCK protein in **Paper IV**. The two isoforms of the ROCK enzymes, ROCK1 and ROCK2 belong to the serine-threonine kinase family and are activated by their upstream regulator, RhoA.[320] Overall, the two ROCK proteins are 64% homologous, but their kinase regions share over 90% similar structure.[321] Interestingly, even with the very similar kinase regions, ROCK1 and ROCK2 have been shown to perform independent roles, both in the periphery and in the CNS.[175–178] The RhoA/ROCK signaling pathway, activated prominently but not exclusively by endogenous Nogo ligands, is a potent regulator of actin dynamics and restrictor of neurite outgrowth,[322] but also performs cell type specific actions.[323,324] Nogo protein activation of RhoA/ROCK in microglia has recently been attributed to promoting an inflammatory response[4,325]; hence our work in **Paper IV** sought to expand on this understudied role of this ubiquitous signaling system.

There is a wide variety of pharmacologic tools to disrupt ROCK activity, including over 170 ROCK inhibitors described.[326] Most of these drugs work by blocking the ATP binding site on ROCK's kinase region, thereby inactivating the catalytic region of the protein.[327] Even with the large quantity of ROCK inhibitors produced, only a select few have reached approval for clinical use. In the US, the FDA approved belumosudil for the treatment of chronic graft-versus-host disease[328] and netarsudil for glaucoma.[329] Japan, China, and Korea have also approved the ROCK inhibitors, ripasudil and netarsudil, for glaucoma[329] and fasudil for treating vasospasms associated with cerebral ischemia.[330] Fasudil is the earliest ROCK inhibitors approved for clinical use.

In **Paper IV**, we first sought to establish the IMG cell line, from **Paper III**, as a viable model for PMg, which I introduced in **Paper II**. Adding to our work in showing IMG cells express P2Ry12 (from **Paper III**), we showed that IMG cells also express the canonical microglial marker TMEM119. We then observed the IMG cell and the PMg response to varying doses of LPS. Both cell types dose-dependently produced increasing amounts of TNF-α in response to increasing doses of LPS, with overall secretion patterns of other cytokines being similar, especially pro-inflammatory immune regulating cytokines IL-6 and IL-12p70. We screened media from LPS treated IMG and PMg for 10 cytokines (TNF-α, IL-6, IL-2, IL-4, IL-5, IL-10, IL-12p70, KC/GRO, IL-1β, and IFNγ) and showed that both cell types produce eight of the analytes. No detectable production of IFNγ or IL-4 were observed from either cell type. Although the original characterization of IMG cells showed them to be comparable to PMg,[272] this is the first broad analysis of cytokine protein production. Our analyses were performed on cells treated with a submaximal dose of LPS (10 ng/mL), so it is likely the low or absent detection of several of the cytokines would be markedly enhanced by a more potent LPS challenge.

After this initial characterization of IMG and PMg, we assessed the influence of ROCK on the LPS induced inflammation response. In our studies, we first utilized the pan-kinase inhibitor Y27632-dihydrochloride (Y27632), which confers its highest inhibition properties on ROCK1 and ROCK2, but

also has potent activity on other kinases, including PKA, protein kinase C (PKC), and myosin light chain kinase (MLCK) (Figure 17A). Although it has other kinase inhibition activity, Y27632 is widely referred to as a ROCK inhibitor in the literature. Against a 10 ng/mL LPS challenge, preincubation of IMG (Figure 17Bi) and PMg (Figure 17Bii) cells with increasing doses of Y27632 dose-dependently decreased the amount of TNF-α produced in both. Y27632 also significantly reduced IL-6, KC/GRO, and IL-12p70, the most prominently produced pro-inflammatory cytokines, with minimal changes in the other cytokines screened. These had low expression even without the drug treatment. Intracellular production of the pro-inflammatory protein inducible nitric oxide synthase (iNOS), evaluated via Western blot, was also significantly reduced by Y27632 pre-treatment in IMG cells (Figure 17C). Our results cross-validate other studies using Y27632, showing anti-inflammatory effects of the drug.[331–334]

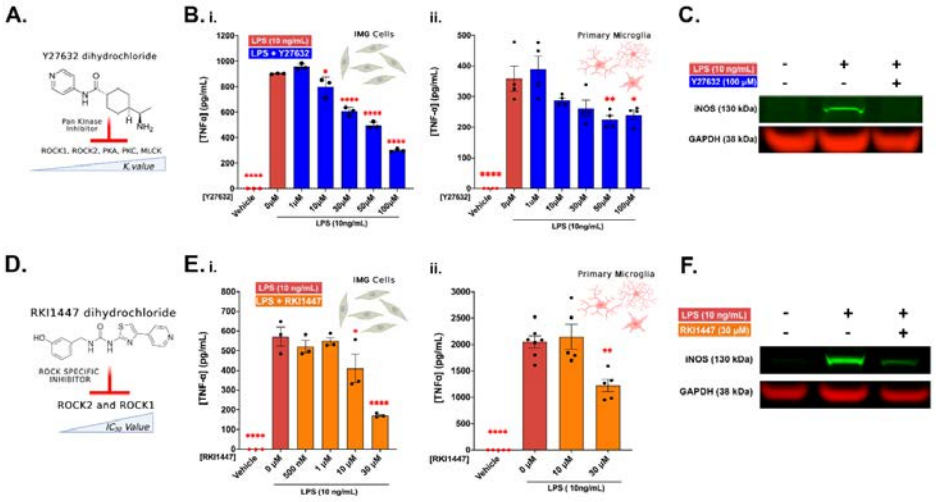

Figure 17. Anti-inflammatory effects of Y27632 and RKI1447: ROCK1 and ROCK2 inhibition is sufficient to produce anti-inflammatory effects. *Analyte samples with concentrations below assay detection limit are shown as red data points and are assumed equivalent to 0 pg/mL to allow for statistical analysis. In B and E, One-way ANOVA with Dunnett's comparison (vs. 0 μM doses) and Tukey's comparison, respectively, were used. Error bars represent mean ±SEM; (*= p<.05, **=p<.01, and ****=p<.0001). A and D were created using BioRender.com.*

As pan-kinase inhibition was effective at mitigating LPS induced inflammatory responses in IMG and PMg, we sought to evaluate if ROCK1 and ROCK2 specific inhibition was sufficient to produce the same observed effect. To probe this, we used the highly specific ROCK2/ROCK1 inhibitor RKI1447-dihydrohloride (RKI1447)[335] (Figure 17D). RKI1447 is a relatively new ROCK inhibitor that has not previously been evaluated for efficacy in nervous system tissue. It was formerly shown to be anti-inflammatory in a mouse model of liver disease[336] and ameliorated LPS induced lung tissue damage in a model of ventilator-associated lung injury.[337] We performed dose-response relationship assays with RKI1447 pretreatments against the 10 ng/mL LPS challenge and, indeed, observed a similar anti-inflammatory effects in IMG (Figure 17Ei and 17F) and PMg (Figure 17Eii) cells as determined with Y27632.

ROCK activation initiates a variety of downstream signaling pathways, most prominently terminating with the phosphorylation (Ser3) of cofilin, a protein intricately involved in actin cytoskeleton rearrangement and turnover. The active form of cofilin is un-phosphorylated and has been linked to neurodegenerative disease[338] and neuroinflammation.[184,339,340] Phosphatases, such as SSH, restore cofilin activation.[341] Our studies show that LPS potently activates cofilin in IMG cells, reducing the ratio of phosphorylated (p)-cofilin to total cofilin. ROCK inhibition by either or Y27632 (Figure 18A and B) or RKI1147 (Figure 18A and C) inhibited this LPS induced effect. This result is counterintuitive in that ROCK inhibition would also infer increased blockade of cofilin phosphorylation.[182] This is the case on shorter timescales (< 6 h),[342] but our 24 h treatments show the opposite result. This implies that ROCK inhibition is blocking another pathway affected by the LPS treatment (Figure 18D). Nucleotide-binding oligomerization domain-containing protein 1 (NOD1), may be a likely candidate affected by these treatments, as it participates in LPS induced inflammation,[343] is linked to SSH activity, and is influenced by ROCK signaling.[344]

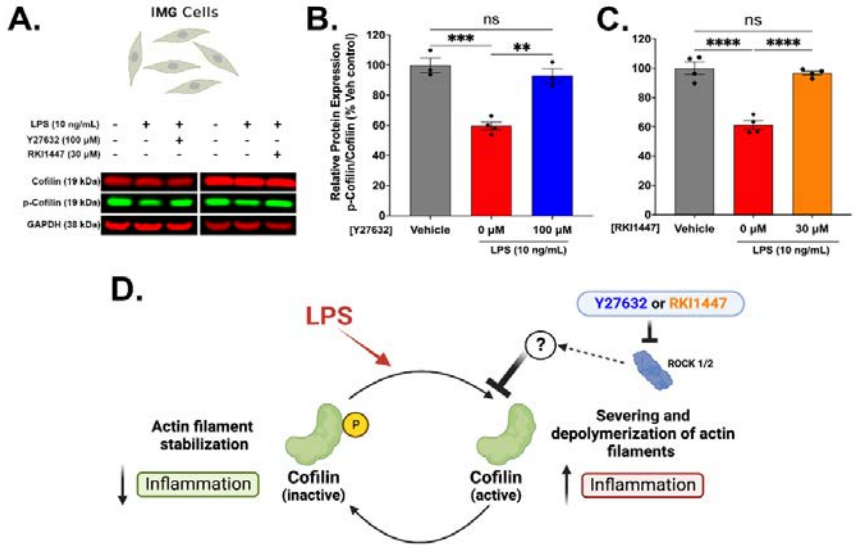

Figure 18. ROCK inhibition blocks cofilin activation induced by LPS. *One-way ANOVA with Tukey's multiple comparison was used for statistical analysis with error bars representing mean ±SEM; **=p<.01, ***=p<.001, and ****=p<.0001. (D) Created using BioRender.com.*

Further interrogation of ROCK signaling and inflammation brought additional questions as to how the RhoA/ROCK pathway influences pro-inflammatory gene production. We decided to apply potent activators of RhoA, Nogo-P4[325] and Narc,[345] alone and in conjunction with a LPS challenge, to see if RhoA activation influenced inflammation (Figure 19A). Nogo-P4 is a commercially available peptide that mimics the active regions shared by Nogo-A, -B, and -C, the prominent ligands involved in the Nogo signaling system. Neither Nogo-P4 nor Narc induced an inflammatory response alone; however, both exacerbated cytokine production induced by the LPS stimulus (Figure 19B and C). Our results are contradictory to a study showing Nogo-P4 induces inflammatory responses in BV-2 microglia,[4] but are in line with a study that showed Narc alone not inducing inflammatory proteins.[346] In our studies, ROCK inhibition blocked the Narc increases in TNF-α and IL-6 produced in conjunction with the LPS

challenge (Figure 19 Ci. and Cii.), implying the exacerbated effect was ROCK mediated. Interestingly, other studies have shown an anti-inflammatory effect from Narc treatments;[345,347–349] however those studies used much higher doses of Narc and did not normalize cytokine levels to cell viabilities, an important factor as the compound is highly cytotoxic. RhoA has divergent roles depending on the activation state of the cell, which is in line with our findings.[350] Representative images of various treatments from Figure 19C are shown in Figure 19D. Clear retraction of microglial processes occur in the Narc and Narc + LPS treatments. Processes and viability of cells is restored with either Y27632 or RKI1447 treatment, further validating a the RhoA/ROCK mediated increases in cytokine levels observed above. Clearly, there are multiple pathways being engaged that we did not probe for, but nonetheless, this should be a ripe area of inquiry in the future.

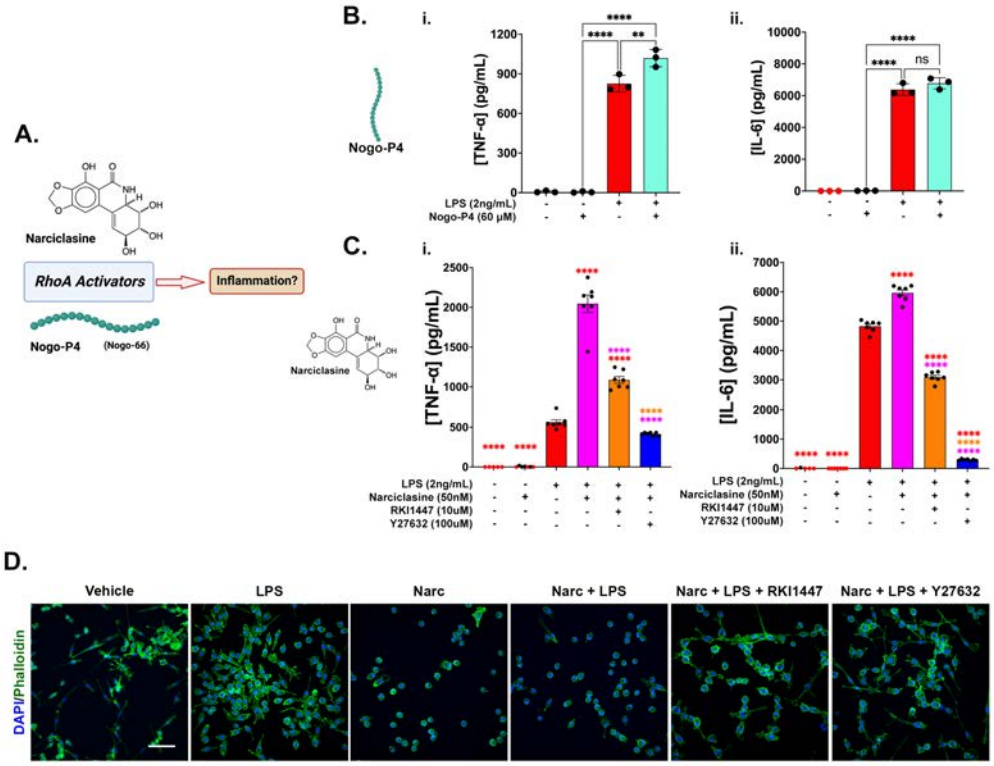

Figure 19. RhoA activation alone in IMG cells does not induce an inflammatory response but exacerbates that response in the presence of a submaximal LPS challenge. *(A) Narciclasine (Narc) and Nogo-P4 are RhoA activators (B) TNF-α (i) and IL-6 (ii) secretion in IMG cells treated with Nogo-P4, LPS, and Nogo-P4 + LPS (n=3/group). (C) Narc (50 nM) ±LPS (2 ng/mL) and Narc (50 nM) + LPS (2 ng/mL) + Y27632 (100 μM) or RKI1447 (10 μM). (n=7 or 8/group) TNF-α (i) and IL-6 (i) ELISA data. (D) Representative images (40x mag) of IMG cells stained for phalloidin (filamentous actin) and DAPI from all treatment groups from C (scale bar= 50 mm). One way ANOVA was used for B and D-F with Tukey's multiple comparison tests used for statistical analysis. Analyte samples with concentrations below assay detection limit are shown in red and are assumed equivalent to 0 pg/mL to allow for statistical analysis. Error bars represent mean ± SEM. (**=p<0.01, and ****=p<0.0001 with color of asterisk corresponding to comparison group in C). (A) was created using BioRender.com.*

We further sought to understand the mechanisms by which ROCK inhibitors block inflammation in microglia by evaluating how Y27632 and RKI1447 modulated LPS induced NF-κB nuclear translocation. Previous studies have established ROCK inhibitors' ability to block NF-κB nuclear translocation,[351–354] but our studies are the first evaluating these effects in microglia. NF-κB is a transcription factor responsible for regulating a wide variety of proinflammatory genes. In order to induce pro-inflammatory gene production, it enters the nucleus rapidly after a stimulus such as LPS employed in our inflammation model.[13] Using an immunochemical approach, we were able to visualize and analyze NF-κB nuclear localization at single cell resolution (Figure 20A). The images shown are from 1 h pretreatment with ROCK inhibitors and 15 minutes after the LPS challenge, allowing capture of the rapid induction of NF-κB. Both Y27632 and RKI1447 potently inhibit NF-κB nuclear translocation (Figure 20B). We also analyzed NF-κB-dependent proinflammatory gene production, including TNF-α, IL-6, and iNOS via RT-qPCR, and verified both ROCK inhibitor treatments reduced expression levels of each (Figure 20C).

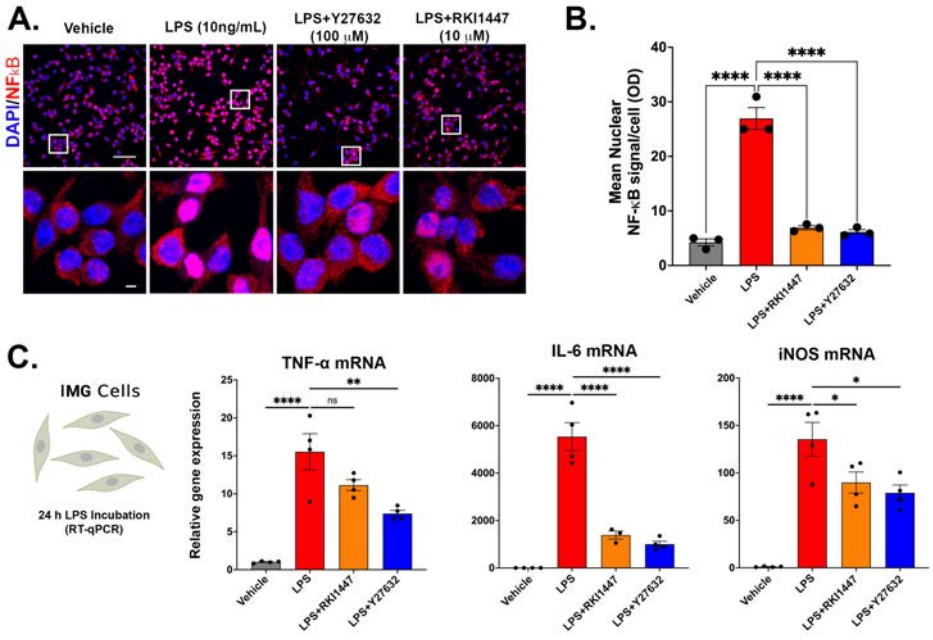

Figure 20. ROCK inhibition blocks NF-κB nuclear translocation and pro-inflammatory gene production in IMG cells. *White boxes represent insets pictured in the bottom immunochemistry panel. One-way ANOVA were used in B and C using Tukey's and Dunnett's (vs. LPS treatment) multiple comparison tests respectively. Error bars represent mean ±SEM (*= p<.05, **=p<.01, and ****=p<.0001).*

An open question is whether ROCK1 or ROCK2 has more influence on the inhibitory effects of ROCK inhibitors. In our studies, we show that IMG cells express equal levels of ROCK1 and ROCK2 mRNA; however microglia have been shown to express higher levels of ROCK1 than other cell types of the brain.[74] We utilized an siRNA approach to knock down (KD) ROCK1 and ROCK2, separately and together, to see if these treatments affected the LPS challenge (2 ng/mL). We achieved roughly 40% reductions in both ROCK1 and ROCK2 with these treatments and applied the NF-κB assay shown above. We found that ROCK1 did decrease levels of NF-κB nuclear localization in IMG cells, but

ROCK2 and the combined siRNA treatment *significantly* reduced these levels (Fig. 21A). We further probed the media from these treatments and found the single knockdown of either protein had no effect on IL-6 or TNF-α cytokine production (Fig. 21B and C), although the combined siRNA treatment did significantly reduce IL-6 levels (Fig. 21B). These studies imply *both* ROCK1 and ROCK2 participate in microglial inflammation responses.

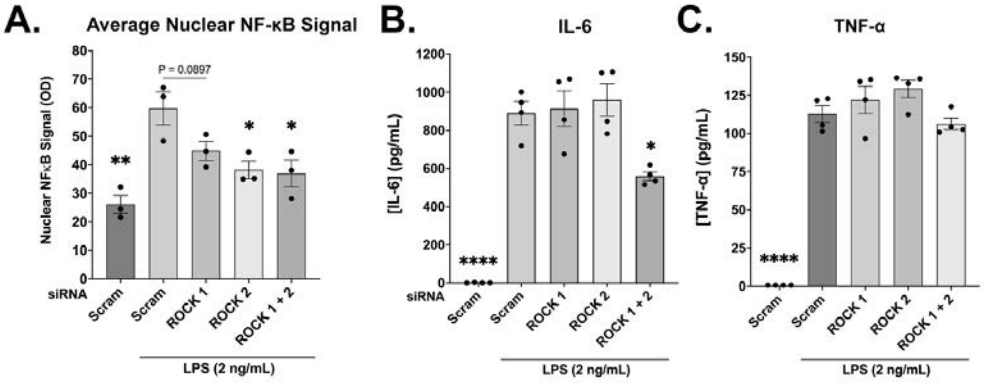

Figure 21. ROCK1 and ROCK2 participate in LPS induced inflammation. *One-way ANOVA with Dunnett's (vs. Scrambled + LPS treatment) multiple comparison tests were used for statistical analyses. Error bars represent mean ±SEM (*= p<.05, **=p<.01, and ****=p<.0001).*

We lastly probed previously published RNAseq data evaluating various microglial cells found in an AD mouse model.[355] The authors of this study identified a novel microglial subtype called neurodegenerative microglia (MGnD) that confer particularly damaging roles to brain tissue. These cells were identified by their localization to Aβ plaques and their expression of the gene Clec7a. Our probing of this study's RNAseq data show that Clec7a^{+} microglia in the AD model express high levels of inflammation related genes, CCL3 and CCL4, along with significant elevations of RhoA and cofilin, as compared to age-matched Clec7a^{-} negative microglia from the same mice and age-matched wild type (WT) mice. ROCK1 and ROCK2 expression levels were stable across all microglia. This data suggests that RhoA/ROCK signaling may play a role in the pro-inflammatory pathologic phenotype shown in MGnD.

Our studies into RhoA/ROCK signaling thus identify clear roles for the proteins in the inflammation response; however, there are many open questions to be explored in future studies. For a full summary of results from **Paper IV**, see Figure 22.

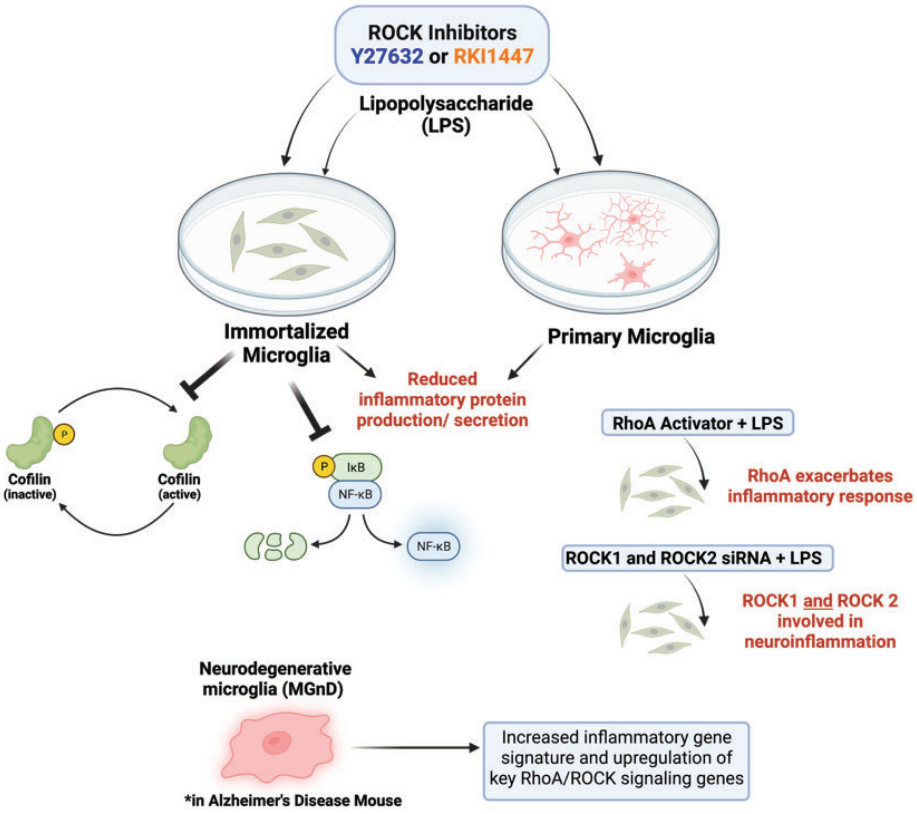

Figure 22. Schematic of results from Paper IV. *Created using BioRender.com.*

4.2.2 Paper V

Microglial Nogo delays recovery from traumatic brain injury in mice

Nogo-A, -B, and -C are prominent reticulon proteins in the brain that confer action on NgR1, among other related receptors, to initiate the activation of the RhoA/ROCK signaling pathway. This signaling axis is a well described mechanism for maintaining stable neuronal connections,[356] but also for negatively regulating axonal repair following injury.[357] As such, many studies have evaluated effects of single or combined germline deletion/knockdown[154,358] or overexpression of Nogo receptors[356,359] or the knockout or knockdown of combinations of the Nogo protein isoforms.[134,155,360–362] In addition, antibody treatments have also targeted Nogo signaling in rodent nervous system injury models.[363,364] Some of these studies produced mixed results[151,358,365,366]; however, genetic or pharmacologic interventions in the Nogo signaling system are generally consistent in improving recovery from injury.[157,361,367,368] With germline deletion of plasticity restricting proteins, such as those in the Nogo signaling system, conferring improved recovery following injury in rodents, cell type specific manipulations of these proteins have provided even more context as to which cell types contribute to improved pathology. For example, oligodendrocyte but not neuronal Nogo KO promoted axonal repair in a model of SCI,[157] with these genetic manipulations also shown to differentially restrict spine density and dendritic branching in the motor cortex.[156] Vajda et al. (2015)[369] posited from their studies that preservation of neuronal Nogo and inhibiting/inactivating oligodendrocyte Nogo produces better outcomes than deletion from both cell types.

While much attention has been dedicated to identifying the roles of Nogo-related proteins in neurite outgrowth and axonal repair, the influence of Nogo proteins on inflammation has recently become of interest.[5] As we showed in **Paper IV,** RhoA/ROCK signaling influences neuroinflammation, with RhoA/ROCK activators exacerbating the LPS induced inflammatory response following a cellular challenge (LPS), suggesting endogenous Nogo may also promote inflammation. Previous studies have shown anti-inflammatory effects of Nogo-A antibodies in a retinal injury model[166] and genetic knockdown of Nogo in PC12 cells, similarly, reduced proinflammatory cytokines (TNF-α and IL-6) produced in response to an LPS challenge.[169] Nogo was also found to regulate pro-inflammatory gene production in peripheral macrophages.[167] Lastly, Nogo/NgR1 signaling was shown to promote inflammation and NF-κB nuclear translocation,[4] although this is contradictory to our findings in **Paper IV**.

With this in mind, investigation of Nogo proteins was necessary, and so we produced a conditional **mi**croglial **No**go **k**nock **o**ut (MinoKO) mouse for **Paper V**. In this mouse, a region of the RTN4 gene (Nogo-66 Exon 4 and 5) common to all three Nogo isoforms was targeted for removal (Figure 11). YFP was inserted into the Cx3Cr1 locus together with CreER and serves as a marker for the specificity of our genetic manipulation. As shown in Figure 23A, YFP is highly colocalized to Iba1 in immunochemical staining, indicating CreER expression is also specific to microglia. As such, microglial Nogo is only deleted in the presence of tamoxifen (TAM), which is delivered via IP injection. mRNA evaluated from microglia isolated via FACS from Floxed control, Cre Control, and MinoKO mice (Figure 23B) confirmed effective deletion of this region of the RTN4 gene (Figure 23C). We characterized these mice both before and after delivery of a CCI TBI, which our laboratory has previously effectively used.[370–373] Our current work extends the previous cell-type specific Nogo deletion studies to microglia and, **to date, these are the first studies evaluating independent roles of Nogo protein in microglia in healthy mice and in a rodent injury model.**

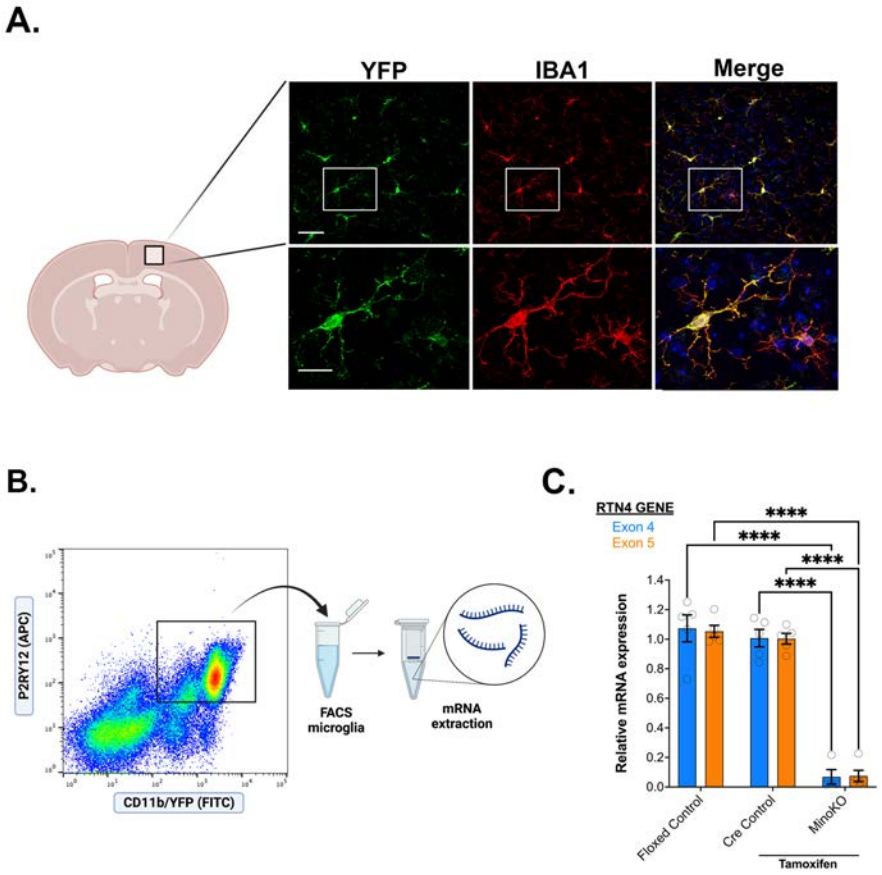

Figure 23.CreER expression is specific to microglia and recombination occurs at target sites of the RTN4 gene*. (A) IHC staining shows specific YFP staining in microglia only. (B) FACS sorted microglia gating strategy. mRNA was isolated from sorted microglia of individual mice and probed for specific regions of the RTN4 gene that were targeted with loxP sites (C) . Two-way ANOVA with Tukey's multiple comparison was used for statistical analysis with error bars representing mean ±SEM; ****=p<.0001. (A) and (B) partially created using BioRender.com.*

Following TAM or oil vehicle administration, mice were singly housed, allowing for continuous activity tracking. All mice showed similar levels of daily activity and no differences were detected between genotypes in evaluations of locomotion (OFT), anxiety (EPM and OFT), working memory (spontaneous alteration cross maze), and spatial memory (MWM). This data is in line with previous studies characterizing behavior in healthy mice with germline Nogo-A deletion.[374] Cage tracking of activity showed that female mice were more active than males, as has been previously described.[375,376] No genotype differences were detected, however. We next induced CCI or sham injuries and evaluated behavior up to two weeks post-injury when brains were collected for pathological assessments (Figure 24A). Injury was consistent across both Control-CCI and MinoKO-CCI mice (Figure 24B and C); however, Control-CCI mice exhibited significantly larger ipsilateral lateral ventricle sizes than sham injured mice (Figure

24D). Lateral ventricle enlargement is typical for TBI patients[377] and is used here as a proxy for injury severity.

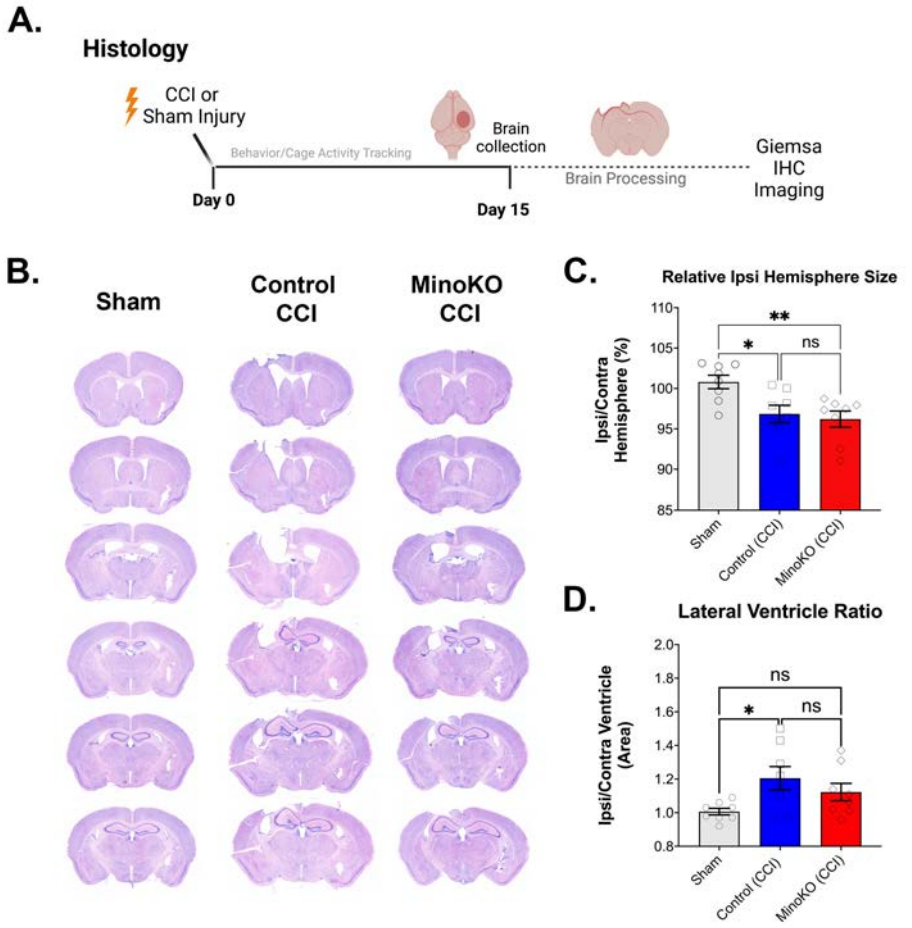

Figure 24. Evaluation of ipsilateral injury size and ventricle size*. (A) Timeline for histology evaluation.(B)Representative Giemsa stained brain slices from Sham and CCI treated animals. Sham group includes animals from all genotypes; Control CCI animals include Floxed and Cre Control CCI treated animals. (B) Differences in size of ipsilateral versus contralateral hemisphere represented as a percentage. (C) CCI significantly induced increased lateral ventricle size in the ipsilateral hemisphere of Control but not MinoKO CCI injured mice. One-way ANOVA with Tukey's multiple comparison was used for statistical analysis with error bars representing mean ±SEM; *=p<.05, and **=p<.01. No differences in injury based upon sex were observed.*

We next evaluated effects of microglial Nogo deletion on microglia and astrocyte activation in the cortex and thalamus. Although microglial and astrocyte activation is most prominent near the area of CCI impact, thalamic microglial activation is also a prominent feature of human TBI, resulting from corticothalamic tract damage.[378,379] Overall microglial coverage, indicated by Iba1 immunoreactivity, in the ipsilateral cortex (Figure 25A and B) and ipsilateral thalamus (Figure 25A and C) is significantly reduced from microglial Nogo deletion. Ipsilateral cortical astrocyte coverage, shown via GFAP immunoreactivity, is also significantly reduced in MinoKO mice (Figure 25A and D). No differences were observed in contralateral tissue distribution on either microglia or astrocytes. Although, astrocytes

and microglia share roles in the brain, which include maintaining homeostasis, regulating blood flow, and providing trophic support to neurons,[380] astrocyte activation and functional states are largely dependent on and, secondary to, microglial signaling and activation.[39]

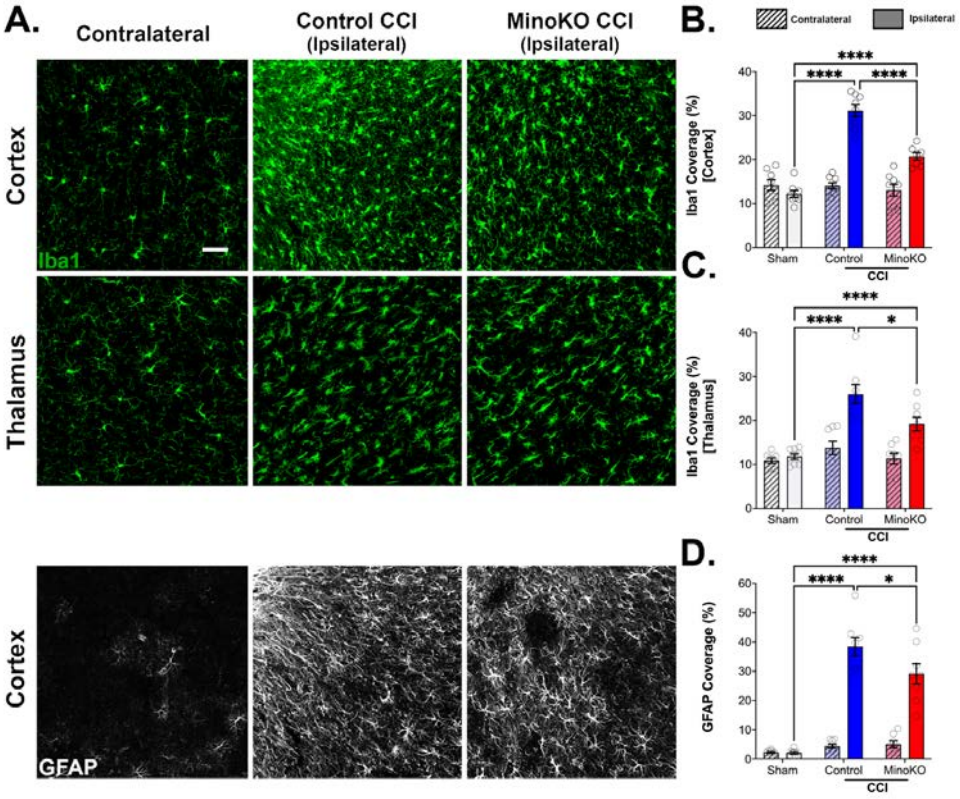

Figure 25. Nogo deletion in microglia reduces ipsilateral microglial and astrocytic immunoreactivity. *(A) Representative immunohistochemistry for cortex and thalamus for Iba1 staining and the cortex for GFAP staining. Cortex (B) and thalamus (C) coverage percentage of Iba1 (microglia) immunoreactivity area in averaged images across all animals. Quantified cortex coverage percentage of GFAP (astrocyte) immunoreactivity area in averaged images across all animals (D). Contralateral brain regions showed no differences. Ipsilateral regions were statistically compared via one-way ANOVA with Tukey's multiple comparison was used for statistical analysis with error bars representing mean ±SEM; *=p<.05 and ****=p<.0001. Measure bar= 50 µm and applies across all images. Contralateral images are from a MinoKO mouse.*

Microglial activation states were previously defined in a dichotomous manner, pro- or -anti-inflammatory (M1 and M2 respectively)[21]; however, microglia are now understood to behave in a context dependent manner with a range of phenotypic responses.[22] In a healthy brain, homeostatic microglia are evenly tiled throughout the brain and possess ramified, long, complex, and motile processes emanating from the cell body.[381] This complexity of morphology is greatly reduced in pathological contexts, such as in moderate TBI, as microglial will retract their processes markedly, if not completely after a strong stimulus.[382] In microglia, reduced process complexity is correlated with

levels of pro-inflammatory protein production, with rounded microglia considered the most pro-inflammatory.[383] A previous study using a rat TBI model, found a role for Nogo protein influencing microglial morphology,[161] with Nogo-A[167] and Nogo-B[168] influencing increased proinflammatory cytokine production. We analyzed individual microglial cell morphologies from Sham, Control-CCI,

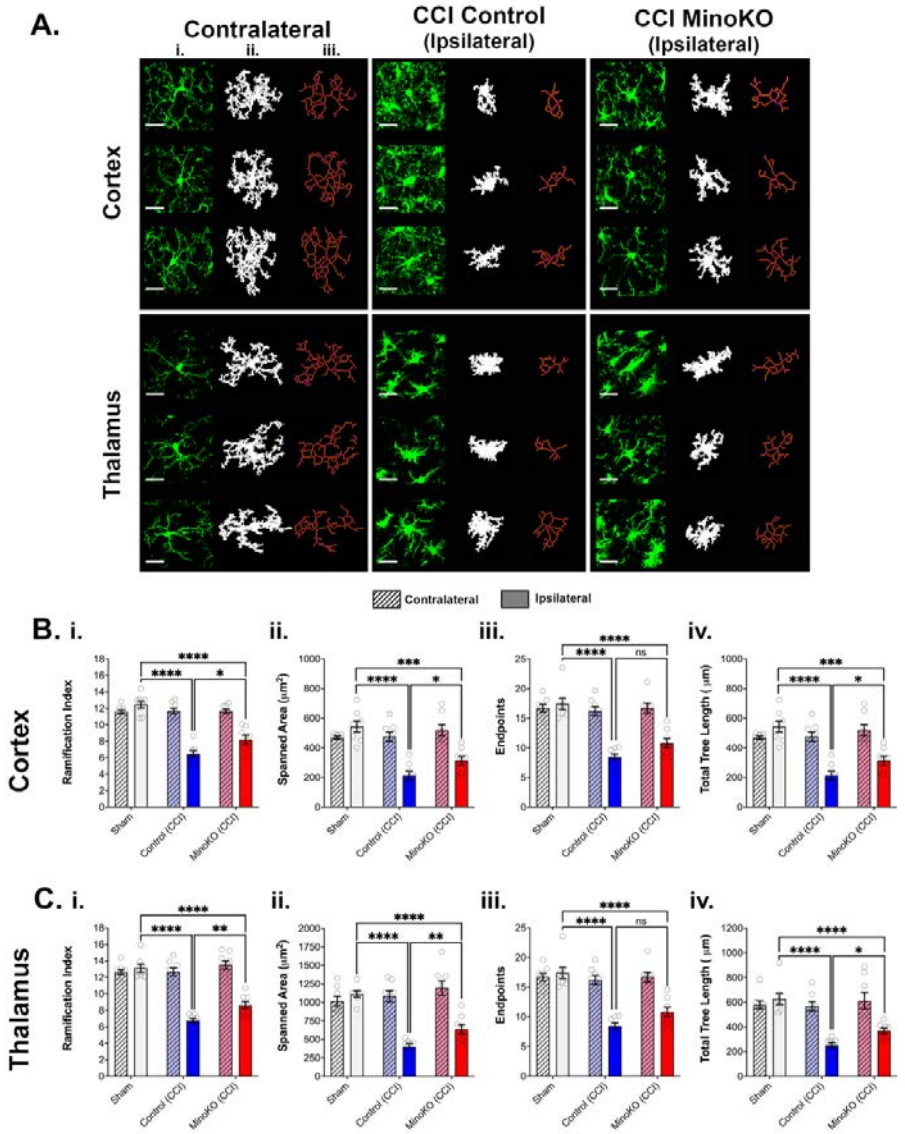

Figure 26. Microglial Nogo deletion increases ipsilateral microglia morphological complexity following CCI injury. *Microglia (Ai) (green) that have been thresholded (Aii) and skeletonized (Aiii) for assessment using MotiQ software across treatment group and regions (A). As no differences were observed in contralateral microglia, as determined via one-way ANOVA, for any parameters assessed (B and C), the contralateral group images are representative across all genotypes. Cortical (B) and thalamic (C) for ramification index (i), spanned area (ii), endpoints (iii), and total tree length (iv). One data point represents the animal mean (20 microglia per region for each animal). Ipsilateral microglia were statistically analyzed via one-way ANOVA with Tukey's multiple comparison with error bars representing mean ±SEM; *=p<.05, **=p<.01, ***=p<.001, and ****=p<.0001. Measure bar= 20 µm.*

and MinoKO-CCI mice via the *MotiQ* software for FIJI and found strong correlation between TBI and reduced microglial complexity. In Figure 26A, Iba1^{+} microglia (i) *Motiq* threshholded (ii) and skeletonized (iii) images are shown from which automated readouts were made. Ipsilateral microglial in the cortex (Figure 26B) and thalamus (Figure 26C) were significantly less ramified (i), spanned smaller regions (ii), showed decreased endpoints (iii), and branch length of processes (iv). Microglia with Nogo deletion reversed all of these trends and showed increased complexity in both the cortex and thalamus. Contralateral microglia showed no differences from one another. Altogether, our data showing decreased microglia and astrocyte immunoreactivity along with the increased complexity of ipsilateral microglia in MinoKO mice, is indicative of a lower inflammatory environment in the brain.

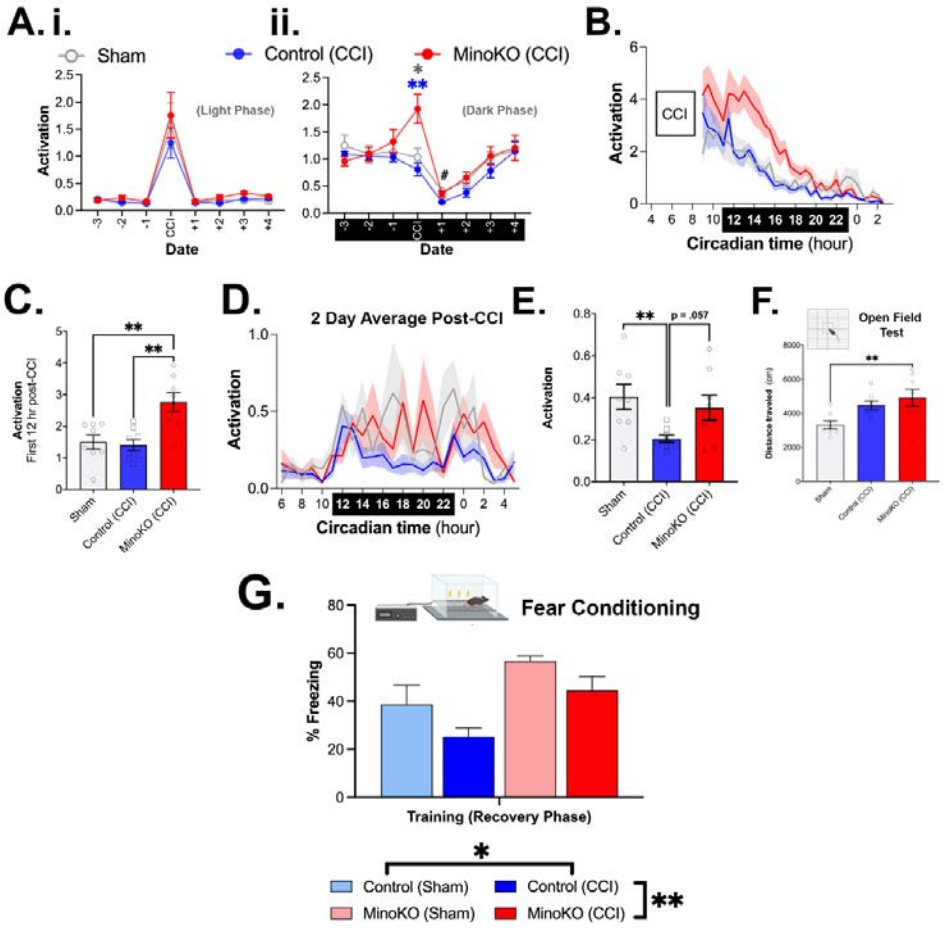

Figure 27. Post CCI behavioral assessment. *Day to day average activation (A) during light (i) and dark (ii) phases, excluding 30 mins surrounding phase transitions; Mixed effects analysis, **= p<0.01 MinoKO (CCI) vs Control (CCI) and *=p<.05 MinoKO (CCI) v. Sham; # p<0.05 Sham vs CCI-control. "CCI" indicates day of CCI treatment (Day 0). B) Home cage activation 12 h following CCI, with each data point representing averaged 2 h blocks of cage activity. C) Total activation in 12 h immediately after CCI. D) Within day activity averaged over the first two days following CCI. E) Dark phase 2 days (~30 h) post-CCI. F) Distance traveled in a 30 min open field test. G) Fear conditioning assessment during the recovery phase of training. Differences in freezing were observed between genotypes and CCI treatment. One way ANOVA with Tukey's multiple comparison was used for statistical analysis with error bars representing mean ±SEM; *=p<.05 and **=p<.01.*

CCI injury induced divergent behavioral outcomes in MinoKO mice not observed prior to injury. Activation, which is a measure of both locomotion and stereotypic behavior such as grooming, was measured continuously while mice were in their home cage. All mice experienced an initial burst of activity following CCI, likely due to buprenorphine administration (Figure 27Ai); however, in MinoKO mice, hyperactivity persisted throughout the first night compared to Sham-injury and Control-CCI mice (Fig. 27Aii-C). Comparing within day activity over two days post CCI (Figure 27D), Control-CCI mice were significantly less active compared to Sham and MinoKO mice. Increased activation in MinoKO-CCI mice remained at nearly significantly higher levels than Control-CCI two days post-CCI (Figure 27E). Seven days after CCI injury, MinoKO mice showed significantly higher distance traveled than the Sham injured group (Figure 27F). No differences were observed in measures of anxiety (EPM). Lastly, a significant effect in freezing levels during a conditioned fear paradigm were observed, indicating CCI injury decreases freezing levels significantly, whereas microglial Nogo deletion increased freezing, regardless of injury (Figure 27G).

In Willi et al. (2009),[374] characterization of total Nogo-A deletion in mice showed increased activity levels resulting from this genetic manipulation. Although, we did not observe this phenotype in healthy mice, our data is in line with findings from the same study showing that amphetamine induced hyperactivity was at significantly higher levels in Nogo-A null mice than WT controls. This result was replicated in a subsequent study.[384] In our injury model, we show a similar pattern of hyperactivity immediately after the CCI injury. Other knock-down studies of Nogo-A show disrupted circadian rhythmicity in these mice when they were in constant darkness, but not normal 12 h dark-light cycles,[385] a finding opposite to Willi et al. (2009).[374] Together, these studies point to a common thread of Nogo-A involvement in daily activity patterns. In Willi et al. (2010),[384] the authors contend that lifelong Nogo-A deletion is responsible for the patterns of behavior they observed. However, our studies 1) replicate these hyperactivity effects with a shorter Nogo deletion time, and 2) show that microglial Nogo deletion is sufficient to produce this phenotype. Willi et al. (2010)[384] also showed differences in freezing patterns of Nogo-A null mice compared to WT controls, providing evidence that microglial Nogo has a strong influence on phenotypes observed in lifelong Nogo-A KO models. Our model is very different in that it deletes all three isoforms of Nogo, perhaps accounting for some of the differences we observed in behavior.

The last piece of evidence that microglial Nogo delays recovery following TBI comes from the elevated body swing test (EBST),[275] which measures the asymmetrical motor function typical of unilaterally brain-injured rodents. We performed this test prior to and 7 and 14 days post-CCI (Figure 28A). Prior to CCI, all mice exhibit symmetrical motor function (Figure 28B), but one week post-CCI, injured mice show preference to swing to the contralateral side of the body, as we have previously shown.[373] In the current studies, MinoKO mice do not exhibit asymmetrical motor function while Control-CCI mice do at 7 days post TBI (Figure 28C). At 14 days post-CCI, the two animal groups are indistinguishable from each other, marking recovery from injury in the CCI-control mice (Figure 28D), albeit at a slower pace than MinoKO mice (that demonstrated recovery re: EBST at 7 days).

Our studies represent a major advancement in the understanding of roles of Nogo in microglia, a previously overlooked Nogo expressing cell type. Importantly, we preserve Nogo expression in other cell types in the brain which has been suggested to help provide even positive benefits in injury recovery.[157,369] We show overall reductions in gliosis in the CCI-injured brain and behavioral data that suggests MinoKO mice recover faster from injury than control mice. A growing body of work has

suggested Nogo influences the inflammation process. Our creation of the MinoKO mouse provides a major opportunity to explore these roles more thoroughly.

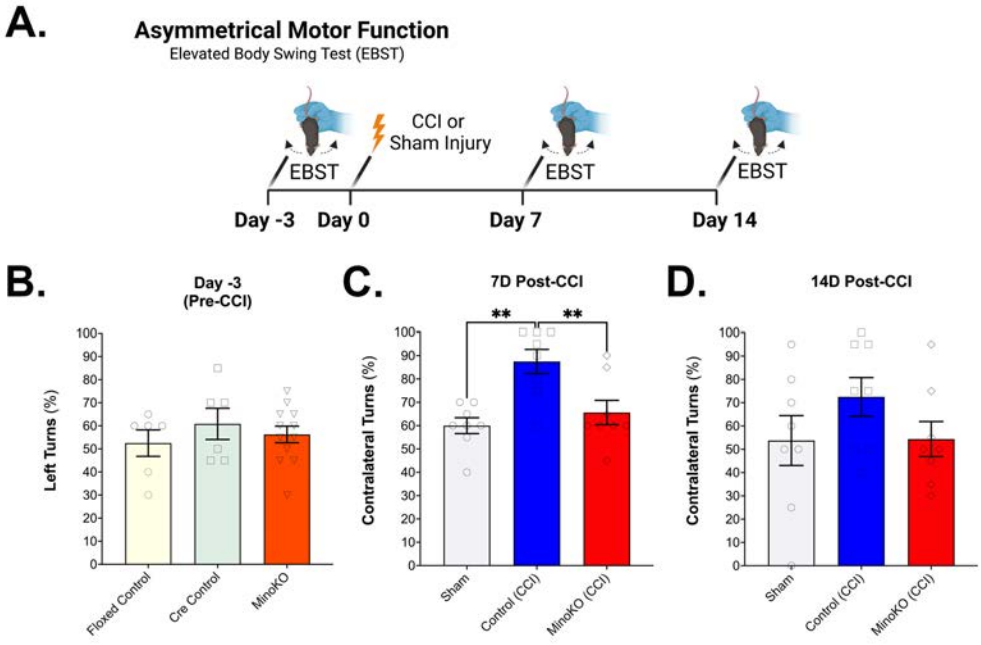

Figure 28. Microglial Nogo deletion prevents asymmetrical motor function following CCI. *A) Timeline of EBST procedures. EBST was performed three days prior to (B) and 7 (C) and 14 days (D) after CCI or sham injury. One way ANOVA with Tukey's multiple comparison was used for statistical analysis with error bars representing mean ±SEM; **=p<.01.*

5 CONCLUSIONS and POINTS of PERSPECTIVE

This thesis covers a broad range of studies in basic preclinical science that advance knowledge of new drug formulations and applications, as well as novel signaling paradigms that influence brain pathology during injury. Herein, we demonstrate utility of novel incretin mimetic formulations towards use in neurological conditions, namely the slow release Exenatide formulation known as PT302 (current drug name is PT320) and an incretin- based triagonist, targeting GLP-1, GIP, and Gcg receptors. As human clinical trials are currently underway or completed, various formulations of incretins have produced mixed results in reaching primary endpoints for treating neurodegenerative conditions. For example, a once weekly formulation of Exenatide, *Bydureon™,* was found to reverse PD symptoms in a 2017 Phase 2 clinical trial reaching its primary endpoints,[84] while PT320,[386] the subject of **Paper I**, and another GLP-1R agonist, NLY01,[387] each failed to meet their primary endpoints in Phase II PD clinical trials. Post-hoc analysis of the successful *Bydureon™* trial indicates that a subset of PD patients earlier in disease progression and age responded most robustly to the treatment.[388] Full analysis of the PT320 and NLY01 trials is not currently available but knowledge of who responds best to treatments is essential for best optimization of clinical trials and moving a drug towards availability to the public. The NLY01 study did find statistically significant improvement in PD symptoms in 95% of patients under the age of 60 receiving the drug, yet another indicator of early intervention and success in treating PD. PT302 also met secondary outcomes of its PD trial. Although PT320 was not successful in meeting primary endpoints for treating PD, it recently met primary endpoints in another Phase II trial, reducing intracranial pressure in patients with idiopathic intracranial hypertension.[87] As more trials are completed and patient populations are optimized, GLP-1R agonists are poised to become future treatments for a broad range of conditions outside of their current use in metabolic diseases. TBI currently lacks treatments and affects large swathes of the world population. While the heterogenous nature of the injury makes it difficult to treat, incretin mimetics will undoubtedly enter clinical trials to treat TBI in the next few years.

The new frontier of incretin mimetics is unimolecular polypharmacy,[52] highlighted in **Paper II,** which involves the combination of the active regions of two or more incretins/secretins into one drug that targets multiple receptors. This may be an avenue to potentiate the protective or reparative cellular signaling not possible with a single receptor agonist. Our studies demonstrated superiority of a triagonist over single GLP-1R agonist in a variety of assays, but much more work is needed to understand which compounds are best suited for human use. Researchers are also currently optimizing these unimolecular multiagonists to bias binding sites on specific receptors and initiate higher levels of signaling, for β-arrestin or cAMP for example, which could perhaps be an avenue of drug design to best treat certain diseases or subsets of patients based on their needs.[389,390] While much of the clinical work with multiagonist incretins is focused on metabolic disease treatment, our work and others are advancing the application of these new drugs for treating neurological conditions. Future studies in our group will move on from working with a TBI model to more focused Tg mouse models of neurodegenerative disease, including AD. As I have demonstrated in this thesis, incretin mimetics are anti-inflammatory. Targeting neuroinflammation to treat chronic neurodegenerative diseases may be the best avenue for positive outcomes from treatment, as we[391] and others[1] have shown.

In parallel to assessing new incretin mimetic formulations for treating neurologic conditions, we also have investigated a biological function of the metabolite of the insulinotropic GLP-1(7-36), GLP-1(9-36) (**Paper III)**. GLP-1R agonists on the market are based upon the structure of GLP-1(7-36), but little is known about the role of its primary metabolite which circulates in the bloodstream at 10-fold higher

concentrations long after GLP-1(7-36) has initiated its function stimulating insulin release.[110] As diabetes patients taking incretin mimetics or incretin boosting drugs (DPP-IV inhibitors) for treatment are less likely to develop a chronic neurodegenerative condition, it possible the circulating metabolite of GLP-1(7-36) may initiate protective signaling in the brain. We found GLP-1(9-36) to confer neuroprotective and anti-inflammatory action on a variety of nervous system cell models in **Paper III**. Our studies partially untangle GLP-1(9-36)'s signaling mechanisms, though more work is needed to fully characterize which receptors and intracellular signaling cascades the metabolite is activating in the nervous system. Cell type specific investigation of these pathways, in microglia rather than neurons, would also be a fruitful avenue of research. Additionally, *in vivo* studies may be revelatory on prophylactic function of GLP-1(9-36). For example, longitudinal studies looking at long-term administration of the metabolite in a mouse PD or AD could provide context to how a properly functioning incretin signaling system, which includes GLP-1(9-36), may affect disease development.

While much of my thesis work focused on incretin mimetics, I also investigated neuroinflammation as a new area of research in the Nogo-signaling field. There has been growing evidence that Nogo protein affects inflammatory processes; hence, I first investigated the canonical signaling pathway affected by this class of proteins–RhoA/ROCK signaling, specifically in microglial cells (**Paper IV**). In **Paper IV** a clear relationship between RhoA/ ROCK signaling is established, using ROCK inhibitors as a molecular tool to block signaling and disrupt the inflammation process. There is growing interest in using ROCK inhibitors to treat neurodegeneration,[392] but like the incretins, optimizing which of the *many* ROCK inhibitors to use is a challenge. A great starting point would be the already clinically approved drugs, but as our studies show, there is promise in newer formulations, such as RKI1447, which has never previously been tested in nervous system tissue. RhoA/ROCK signaling robustly affects cytoskeleton rearrangement and actin dynamics, and although our work shows that ROCK inhibition effectively blocks NF-κB nuclear translocation and cofilin activation, we did not assess intermediate proteins affected by the drug treatments that are conferring this effect. This is an area of research that could be extended from our findings in **Paper IV**. Phosphatases such as SSH may be affected by ROCK inhibition and would be an interesting target to probe in future studies. The role of RhoA in the inflammation process also needs further investigation as we show its activation exacerbates LPS induced inflammation in a ROCK dependent manner. Interestingly, RhoA activation alone did not induce cytokine production in IMG cells. Further research using more specific tools for assessing RhoA activation are needed to identify how this effector molecule is working in microglia. Additional targeted investigation of ROCK1 and ROCK2 roles in inflammation are warranted, as we did not achieve greater than 40% KD of either gene in IMG cells. Manipulations that totally delete each gene together and separately would provide better context to their roles in neuroinflammation. ROCK1 in the brain has been given very little attention in the scientific literature, including in the Nogo-signaling field. Evidence of divergent roles of ROCK1 and ROCK2 make these proteins interesting targets for inquiry.

One main goal of **Paper IV** was the characterization of IMG cells as a model for PMg cell cultures. PMg are costly, time consuming, and require live animals, yet are the gold standard for interrogating microglial specific responses in preclinical research. I have demonstrated, as a proof of concept and extension of their original characterization,[393] IMG cell versatility and biological relevance to serve as a viable model system for microglial research. Very few articles have been published using IMG cells and our work opens the field to this new option for microglia research for drug screening and other applications. Our laboratories at the Karolinska Institutet and National Institutes of Health have

continued using IMG cells as a model system because of their versatility. Their use outside our laboratories will also likely grow - based on the work presented in **Paper IV**.

For my last project, and one of my primary aims when I applied to the PhD program at the Karolinska Institutet, I created a microglial specific Nogo KO mouse and identified a pathological role for the protein in brain injury. This is the first microglial specific Nogo KO mouse model ever created and adds to the broad literature describing plasticity restricting and disease modifying roles of Nogo. When I started my PhD, the Nogo field had begun using cell type specific KO models of Nogo signaling proteins and these were showing specific phenotypes based on which cell type proteins were or were not expressed. Neurons and oligodendrocytes were early targets of cell type specific Nogo-KO models,[156,157,369] while our laboratory has been investigating the effects of forebrain neuron overexpression of NgR1 for the past decade. We have shown profound effects from NgR1 overexpression on memory formation,[356] AD progression,[149] and recovery from TBI.[162] There have been several studies identifying Nogo as a regulator of inflammation,[4,167,169,170,174,394] providing a foundation of evidence to support the creation of a microglial specific Nogo KO mouse. Our findings need to be confirmed with more detailed studies, including cytokine assessments in brain tissue following an inflammatory challenge. LPS administration to MinoKO mice would be informative to this line of inquiry. One useful aspect of MinoKO mice is that Nogo can be deleted at any time, which may have differential effects in development or in old age. Does microglial Nogo impact synaptic pruning and modeling early in life? How does microglia Nogo affect pathology in mouse models of PD? These and many more questions are open for inquiry with the development of this mouse. Lastly, the use of IMG cells would also be a great tool for investigating Nogo roles in microglia, as we demonstrated in **Paper IV** their amenability to genetic manipulation using siRNA technology. Clearly, Nogo-like signaling affects neuroinflammation, perhaps related to the RhoA/ROCK signaling pathways or others. Microglial specific KO of Nogo-related proteins other than Nogo, perhaps MAG or NgR3, as we have shown are expressed in microglia (Fig. 8), may also be a fruitful area of research.

Microglia have received immense attention over the past decade as disease modifying cells. Tools to ablate entire populations of microglial and targeted genetic manipulations such as those used in **Paper V** are illuminating even more roles for this cellular niche in the brain. As I have shown in this thesis, microglia are both important for phenotypic assessment of disease and injury progression, but also can serve as versatile targets for pharmacological interventions.

6 ACKNOWLEDGEMENTS

It is hard to put into the words the gratitude I have for so many people who have come in and out of my life up until this point to make me who I am today and allowed me to reach this pivotal moment. In the past, I struggled at letting life flow and felt an overwhelming sense that a choice in one interest would mean sacrificing another. Often, all that we want is already in front of us. The stress is all in our heads and even I get lost in the brain's tendency to wander *and* wonder.

I grew up on my family farm, surrounded by nature and deep roots in the land my ancestors had tended to. Along with my three siblings (**Darryl**, **Rob**, and **Blakely)** my six first cousins also lived also nearby on the farm, collectively known as "10 cousins" (**Claire**, **Mere**, **Caity**, **Scott**, **Matt**, and **Emma**). You all continue to be a source of inspiration and strength and I am lucky to have you all in my life to this day. Living in a rural area in western Maryland and attending public schools in Garrett County, I never would have imagined myself living in, or buying a house with a concrete backyard in Baltimore or completing a PhD in a faraway place such as Sweden. But here we are. My small hometown of **Accident, MD**, nourished me and supported my endeavors and I am lucky to have had such great public educators in my life. Whether it be my fifth grade elementary teacher, **Rosanne Sherwood**, allowing my brother and I to perform a fish dissection demonstration to the fifth grade class, or **Christine Ashby's** love for math that sparked the same passion for me, I was always lucky to have caring teachers. My high school science teachers **April Slagle, Brian Price,** and **Yolanda Harman** always encouraged curiosity and exposed me to possibilities in science and even exploring science as a career. I still fondly reflect on my time at Northern Garrett High School, especially the science courses you taught.

Jack Shepard gave me my first chance to do neuroscience research in college at **Towson University**, and I can't thank him enough for taking me on and helping me get my first job as a post-bac researcher with the US Army Medical Research Institute of Chemical Defense in 2011 and supporting me in the graduate school application process. At the Army labs, **Patrick McNutt** mentored me and fostered freedom to explore my interests in neuroscience/microscopy and even interests and pursuits outside of science, allowing me to travel abroad for extended periods (on several occasions) and then hiring me back. He actively encouraged I apply to PhD programs and was supportive through the whole endeavor. Thank you! To all the post-docs at the Army during this time that also encouraged me to pursue science (**Phil Beske, Ian Gut**, **Katie Holick**, **Kyle Hubbard**, **Aaron Bradford, Edwin Vazquez-Cintron, Mallory (Stenslik) Strike** and **James Machamer**) you all made a huge difference in my life. Thank you! **Maggie** and **Dom** not sure what I would have done without you on the journey as well. Thanks for your continued support.

While working after college in the army labs, I yearned for an international experience and quit working as a scientist and volunteered with the NGO Urban Light in Thailand. I had the opportunity to help fundraise and work with the organization to raise awareness on human trafficking and exploitation of boys and young men in northern Thailand. **Alezandra Russell**, the founder of the organization, allowed me to make this giant leap and I am forever grateful. Being in Thailand enriched my life immeasurably. While volunteering in Thailand, researchers from the anti-human trafficking NGO *Love146,* **Glenn Miles** and **Jarrett Davis**, allowed me to join them as an assistant researcher investigating the vulnerabilities and needs of young men Urban Light served. Together, we published our findings and I have since published another article with **Glenn,** who also supported my application for entry into the Karolinska PhD Program. Thank you for all you do, Glenn!

Following my work in Thailand, I pursued yoga teacher certification in parallel to my research job at the army. As a yoga teacher, I met my current NIH mentor, **Nigel Greig**, and his wife **Amy Hauck Newman**. They attended my classes regularly, and in one class, I attempted to relate my neuroscience research to yoga, not knowing both ran very established neuroscience labs at the NIH in Baltimore. Nigel chatted with me after class and encouraged me to give him a call if I was ever interested in working in his lab, which focused on drug development to treat neuroinflammation related to neurodegenerative disease. Two years later I made that call and cannot believe the serendipity life has chosen to land with me. I always joke that Nigel and Amy are my science parents, but it is absolutely true. I am not sure what I would have done without having both of you in my life. Your encouragement and support, whether when I was working as a scientist in this PhD program, or volunteering abroad in Thailand beforehand, you have been great friends and mentors. I look forward to continuing our friendship and working with both of you throughout my career. **Nigel,** you have truly given me a lot of room to grow as an independent scientist, treated me as a colleague, and always have been available when I've needed encouragement or ideas. I cannot imagine having a better graduate mentor. You have shown me the importance of caring for people over projects and how that translates to high productivity. I've seen a lot of PhD students dreading the prospects of doing more science after a PhD, but I am still excited to come to work every day and that is largely a product of the supervision you've provided.

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My time at the Karolinska Institutet was enriched with my other lab members including **Alvin**, **Gabriella**, **Katrin** and **Ville**.

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7 REFERENCES

1. Bellenguez, C. *et al.* New insights into the genetic etiology of Alzheimer's disease and related dementias. *Nat. Genet.* **54**, 412–436 (2022).

2. Glotfelty, E. J. *et al.* Incretin Mimetics as Rational Candidates for the Treatment of Traumatic Brain Injury. *ACS Pharmacol. Transl. Sci.* **2**, 66–91 (2019).

3. Yun, S. P. *et al.* Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease. *Nat. Med.* **24**, 931–938 (2018).

4. Fang, Y. *et al.* The Nogo/Nogo Receptor (NgR) Signal Is Involved in Neuroinflammation through the Regulation of Microglial Inflammatory Activation. *J. Biol. Chem.* **290**, 28901–14 (2015).

5. Zhang, N., Cui, Y., Li, Y. & Mi, Y. A Novel Role of Nogo Proteins: Regulating Macrophages in Inflammatory Disease. *Cellular and Molecular Neurobiology* vol. 42 2439–2448 (2022).

6. DiSabato, D. J., Quan, N. & Godbout, J. P. Neuroinflammation: the devil is in the details. *J. Neurochem.* **139**, 136–153 (2016).

7. Augusto-Oliveira, M. *et al.* What Do Microglia Really Do in Healthy Adult Brain? *Cells* **8**, 1293 (2019).

8. Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* **81**, 1–5 (2006).

9. Alarcón-Vila, C., Pizzuto, M. & Pelegrín, P. Purinergic receptors and the inflammatory response mediated by lipids. *Current Opinion in Pharmacology* vol. 47 90–96 (2019).

10. Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* vol. 140 805–820 (2010).

11. Aguilera, G., Colín-González, A. L., Rangel-López, E., Chavarría, A. & Santamaría, A. Redox Signaling, Neuroinflammation, and Neurodegeneration. *Antioxidants and Redox Signaling* vol. 28 1626–1651 (2018).

12. Shabab, T., Khanabdali, R., Moghadamtousi, S. Z., Kadir, H. A. & Mohan, G. Neuroinflammation pathways: a general review. *International Journal of Neuroscience* vol. 127 624–633 (2017).

13. Blackwell, T. S. & Christman, J. W. The Role of Nuclear Factor-κB in Cytokine Gene Regulation. *American Journal of Respiratory Cell and Molecular Biology* vol. 17 3–9 (1997).

14. Ahn, K. S. & Aggarwal, B. B. Transcription factor NF-κB: A sensor for smoke and stress signals. in *Annals of the New York Academy of Sciences* vol. 1056 218–233 (John Wiley & Sons, Ltd, 2005).

15. Zuiderwijk-Sick, E. A. *et al.* Exposure of Microglia to Interleukin-4 Represses NF-κB-Dependent Transcription of Toll-Like Receptor-Induced Cytokines. *Front. Immunol.* **12**, 4743 (2021).

16. Franco, R. & Fernández-Suárez, D. Alternatively activated microglia and macrophages in the central nervous system. *Progress in Neurobiology* vol. 131 65–86 (2015).

17. Lecca, D. *et al.* Boosting phagocytosis and anti-inflammatory phenotype in microglia mediates neuroprotection by PPARγ agonist MDG548 in Parkinson's disease models. *Br. J. Pharmacol.* **175**, 3298–3314 (2018).

18. Lively, S. & Schlichter, L. C. The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *J. Neuroinflammation* **10**, 843 (2013).

19. Pepe, G. *et al.* Selective proliferative response of microglia to alternative polarization signals. *J. Neuroinflammation* **14**, (2017).

20. Corps, K. N., Roth, T. L. & McGavern, D. B. Inflammation and neuroprotection in traumatic brain injury. *JAMA Neurol.* **72**, 355–362 (2015).

21. Ransohoff, R. M. A polarizing question: Do M1 and M2 microglia exist. *Nature Neuroscience* vol. 19 987–991 (2016).

22. Paolicelli, R. C. *et al.* Microglia states and nomenclature: A field at its crossroads. *Neuron* vol. 110 3458–3483 (2022).

23. Tang, Y. & Le, W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Molecular Neurobiology* vol. 53 1181–1194 (2016).

24. De Biase, L. M. *et al.* Local Cues Establish and Maintain Region-Specific Phenotypes of Basal Ganglia Microglia. *Neuron* **95**, 341-356.e6 (2017).

25. Hammond, T. R. *et al.* Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity* **50**, 253-271.e6 (2019).

26. Li, Q. *et al.* Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing. *Neuron* **101**, 207-223.e10 (2019).

27. Masuda, T. *et al.* Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature* **566**, 388–392 (2019).

28. Keren-Shaul, H. *et al.* A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* **169**, 1276-1290.e17 (2017).

29. Krasemann, S. *et al.* The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity* **47**, 566-581.e9 (2017).

30. Kanazawa, H., Ohsawa, K., Sasaki, Y., Kohsaka, S. & Imai, Y. Macrophage/microglia-specific protein Iba1 enhances membrane ruffling and Rac activation via phospholipase C-γ-dependent pathway. *J. Biol. Chem.* **277**, 20026–20032 (2002).

31. Lou, N. *et al.* Purinergic receptor P2RY12-dependent microglial closure of the injured blood-brain barrier. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 1074–1079 (2016).

32. Badimon, A. *et al.* Negative feedback control of neuronal activity by microglia. *Nature* **586**, 417–423 (2020).

33. Zrzavy, T. *et al.* Loss of 'homeostatic' microglia and patterns of their activation in active multiple sclerosis. *Brain* **140**, 1900–1913 (2017).

34. Wolf, Y., Yona, S., Kim, K. W. & Jung, S. Microglia, seen from the CX3CR1 angle. *Front. Cell. Neurosci.* **0**, 26 (2013).

35. Bedolla, A. M. *et al.* Finding the right tool: a comprehensive evaluation of microglial inducible cre mouse models. *bioRxiv* 2023.04.17.536878 (2023) doi:10.1101/2023.04.17.536878.

36. Scherbel, U. *et al.* Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8721–6 (1999).

37. Rolls, A., Shechter, R. & Schwartz, M. The bright side of the glial scar in CNS repair. *Nat. Rev. Neurosci.* (2009) doi:10.1038/nrn2591.

38. Norden, D. M., Muccigrosso, M. M. & Godbout, J. P. Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. *Neuropharmacology* **96**, 29–41 (2015).

39. Liddelow, S. A. *et al.* Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541**, 481–487 (2017).

40. Guttenplan, K. A. *et al.* Neurotoxic reactive astrocytes induce cell death via saturated lipids. *Nature* **599**, 102–107 (2021).

41. Clarke, L. E. *et al.* Normal aging induces A1-like astrocyte reactivity. *Proc. Natl. Acad. Sci.* **115**, E1896–E1905 (2018).

42. Escartin, C. *et al.* Reactive astrocyte nomenclature, definitions, and future directions. *Nature Neuroscience* vol. 24 312–325 (2021).

43. Kopp, K. O., Glotfelty, E. J., Li, Y. & Greig, N. H. Glucagon-like peptide-1 (GLP-1) receptor agonists and neuroinflammation: Implications for neurodegenerative disease treatment. *Pharmacol. Res.* **186**, 106550 (2022).

44. Capozzi, M. E., DiMarchi, R. D., Tschöp, M. H., Finan, B. & Campbell, J. E. Targeting the Incretin/Glucagon System with Triagonists to Treat Diabetes. *Endocr. Rev.* **39**, 719–738 (2018).

45. Kedia, N. Treatment of severe diabetic hypoglycemia with glucagon: an underutilized therapeutic approach. *Diabetes, Metab. Syndr. Obes. Targets Ther.* **4**, 337 (2011).

46. Deacon, C. F. Circulation and degradation of GIP and GLP-1. *Hormone and Metabolic Research* vol. 36 761–765 (2004).

47. Pospisilik, J. A. *et al.* Metabolism of glucagon by dipeptidyl peptidase IV (CD26). *Regul. Pept.* **96**, 133–141 (2001).

48. Edholm, T. *et al.* Differential incretin effects of GIP and GLP-1 on gastric emptying, appetite, and insulin-glucose homeostasis. *Neurogastroenterol. Motil.* **22**, (2010).

49. Bai, L. *et al.* Genetic Identification of Vagal Sensory Neurons That Control Feeding. *Cell* **179**, 1129-1143.e23 (2019).

50. Zimmerman, C. A. *et al.* A gut-to-brain signal of fluid osmolarity controls thirst satiation. *Nature* vol. 568 98–102 (2019).

51. Eng, J., Kleinman, W. A., Singh, L., Singh, G. & Raufman, J. P. Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom: Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* **267**, 7402–7405 (1992).

52. Tschöp, M. H. *et al.* Unimolecular Polypharmacy for Treatment of Diabetes and Obesity. *Cell Metab.* **24**, 51–62 (2016).

53. Göke, R., Larsen, P. J., Mikkelsen, J. D. & Sheikh, S. P. Distribution of GLP-1 Binding Sites in the Rat Brain: Evidence that Exendin-4 is a Ligand of Brain GLP-1 Binding Sites. *Eur. J. Neurosci.* **7**, 2294–2300 (1995).

54. Shughrue, P. J., Lane, M. V. & Merchenthaler, I. Glucagon-like peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus. *Endocrinology* **137**, 5159–5162 (1996).

55. Wei, Y. & Mojsov, S. Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences. *FEBS Lett.* **358**, 219–224 (1995).

56. Satoh, F. *et al.* Characterization of human and rat glucagon-like peptide-1 receptors in the neurointermediate lobe: Lack of coupling to either stimulation or inhibition of adenylyl cyclase. *Endocrinology* **141**, 1301–1309 (2000).

57. Perry, T. *et al.* A novel neurotrophic property of glucagon-like peptide 1: a promoter of nerve growth factor-mediated differentiation in PC12 cells. *J. Pharmacol. Exp. Ther.* **300**, 958–66 (2002).

58. Perry, T., Haughey, N. J., Mattson, M. P., Egan, J. M. & Greig, N. H. Protection and Reversal of Excitotoxic Neuronal Damage by Glucagon-Like Peptide-1 and Exendin-4. *J. Pharmacol. Exp. Ther.* **302**, 881–888 (2002).

59. Gilman, C. P. *et al.* Glucagon-like peptide 1 modulates calcium responses to glutamate and membrane depolarization in hippocampal neurons. *J. Neurochem.* **87**, 1137–1144 (2003).

60. Perry, T. *et al.* Glucagon-like peptide-1 decreases endogenous amyloid-beta peptide (Abeta) levels and protects hippocampal neurons from death induced by Abeta and iron. *J. Neurosci. Res.* **72**, 603–612 (2003).

61. Perry, T. A. & Greig, N. H. The glucagon-like peptides: A double-edged therapeutic sword? *Trends in Pharmacological Sciences* vol. 24 377–383 (2003).

62. Greig, N. H. *et al.* New therapeutic strategies and drug candidates for neurodegenerative diseases: p53 and TNF-α inhibitors, and GLP-1 receptor agonists. *Ann. N. Y. Acad. Sci.* **1035**, 290–315 (2004).

63. Hölscher, C. Brain insulin resistance: role in neurodegenerative disease and potential for targeting. *Expert Opinion on Investigational Drugs* vol. 29 333–348 (2020).

64. Zabala, A. *et al.* Risk of first stroke in people with type 2 diabetes and its relation to glycaemic control: A nationwide observational study. *Diabetes, Obes. Metab.* **22**, 182–190 (2020).

65. Pyke, C. *et al.* GLP-1 receptor localization in monkey and human tissue: Novel distribution revealed with extensively validated monoclonal antibody. *Endocrinology* **155**, 1280–1290 (2014).

66. Katsurada, K. *et al.* Central Glucagon-like Peptide-1 Receptor Signaling via Brainstem Catecholamine Neurons Counteracts Hypertension in Spontaneously Hypertensive Rats. *Sci. Rep.* **9**, (2019).

67. Baggio, L. L. & Drucker, D. J. Glucagon-like peptide-1 receptors in the brain: Controlling food intake and body weight. *Journal of Clinical Investigation* vol. 124 4223–4226 (2014).

68. Alvarez, E. *et al.* The expression of GLP-1 receptor mRNA and protein allows the effect of GLP-1 on glucose metabolism in the human hypothalamus and brainstem. *J. Neurochem.* **92**, 798–806 (2005).

69. Ma, L. *et al.* Microglial Activation of GLP-1R Signaling in Neuropathic Pain Promotes Gene Expression Adaption Involved in Inflammatory Responses. *Neural Plast.* **2021**, (2021).

70. Adriaenssens, A. E. *et al.* Glucose-Dependent Insulinotropic Polypeptide Receptor-Expressing Cells in the Hypothalamus Regulate Food Intake. *Cell Metab.* **30**, 987-996.e6 (2019).

71. Nyberg, J. *et al.* Glucose-dependent insulinotropic polypeptide is expressed in adult hippocampus and induces progenitor cell proliferation. *J. Neurosci.* **25**, 1816–1825 (2005).

72. Campos, R. V, Lee, Y. C. & Drucker, D. J. Divergent tissue-specific and developmental expression of receptors for

glucagon and glucagon-like peptide-1 in the mouse. *Endocrinology* **134**, 2156–2164 (1994).

73. Reiner, D. J. *et al.* Astrocytes regulate GLP-1 receptor-mediated effects on energy balance. *J. Neurosci.* **36**, 3531–3540 (2016).

74. Zhang, Y. *et al.* Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron* **89**, 37–53 (2016).

75. Zhang, Y. *et al.* An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. *J. Neurosci.* **34**, 11929–11947 (2014).

76. Olah, M. *et al.* A transcriptomic atlas of aged human microglia. *Nat. Commun.* **9**, 539 (2018).

77. Darmanis, S. *et al.* A survey of human brain transcriptome diversity at the single cell level. *Proc. Natl. Acad. Sci.* **112**, 7285–90 (2015).

78. Abraham, M. A. & Lam, T. K. T. Glucagon action in the brain. *Diabetologia* vol. 59 1367–1371 (2016).

79. Brundin, P. *et al.* Linked clinical trials-the development of new clinical learning studies in Parkinson's disease using screening of multiple prospective new treatments. *Journal of Parkinson's Disease* vol. 3 231–239 (2013).

80. Athauda, D. & Foltynie, T. The glucagon-like peptide 1 (GLP) receptor as a therapeutic target in Parkinson's disease: mechanisms of action. *Drug Discov. Today* **21**, 802–818 (2016).

81. Kim, D. S. *et al.* A New Treatment Strategy for Parkinson's Disease through the Gut-Brain Axis: The Glucagon-Like Peptide-1 Receptor Pathway. *Cell Transplant.* **26**, 1560–1571 (2017).

82. Glotfelty, E. J., Olson, L., Karlsson, T. E., Li, Y. & Greig, N. H. Glucagon-like peptide-1 (GLP-1)-based receptor agonists as a treatment for Parkinson's disease. *Expert Opinion on Investigational Drugs* vol. 29 595–602 (2020).

83. Salcedo, I., Tweedie, D., Li, Y. & Greig, N. H. Neuroprotective and neurotrophic actions of glucagon-like peptide-1: An emerging opportunity to treat neurodegenerative and cerebrovascular disorders. *British Journal of Pharmacology* vol. 166 1586–1599 (2012).

84. Athauda, D. *et al.* Exenatide once weekly versus placebo in Parkinson's disease: a randomised, double-blind, placebo-controlled trial. *Lancet* **390**, 1664–1675 (2017).

85. Aviles-Olmos, I., Limousin, P., Lees, A. & Foltynie, T. Parkinson's disease, insulin resistance and novel agents of neuroprotection. *Brain* **136**, 374–384 (2013).

86. Yu, S.-J. *et al.* PT320, sustained release Exendin-4, mitigates L-DOPA-induced dyskinesia in a rat 6-hydroxydopamine model of Parkinson's disease. *Front. Neurosci.* **14**, (2020).

87. Mitchell, J. L. *et al.* The effect of GLP-1RA exenatide on idiopathic intracranial hypertension: a randomized clinical trial. *Brain* (2023) doi:10.1093/brain/awad003.

88. Svenningsson, P. *et al.* Reduced incidence of Parkinson's disease after dipeptidyl peptidase-4 inhibitors—A nationwide case-control study. *Movement Disorders* vol. 31 1422–1423 (2016).

89. Malhotra, K. *et al.* GLP-1 receptor agonists in diabetes for stroke prevention: a systematic review and meta-analysis. *J. Neurol.* **1**, 3 (2020).

90. Barkas, F., Elisaf, M. & Milionis, H. Protection against stroke with glucagon-like peptide 1 receptor agonists: a systematic review and meta-analysis. *European Journal of Neurology* vol. 26 559–565 (2019).

91. Frias, J. P. *et al.* The Sustained Effects of a Dual GIP/GLP-1 Receptor Agonist, NNC0090-2746, in Patients with Type 2 Diabetes. *Cell Metab.* **26**, 343-352.e2 (2017).

92. Ambery, P. *et al.* MEDI0382, a GLP-1 and glucagon receptor dual agonist, in obese or overweight patients with type 2 diabetes: a randomised, controlled, double-blind, ascending dose and phase 2a study. *Lancet* **391**, 2607–2618 (2018).

93. Parker, V. E. R. *et al.* Efficacy, Safety, and Mechanistic Insights of Cotadutide, a Dual Receptor Glucagon-Like Peptide-1 and Glucagon Agonist. *J. Clin. Endocrinol. Metab.* **105**, 803–820 (2020).

94. Goebel, B. *et al.* Effects of the Novel Dual GLP-1R/GCGR Agonist SAR425899 on Postprandial Glucose Metabolism in Overweight/Obese Subjects with Type 2 Diabetes. *Diabetes* **67**, 72-OR (2018).

95. Li, Y. *et al.* Neurotrophic and neuroprotective effects of a monomeric GLP-1/GIP/Gcg receptor triagonist in cellular and rodent models of mild traumatic brain injury. *Exp. Neurol.* **324**, 113113 (2020).

96. Jiao, J.-J. *et al.* GLP-1/GIP/Gcg receptor Triagonist improves the cognitive behaviors in triple-transgenic mice of Alzheimer's disease. *Sheng Li Xue Bao* **69**, 135–145 (2017).

97. Li, T. *et al.* A novel GLP-1/GIP/Gcg triagonist reduces cognitive deficits and pathology in the 3xTg mouse model of Alzheimer's disease. *Hippocampus* **28**, 358–372 (2018).

98. Mullins, R. J. *et al.* A Pilot Study of Exenatide Actions in Alzheimer's Disease. *Curr. Alzheimer Res.* **16**, 741–752 (2019).

99. Aviles-Olmos, I. *et al.* Exenatide and the treatment of patients with Parkinson's disease. *J. Clin. Invest.* **123**, 2730–2736 (2013).

100. Igoillo-Esteve, M. *et al.* Exenatide induces frataxin expression and improves mitochondrial function in Friedreich ataxia. *JCI Insight* **5**, (2020).

101. Gejl, M. *et al.* In Alzheimer's disease, 6-month treatment with GLP-1 analog prevents decline of brain glucose metabolism: Randomized, placebo-controlled, double-blind clinical trial. *Front. Aging Neurosci.* **8**, (2016).

102. Hogg, E. *et al.* A Phase II, Randomized, Double-Blinded, Placebo-Controlled Trial of Liraglutide in Parkinson's Disease. *SSRN Electron. J.* (2022) doi:10.2139/SSRN.4212371.

103. Sterling, J., Hua, P., Dunaief, J. L., Cui, Q. N. & VanderBeek, B. L. Glucagon-like peptide 1 receptor agonist use is associated with reduced risk for glaucoma. *Br. J. Ophthalmol.* **107**, 215–220 (2023).

104. Donnelly, D. The structure and function of the glucagon-like peptide-1 receptor and its ligands. *British Journal of Pharmacology* vol. 166 27–41 (2012).

105. Ørskov, C., Rabenhøj, L., Wettergren, A., Kofod, H. & Holst, J. J. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. *Diabetes* **43**, 535–539 (1994).

106. Ørskov, C., Wettergren, A. & Holst, J. J. Biological effects and metabolic rates of glucagonlike peptide-1 7-36 amide and glucagonlike peptide-1 7-37 in healthy subjects are indistinguishable. *Diabetes* **42**, 658–661 (1993).

107. Drucker, D. J., Philippe, J., Mojsov, S., Chick, W. L. & Habener, J. F. Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3434–3438 (1987).

108. Mojsov, S., Weir, G. C. & Habener, J. F. Insulinotropin: Glucagon-like peptide I (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. *J. Clin. Invest.* **79**, 616–619 (1987).

109. Li, Y. *et al.* The metabolite GLP-1 (9-36) is neuroprotective and anti-inflammatory in cellular models of neurodegeneration. *J. Neurochem.* **159**, 867–886 (2021).

110. Knudsen, L. B. & Pridal, L. Glucagon-like peptide-1-(9-36) amide is a major metabolite of glucagon-like peptide-1-(7-36) amide after in vivo administration to dogs, and it acts as an antagonist on the pancreatic receptor. *Eur. J. Pharmacol.* **318**, 429–435 (1996).

111. Elahi, D. *et al.* GLP-1 (9-36) amide, cleavage product of GLP-1 (7-36) amide, is a glucoregulatory peptide. *Obesity* **16**, 1501–1509 (2008).

112. Tomas, E., Stanojevic, V. & Habener, J. F. GLP-1 (936) amide metabolite suppression of glucose production in isolated mouse hepatocytes. *Horm. Metab. Res.* **42**, 657–662 (2010).

113. Deacon, C. F., Plamboeck, A., Møller, S. & Holst, J. J. GLP-1-(9-36) amide reduces blood glucose in anesthetized pigs by a mechanism that does not involve insulin secretion. *Am. J. Physiol. - Endocrinol. Metab.* **282**, E873–E879 (2002).

114. Meier, J. J. *et al.* The glucagon-like peptide-1 metabolite GLP-1-(9-36) amide reduces postprandial glycemia independently of gastric emptying and insulin secretion in humans. *Am. J. Physiol. - Endocrinol. Metab.* **290**, E1118–E1123 (2006).

115. Rolin, B., Deacon, C. F., Carr, R. D. & Ahrén, B. The major glucagon-like peptide-1 metabolite, GLP-1-(9-36)-amide, does not affect glucose or insulin levels in mice. *Eur. J. Pharmacol.* **494**, 283–288 (2004).

116. Hou, J. *et al.* Liraglutide, a long-acting GLP-1 mimetic, and its metabolite attenuate inflammation after intracerebral hemorrhage. *J. Cereb. Blood Flow Metab.* **32**, 2201–2210 (2012).

117. Guida, C. *et al.* GLP-1(9-36) mediates the glucagonostatic effect of GLP-1 by promiscuous activation of the glucagon receptor. *bioRxiv* 785667 (2020) doi:10.1101/785667.

118. Ma, T. *et al.* Glucagon-like peptide-1 cleavage product GLP-1(9-36) amide rescues synaptic plasticity and memory

deficits in Alzheimer's disease model mice. *J. Neurosci.* **32**, 13701–13708 (2012).

119. Day, S. M., Yang, W., Ewin, S., Zhou, X. & Ma, T. Glucagon-like peptide-1 cleavage product GLP-1 (9-36) amide enhances hippocampal long-term synaptic plasticity in correlation with suppression of Kv4.2 expression and eEF2 phosphorylation. *Hippocampus* **27**, 1264–1274 (2017).

120. Gault, V. A. & Hölscher, C. GLP-1 agonists facilitate hippocampal LTP and reverse the impairment of LTP induced by beta-amyloid. *Eur. J. Pharmacol.* **587**, 112–117 (2008).

121. Issa, N. P., Trachtenberg, J. T., Chapman, B., Zahs, K. R. & Stryker, M. P. The critical period for ocular dominance plasticity in the Ferret's visual cortex. *J. Neurosci.* **19**, 6965–78 (1999).

122. Grutzendler, J., Kasthuri, N. & Gan, W.-B. Long-term dendritic spine stability in the adult cortex. *Nature* **420**, 812–816 (2002).

123. Feldman, D. E. Synaptic Mechanisms for Plasticity in Neocortex. *Annu. Rev. Neurosci.* **32**, 33–55 (2009).

124. Holtmaat, A. & Svoboda, K. Experience-dependent structural synaptic plasticity in the mammalian brain. *Nature Reviews Neuroscience* vol. 10 647–658 (2009).

125. Bavelier, D., Levi, D. M., Li, R. W., Dan, Y. & Hensch, T. K. Removing Brakes on Adult Brain Plasticity: From Molecular to Behavioral Interventions. *J. Neurosci.* **30**, 14964–14971 (2010).

126. McKerracher, L. & Winton, M. J. Nogo on the Go. *Neuron* vol. 36 345–348 (2002).

127. Dodd, D. A. *et al.* Nogo-A, -B, and -C are found on the cell surface and interact together in many different cell types. *J. Biol. Chem.* **280**, 12494–12502 (2005).

128. Mironova, Y. A. & Giger, R. J. Where no synapses go: gatekeepers of circuit remodeling and synaptic strength. *Trends Neurosci.* **36**, 363–73 (2013).

129. Karlsson, T. E., Wellfelt, K. & Olson, L. Spatiotemporal and Long Lasting Modulation of 11 Key Nogo Signaling Genes in Response to Strong Neuroexcitation. *Front. Mol. Neurosci.* **10**, 94 (2017).

130. Kempf, A. *et al.* The Sphingolipid Receptor S1PR2 Is a Receptor for Nogo-A Repressing Synaptic Plasticity. *PLoS Biol.* **12**, e1001763 (2014).

131. Fujita, Y. & Yamashita, T. Axon growth inhibition by RhoA/ROCK in the central nervous system. *Frontiers in Neuroscience* vol. 8 338 (2014).

132. Liebscher, T. *et al.* Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. *Ann. Neurol.* **58**, 706–719 (2005).

133. Tsai, S. Y. *et al.* Intrathecal treatment with anti-Nogo-A antibody improves functional recovery in adult rats after stroke. *Exp. Brain Res.* **182**, 261–266 (2007).

134. Yang, Y. *et al.* Silencing Nogo-A promotes functional recovery in demyelinating disease. *Ann. Neurol.* **67**, 498–507 (2010).

135. Li, S. Blockade of Nogo-66, Myelin-Associated Glycoprotein, and Oligodendrocyte Myelin Glycoprotein by Soluble Nogo-66 Receptor Promotes Axonal Sprouting and Recovery after Spinal Injury. *J. Neurosci.* **24**, 10511–10520 (2004).

136. Sozmen, E. G. *et al.* Nogo receptor blockade overcomes remyelination failure after white matter stroke and stimulates functional recovery in aged mice. *Proc. Natl. Acad. Sci.* **113**, E8453–E8462 (2016).

137. Masliah, E. *et al.* Genetic deletion of Nogo/Rtn4 ameliorates behavioral and neuropathological outcomes in amyloid precursor protein transgenic mice. *Neuroscience* **169**, 488–494 (2010).

138. Niederöst, B., Oertle, T., Fritsche, J., McKinney, R. A. & Bandtlow, C. E. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *J. Neurosci.* **22**, 10368–10376 (2002).

139. Cramer, S. C., Abila, B., Scott, N. E., Simeoni, M. & Enney, L. A. Safety, pharmacokinetics, and pharmacodynamics of escalating repeat doses of GSK249320 in patients with stroke. *Stroke* **44**, 1337–1342 (2013).

140. Meininger, V. *et al.* Safety, pharmacokinetic, and functional effects of the Nogo-A monoclonal antibody in amyotrophic lateral sclerosis: A randomized, first-in-human clinical trial. *PLoS One* **9**, e97803 (2014).

141. Mi, S., Blake Pepinsky, R. & Cadavid, D. Blocking LINGO-1 as a therapy to promote CNS repair: From concept to the clinic. *CNS Drugs* **27**, 493–503 (2013).

142. Bandtlow, C. E. *et al.* Increased expression of Nogo-A in hippocampal neurons of patients with temporal lobe epilepsy. *Eur. J. Neurosci.* **20**, 195–206 (2004).

143. Satoh, J. I., Onoue, H., Arima, K. & Yamamura, T. Nogo-A and Nogo receptor expression in demyelinating lesions of multiple sclerosis. *J. Neuropathol. Exp. Neurol.* **64**, 129–138 (2005).

144. Novak, G., Kim, D., Seeman, P. & Tallerico, T. Schizophrenia and Nogo: Elevated mRNA in cortex, and high prevalence of a homozygous CAA insert. *Mol. Brain Res.* **107**, 183–189 (2002).

145. Gil, V. *et al.* Nogo-A expression in the human hippocampus in normal aging and in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **65**, 433–444 (2006).

146. Eyer, G. C. *et al.* Co-expression of nogo-a in dopaminergic neurons of the human substantia nigra pars compacta is reduced in parkinson's disease. *Cells* **10**, 3368 (2021).

147. Zhu, H. Y. *et al.* Increased expression of the Nogo receptor in the hippocampus and its relation to the neuropathology in Alzheimer's disease. *Hum. Pathol.* **38**, 426–434 (2007).

148. Park, J. H. *et al.* Alzheimer precursor protein interaction with the Nogo-66 receptor reduces amyloid-beta plaque deposition. *J. Neurosci.* **26**, 1386–95 (2006).

149. Karlsson, T. E., Karlén, A., Olson, L. & Josephson, A. Neuronal overexpression of nogo receptor 1 in APPswe/PSEN1(ΔE9) mice impairs spatial cognition tasks without influencing plaque formation. *J. Alzheimer's Dis.* **33**, 145–155 (2013).

150. Joset, A., Dodd, D. A., Halegoua, S. & Schwab, M. E. Pincher-generated Nogo-A endosomes mediate growth cone collapse and retrograde signaling. *J. Cell Biol.* **188**, 271–85 (2010).

151. Teng, F. Y. H. & Tang, B. L. Why do Nogo/Nogo-66 receptor gene knockouts result in inferior regeneration compared to treatment with neutralizing agents? *Journal of Neurochemistry* vol. 94 865–874 (2005).

152. Dimou, L. *et al.* Nogo-A-deficient mice reveal strain-dependent differences in axonal regeneration. *J. Neurosci.* **26**, 5591–603 (2006).

153. Steward, O., Zheng, B., Banos, K. & Yee, K. M. Response to: Kim et al., 'Axon Regeneration in Young Adult Mice Lacking Nogo-A/B.' Neuron 38, 187-199. *Neuron* vol. 54 191–195 (2007).

154. Dickendesher, T. L. *et al.* NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans. *Nat. Neurosci.* **15**, 703–712 (2012).

155. Zheng, B. *et al.* Lack of enhanced spinal regeneration in Nogo-deficient mice. *Neuron* **38**, 213–224 (2003).

156. Zemmar, A. *et al.* Oligodendrocyte- and Neuron-Specific Nogo-A Restrict Dendritic Branching and Spine Density in the Adult Mouse Motor Cortex. *Cereb. Cortex* **28**, 1–9 (2017).

157. Meves, J. M., Geoffroy, C. G., Kim, N. D., Kim, J. J. & Zheng, B. Oligodendrocytic but not neuronal Nogo restricts corticospinal axon sprouting after CNS injury. *Exp. Neurol.* **309**, 32–43 (2018).

158. Yan, J. *et al.* Nogo-66 inhibits adhesion and migration of microglia via GTPase Rho pathway in vitro. *J. Neurochem.* **120**, 721–731 (2012).

159. Ukai, J. *et al.* Nogo receptor 1 is expressed in both primary cultured glial cells and neurons. *Nagoya J. Med. Sci.* **78**, 303–311 (2016).

160. Spangenberg, E. *et al.* Sustained microglial depletion with CSF1R inhibitor impairs parenchymal plaque development in an Alzheimer's disease model. *Nat. Commun.* **10**, 3758 (2019).

161. Ziebell, J. M., Ray-Jones, H. & Lifshitz, J. Nogo presence is inversely associated with shifts in cortical microglial morphology following experimental diffuse brain injury. *Neuroscience* **359**, 209–223 (2017).

162. Lai, J.-H. *et al.* Role of Nogo Receptor-1 for Recovery of Balance, Cognition, and Emotion after Mild Traumatic Brain Injury in Mice. *J. Neurotrauma* neu.2018.5949 (2018) doi:10.1089/neu.2018.5949.

163. Jeong, Y. H., Park, J. S., Kim, D. H., Kang, J. L. & Kim, H. S. Anti-inflammatory mechanism of lonchocarpine in LPS- or poly(I:C)-induced neuroinflammation. *Pharmacol. Res.* **119**, 431–442 (2017).

164. Kang, S. S. *et al.* Microglial translational profiling reveals a convergent APOE pathway from aging, amyloid, and tau. *J. Exp. Med.* jem.20180653 (2018) doi:10.1084/jem.20180653.

165. Liu, G. *et al.* Expression of Nogo receptor 1 in microglia during development and following traumatic brain injury. *Brain Res.* **1627**, 41–51 (2015).

166. Baya Mdzomba, J. *et al.* Nogo-A-targeting antibody promotes visual recovery and inhibits neuroinflammation after retinal injury. *Cell Death Dis.* **11**, 1–16 (2020).

167. Arif Ullah, H. M. *et al.* Nogo-A is critical for pro-inflammatory gene regulation in myocytes and macrophages. *Cells* **10**, 1–20 (2021).

168. Zhu, Y. *et al.* Nogo-B Facilitates LPS-Mediated Immune Responses by Up-Regulation of TLR4-signaling in Macrophage RAW264.7. *Cell. Physiol. Biochem.* **41**, 265–273 (2017).

169. Zhong, J. *et al.* Effects of Nogo-A silencing on TNF- α and IL-6 secretion and TH downregulation in lipopolysaccharide-stimulated PC12 cells. *Biomed Res. Int.* **2015**, 817914 (2015).

170. Yu, J. *et al.* Reticulon 4B (Nogo-B) is necessary for macrophage infiltration and tissue repair. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 17511–17516 (2009).

171. Wang, C. *et al.* Inhibition of the Rho/Rho kinase pathway prevents lipopolysaccharide-induced hyperalgesia and the release of TNF-α and IL-1β in the mouse spinal cord. *Sci. Rep.* **5**, 14553 (2015).

172. Tönges, L. *et al.* Rho kinase inhibition modulates microglia activation and improves survival in a model of amyotrophic lateral sclerosis. *Glia* **62**, 217–232 (2014).

173. Chimini, G. & Chavrier, P. Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. *Nature Cell Biology* vol. 2 E191–E196 (2000).

174. Fang, Y. *et al.* The blockage of the Nogo/NgR signal pathway in microglia alleviates the formation of Aβ plaques and tau phosphorylation in APP/PS1 transgenic mice. *J. Neuroinflammation* **13**, 56 (2016).

175. Newell-Litwa, K. A. *et al.* ROCK 1 and 2 differentially regulate actomyosin organization to drive cell and synaptic polarity. *J. Cell Biol.* **210**, 225–242 (2015).

176. Shi, J. *et al.* Distinct roles for ROCK1 and ROCK2 in the regulation of cell detachment. *Cell Death Dis.* **4**, e483–e483 (2013).

177. Mertsch, S. & Thanos, S. Opposing signaling of ROCK1 and ROCK2 determines the switching of substrate specificity and the mode of migration of glioblastoma cells. *Mol. Neurobiol.* **49**, 900–915 (2014).

178. Greathouse, K. M. *et al.* Distinct and complementary functions of rho kinase isoforms ROCK1 and ROCK2 in prefrontal cortex structural plasticity. *Brain Struct. Funct.* **223**, 4227–4241 (2018).

179. Nakagawa, O. *et al.* ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.* **392**, 189–193 (1996).

180. Kempf, A. & Schwab, M. E. Nogo-A represses anatomical and synaptic plasticity in the central nervous system. *Physiology* vol. 28 151–163 (2013).

181. Hsieh, S. H.-K., Ferraro, G. B. & Fournier, A. E. Myelin-associated inhibitors regulate cofilin phosphorylation and neuronal inhibition through LIM kinase and slingshot phosphatase. *J. Neurosci.* **26**, 1006–1015 (2006).

182. Sumi, T., Matsumoto, K., Takai, Y. & Nakamura, T. Cofilin phosphorylation and actin cytoskeletal dynamics regulated by Rho- and Cdc42-activated LIM-kinase 2. *J. Cell Biol.* **147**, 1519–1532 (1999).

183. Alhadidi, Q. & Shah, Z. A. Cofilin Mediates LPS-Induced Microglial Cell Activation and Associated Neurotoxicity Through Activation of NF-κB and JAK–STAT Pathway. *Mol. Neurobiol.* **55**, 1676–1691 (2018).

184. Alhadidi, Q., Nash, K. M., Alaqel, S., Sayeed, M. S. Bin & Shah, Z. A. Cofilin Knockdown Attenuates Hemorrhagic Brain Injury-induced Oxidative Stress and Microglial Activation in Mice. *Neuroscience* **383**, 33–45 (2018).

185. Luciani, P. *et al.* Differentiating effects of the glucagon-like peptide-1 analogue exendin-4 in a human neuronal cell model. *Cell. Mol. Life Sci.* **67**, 3711–3723 (2010).

186. Chhor, V. *et al.* Role of microglia in a mouse model of paediatric traumatic brain injury. *Brain. Behav. Immun.* **63**, 197–209 (2017).

187. Homsi, S. *et al.* Blockade of acute microglial activation by minocycline promotes neuroprotection and reduces locomotor hyperactivity after closed head injury in mice: A twelve-week follow-up study. *J. Neurotrauma* **27**, 911–921 (2010).

188. Bader, M. *et al.* Pharmacokinetics and efficacy of PT302, a sustained-release Exenatide formulation, in a murine model of mild traumatic brain injury. *Neurobiol. Dis.* **124**, 439–453 (2018).

189. Bye, N. *et al.* Transient neuroprotection by minocycline following traumatic brain injury is associated with

attenuated microglial activation but no changes in cell apoptosis or neutrophil infiltration. *Exp. Neurol.* **204**, 220–233 (2007).

190. Baratz, R. *et al.* Transiently lowering tumor necrosis factor-aα synthesis ameliorates neuronal cell loss and cognitive impairments induced by minimal traumatic brain injury in mice. *J. Neuroinflammation* **12**, 45 (2015).

191. Singh, K., Trivedi, R., Devi, M. M., Tripathi, R. P. & Khushu, S. Longitudinal changes in the DTI measures, anti-GFAP expression and levels of serum inflammatory cytokines following mild traumatic brain injury. *Exp. Neurol.* **275**, 427–435 (2016).

192. Kane, M. J. *et al.* A mouse model of human repetitive mild traumatic brain injury. *J Neurosci Methods J Neurosci Methods. January* **15**, 41–49 (2012).

193. Baratz, R. *et al.* Tumor necrosis factor-α synthesis inhibitor, 3,6′- dithiothalidomide, reverses behavioral impairments induced by minimal traumatic brain injury in mice. *J. Neurochem.* **118**, 1032–1042 (2011).

194. Shohami, E., Gallily, R., Mechoulam, R., Bass, R. & Ben-Hur, T. Cytokine production in the brain following closed head injury: Dexanabinol (HU-211) is a novel TNF-α inhibitor and an effective neuroprotectant. in *Journal of Neuroimmunology* vol. 72 169–177 (Elsevier, 1997).

195. Shohami, E., Novikov, M., Bass, R., Yamin, A. & Gallily, R. Closed head injury triggers early production of TNFα and IL-6 by brain tissue. *J. Cereb. Blood Flow Metab.* **14**, 615–619 (1994).

196. Li, Y. *et al.* Liraglutide is neurotrophic and neuroprotective in neuronal cultures and mitigates mild traumatic brain injury in mice. *J. Neurochem.* **135**, 1203–1217 (2015).

197. Tashlykov, V. *et al.* Apoptotic changes in the cortex and hippocampus following minimal brain trauma in mice. *Brain Res.* **1130**, 197–205 (2007).

198. Rachmany, L. *et al.* Cognitive Impairments Accompanying Rodent Mild Traumatic Brain Injury Involve p53-Dependent Neuronal Cell Death and Are Ameliorated by the Tetrahydrobenzothiazole PFT-α. *PLoS One* **8**, e79837 (2013).

199. Yaka, R. *et al.* D-cycloserine improves functional recovery and reinstates long-term potentiation (LTP) in a mouse model of closed head injury. *FASEB J.* **21**, 2033–2041 (2007).

200. Shimon, M. Ben *et al.* Recovery from trauma induced amnesia correlates with normalization of thrombin activity in the mouse hippocampus. *PLoS One* **12**, e0188524 (2017).

201. Esen, F. *et al.* Effects of magnesium administration on brain edema and blood-brain barrier breakdown after experimental traumatic brain injury in rats. *J. Neurosurg. Anesthesiol.* **15**, 119–125 (2003).

202. Habgood, M. D. *et al.* Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. *Eur. J. Neurosci.* **25**, 231–238 (2007).

203. Tweedie, D. *et al.* Cognitive impairments induced by concussive mild traumatic brain injury in mouse are ameliorated by treatment with phenserine via multiple non-cholinergic and cholinergic mechanisms. *PLoS One* **11**, e0156493 (2016).

204. Schwarzbold, M. L. *et al.* Effects of Traumatic Brain Injury of Different Severities on Emotional, Cognitive, and Oxidative Stress-Related Parameters in Mice. *J. Neurotrauma* **27**, 1883–1893 (2010).

205. Kuehn, R. *et al.* Rodent Model of Direct Cranial Blast Injury. *J. Neurotrauma* **28**, 2155–2169 (2011).

206. Svetlov, S. I. *et al.* Morphologic and biochemical characterization of brain injury in a model of controlled blast overpressure exposure. *J. Trauma - Inj. Infect. Crit. Care* **69**, 795–804 (2010).

207. Sajja, V. S. S. S., Galloway, M., Ghoddoussi, F., Kepsel, A. & Vandevord, P. Effects of blast-induced neurotrauma on the nucleus accumbens. *J. Neurosci. Res.* **91**, 593–601 (2013).

208. Du, X. *et al.* Effects of antioxidant treatment on blast-induced brain injury. *PLoS One* **8**, e80138 (2013).

209. Säljö, A., Bao, F., Hamberger, A., Haglid, K. G. & Hansson, H. A. Exposure to short-lasting impulse noise causes microglial and astroglial cell activation in the adult rat brain. *Pathophysiology* **8**, 105–111 (2001).

210. Sajja, V. S. S. S., Ereifej, E. S. & VandeVord, P. J. Hippocampal vulnerability and subacute response following varied blast magnitudes. *Neurosci. Lett.* **570**, 33–37 (2014).

211. Cho, H. J., Sajja, V. S. S. S., VandeVord, P. J. & Lee, Y. W. Blast induces oxidative stress, inflammation, neuronal loss and subsequent short-term memory impairment in rats. *Neuroscience* **253**, 9–20 (2013).

212. Kochanek, P. M. *et al.* Screening of biochemical and molecular mechanisms of secondary injury and repair in the brain after experimental blast-induced traumatic brain injury in rats. *J. Neurotrauma* **30**, 920–37 (2013).

213. Heim, L. R. *et al.* The Invisibility of Mild Traumatic Brain Injury: Impaired Cognitive Performance as a Silent Symptom. *J. Neurotrauma* **34**, (2017).

214. Rachmany, L. *et al.* Exendin-4 attenuates blast traumatic brain injury induced cognitive impairments, losses of synaptophysin and in vitro TBI-induced hippocampal cellular degeneration. *Sci. Rep.* **7**, 3735 (2017).

215. Tweedie, D. *et al.* Blast traumatic brain injury-induced cognitive deficits are attenuated by preinjury or postinjury treatment with the glucagon-like peptide-1 receptor agonist, exendin-4. *Alzheimer's Dement.* **12**, 34–48 (2016).

216. Vogel, E. W. *et al.* Isolated Primary Blast Inhibits Long-Term Potentiation in Organotypic Hippocampal Slice Cultures. *J. Neurotrauma* **33**, 652–661 (2016).

217. Goldstein, L. E. *et al.* Chronic Traumatic Encephalopathy in Blast-Exposed Military Veterans and a Blast Neurotrauma Mouse Model. *Sci. Transl. Med.* **4**, (2012).

218. Readnower, R. D. *et al.* Increase in blood-brain barrier permeability, oxidative stress, and activated microglia in a rat model of blast-induced traumatic brain injury. *J. Neurosci. Res.* **88**, 3530–3539 (2010).

219. Abdul-Muneer, P. M. *et al.* Induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast. *Free Radic. Biol. Med.* **60**, 282–291 (2013).

220. Chio, C. C. *et al.* Etanercept attenuates traumatic brain injury in rats by reducing early microglial expression of tumor necrosis factor-α. *BMC Neurosci.* **14**, 33 (2013).

221. Gao, W. *et al.* VEGI attenuates the inflammatory injury and disruption of blood-brain barrier partly by suppressing the TLR4/NF-κB signaling pathway in experimental traumatic brain injury. *Brain Res.* **1622**, 230–239 (2015).

222. Cao, T., Thomas, T. C., Ziebell, J. M., Pauly, J. R. & Lifshitz, J. Morphological and genetic activation of microglia after diffuse traumatic brain injury in the rat. *Neuroscience* **225**, 65–75 (2012).

223. Skovira, J. W. *et al.* Cell cycle inhibition reduces inflammatory responses, neuronal loss, and cognitive deficits induced by hypobaria exposure following traumatic brain injury. *J. Neuroinflammation* **13**, 299 (2016).

224. Witcher, K. G. *et al.* Traumatic brain injury-induced neuronal damage in the somatosensory cortex causes formation of rod-shaped microglia that promote astrogliosis and persistent neuroinflammation. *Glia* **66**, 2719–2736 (2018).

225. Di Giovanni, S. *et al.* Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. *Proc. Natl. Acad. Sci.* **102**, (2005).

226. Fan, L. *et al.* Experimental brain injury induces differential expression of tumor necrosis factor-α mRNA in the CNS. *Mol. Brain Res.* **36**, 287–291 (1996).

227. Muccigrosso, M. M. *et al.* Cognitive deficits develop 1 month after diffuse brain injury and are exaggerated by microglia-associated reactivity to peripheral immune challenge. *Brain. Behav. Immun.* **54**, 95–109 (2016).

228. Spain, A. *et al.* Mild Fluid Percussion Injury in Mice Produces Evolving Selective Axonal Pathology and Cognitive Deficits Relevant to Human Brain Injury. *J. Neurotrauma* **27**, 1429–1438 (2010).

229. Bhowmick, S., D'Mello, V., Ponery, N. & Abdul-Muneer, P. M. Neurodegeneration and sensorimotor deficits in the mouse model of traumatic brain injury. *Brain Sci.* **8**, (2018).

230. Shojo, H., Borlongan, C. V. & Mabuchi, T. Genetic and histological alterations reveal key role of prostaglandin synthase and cyclooxygenase 1 and 2 in traumatic brain injury–induced neuroinflammation in the cerebral cortex of rats exposed to moderate fluid percussion injury. *Cell Transplant.* **26**, 1301–1313 (2017).

231. Rink, A. *et al.* Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. *Am. J. Pathol.* **147**, 1575–83 (1995).

232. Van Bregt, D. R. *et al.* Substantia nigra vulnerability after a single moderate diffuse brain injury in the rat. *Exp. Neurol.* **234**, 8–19 (2012).

233. Sanders, M. J., Sick, T. J., Perez-Pinzon, M. A., Dietrich, W. D. & Green, E. J. Chronic failure in the maintenance of long-term potentiation following fluid percussion injury in the rat. *Brain Res.* **861**, 69–76 (2000).

234. Chen, Y.-H. *et al.* Profound deficits in hippocampal synaptic plasticity after traumatic brain injury and seizure is ameliorated by prophylactic levetiracetam. *Oncotarget* **9**, 11515–11527 (2018).

235. Wu, A., Ying, Z. & Gomez-Pinilla, F. Dietary curcumin counteracts the outcome of traumatic brain injury on oxidative stress, synaptic plasticity, and cognition. *Exp. Neurol.* **197**, 309–317 (2006).

236. Tomura, S. *et al.* Effect of Decompressive Craniectomy on Aquaporin-4 Expression after Lateral Fluid Percussion Injury in Rats. *J. Neurotrauma* **28**, 237–243 (2011).

237. Karklin Fontana, A. C., Fox, D. P., Zoubroulis, A., Valente Mortensen, O. & Raghupathi, R. Neuroprotective Effects of the Glutamate Transporter Activator (R)-(−)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) following Traumatic Brain Injury in the Adult Rat. *J. Neurotrauma* **33**, 1073–1083 (2016).

238. Hinzman, J. M. *et al.* Diffuse Brain Injury Elevates Tonic Glutamate Levels and Potassium-Evoked Glutamate Release in Discrete Brain Regions at Two Days Post-Injury: An Enzyme-Based Microelectrode Array Study. *J. Neurotrauma* **27**, 889–899 (2010).

239. Sun, F. Y. & Faden, A. I. Pretreatment with antisense oligodeoxynucleotides directed against the NMDA-R1 receptor enhances survival and behavioral recovery following traumatic brain injury in rats. *Brain Res.* **693**, 163–168 (1995).

240. Okiyama, K. *et al.* The Sodium Channel Blocker and Glutamate Release Inhibitor BW1003C87 and Magnesium Attenuate Regional Cerebral Edema Following Experimental Brain Injury in the Rat. *J. Neurochem.* **64**, 802–809 (1995).

241. Matsushita, Y., Shima, K., Nawashiro, H. & Wada, K. Real-Time Monitoring of Glutamate Following Fluid Percussion Brain Injury With Hypoxia in the Rat. *J. Neurotrauma* **17**, 143–153 (2000).

242. Shitaka, Y. *et al.* Repetitive closed-skull traumatic brain injury in mice causes persistent multifocal axonal injury and microglial reactivity. *J. Neuropathol. Exp. Neurol.* **70**, 551–567 (2011).

243. Loane, D. J., Kumar, A., Stoica, B. A., Cabatbat, R. & Faden, A. I. Progressive neurodegeneration after experimental brain trauma: association with chronic microglial activation. *J. Neuropathol. Exp. Neurol.* **73**, 14–29 (2014).

244. Sandhir, R., Onyszchuk, G. & Berman, N. E. J. Exacerbated glial response in the aged mouse hippocampus following controlled cortical impact injury. *Exp. Neurol.* **213**, 372–380 (2008).

245. Zhang, Q.-G. *et al.* Critical Role of NADPH Oxidase in Neuronal Oxidative Damage and Microglia Activation following Traumatic Brain Injury. *PLoS One* **7**, e34504 (2012).

246. Turtzo, L. C. *et al.* Macrophagic and microglial responses after focal traumatic brain injury in the female rat. *J. Neuroinflammation* **11**, 82 (2014).

247. Isaksson, J., Hillered, L. & Olsson, Y. Cognitive and histopathological outcome after weight-drop brain injury in the rat: influence of systemic administration of monoclonal antibodies to ICAM-1. *Acta Neuropathol.* **102**, 246–256 (2001).

248. d'Avila, J. C. *et al.* Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. *J. Neuroinflammation* **9**, 521 (2012).

249. Huang, Y.-N. *et al.* Neuroprotective effects of pifithrin-α against traumatic brain injury in the striatum through suppression of neuroinflammation, oxidative stress, autophagy, and apoptosis. *Sci. Rep.* **8**, 2368 (2018).

250. He, J., Evans, C.-O., Hoffman, S. W., Oyesiku, N. M. & Stein, D. G. Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. *Exp. Neurol.* **189**, 404–412 (2004).

251. Clark, R. S. B. *et al.* Caspase-3 mediated neuronal death after traumatic brain injury in rats. *J. Neurochem.* **74**, 740–753 (2000).

252. Clark, R. S. *et al.* boc-Aspartyl(OMe)-fluoromethylketone attenuates mitochondrial release of cytochrome c and delays brain tissue loss after traumatic brain injury in rats. *J. Cereb. Blood Flow Metab.* **27**, 316–326 (2007).

253. Choi, B. Y. *et al.* Prevention of traumatic brain injury-induced neuronal death by inhibition of NADPH oxidase activation. *Brain Res.* **1481**, 49–58 (2012).

254. Ansari, M. A., Roberts, K. N. & Scheff, S. W. Oxidative stress and modification of synaptic proteins in hippocampus after traumatic brain injury. *Free Radic. Biol. Med.* **45**, 443–452 (2008).

255. Albensi, B. C., Sullivan, P. G., Thompson, M. B., Scheff, S. W. & Mattson, M. P. Cyclosporin ameliorates traumatic brain-injury-induced alterations of hippocampal synaptic plasticity. *Exp. Neurol.* **162**, 385–389 (2000).

256. Hakon, J., Ruscher, K., Romner, B. & Tomasevic, G. Preservation of the blood brain barrier and cortical neuronal tissue by liraglutide, a long acting glucagon-like-1 analogue, after experimental traumatic brain injury. *PLoS One* **10**,

e0120074 (2015).

257. Başkaya, M. K., Rao, A. M., Doğan, A., Donaldson, D. & Dempsey, R. J. The biphasic opening of the blood-brain barrier in the cortex and hippocampus after traumatic brain injury in rats. *Neurosci. Lett.* **226**, 33–36 (1997).

258. Faul, M. & Coronado, V. Epidemiology of traumatic brain injury. in *Handbook of Clinical Neurology* 3–13 (2015). doi:10.1016/B978-0-444-52892-6.00001-5.

259. Faul, M., Xu, L., Wald, M. M. & Coronado, V. G. *Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths, 2002-2006*. www.cdc.gov/TraumaticBrainInjury (2010).

260. Collins, N. C. *et al.* Are boys and girls that different? An analysis of traumatic brain injury in children. *Emerg. Med. J.* **30**, 675–678 (2013).

261. Taylor, C. A., Bell, J. M., Breiding, M. J. & Xu, L. Traumatic Brain Injury–Related Emergency Department Visits, Hospitalizations, and Deaths — United States, 2007 and 2013. *MMWR. Surveill. Summ.* **66**, 1–16 (2017).

262. Susman, M. *et al.* Traumatic brain injury in the elderly: Increased mortality and worse functional outcome at discharge despite lower injury severity. *J. Trauma* **53**, 219–224 (2002).

263. LeBlanc, J., Guise, E. de, Gosselin, N. & Feyz, M. Comparison of functional outcome following acute care in young, middle-aged and elderly patients with traumatic brain injury. *Brain Inj.* **20**, 779–790 (2006).

264. Gupte, R., Brooks, W., Vukas, R., Pierce, J. & Harris, J. Sex Differences in Traumatic Brain Injury: What We Know and What We Should Know. *Journal of Neurotrauma* vol. 36 3063–3091 (2019).

265. Zárate, S., Stevnsner, T. & Gredilla, R. Role of estrogen and other sex hormones in brain aging. Neuroprotection and DNA repair. *Frontiers in Aging Neuroscience* vol. 9 430 (2017).

266. Soldin, O. P. & Mattison, D. R. Sex differences in pharmacokinetics and pharmacodynamics. *Clinical Pharmacokinetics* vol. 48 143–157 (2009).

267. Yona, S. *et al.* Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity* **38**, 79–91 (2013).

268. Vorhees, C. V. & Williams, M. T. Morris water maze: Procedures for assessing spatial and related forms of learning and memory. *Nat. Protoc.* **1**, 848–858 (2006).

269. Golde, W. T., Gollobin, P. & Rodriguez, L. L. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. *Lab Anim. (NY).* **34**, 39–43 (2005).

270. Murakami, H., Takanaga, H., Matsuo, H., Ohtani, H. & Sawada, Y. Comparison of blood-brain barrier permeability in mice and rats using in situ brain perfusion technique. *Am. J. Physiol. Circ. Physiol.* **279**, H1022–H1028 (2000).

271. Pan, W., Kastin, A. J., Rigai, T., McLay, R. & Pick, C. G. Increased hippocampal uptake of tumor necrosis factor α and behavioral changes in mice. *Exp. Brain Res.* **149**, 195–199 (2003).

272. McCarthy, R. C. *et al.* Characterization of a novel adult murine immortalized microglial cell line and its activation by amyloid-beta. *J. Neuroinflammation* **13**, 21 (2016).

273. Lee, J. K. & Tansey, M. G. Microglia isolation from adult mouse brain. *Methods Mol. Biol.* **1041**, 17–23 (2013).

274. Schindelin, J. *et al.* Fiji: An open-source platform for biological-image analysis. *Nature Methods* vol. 9 676–682 (2012).

275. Borlongan, C. V., Randall, T. S., Cahill, D. W. & Sanberg, P. R. Asymmetrical motor behavior in rats with unilateral striatal excitotoxic lesions as revealed by the elevated body swing test. *Brain Res.* **676**, 231–234 (1995).

276. Babagana, M. *et al.* Hedgehog dysregulation contributes to tissue-specific inflammaging of resident macrophages. *Aging (Albany. NY).* **13**, 19207–19229 (2021).

277. Wang, J.-Y. *et al.* Pomalidomide mitigates neuronal loss, neuroinflammation, and behavioral impairments induced by traumatic brain injury in rat. *J. Neuroinflammation* **13**, 168 (2016).

278. Rachmany, L. *et al.* Exendin-4 induced glucagon-like peptide-1 receptor activation reverses behavioral impairments of mild traumatic brain injury in mice. *Age (Omaha).* **35**, 1621–1636 (2013).

279. Tweedie, D. *et al.* Exendin-4, a glucagon-like peptide-1 receptor agonist prevents mTBI-induced changes in hippocampus gene expression and memory deficits in mice. *Exp. Neurol.* **239**, 170–182 (2013).

280. Li, Y. *et al.* Pharmacokinetics of Exenatide in nonhuman primates following its administration in the form of

sustained-release PT320 and Bydureon. *Sci. Rep.* **9**, 1–10 (2019).

281. Tomlinson, B., Hu, M., Zhang, Y., Chan, P. & Liu, Z. M. Investigational glucagon-like peptide-1 agonists for the treatment of obesity. *Expert Opinion on Investigational Drugs* vol. 25 1167–1179 (2016).

282. Zhao, P. *et al.* Activation of the GLP-1 receptor by a non-peptidic agonist. *Nature* **577**, 432–436 (2020).

283. Freeman, J. L., Agolory, J. & Valcarce, C. Preclinical findings with oral GLP-1 receptor agonist TTP273 reinforce importance of neuro-enteroendocrine signaling. in *Diabetes* vol. 65 A309–A309 (2016).

284. Li, Y. *et al.* GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. *Proc. Natl. Acad. Sci.* **106**, 1285–90 (2009).

285. Greig, N. H. *et al.* Incretin mimetics as pharmacologic tools to elucidate and as a new drug strategy to treat traumatic brain injury. *Alzheimer's Dement.* **10**, S62-75 (2014).

286. Jalewa, J., Sharma, M. K., Gengler, S. & Hölscher, C. A novel GLP-1/GIP dual receptor agonist protects from 6-OHDA lesion in a rat model of Parkinson's disease. *Neuropharmacology* **117**, 238–248 (2017).

287. Shi, L., Zhang, Z., Li, L. & Hölscher, C. A novel dual GLP-1/GIP receptor agonist alleviates cognitive decline by re-sensitizing insulin signaling in the Alzheimer icv. STZ rat model. *Behav. Brain Res.* **327**, 65–74 (2017).

288. Yuan, Z. *et al.* A novel GLP-1/GIP dual agonist is more effective than liraglutide in reducing inflammation and enhancing GDNF release in the MPTP mouse model of Parkinson's disease. *Eur. J. Pharmacol.* **812**, 82–90 (2017).

289. Tamargo, I. A. *et al.* Novel GLP-1R/GIPR co-agonist "twincretin" is neuroprotective in cell and rodent models of mild traumatic brain injury. *Exp. Neurol.* **288**, 176–186 (2017).

290. Frias, J. P. *et al.* Efficacy and safety of LY3298176, a novel dual GIP and GLP-1 receptor agonist, in patients with type 2 diabetes: a randomised, placebo-controlled and active comparator-controlled phase 2 trial. *Lancet* (2018) doi:10.1016/S0140-6736(18)32260-8.

291. Finan, B. *et al.* Unimolecular dual incretins maximize metabolic benefits in rodents, monkeys, and humans. *Sci. Transl. Med.* **5**, (2013).

292. Tillner, J. *et al.* A novel dual glucagon-like peptide and glucagon receptor agonist SAR425899: Results of randomized, placebo-controlled first-in-human and first-in-patient trials. *Diabetes, Obesity and Metabolism* (2018) doi:10.1111/dom.13494.

293. Taylor, N. P. Lilly's tirzepatide beats Novo's semaglutide in diabetes phase 3, but another study muddies picture | Fierce Biotech. *Fierce Biotech* https://www.fiercebiotech.com/biotech/lilly-s-tirzepatide-beats-novo-s-semaglutide-diabetes-phase-3-but-another-study-muddies (2021).

294. Khajavi, N. *et al.* An incretin-based tri-agonist promotes superior insulin secretion from murine pancreatic islets via PLC activation. *Cell. Signal.* **51**, 13–22 (2018).

295. Finan, B. *et al.* A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. *Nat. Med.* **21**, 27–36 (2015).

296. Bossart, M. *et al.* Effects on weight loss and glycemic control with SAR441255, a potent unimolecular peptide GLP-1/GIP/GCG receptor triagonist. *Cell Metab.* **34**, 59-74.e10 (2022).

297. Tai, J., Liu, W., Li, Y., Li, L. & Hölscher, C. Neuroprotective effects of a triple GLP-1/GIP/glucagon receptor agonist in the APP/PS1 transgenic mouse model of Alzheimer's disease. *Brain Res.* **1678**, 64–74 (2018).

298. Lee, B., Butcher, G. Q., Hoyt, K. R., Impey, S. & Obrietan, K. Activity-dependent neuroprotection and cAMP response element-binding protein (CREB): Kinase coupling, stimulus intensity, and temporal regulation of CREB phosphorylation at serine 133. *J. Neurosci.* **25**, 1137–1148 (2005).

299. Bruno, B. J., Miller, G. D. & Lim, C. S. Basics and recent advances in peptide and protein drug delivery. *Therapeutic Delivery* vol. 4 1443–1467 (2013).

300. Bader, M. *et al.* Neuroprotective Effects and Treatment Potential of Incretin Mimetics in a Murine Model of Mild Traumatic Brain Injury. *Front. Cell Dev. Biol.* **7**, 356 (2020).

301. Eakin, K. *et al.* Exendin-4 ameliorates traumatic brain injury-induced cognitive impairment in rats. *PLoS One* **8**, e82016 (2013).

302. Cheong, J. L. Y., de Pablo-Fernandez, E., Foltynie, T. & Noyce, A. J. The Association Between Type 2 Diabetes Mellitus and Parkinson's Disease. *J. Parkinsons. Dis.* **10**, 775–789 (2020).

303. Stanciu, G. D. *et al.* Link between Diabetes and Alzheimer's Disease Due to the Shared Amyloid Aggregation and Deposition Involving Both Neurodegenerative Changes and Neurovascular Damages. *J. Clin. Med.* **9**, 1713 (2020).

304. Brauer, R. *et al.* Diabetes medications and risk of Parkinson's disease: a cohort study of patients with diabetes. *Brain* **143**, 3067–3076 (2020).

305. Craft, S. *et al.* Safety, Efficacy, and Feasibility of Intranasal Insulin for the Treatment of Mild Cognitive Impairment and Alzheimer Disease Dementia: A Randomized Clinical Trial. *JAMA Neurol.* **77**, 1099–1109 (2020).

306. Nowell, J., Blunt, E. & Edison, P. Incretin and insulin signaling as novel therapeutic targets for Alzheimer's and Parkinson's disease. *Molecular Psychiatry* vol. 28 217–229 (2023).

307. Rowlands, J., Heng, J., Newsholme, P. & Carlessi, R. Pleiotropic Effects of GLP-1 and Analogs on Cell Signaling, Metabolism, and Function. *Front. Endocrinol. (Lausanne).* **9**, 672 (2018).

308. Holst, J. J., Vilsbøll, T. & Deacon, C. F. The incretin system and its role in type 2 diabetes mellitus. *Molecular and Cellular Endocrinology* vol. 297 127–136 (2009).

309. Cereda, E. *et al.* Diabetes and risk of Parkinson's disease: A systematic review and meta-analysis. *Diabetes Care* vol. 34 2614–2623 (2011).

310. Li, Y., Tweedie, D., Mattson, M. P., Holloway, H. W. & Greig, N. H. Enhancing the GLP-1 receptor signaling pathway leads to proliferation and neuroprotection in human neuroblastoma cells. *J. Neurochem.* **113**, 1621–31 (2010).

311. Lewerenz, J. & Maher, P. Chronic glutamate toxicity in neurodegenerative diseases-What is the evidence? *Frontiers in Neuroscience* vol. 9 469 (2015).

312. Ban, K. *et al.* Glucagon-Like Peptide (GLP)-1(9-36)amide-mediated cytoprotection is blocked by exendin(9-39) yet does not require the known GLP-1 receptor. *Endocrinology* **151**, 1520–1531 (2010).

313. Huang, J. *et al.* Glucagon-like peptide-1 cleavage product GLP-1(9–36) reduces neuroinflammation from stroke via the activation of insulin-like growth factor 1 receptor in astrocytes. *Eur. J. Pharmacol.* **887**, 173581 (2020).

314. Janabi, N., Peudenier, S., Héron, B., Ng, K. H. & Tardieu, M. Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neurosci. Lett.* **195**, 105–108 (1995).

315. Li, N., Lu, J. & Willars, G. B. Allosteric Modulation of the Activity of the Glucagon-like Peptide-1 (GLP-1) Metabolite GLP-1 9-36 Amide at the GLP-1 Receptor. *PLoS One* **7**, e47936 (2012).

316. Wootten, D. *et al.* Allosteric modulation of endogenous metabolites as an avenue for drug discovery. *Mol. Pharmacol.* **82**, 281–290 (2012).

317. Wootten, D. *et al.* The Extracellular Surface of the GLP-1 Receptor Is a Molecular Trigger for Biased Agonism. *Cell* **165**, 1632–1643 (2016).

318. Wootten, D. & Miller, L. J. Structural basis for allosteric modulation of class b g protein-coupled receptors. *Annual Review of Pharmacology and Toxicology* vol. 60 89–107 (2020).

319. Kuc, R. E. *et al.* Characterization of [125I]GLP-1(9-36), a novel radiolabeled analog of the major metabolite of glucagon-like peptide 1 to a receptor distinct from GLP1-R and function of the peptide in murine aorta. *Life Sci.* **102**, 134–138 (2014).

320. Leung, T., Manser, E., Tan, L. & Lim, L. A novel serine/threonine kinase binding the ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* **270**, 29051–29054 (1995).

321. Sakakibara, T. *et al.* Imbalance of interneuron distribution between neocortex and basal ganglia: Consideration of epileptogenesis of focal cortical dysplasia. *J. Neurol. Sci.* **323**, 128–133 (2012).

322. Heasman, S. J. & Ridley, A. J. Mammalian Rho GTPases: New insights into their functions from in vivo studies. *Nature Reviews Molecular Cell Biology* vol. 9 690–701 (2008).

323. Wikström, K., Kavanagh, D. J., Reid, H. M. & Kinsella, B. T. Differential regulation of RhoA-mediated signaling by the TPα and TPβ isoforms of the human thromboxane A2 receptor: Independent modulation of TPα signaling by prostacyclin and nitric oxide. *Cell. Signal.* **20**, 1497–1512 (2008).

324. Xiang, S. Y., Dusaban, S. S. & Brown, J. H. Lysophospholipid receptor activation of RhoA and lipid signaling pathways. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* vol. 1831 213–222 (2013).

325. Fang, Y. *et al.* The adhesion and migration of microglia to β-amyloid (Aβ) is decreased with aging and inhibited by

Nogo/NgR pathway. *J. Neuroinflammation* **15**, 1–16 (2018).

326. Feng, Y., Lograsso, P. V., Defert, O. & Li, R. Rho Kinase (ROCK) Inhibitors and Their Therapeutic Potential. *Journal of Medicinal Chemistry* vol. 59 2269–2300 (2016).

327. Liu, Y. & Gray, N. S. Rational design of inhibitors that bind to inactive kinase conformations. *Nature Chemical Biology* vol. 2 358–364 (2006).

328. Blair, H. A. Belumosudil: First Approval. *Drugs* vol. 81 1677–1682 (2021).

329. Berrino, E. & Supuran, C. T. Rho-kinase inhibitors in the management of glaucoma. *Expert Opinion on Therapeutic Patents* vol. 29 817–827 (2019).

330. Shibuya, M., Hirai, S., Seto, M., Satoh, S. I. & Ohtomo, E. Effects of fasudil in acute ischemic stroke: Results of a prospective placebo-controlled double-blind trial. *J. Neurol. Sci.* **238**, 31–39 (2005).

331. Cui, G. *et al.* ROCK mediates the inflammatory response in thrombin induced microglia. *Neurosci. Lett.* **554**, 82–87 (2013).

332. Shinozaki, Y., Danjo, Y. & Koizumi, S. Microglial ROCK is essential for chronic methylmercury-induced neurodegeneration. *J. Neurochem.* **151**, 64–78 (2019).

333. Scheiblich, H. & Bicker, G. Regulation of Microglial Phagocytosis by RhoA/ROCK-Inhibiting Drugs. *Cell. Mol. Neurobiol.* **37**, 461–473 (2017).

334. Villar-Cheda, B. *et al.* Involvement of microglial RhoA/Rho-Kinase pathway activation in the dopaminergic neuron death. Role of angiotensin via angiotensin type 1 receptors. *Neurobiol. Dis.* **47**, 268–279 (2012).

335. Patel, R. A. *et al.* RKI-1447 is a potent inhibitor of the Rho-associated ROCK kinases with anti-invasive and antitumor activities in breast cancer. *Cancer Res.* **72**, 5025–5034 (2012).

336. Wang, J. & Jiang, W. The effects of RKI-1447 in a mouse model of nonalcoholic fatty liver disease induced by a high-fat diet and in HepG2 human hepatocellular carcinoma cells treated with oleic acid. *Med. Sci. Monit.* **26**, e919220-1 (2020).

337. Wang, Y. *et al.* Upregulation of sphingosine kinase 1 contributes to ventilator-associated lung injury in a two-hit model. *Int. J. Mol. Med.* **44**, 2077–2090 (2019).

338. Namme, J. N., Bepari, A. K. & Takebayashi, H. Cofilin signaling in the CNS physiology and neurodegeneration. *International Journal of Molecular Sciences* vol. 22 10727 (2021).

339. Alsegiani, A. S. & Shah, Z. A. The role of cofilin in age-related neuroinflammation. *Neural Regeneration Research* vol. 15 1451–1459 (2020).

340. Alaqel, S. I. *et al.* Synthesis and Development of a Novel First-in-Class Cofilin Inhibitor for Neuroinflammation in Hemorrhagic Brain Injury. *ACS Chem. Neurosci.* **13**, 1014–1029 (2022).

341. Endo, M. *et al.* Control of growth cone motility and morphology by LIM kinase and slingshot via phosphorylation and dephosphorylation of cofilin. *J. Neurosci.* **23**, 2527–2537 (2003).

342. Zhang, Z. *et al.* Direct Rho-associated kinase inhibiton induces cofilin dephosphorylation and neurite outgrowth in PC-12 cells. *Cell. Mol. Biol. Lett.* **11**, 12–29 (2006).

343. Bi, D. *et al.* Recognition of Lipopolysaccharide and activation of NF-κB by cytosolic sensor NOD1 in teleost fish. *Front. Immunol.* **9**, 1413 (2018).

344. Bielig, H. *et al.* The Cofilin Phosphatase Slingshot Homolog 1 (SSH1) Links NOD1 Signaling to Actin Remodeling. *PLoS Pathog.* **10**, e1004351 (2014).

345. Lefranc, F. *et al.* Narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma cells. *Mol. Cancer Ther.* **8**, 1739–1750 (2009).

346. Duan, Q. *et al.* Narciclasine attenuates LPS-induced acute lung injury in neonatal rats through suppressing inflammation and oxidative stress. *Bioengineered* **11**, 801–810 (2020).

347. Zhao, D. *et al.* Narciclasine inhibits LPS-induced neuroinflammation by modulating the Akt/IKK/NF-κB and JNK signaling pathways. *Phytomedicine* **85**, 153540 (2021).

348. Kingsley, M. K., Bhat, B. V., Badhe, B. A., Dhas, B. B. & Parija, S. C. Narciclasine improves outcome in sepsis among neonatal rats via inhibition of calprotectin and alleviating inflammatory responses. *Sci. Rep.* **10**, 1–14 (2020).

349. Shen, C. Y., Xu, X. L., Yang, L. J. & Jiang, J. G. Identification of narciclasine from Lycoris radiata (L'Her.) Herb. and its inhibitory effect on LPS-induced inflammatory responses in macrophages. *Food Chem. Toxicol.* **125**, 605–613 (2019).

350. Fessler, M. B. *et al.* Dual role for RhoA in suppression and induction of cytokines in the human neutrophil. *Blood* **109**, 1248–1256 (2007).

351. Zhang, Y. *et al.* Rho Kinase Inhibitor Y27632 Improves Recovery After Spinal Cord Injury by Shifting Astrocyte Phenotype and Morphology via the ROCK/NF-κB/C3 Pathway. *Neurochem. Res.* **47**, 3733–3744 (2022).

352. Liu, H. *et al.* ROCK inhibitor fasudil reduces the expression of inflammatory factors in LPS-induced rat pulmonary microvascular endothelial cells via ROS/NF-κB pathway. *BMC Pharmacol. Toxicol.* **23**, 1–14 (2022).

353. Chen, J. *et al.* Inhibition of AGEs/RAGE/Rho/ROCK pathway suppresses non-specific neuroinflammation by regulating BV2 microglial M1/M2 polarization through the NF-κB pathway. *J. Neuroimmunol.* **305**, 108–114 (2017).

354. Shimizu, S. *et al.* Involvement of nuclear factor-kB activation through RhoA/Rho-kinase pathway in LPS-induced IL-8 production in human cervical stromal cells. *Mol. Hum. Reprod.* **13**, 41–47 (2007).

355. Mazaheri, F. *et al.* TREM 2 deficiency impairs chemotaxis and microglial responses to neuronal injury. *EMBO Rep.* **18**, 1186–1198 (2017).

356. Karlén, A. *et al.* Nogo receptor 1 regulates formation of lasting memories. *Proc. Natl. Acad. Sci.* **106**, 20476–20481 (2009).

357. Buchli, A. D. & Schwab, M. E. Inhibition of Nogo: A key strategy to increase regeneration, plasticity and functional recovery of the lesioned central nervous system. *Ann. Med.* **37**, 556–567 (2005).

358. Zheng, B. *et al.* Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 1205–1210 (2005).

359. Karlsson, T. E. *et al.* NgR1: A Tunable Sensor Regulating Memory Formation, Synaptic, and Dendritic Plasticity. *Cereb. Cortex* **26**, 1804–1817 (2016).

360. Kim, J. E., Li, S., Grand Pré, T., Qiu, D. & Strittmatter, S. M. Axon regeneration in young adult mice lacking Nogo-A/B. *Neuron* **38**, 187–199 (2003).

361. Lee, J. K. *et al.* Assessing Spinal Axon Regeneration and Sprouting in Nogo-, MAG-, and OMgp-Deficient Mice. *Neuron* **66**, 663–670 (2010).

362. Simonen, M. *et al.* Systemic deletion of the myelin-associated outgrowth inhibitor Nogo-A improves regenerative and plastic responses after spinal cord injury. *Neuron* **38**, 201–211 (2003).

363. Gonzenbach, R. R. *et al.* Delayed Anti-Nogo-A Antibody Application after Spinal Cord Injury Shows Progressive Loss of Responsiveness. *J. Neurotrauma* **29**, 567–578 (2012).

364. Zörner, B. & Schwab, M. E. Anti-Nogo on the go: From animal models to a clinical trial. *Annals of the New York Academy of Sciences* vol. 1198 E22–E34 (2010).

365. Geoffroy, C. G. *et al.* Effects of PTEN and Nogo Codeletion on Corticospinal Axon Sprouting and Regeneration in Mice. *J. Neurosci.* **35**, 6413–6428 (2015).

366. Cafferty, W. B. J., Duffy, P., Huebner, E. & Strittmatter, S. M. MAG and OMgp synergize with Nogo-A to restrict axonal growth and neurological recovery after spinal cord trauma. *J. Neurosci.* **30**, 6825–6837 (2010).

367. Wang, X. *et al.* Recovery from chronic spinal cord contusion after nogo receptor intervention. *Ann. Neurol.* **70**, 805–821 (2011).

368. Raineteau, O., Fouad, K., Noth, P., Thallmair, M. & Schwab, M. E. Functional switch between motor tracts in the presence of the mAB IN-1 in the adult rat. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6929–6934 (2001).

369. Vajda, F. *et al.* Cell type-specific Nogo-A gene ablation promotes axonal regeneration in the injured adult optic nerve. *Cell Death Differ.* **22**, 323–335 (2015).

370. Hsueh, S. C. *et al.* N-Adamantyl Phthalimidine: A New Thalidomide-like Drug That Lacks Cereblon Binding and Mitigates Neuronal and Synaptic Loss, Neuroinflammation, and Behavioral Deficits in Traumatic Brain Injury and LPS Challenge. *ACS Pharmacol. Transl. Sci.* **4**, 980–1000 (2021).

371. Greig, N. H. *et al.* (−)-Phenserine tartrate (PhenT) as a treatment for traumatic brain injury. *CNS Neurosci. Ther.* cns.13274 (2019) doi:10.1111/cns.13274.

372. Hsueh, S. C. *et al.* Activity of a Novel Anti-Inflammatory Agent F-3,6′-dithiopomalidomide as a Treatment for Traumatic Brain Injury. *Biomedicines* **10**, 2449 (2022).

373. Hsueh, S. C. *et al.* (-)-Phenserine Ameliorates Contusion Volume, Neuroinflammation, and Behavioral Impairments Induced by Traumatic Brain Injury in Mice. *Cell Transplant.* **28**, 1183–1196 (2019).

374. Willi, R., Aloy, E. M., Yee, B. K., Feldon, J. & Schwab, M. E. Behavioral characterization of mice lacking the neurite outgrowth inhibitor Nogo-A. *Genes, Brain Behav.* **8**, 181–192 (2009).

375. Konhilas, J. P. *et al.* Diet and sex modify exercise and cardiac adaptation in the mouse. *Am. J. Physiol. - Hear. Circ. Physiol.* **308**, H135–H145 (2015).

376. Oydanich, M. *et al.* Mechanisms of sex differences in exercise capacity. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **316**, R832–R838 (2019).

377. Levin, H. S., Meyers, C. A., Grossman, R. G. & Sarwar, M. Ventricular Enlargement After Closed Head Injury. *Arch. Neurol.* **38**, 623–629 (1981).

378. Ramlackhansingh, A. F. *et al.* Inflammation after trauma: Microglial activation and traumatic brain injury. *Ann. Neurol.* **70**, 374–383 (2011).

379. Scott, G. *et al.* Thalamic inflammation after brain trauma is associated with thalamo-cortical white matter damage. *J. Neuroinflammation* **12**, 224 (2015).

380. Garland, E. F., Hartnell, I. J. & Boche, D. Microglia and Astrocyte Function and Communication: What Do We Know in Humans? *Frontiers in Neuroscience* vol. 16 127 (2022).

381. Moca, E. N. *et al.* Microglia Drive Pockets of Neuroinflammation in Middle Age. *J. Neurosci.* **42**, 3896–3918 (2022).

382. Morrison, H., Young, K., Qureshi, M., Rowe, R. K. & Lifshitz, J. Quantitative microglia analyses reveal diverse morphologic responses in the rat cortex after diffuse brain injury. *Sci. Rep.* **7**, 1–12 (2017).

383. Karperien, A., Ahammer, H. & Jelinek, H. F. Quantitating the subtleties of microglial morphology with fractal analysis. *Frontiers in Cellular Neuroscience* vol. 7 1–34 (2013).

384. Willi, R. *et al.* Constitutive genetic deletion of the growth regulator nogo-A induces schizophrenia-related endophenotypes. *J. Neurosci.* **30**, 556–567 (2010).

385. Petrasek, T. *et al.* Nogo-A-deficient transgenic rats show deficits in highe cognitive functions, decreased anxiety, and altered circadian activity patterns. *Front. Behav. Neurosci.* **8**, (2014).

386. Chang, M. Despite failing phase 2a clinical trials, Peptron not giving up on Parkinson's treatment. *Korea Biomedical Review* https://www.koreabiomed.com/news/articleView.html?idxno=20071 (2022).

387. Wexler, M. NLY01 fails to slow motor symptom progression in Phase 2 trial. *Parkinson's News Today* https://parkinsonsnewstoday.com/news/nly01-fails-slow-motor-symptom-progression-phase-2-trial/ (2023).

388. Athauda, D. *et al.* Post hoc analysis of the Exenatide-PD trial—Factors that predict response. *Eur. J. Neurosci.* **49**, 410–421 (2019).

389. Koole, C. *et al.* Minireview: Signal Bias, Allosterism, and Polymorphic Variation at the GLP-1R: Implications for Drug Discovery. *Mol. Endocrinol.* **27**, 1234–1244 (2013).

390. Knerr, P. J. *et al.* Next generation GLP-1/GIP/glucagon triple agonists normalize body weight in obese mice. *Mol. Metab.* **63**, 101533 (2022).

391. Lecca, D. *et al.* Role of chronic neuroinflammation in neuroplasticity and cognitive function: A hypothesis. *Alzheimer's Dement.* **18**, 2327–2340 (2022).

392. Koch, J. C. *et al.* ROCK inhibition in models of neurodegeneration and its potential for clinical translation. *Pharmacology and Therapeutics* vol. 189 1–21 (2018).

393. Williams, G. *et al.* Ganglioside inhibition of neurite outgrowth requires Nogo receptor function: Identification of interaction sites and development of novel antagonists. *J. Biol. Chem.* **283**, 16641–16652 (2008).

394. Hu, F. *et al.* Nogo-A promotes inflammatory heat hyperalgesia by maintaining TRPV-1 function in the rat dorsal root ganglion neuron. *FASEB J.* **33**, 668–682 (2019).