Developing novel viral vectors to facilitate gene therapies for neuropathic pain

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Abstract

Neuropathic pain (NeuP) is a chronic pain condition arising following injury or disease to the somatosensory system and affects 7-10% of the population worldwide. Despite its prevalence, currently available treatments for NeuP have limited efficacy and are often complicated by issues such as addiction. Spontaneous activity, a hallmark of NeuP, occurs in both injured and intact primary afferents and their relative contribution to pain remains a controversial subject, with the majority of evidence pointing towards an important role for injured afferents. Adeno associated viral (AAV) vector-based gene therapies are an attractive option for treating NeuP, however, their use is limited by immunogenicity and dorsal root ganglion (DRG) toxicity concerns. In this thesis, we designed viral vectors capable of delivering therapeutic transgenes and restricting their expression to injured, but not intact primary afferents. To achieve this, we initially developed a pipeline of *in vitro* and *in vivo* model systems to test the targeting of our vectors. Following this, we first investigated the capacity of the endogenous activating transcription factor 3 (ATF3) P1 promoter to target AAV gene expression. While the ATF3P1 AAV9 selectively limited transgene expression to injured sensory neurons *in vitro*, these results were not replicated *in vivo*. We next generated an epigenomic atlas of naïve DRG neurons using single nucleus Assay for Transposase Accessible Chromatin sequencing (snATAC-seq) and gained insight into the epigenomic signatures of injured and intact primary afferents. This data provides a resource for exploring the epigenomic mechanisms governing gene expression in the DRG and can be used to identify putative regulatory elements capable of targeting AAV gene expression to injured primary afferents. Finally, we trialled a microRNA (miRNA)-based approach, whereby miR-182 target sites were introduced into an AAV-PHP.S vector and successfully restricted expression to injured primary afferents *in vivo*.

Table of Contents

List of Tables

List of figures

Acknowledgments

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Declaration

I declare that any work presented in this thesis is my own, unless otherwise specified. All intrathecal injections were performed by Dr Greg Weir who also carried out perfusions, as did Dr Andrew Cooper. Avil^{FlpO};Atf3^{CreERT2};RC::FLTG mice were characterised by Dr Andrew Cooper.

No part of this thesis has been submitted for any other degree at the University of Glasgow or any other institution.

List of Abbreviations

3’ UTR	3’ untranslated region
A/A	Antibiotic/antimycotic
AAV	Adeno associated virus
AAV1	AAV serotype 1
AGO	Argonaute
ALK	Anaplastic lymphoma kinase
ATAC	Assay for transposase accessible chromatin followed by sequencing
ATF3	Activating transcription factor 3
BSA	Bovine serum albumin
CAG	Chicken ß-actin
CCI	Chronic constriction injury
CEPT	Chroman 1, emricasan, polyamines, trans-ISRIB
CFA	Complete Freud’s adjuvant
CGRP	Calcitonin-gene related peptide
ChIP-seq	chromatin immunoprecipitation sequencing
CNS	Central nervous system
CPP	Conditioned place preference
CREATE	Cre recombination-based AAV targeted evolution
CREB	Cyclic AMP responsive element binding
CRISPR	Clustered regularly interspaced short palindromic repeats
DARs	Differentially accessible regions
DEGs	Differentially expressed genes
DIV	Day of differentiation
DRG	Dorsal root ganglia
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FDR	False discovery rate
FISH	Fluorescence *in situ* hybridisation
GO	Gene ontology
HBSS	Hank’s balanced salt solution
hDRG	Human dorsal root ganglia
HTMRs	High-threshold mechanoreceptors
i.p.	Intraperitoneal

iPSC	Induced pluripotent stem cells
iSNs	iPSC-derived sensory neurons
ITRs	Inverted terminal repeats
KLF	Kruppel like factors
LSI	Latent semantic indexing
LTRMs	Low-threshold mechanoreceptors
mDRG	Mouse DRG
miRISC	MiRNA-induced silencing complex
miRNA	MicroRNA
MOI	Multiplicity of infection
MREs	MiRNA response elements
MRGPRs	Mas-related G protein-coupled receptors
NeuP	Neuropathic pain
NF200	Neurofilament 200
NGF	Nerve growth factor
NHP	Nonhuman primate
NNT	Numbers needed to treat
NpHR	Halorhodopsin
Nppb	Natriuretic polypeptide B
OTC	Ornithine transcarbamylase
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PNS	Peripheral nervous system
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PSAM	Pharmacologically selective actuator module
PV	Parvalbumin
rAAVs	Recombinant AAVs
RAGs	Regeneration-associated genes
RA-LTMRs	Rapidly acting low-threshold mechanoreceptors
RNAi	RNA interference
SA-LTMRs	Slowly adapting low-threshold mechanoreceptors
sCAG	Short CAG
scATAC-seq	Single cell Assay for Transposase Accessible Chromatin sequencing
scRNA-seq	Single cell RNA sequencing

SD	Standard deviation
SEM	Standard error mean
SGCs	Satellite glial cells
snATAC-seq	Single nucleus Assay for Transposase Accessible Chromatin sequencing
SNI	Spared nerve injury
SNL-	Spinal nerve ligation
SNRIs	Serotonin-noradrenaline reuptake inhibitors
SP	Substance P
Sprr1a	Small proline-rich repeat protein 1A
Sst	Somatostatin
SVD	Singular value decomposition
TCAs	Tricycling antidepressants
TF	Transcription factors
TF-IDF	Term frequency-inverse document frequency
TG	Trigeminal ganglia
Th	Tyrosine hydroxylase
TrkA	Tyrosine kinase receptor
TrkC	Tropomyosin receptor kinase C
TRPA1	Transient receptor potential ankyrin 1
Trpm8	Transient receptor potential melastatin 8
TRPV1	Transient receptor potential vanilloid 1
TSS	Transcription start site
TUJI	ß-tubulin III
UMAP	Uniform manifold approximation and projection
Vglut3	Vesicular glutamate transporter 3

Chapter 1 General Introduction

Pain is “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (Raja *et al.*, 2020). The sensory component of pain is termed nociception, defined as the process of detecting, encoding, and transmitting noxious stimuli. Critically, nociception is not the same as pain and it is the processing of these noxious stimuli in higher brain centres that results in the combined emotional and physical experience of pain. In a healthy state, acute pain in the presence of injury or threat serves a protective function and its importance becomes apparent when examining cases of individuals with loss of pain disorders. While extremely rare, these disorders can be life-threatening due to lack of pain avoidance behaviours and resulting injuries which remain undetected and can lead to permanent health problems (Lischka *et al.*, 2022).

Pain persisting in the absence of a noxious stimulus likely no longer serves a protective purpose. Chronic pain is clinically defined as pain lasting or recurring for longer than three months and is an umbrella term encompassing different types of clinically relevant chronic pain conditions (Treede *et al.*, 2019). Chronic pain affects between one third and one half of people living in the UK and poses a substantial socioeconomic burden on society, with chronic pain conditions found amongst the top 10 leading causes of disability (Fayaz *et al.*, 2016; Vos *et al.*, 2017; James *et al.*, 2018; Treede *et al.*, 2019). It has high comorbidity with other chronic diseases, such as depression, and as a result patients experience a lower quality of life. Chronic pain is a complex condition, and its treatment remains elusive, with many patients receiving inadequate pain management due to lack of efficacy and off-target effects (Breivik *et al.*, 2006). The work presented in this thesis aimed to develop viral vectors capable of transducing specific primary afferent populations, thus facilitating the targeted delivery of gene therapies for neuropathic pain.

In order to develop better treatments, it is first necessary to understand how the healthy somatosensory nervous system operates and appreciate the pathological changes which drive chronic pain. There have been major strides forward in our understanding of both topics in recent years.

1.1 Anatomy of the somatosensory system

The somatosensory system is responsible for relaying sensory information to and from the brain. Sensory stimuli are initially detected by the specialised nerve endings of primary afferent fibres, the cell bodies of which originate in the dorsal root ganglia (DRG) or trigeminal ganglia (TG) and innervate the body and head, respectively. Non-neuronal cells and specialised end-organ structures, such as keratinocytes, Schwann cells and Meissner corpuscles, also participate in sensory signal transduction by interacting with sensory neurons (Abdo *et al.*, 2019; Neubarth *et al.*, 2020; Mikesell *et al.*, 2022; Erbacher *et al.*, 2024; Ojeda-Alonso *et al.*, 2024). Following detection, signals are transmitted to the dorsal horn of the spinal cord where they are gated and modified by complex interneuron circuitry, before being relayed to multiple higher brain centres, resulting in the conscious experience of pain (Todd, 2010; Garland, 2012).

1.1.1 Primary afferent heterogeneity

The project described in this thesis is concerned with targeting specific primary afferent populations, the heterogeneity of which has long been known (Table 1.1). Primary afferent neurons have pseudo-unipolar morphology, with a distal branch projecting to peripheral tissues and a proximal branch terminating on second order neurons in the central nervous system (CNS). Sensory stimuli are transduced in the form of action potentials and primary afferents have traditionally been classified according to their degree of myelination and axonal conduction velocity as Aβ-, Aδ- and C-fibres. Aβ-fibres have large cell bodies and heavily myelinated processes, leading to high conduction velocities. They are mostly classified as low threshold mechanoreceptors (A-LTMRs), playing a role in discriminative touch and vibration sensation, while some studies have also identified Aβ-fibres responsive to noxious stimuli (Zotterman, 1939; Johnson and Hsiao, 1992; Djouhri and Lawson, 2004; Vaden and Gu, 2023). Aδ-fibres have relatively smaller cell body sizes and lighter myelination, resulting in slower conduction velocities while C-fibres are the most abundant afferent type, characterised by lack of myelination and small cell body size (Li *et al.*, 2011). The majority of Aδ- and C-fibres are thought to be nociceptors, a specialised class of primary afferents that is preferentially responsive to noxious stimuli, however two subsets of them also responds to weaker, innocuous stimuli (e.g. C-

LTMRs and Aδ-LTMRs) (Zotterman, 1939; Leem, Willis and Chung, 1993; Abraira and Ginty, 2013; Rutlin *et al.*, 2014). Aδ-nociceptors can be further categorised according to their mechanical and heat thresholds, and are thought to mediate acute, fast pain (Cain, Khasabov and Simone, 2001). C-fibre nociceptors are the most common nociceptor type and are primarily polymodal, responding to noxious mechanical, thermal and chemical stimuli and mediating poorly localised, slow pain (Schmidt *et al.*, 1995; Kumazawa, 1996; Schmelz, 2000; Chisholm *et al.*, 2018). A population of C-fibres, known as C-LTMRs, is responsible for the detection of low-threshold mechanical stimuli, such as stroking of hairy skin (Olausson *et al.*, 2007). Finally, approximately 15-20% of C-fibres are known as "silent" nociceptors due to their insensitivity to mechanical stimuli but can become sensitised after injury or inflammation (Schmidt *et al.*, 1995; Michaelis, Häbler and Jänig, 1996; Prato *et al.*, 2017).

In addition to their anatomical and physiological properties, primary afferents can be categorised by their neurochemistry. Historically, DRG neurons were divided into large light (myelinated) and small dark (unmyelinated) neurons depending on their ability to bind neurofilament 200 (NF200) (Lawson and Waddell, 1991). Nociceptors can be distinguished into peptidergic and non-peptidergic fibres. Peptidergic nociceptors release neuropeptides such as calcitonin-gene related peptide (CGRP) and substance P (SP) and comprise of Aδ- and C-fibres, based on conduction velocity (Hökfelt, J.-O. Kellerth, *et al.*, 1975; Wiesenfeld-Hallin *et al.*, 1984; McCarthy and Lawson, 1990). These fibres are further characterised by the expression of the tyrosine kinase receptor (TrkA), bound by nerve growth factor (NGF), and transient receptor potential vanilloid 1 (TRPV1), a receptor responsive to heat and activated by compounds such as capsaicin (Otten, 1984; Cavanaugh *et al.*, 2011). In contrast, non-peptidergic neurons do not release neuropeptides and instead bind the plant lectin isolectin B4 (IB4) (Silverman and Kruger, 1990; Stucky and Lewin, 1999). All non-peptidergic nociceptors are C-fibres and express the tyrosine kinase glial cell line-derived neurotrophic factor receptor Ret as well as the purinergic receptor $P2X_3$ (Chen *et al.*, 1995; Lawson, McCarthy and Prabhakar, 1996; Bennett *et al.*, 1998). A large subset of non-peptidergic fibres have also been found to express mas-related G protein-coupled receptors (MRGPRs) (Dong *et al.*, 2001).

1.1.2 Molecular classification of primary afferents

Advances in single cell transcriptomics have allowed for the unbiased classification of DRG neurons, furthering our ability to discriminate sensory neuron subtypes in more granular detail. Usoskin *et al.* (2015) was the first to use single cell RNA-sequencing (scRNA-seq) to analyse 622 mouse DRG (mDRG) neurons. They identified 11 distinct neuronal classes, five of which consisted of neurofilament heavy chain expressing neurons and were further subcategorised into LTMRs (NF1-3) and proprioceptors (NF4-5). Peptidergic neurons were identified based on *Calca* (encoding CGRP) and *Tac1* (encoding SP) expression and further divided into PEP1 and PEP2 depending on the presence or absence of *Trpv1* expression. Non-peptidergic nociceptors were divided into three subpopulations (NP1-3), all of which expressed the $P2X_3$ receptor. NP1 neurons were defined by *Mrgprd* expression, NP2 by *Mrgpra3* and NP3 by somatostatin (*Sst*). Finally, neurons expressing tyrosine hydroxylase (*Th*) and the vesicular glutamate transporter 3 (*Vglut3*) were also identified and labelled as unmyelinated low threshold mechonodetectors (C-LTMRs) (Usoskin *et al.*, 2015).

Following Usoskin *et al.* (2015), more comprehensive studies have since been carried out, sequencing more neurons at a greater depth. Sharma *et al.* (2020) sequenced DRG neurons across several developmental stages to investigate the mechanisms of sensory neuron development in mice. They found sensory neurons begin from a transcriptionally unspecialised state, before they acquire specific identities following co-expression of different transcription factors (TFs). Adult DRG neurons were assigned into 15 classes, further subdividing previously identified clusters. Six instead of two peptidergic populations were detected and myelinated neurons were categorised into Aß-field and Aß- and Aδ-LTMRs. Cold thermoreceptors were also captured and defined by transient receptor potential metastatin 8 (*Trpm8*) expression. Perhaps the most comprehensive dataset to date was generated by Qi *et al.* (2024), who sequenced approximately 40,000 neurons and organised them in at least 12 transcriptionally, morphologically and physiologically defined classes overlapping those described by Sharma *et al.* (2020). Using this information, Qi *et al.* (2024) developed new genetic tools for targeting specific DRG neuron subtypes, such as the $Cysltr2^{Cre}$ allele, and was the first to attempt linking transcriptomic profiles with functional properties. Neuronal classes were found to have morphologically distinguishable cutaneous

endings with varying branching patterns and different responses to temperature. Cooling, for instance, activated C-LTMRs only during the temperature decrease while Trpm8-expressing neurons were activated both throughout the duration of cooling and as well as when exposed to a low temperature (Qi *et al.*, 2024).

Much of the already described literature is derived from rodent studies, which have underpinned our understanding until recently. However, with increased tissue availability and optimised processing protocols, we are now in an exciting time where human DRG (hDRG) neurons can and have been profiled. In general, sensory neurons appear to be well-conserved across species (Kupari *et al.*, 2021; Nguyen *et al.*, 2021; Jung *et al.*, 2023; Bhuiyan *et al.*, 2024; Yu *et al.*, 2024). Yu *et al.* (2024) identified 16 distinct classes of human DRG neurons, most of which are also found in mouse and macaque and assessed their functional properties using single cell *in vivo* electrophysiological recordings. Key differences in gene expression, however, have also been observed, primarily in nociceptive populations, suggesting nociceptors may have divergent functions across species (Jung *et al.*, 2023; Bhuiyan *et al.*, 2024; Yu *et al.*, 2024).

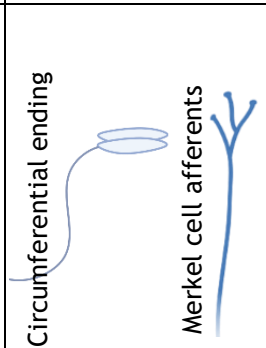
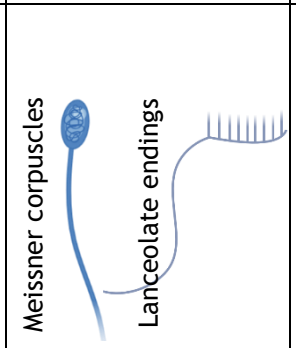
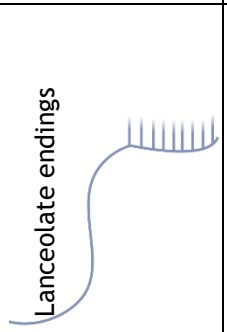
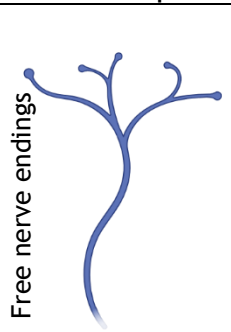
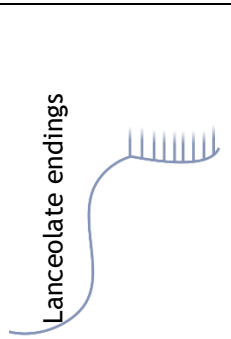
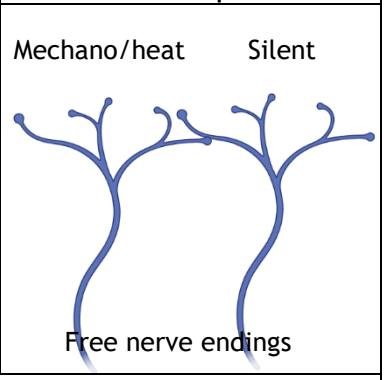
Fibre class		Aβ-fibres		Aδ-fibres		C-fibres		
Subclass		SA-LTMRs	RA-LTMRs	Aδ-LTMRs	Aδ-nociceptors	C-LTMRs	C-nociceptors	
Sensory ending								
Skin nerve responses	Conduction velocity	>10m/s		10>1.2m/s		<1.2m/s		
	Heat	No		No	Subpop	No	Subpop	
	Cold	No		No		Subpop		
	Vibration	Yes		Yes	No	No		
	Force	Yes	No	No	Yes	Yes		
	Algogens	No		No	Subpop	No	Subpop	
	Velocity	Yes		Yes	No	Yes	No	
Markers		S100β		TrkB	Nav1.8	Nav1.8	Nav1.8	TrkA
		TrkC	TrkB	Nav1.5	CGRP	TH	MRGPRD	CHRNA3
		Ret	Calbindin	Cav3.2	TrkA	VGLUT3	CGRP	CGRP
		S100a16	VGLUT1	S100β	NPY2R	TAFA4	TRPV1	

Table 1.1 Summary of primary afferent classification based on myelination, sensory ending biology, conduction velocity and responsiveness to different sensory modalities. *Canonical markers for each population are also provided. (Adapted from a presentation from Dr Steven Middleton, University of Oxford, with permission). CGRP; calcitonin gene-related peptide, MRGPRD; mas-related G protein-coupled receptor D, Npy2; neuropeptide Y receptor type 2, RA-LTMRs; rapidly adapting-low threshold mechanoreceptors, SA-LTMRs; slowly adapting-low threshold*

mechanoreceptors, Subpop; subpopulation, TAFA4; TAFA chemokine like family member 4, TH; tyrosine hydroxylase, TrkA; tropomyocin receptor kinase A, TrkB; tropomyocin receptor kinase B, TrkC; tropomyocin receptor kinase C, TRPV1; transient receptor potential vanilloid 1, VGLUT1; vesicular glutamate transporter 1, VGLUT3; vesicular glutamate transporter 3

1.1.3 Epigenomic profiling of primary afferents

Chromatin accessibility is highly correlated to gene expression and recent studies have shed light on the epigenomic landscape of hDRG neurons using techniques such as assay for transposase accessible chromatin followed by sequencing (ATAC-seq) (Sharif *et al.*, 2023; Franco-Enzástiga *et al.*, 2025). Bulk ATAC-seq performed in human lumbar DRGs discovered thousands of DARs, mostly located on the X chromosome in females and on autosomal chromosomes in males, suggesting reduced X inactivation or a dormant transcriptional state in female hDRG. Analysis of TF binding motifs within differentially accessible regions (DARs) shows enrichment for TFs from the activating protein 1 family, such as JUN and FOS, in males and from the EGR and SP families in females. These TFs have open chromatin binding sites in a variety of genes with roles in pain, such as *PIEZO2*, *TACR1*, and signalling related genes like *CACNA1G* (Franco-Enzástiga *et al.*, 2025). Spatial ATAC-seq data from the same study found DARs and TF binding sites largely consistent with those from bulk ATAC-seq. Interestingly, the top 100 DARs were enriched for glutamatergic, centromere, and interferon-related genes in females and voltage-gated Ca^{2+} channel related genes and *TRPV1/3/4* in males (Franco-Enzástiga *et al.*, 2025). Collectively, this epigenomic data suggests females and males have sexually distinct chromatin accessibility profiles and transcriptional programmes potentially contributing to sex-related differences in pain and response to injury.

A human and mouse TG cell atlas is currently available and was created using scRNA-seq and single nucleus ATAC-seq (snATAC-seq). This atlas maps putative gene regulatory elements likely to affect cell type-specific gene expression within distinct TG cell types and was used to identify cell types implicated in human genetic variations associated with migraine susceptibility and two mouse models of headache (Yang *et al.*, 2022). While we are beginning to have a clearer picture of chromatin accessibility in naïve DRG neurons, there is to date no single cell atlas of the naïve mDRG, nor is there a single cell dataset to interrogate chromatin accessibility in mDRG neurons following nerve injury.

1.1.4 Functional classification of primary afferents

Primary afferents can be categorised based on their responsiveness to different sensory modalities and the expression of relevant molecular transducers. The TRPV1 channel is responsive to capsaicin and low pH and has a thermal activation threshold of approximately 43°C, pointing towards a role in noxious heat signalling (Caterina *et al.*, 1997). TRPV1 expression is seen in the majority of heat sensitive nociceptors and mice lacking the channel show complete loss of capsaicin sensitivity as well as impairment in their ability to detect and respond to noxious heat (Caterina *et al.*, 2000). TRPV1-mediated responses are enhanced by proalgesic and proinflammatory mediators and the channel is crucial for the development of heat hypersensitivity following injury or inflammation (Caterina *et al.*, 2000; Davis *et al.*, 2000). Thus, TRPV1 is regarded as essential for acute heat sensation. The modest deficits in acute heat sensing observed in knockout mice, however, suggest TRPV1 is not the sole mediator of heat sensation. Other members of the TRP ion channel family are also implicated, as indicated by the reduced heat sensitivity observed in TRPM3-knockout mice (Vriens *et al.*, 2011). Combined elimination of TRPV1, TRPM3 and transient receptor potential ankyrin 1 (TRPA1) in knockout mice has been successful in abolishing noxious heat sensation, suggesting all three channels are important for the generation of heat-evoked pain responses (Vandewauw *et al.*, 2018). The transcriptomic profiling of primary afferents has confirmed the expression of *Trpv1* in peptidergic afferents, but it has also detected in non-peptidergic *Sst*-expressing clusters implicated in inflammatory hyperalgesia (Emery and Ernfors, 2018). Other TRPV ion channels are also implicated in sensing heath within the warm (mid-30°C) and very hot (> 50°C) range (Lumpkin and Caterina, 2007). Some, such as TRPV4, are more highly expressed in keratinocytes and epithelial cells, indicating heat sensation is likely dependent on the interplay between sensory neurons and non-neuronal cells in the skin (Chung, Lee and Caterina, 2003).

A small fraction of DRG neurons are made up of cold-sensing primary afferents responding to cooling agents such as menthol and eucalyptol. These fibres have an activation threshold of roughly 25°C, matching with the thermal activation threshold of the TRPM8 channel, the main candidate for cold sensation. TRPM8-knockout mice show substantial decreases in sensitivity to cold temperatures and ablation of TRPM8-expressing primary afferents leads to complete loss of

innocuous cold sensation. While sensitivity to cool temperatures is observed, noxious cold stimuli are still aversive to TRPM8-knockout mice, suggesting that the channel in not the only mediator of cold sensation (Bautista *et al.*, 2007; Colburn *et al.*, 2007; Dhaka *et al.*, 2007). TRPA1 is also activated by menthol, although higher concentrations are required, and is therefore thought to play a role in sensing cold. Knockout and ablation studies, however, have returned mixed results and lack conclusive evidence for the role of TRPA1 in cold detection (Bautista *et al.*, 2006; Kwan *et al.*, 2006; Karashima *et al.*, 2009; Zhou *et al.*, 2013). TRPM8 has been shown to mark all cold-sensing primary afferents which are subdivided into three functional classes, a classification supported by RNA-seq data (Trpm8.1, 8.2, 8.3) (Yarmolinsky *et al.*, 2016; Zeisel *et al.*, 2018).

Mechanical stimuli are quite diverse, thus requiring a variety of mechanoreceptors for their detection. Mechanoreceptors can be classified into high- and low-threshold depending on the intensity of stimulus they respond to. High-threshold mechanoreceptors (HTMRs) include primarily polymodal Aδ- and C-fibres terminating as free nerve endings in the skin and detecting noxious mechanical and thermal stimuli (Handler and Ginty, 2021). LTMRs, on the other hand, detect innocuous mechanical stimuli (Handler and Ginty, 2021). C-LTMRs innervate hairy skin by forming lanceolate endings associated with hair follicles and mediate the affective component of pleasant touch. They respond to low mechanical stimulation, such as soft brush stroking, and are the only sensory neurons expressing TH and VGLUT3, in line with RNA-seq evidence (Seal *et al.*, 2009; Li *et al.*, 2011; Usoskin *et al.*, 2015). Aδ-LTMRs also form lanceolate endings and are sensitive to hair inflections and stroking (Li *et al.*, 2011; Handler and Ginty, 2021). Slowly adapting LTMRs (SA-LTMRs) consist of Aβ-fibres innervating both hairy and glabrous skin by forming complexes with Merkel cells and can be distinguished based on their firing pattern during sustained indentation. These fibres express tropomyosin receptor kinase C (TrkC) and Ret and respond to high frequence stimulation (Iggo and Muir, 1969; Zimmerman, Bai and Ginty, 2014; Bai *et al.*, 2015). Finally, rapidly acting Aβ-LTMRs (RA-LTMRs) terminate in Pacinian and Meissner corpuscles in the skin and respond to the onset/end of static indentation and light forces associated with small movements on the surface of the skin respectively (Li *et al.*, 2011; Bai *et al.*, 2015; Handler and Ginty, 2021).

Primary afferents are capable of detecting a diverse host of chemical stimuli. Many exogenous compounds, such as capsaicin, formalin and mustard oil, mediate their effects by acting on TRP ion channels expressed by sensory neurons, such as TRPV1 and TRPA1 (Caterina *et al.*, 1997; McNamara *et al.*, 2007; Merrill *et al.*, 2008). The TRPA1 channel is also activated by other irritants, including compounds forming bonds with thiol groups as well as some general anaesthetics and chemotherapeutic agents (Blair *et al.*, 2016; Ton *et al.*, 2017; Marcotti *et al.*, 2023). Primary afferents also express receptors for endogenous mediators, such as the $P2X_3$ receptor and acid-sensing ionic channel for ATP and protons respectively (Bradbury, Burnstock and McMahon, 1998; Chen *et al.*, 1998). Some endogenous compounds, such as bradykinin and inflammatory prostaglandins, act indirectly by activating receptors linked to second messenger systems while other mediators, including neuropeptides such as CGRP and SP, are released by the sensory neurons themselves (Hökfelt, J. O. Kellerth, *et al.*, 1975; Iyengar, Ossipov and Johnson, 2017; Jang, Kim and Hwang, 2020). These are only some of the chemical activators acting on primary afferents, showcasing that their expression or expression of their receptors can be used to further functionally classify neurons (Michaela and Peter W., 1996).

Transcriptomic data can be used to predict the functional properties of primary afferents with the most recent studies attempting to link functional properties to transcriptionally distinct populations. Care should be taken, however, when correlating gene expression to function of a particular neuron subtype. For instance, itch populations are identified based on *Sst* and natriuretic polypeptide B (*Nppb*) expression in transcriptomic studies, but these are not expressed by all itch-sensing afferents. MRGPRD- and MRGPRA3-expressing neurons also mediate itch despite not expressing transcriptomic “itch markers” (Han *et al.*, 2013; Guo *et al.*, 2023). Furthermore, MRGPRA3 neurons are activated by noxious heat despite TRPV1 being expressed by a small proportion of this cell type and the complete lack of TRPM3 and TRPA1 expression (Xing *et al.*, 2020). It is unlikely that molecularly distinct neuronal subtypes encode most sensory modalities in a strictly “labelled line” fashion, and instead it is probable that there is a summation of activity and inactivity from different fibres (Kupari and Ernfors, 2023). For instance, simultaneous inhibition and activation of cold and heat sensing C-fibres respectively is required to encode warm perception, highlighting

an unexpected role for the cool-sensing TRPM8 channel in heat sensation Paricio-Montesinos *et al.*, 2020).

1.1.5 Central anatomy of nociception

Primary afferent fibres have peripheral and central terminals originating from a common axon owing to their pseudo-unipolar morphology. This allows sensory neurons to both detect sensory stimuli and relay the information to the CNS by terminating in the dorsal horn of the spinal cord. Depending on their functional properties, primary afferents innervate anatomically and electrophysiologically distinct laminae. A- and C-LTMRs transducing tactile information can be seen throughout lamina II-V and terminate mostly in deeper laminae. In contrast, C-nociceptive fibres project superficially in laminae I and II while Aδ nociceptors project to laminae I and V (Todd, 2010)(Todd, 2010). Accordingly, spinal cord neurons within lamina I are responsive to noxious stimuli from Aδ- and C-fibres and neurons in lamina V receive a combination of innocuous and noxious stimuli from direct Aß- and Aδ-fibres and indirect C-fibre inputs (Basbaum *et al.*, 2009). The dorsal horn is composed of large populations of excitatory (75%) and inhibitory (25%) interneurons, important for gating incoming primary afferent signals and maintaining normal nociceptive processing. Projection neurons represent the minority of spinal neurons and are mainly concentrated in lamina I, with some neurons found in laminae III-IV and very few in lamina II (Todd, 2010). Deep sequencing coupled with *in situ* hybridisation has revealed the complex heterogeneity and laminar distribution of projection neurons. Three transcriptionally distinct neuronal clusters have been found located in laminae I-III, representing antenna, nociceptive and cold-sensing cells, while two clusters found in laminae IV-V are thought to have wide dynamic range receptive fields and are defined by *Glra1* (encoding for the glycine receptor α1 subunit) expression (Bell *et al.*, 2024). Projection neurons form pathways relaying nociceptive signals to supraspinal sites, such as the spinothalamic and spinoreticulothalamic tracts terminating in the thalamus and brainstem respectively. Supraspinal targets for lamina I projection neurons include the lateral parabrachial area, the periaqueductal grey matter and the caudal ventrolateral medulla, all of which are believed to receive information from pain specific projections (Todd, 2010). The experience of pain is the result of activation of several structures associated both with the qualitative and

emotional aspects of pain, such as the somatosensory cortex, anterior cingulate cortex, basal ganglia, prefrontal cortex and amygdala (Schweinhardt and Bushnell, 2010).

Much like in the DRG, single cell transcriptomic techniques have been used to examine neuron populations in the spinal cord. Several papers profiling mouse spinal cord neurons are available, covering a wide range of ages, tissue location and circuit features and providing insight into spinal cord cell types (Häring *et al.*, 2018; Rosenberg *et al.*, 2018; Sathyamurthy *et al.*, 2018; Zeisel *et al.*, 2018; Baek *et al.*, 2019; Delile *et al.*, 2019; Mona *et al.*, 2020; Alkaslasi *et al.*, 2021; Blum *et al.*, 2021; Skinnider *et al.*, 2021; Yadav *et al.*, 2023). A harmonised atlas created by Russ *et al.* (2021) integrated the first six publicly available postnatal spinal cord datasets to address the lack of a cell type atlas for the spinal cord. They identified 69 neuronal cell types comprised of 20 dorsal excitatory, 10 dorsal inhibitory, 10 deep dorsal/mid excitatory, 7 deep dorsal/mid inhibitory, 8 ventral excitatory, 6 ventral inhibitory, 3 cholinergic motoneuron and one cerebrospinal fluid contacting neuron clusters. Extensive molecular differences were identified between dorsal and ventral neurons, mirroring the anatomical division of the spinal cord, and the expression of plasticity and structural stability genes was noted in the dorsal and ventral horn respectively. A panel of dozens of marker genes for all neuronal types was also created, providing a valuable resource for correlating transcriptomic and functional data (Russ *et al.*, 2021). In humans, slightly fewer (21-35) neuronal clusters have been identified but a similar organisation based on anatomical location is noted (Yadav *et al.*, 2023; Zhang *et al.*, 2024). Most neuronal types appear to be well-conserved between humans and mice, and dorsal neurons cluster into distinct subtypes while ventral cells show overlapping gene expression (Yadav *et al.*, 2023; Zhang *et al.*, 2024).

As illustrated, neurons at all levels of the somatosensory nervous system are incredibly diverse. However, technological advances and large-scale studies have massively accelerated our understanding and ability to classify them. We now need to apply the knowledge gained to target these neuronal types in order to fully elucidate their functional contribution and potentially advance therapeutic options. The focus of this work is primary afferent dysfunction and targeting following nerve injury. Substantial plasticity in local spinal cord circuitry,

descending modulation and higher brain centres occurs and contributes to chronic pain pathogenesis. These are indeed areas of fundamental importance for our understanding of chronic pain, however, are not within the scope of this project.

1.2 The burden of neuropathic pain

Neuropathic pain (NeuP) develops following injury or disease of the somatosensory nervous system, occurring either peripherally or centrally, and accounts for approximately 25% of chronic pain cases (Bouhassira *et al.*, 2008). Despite advances in our understanding of NeuP pathophysiology, currently available analgesics remain ineffective and are poorly tolerated, highlighting the need for safer, more efficacious pain treatments.

Research into the epidemiology of NeuP has been challenging, largely due to the lack of consensus on the definition of the disease and of validated diagnostic tools that could be used to survey the general population (Bouhassira, 2019). The recent development of screening tools in the form of questionnaires, such as the Douleur Neuropathique 4 and the Leeds Assessment of Neuropathic Pain Symptoms pain scale, has enabled the estimation of the general prevalence for NeuP. NeuP affects 7-10% of the population worldwide and its prevalence is predicted to increase due to the ageing population, improved cancer survival rates and increased incidence of diabetes (van Hecke *et al.*, 2015; Bouhassira, 2019). The condition is more prevalent in women than men (8% vs 5.7%) and in older patients (> 50 years of age), and affects primarily the upper and lower limbs, lower back and neck (Bouhassira *et al.*, 2008). A NeuP diagnosis is associated with worse health-related quality of life, with sleep disturbances, anxiety and depression frequently reported by patients (Attal *et al.*, 2011).

1.2.1 Characteristics of neuropathic pain

The nociceptive system is highly dynamic, especially in an injured state, and distinguishing between nociceptive and neuropathic pain is sometimes challenging. Patients with NeuP often complain of both intermittent and spontaneous pain in the form of shooting, burning or tingling pains, pins and needles, and squeezing or freezing pain (Bouhassira *et al.*, 2004; Baron, Binder

and Wasner, 2010). Evoked pain can also occur in response to mechanical and thermal stimuli and is defined by hyperalgesia, an increase in painful response following a noxious stimulus, and allodynia, pain triggered by an innocuous stimulus. Not all symptoms of NeuP are painful, with patients reporting both dysesthesia, defined as unpleasant abnormal sensations, and paraesthesia, non-unpleasant abnormal sensations (Finnerup, Kuner and Jensen, 2020).

NeuP is organised into peripheral and central depending on the location of the lesion or disease and is further classified based on the underlying aetiology (Scholz *et al.*, 2019). Some of the most common causes of NeuP include trauma, neurological diseases (e.g. multiple sclerosis), vascular (e.g. stroke), infectious (e.g. HIV, herpes) and metabolic (e.g. diabetes mellitus) diseases, medication (e.g. chemotherapeutics) and hereditary syndromes (e.g. erythromelalgia) (Colloca *et al.*, 2017). There are currently no specific biomarkers that can be used to identify NeuP, and diagnosis relies on a combination of psychophysical examinations and questionnaires. As a result, discrepancies in diagnoses given by clinicians are not uncommon and can account for the different NeuP prevalence rates reported in different countries. For instance, NeuP affects between 8.2-9.3% of the population in the UK but its prevalence is estimated to be 6.5% in Japan (Torrance *et al.*, 2006, 2013; Sumitani *et al.*, 2018; Baskozos *et al.*, 2023). There is a hierarchical system currently in place dividing NeuP into three classes. In patients with a history of neurological lesion or disease and pain distribution that is neuroanatomically plausible, the pain is termed “possible” NeuP. If further evidence is obtained via clinical examination, the pain is described as “probable” NeuP and if the lesion or disease can be confirmed, the pain is upgraded to “confirmed” NeuP (Finnerup *et al.*, 2016). Several screening tools have been developed to identify NeuP conditions or symptoms in other chronic pain syndromes. These include patient-reported questionnaires, qualitative sensory tests used to determine the loss or gain of function of different afferent fibre classes, neurophysiological techniques and skin or nerve biopsies (Pfau *et al.*, 2012; Colloca *et al.*, 2017).

Many NeuP patients do not receive appropriate treatment for their condition (Attal *et al.*, 2011; Torrance *et al.*, 2013). Pharmacotherapies for NeuP include tricyclic antidepressants (TCAs), serotonin-noradrenaline reuptake inhibitors (SNRIs), antiepileptic medication, opioids and topical anaesthetics. Although

opioids can be effective in treating NeuP, they are not the first choice of treatment due to their adverse reactions and high addiction potential (Dowell, Haegerich and Chou, 2016). Antidepressants and anticonvulsants are used as a first-line treatment instead, followed by topical anaesthetics and opioids as second and third (Finnerup *et al.*, 2015). Clinical trials for these treatments report modest effects with the Numbers Needed to Treat (NNT) for 50% pain relief being 3.6 for TCAs, 6.4 for SNRIs, 7.7 for pregabalin and 7.2 for gabapentin (Finnerup *et al.*, 2015). This inadequate response to treatment and adverse effects associated with most of these pharmacotherapies highlight the need for novel, more efficacious and targeted treatments.

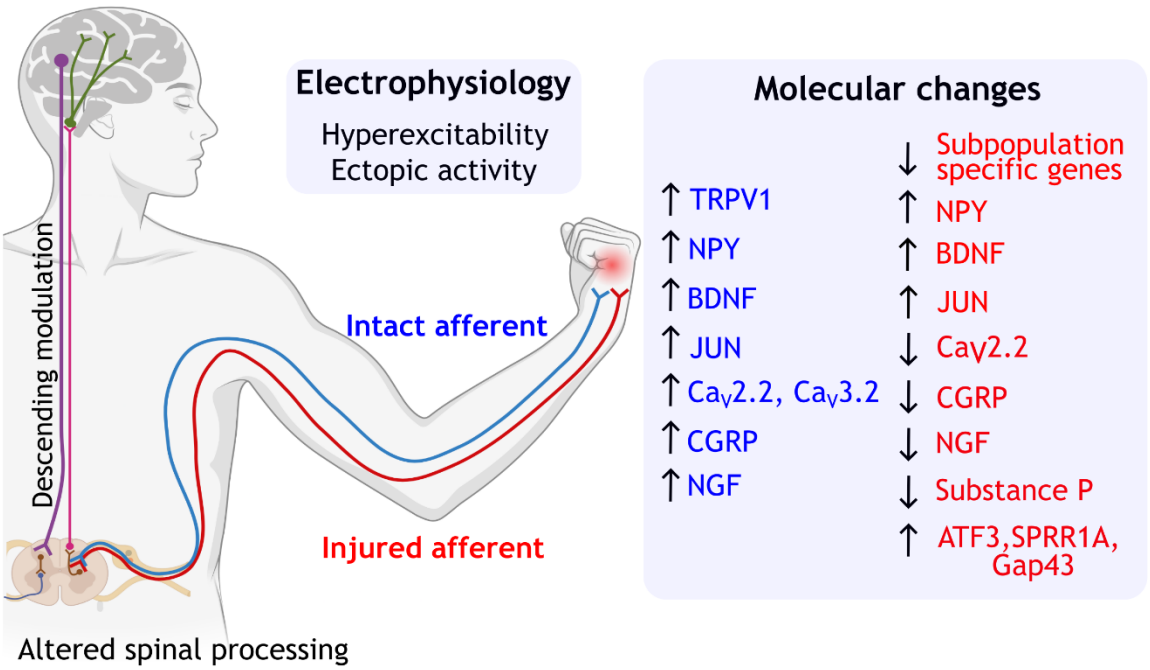
1.2.2 Pathophysiology of NeuP

Certain symptoms are consistent across NeuP conditions, suggesting that there are shared mechanisms underlying their development, irrespective of disease aetiology. Spontaneous activity, for instance, is a hallmark of NeuP and has been detected in many different models of the disease, including nonregenerative nerve ligation, regenerative nerve crush and chemotherapy-, neuritis-, or ischaemia-induced NeuP, in preclinical models as well as in patients (Serra *et al.*, 2012; Choi *et al.*, 2024). Despite similarities, some characteristics do differ between conditions, suggesting that we should always be cautious about generalising findings across different nerve insults (Attal *et al.*, 2008). For example, transcriptomic analysis of mDRG neurons after traumatic injury describes a loss of transcriptional identity and the initiation of a common transcriptional injury programme characterised by activating transcription factor 3 (Atf3) expression in almost all damaged neurons. In contrast, neurons from chemotherapy-induced neuropathy and inflammatory pain models, do not show similar transcriptomic signatures and instead more closely resemble naïve DRG neurons (Renthal *et al.*, 2020). This is despite the finding that spontaneous activity occurs in DRG neurons under both scenarios (Xie *et al.*, 2005; Li *et al.*, 2017).

Some of the most common causes of NeuP, including diabetic polyneuropathy and post-herpetic neuralgia, can injure primary afferents of the peripheral nervous system (PNS) and lead to the development of peripheral NeuP. Hyperexcitability and ectopic activity in sensory neurons are critical for

peripheral NeuP. This was best demonstrated in a study using peripheral nerve block in NeuP patients to selectively silence primary afferent activity, leading to complete and rapid abolition of spontaneous and evoked pain (Haroutounian *et al.*, 2014). Most nerve lesions induce damage in a proportion of primary afferents and result in co-mingling of injured and intact fibres. Spontaneous activity occurs in both neuronal populations and their relative contribution to pain has remained a controversial subject for several decades (Campbell and Meyer, 2006; Chen *et al.*, 2019; Chao *et al.*, 2021). A summary of changes occurring in injured and intact primary afferents following injury is provided in Figure 1.1. Administration of local anaesthetics to the site of nerve injury in patients relieves ongoing pain as well as mechanical and thermal hyperalgesia (Gracely, Lynch and Bennett, 1992). Further evidence of injured afferent contribution to NeuP has been provided by studies conducted using the rat L5 spinal nerve ligation (SNL) model. Following SNL, spontaneous activity develops in injured primary afferents. Administration of tetrodotoxin or anaesthetics in L5 DRG reduced or completely alleviated tactile allodynia and mechanical hyperalgesia (Lyu *et al.*, 2000; Sukhotinsky *et al.*, 2004).

Intact afferent signalling is required for the perception of mechanical and thermal stimuli after nerve injury in cases of denervation. Intact myelinated and unmyelinated fibres innervating affected areas have been shown to develop spontaneous activity and become hyperexcitable following nerve injury (Djouhri *et al.*, 2006, 2012). Mechanical and thermal sensitisation of intact nociceptors has also been observed in uninjured, unmyelinated nociceptors (Shim *et al.*, 2005).



	References	
	Intact	**Injured**
Evoked hypersensitivities	Li et al. (2000)	Sheen and Chung (1993)
	Jang et al. (2007)	Lyu et al. (2000)
	Yang et al. (2018)	Sukhotinsky et al. (2004)
Spontaneous pain	Ali et al. (1999)	Yoon, Na and Chung (1996)
	Djouhri et al. (2006)	Liu et al. (2000)
	Chen et al. (2019)	Chao et al. (2021)

Figure 1.1 Changes occurring in injured and intact primary afferents following peripheral nerve injury. *Both damaged and uninjured fibres become sensitised and develop spontaneous activity after injury. This is reflected in a host of molecular changes, such as the dysregulated expression of Ca^{2+} channels in both populations (Chen et al., 2018; Yang et al., 2018). Increased expression of nociceptive markers, such as TRPV1 and CGRP, as well as of factors such as NPY, BDNF and NGF has also been observed in uninjured fibres. Only some examples of the molecular changes taking place in injured primary afferents are shown here (Michael et al., 1999; Fukuoka et al., 2001; Obata et al., 2006; Weissner et al., 2006; Nitzan-Luques, Devor and Tal, 2011; Renthal et al., 2020). Blue and red text denote changes in intact and injured afferents*

respectively. While recent evidence suggests damaged afferents drive spontaneous pain and uninjured neurons drive evoked hypersensitivities, different studies have implicated both populations to either pain behaviour. BDNF; brain-derived neurotrophic factor, CGRP; calcitonin gene-related peptide, NGF; nerve growth factor, NPY; neuropeptide Y, TRPV1; transient receptor potential vanilloid 1

The absolute contribution of injured compared to intact fibres to the different components of NeuP is not fully resolved, however, the weight of evidence suggests an important role for injured afferents. Using DRG field stimulation to block spontaneous activity in rat L5 DRG resulted in the reduction of mechanical hyperalgesia and tactile allodynia behaviours as well as spontaneous pain. In contrast, DRG field stimulation of uninjured L4 DRG abolished evoked pain behaviours but not ongoing pain (Chao *et al.*, 2021). In addition to the literature, our lab has data supporting the role of injured afferents in spontaneous pain. Chemogenetic silencing of genetically identified injured primary afferents (based on the ATF3CreERT2 mouse line (Denk *et al.*, 2015)) attenuates ongoing pain as assessed by conditioned place preference (CPP) to gabapentin, but does not alter evoked hypersensitivities (Dr Andrew Cooper, unpublished data). On the assumption that directly injured afferents underlie spontaneous pain, selective targeting of these neurons offers the potential to treat pathological pain, while not impacting normal somatosensation transduced by intact afferents.

1.2.3 Transcriptomic and epigenomic changes in DRG neurons following injury

Peripheral nerve injury induces an array of transcriptional changes in DRG neurons to facilitate axonal regeneration, cell repair and stress, as well as the development of pathological hyperexcitability underlying NeuP. Transcriptomic analysis of thousands of neurons following peripheral nerve damage using RNA-seq has uncovered a common injury programme initiated in 80-93% of affected neurons only one day post injury (Renthal *et al.*, 2020; Zhang *et al.*, 2022). More specifically, injured DRG and TG neurons highly express regeneration-associated genes (RAGs), including *Atf3*, *Sprr1a*, *Gap43* and *Sox11*, while neuron subtype specific genes are downregulated, leading to a loss of identity. Gene expression changes affecting neuronal excitability also take place, including the

downregulation of potassium channels, upregulation of calcium channels like *Cacna2d1*, and dysregulation of neuropeptide expression (Hu *et al.*, 2016; Nguyen, Le Pichon and Ryba, 2019; Palmisano *et al.*, 2019; Renthal *et al.*, 2020; K. Wang *et al.*, 2021; Zhang *et al.*, 2022; Barry *et al.*, 2023). Renthal *et al.* (2020) suggested that this transcriptional reprogramming is primarily mediated by *Atf3*, a TF critical to the initiation of the injury transcriptional response. This is, however, likely to occur indirectly since *Atf3* binding motifs are not enriched in differentially expressed genes (DEGs). Gene ontology (GO) analysis of top DEGs in injured neurons reveals pathways related to axonal regeneration, receptor signalling, cell proliferation, apoptotic processes and inflammatory response (Hu *et al.*, 2016; Renthal *et al.*, 2020; K. Wang *et al.*, 2021; Zhang *et al.*, 2022; Barry *et al.*, 2023). Many of these pathways continue being enriched four weeks post injury (K. Wang *et al.*, 2021; Barry *et al.*, 2023).

The composition of DRG is quite heterogeneous with sensory neurons varying in size, conduction velocity, gene expression profiles and target innervation. As a result, some differences in their responses to injury are expected, beyond their shared injury-induced transcriptional programme (Hu *et al.*, 2016; Renthal *et al.*, 2020; K. Wang *et al.*, 2021; Zhang *et al.*, 2022; Barry *et al.*, 2023). A study by Barry *et al.* (2023) found over 400 DEGs regulated in opposing directions in *Scn10a*+, peptidergic and nonpeptidergic nociceptors, C-LTMRs and Aβ- and Aδ-LTMRs, suggesting transcriptionally distinct programmes are activated in different neuron subtypes.

There is a strong link between gene expression and chromatin accessibility, meaning the marked transcriptional changes after injury can serve as an indication of the likely epigenetic alterations and can provide hints as to what genes and pathways are likely to be dysregulated. Epigenomic DRG studies have provided insight into the chromatin accessibility landscape in rodent DRG after injury. Using bulk ATAC-seq in combination with chromatin immunoprecipitation sequencing (ChIP-seq) and RNA-seq, Palmisano *et al.* (2019) found a significant gain in chromatin accessibility in mDRG neurons at very early time points (24 hours) following sciatic nerve axotomy. Pathways associated with calcium channel activity, chromatin remodelling, cell signalling, and axon transport were enriched, in line with transcriptomic changes following injury. In addition, chromatin was more relaxed in areas containing injury gene promoters,

potentially to allow for the expression of RAGs, and different TFs were recruited in response to peripheral and central axonal injury. Overall, distinct chromatin accessibility and histone acetylation signatures correlating with gene expression can be detected after peripheral, but not central, axotomy (Palmisano *et al.*, 2019).

Epigenomic analyses of rat DRG have also identified differences in chromatin accessibility between models of neuropathic (chronic constriction injury, CCI) and inflammatory (complete Freud's adjuvant, CFA) pain (Stephens *et al.*, 2021). GO analysis of DARs after CCI shows enrichment for functions linked to neurotransmitter receptor activity, regulation of postsynaptic membrane potential and enhancer binding. In comparison, potassium ion transmembrane transport and receptor serine/threonine kinase binding pathways are enriched in CFA animals, combined with a wider induction of TF remodelling (Stephens *et al.*, 2021). When comparing chromatin accessibility data from naïve and CCI rat DRG, Stephens *et al.* (2024) found hundreds of genes and promoters significantly more accessible in CCI rats and enrichment for pain and sensory related processes. Integration with RNA-seq data showed a correlation between regions with increased chromatin accessibility and genes upregulated after CCI. Cloning of some of these DARs in vectors and introduction to the immortalised rat nociceptor cell line, 50B11, led to both up- and downregulation of expression. This suggests DARs are associated with both enhancers and repressors, further supporting the notion that changes in DNA accessibility can alter gene expression in nociception (Stephens *et al.*, 2024). In common with Palmisano *et al.* (2019), these studies relied upon bulk ATAC-seq, which does not indicate the cell types (e.g. neuronal vs non-neuronal, different subpopulations of sensory neurons, etc) contributing to the observed signals. The heterogeneity of transcriptional responses across subpopulations also highlights the importance of profiling chromatin accessibility at single cell level.

1.2.4 MicroRNAs in neuropathic pain

MicroRNAs (miRNAs) are short non-coding RNAs (~21-23 nucleotides long) first identified in the nematode *Caenorhabditis elegans* (Lee, Feinbaum and Ambros, 1993). Most miRNAs are intragenic and transcribed primarily from introns into hairpin-containing primary miRNAs (pri-miRNA). MiRNAs are then processed into

precursor miRNA (pre-miRNA) by the microcompressor complex and exported to the cytoplasm where they are further processed to remove the terminal loop and produce mature miRNA duplexes (Denli *et al.*, 2004). These consist of the 5p and 3p strands, both of which interact with the Argonaute (AGO) family of proteins. The strand with the lowest 5' stability or 5' uracil content is normally preferentially loaded into an AGO protein complex and is termed the guide strand while the unloaded, or passenger, strand is degraded (O'Brien *et al.*, 2018).

MiRNAs regulate the expression of almost all protein-coding genes and therefore play a vital role in numerous biological processes (Jonas and Izaurralde, 2015). They primarily control gene expression post-transcriptionally by binding to the 3' untranslated region (3' UTR) of their target mRNA to induce translational repression, although miRNA-mediated activation and miRNA buffers have also been reported (Vasudevan and Steitz, 2007; Truesdell *et al.*, 2012; Nishi *et al.*, 2013; Xu *et al.*, 2014; Chaluvally-Raghavan *et al.*, 2016; Miao *et al.*, 2016; Ramchandran and Chaluvally-Raghavan, 2017; Xiao *et al.*, 2017). The gene silencing effects are mediated by the miRNA-induced silencing complex (miRISC), composed of the guide strand and AGO protein. The target specificity of miRISC is the result of interactions with specific sequences on target mRNA known as miRNA response elements (MREs). The degree of complementarity between these MREs and nucleotides 2-8 of the guide strand determines how miRISC will regulate its target. Perfect complementarity induces AGO2 endonuclease activity and mRNA cleavage, leading to rapid target degradation and the prevention of translation, and is rare in animals (Jo *et al.*, 2015). Most miRNA and MREs interactions are not fully complementary, resulting in translational inhibition and target decay taking place in the form of mRNA deadenylation, decapping and finally degradation mediated by miRISC (Behm-Ansmant *et al.*, 2006; Braun *et al.*, 2012; O'Brien *et al.*, 2018). Ultimately, the effect of miRNAs on gene expression depends on multiple factors, including miRNA abundance and localisation, cell type, and cell state.

Dysregulations in miRNA expression have been reported in experimental animal models as well as patients with NeuP conditions, suggesting these small noncoding RNAs contribute to the development and maintenance of NeuP (Bali *et al.*, 2021; Kovanur Sampath *et al.*, 2023; Zhao *et al.*, 2023). NeuP is

characterised by neuronal hyperexcitability and ectopic activity facilitated by changes in the expression of ion channels, several of which constitute miRNA targets (Figure 1.2). For instance, members of the miRNA-183 (miR-183) family (discussed further in Chapter 5) help regulate neuronal activity under normal physiological conditions by suppressing the expression of the voltage-gated sodium channels Nav1.3, Nav1.7 and Nav1.8 and the calcium channel subunits *Cacna2d1* and *Cacna2d2* (Chen *et al.*, 2014; Peng *et al.*, 2017; Cai *et al.*, 2018; Tao *et al.*, 2021). Other miRNAs, such as miR-7a, miR-30b and miR-384-5p, are also implicated in the regulation of ion channel expression as indicated by changes in neuron excitability and the downregulation of these miRNAs in the DRG following nerve injury (Sakai *et al.*, 2013; Shao *et al.*, 2016; Li *et al.*, 2019; Ye *et al.*, 2020). MiRNAs can also be upregulated in injured primary afferents, as is the case with the miR-17-92 cluster. Its elevated levels contribute to the development of mechanical hypersensitivity by supressing the expression of potassium channels and reducing outward potassium currents (Sakai *et al.*, 2017). PNS neurons that survive injury are capable of regenerating, a process that is regulated by numerous factors, including miRNAs. Changes in the expression of certain miRNAs, such as miR-210, promote neuronal survival and regeneration by inhibiting apoptosis, while other miRNAs bind to intrinsic inhibitors of axonal growth (Hu *et al.*, 2016; van Battum *et al.*, 2018). Some miRNAs can also regulate nerve regrowth by interacting with regeneration associated genes, such as ATF3 and SPRR1A bound by miR-183 and miR-155, respectively (Gaudet *et al.*, 2016; Peng *et al.*, 2017). In contrast, many miRNAs can also negatively impact regeneration via the inhibition of axonal growth signalling pathways (miR-455-5p) or by forming mutual negative feedback regulatory loops (miR-138) (Liu *et al.*, 2013; Su *et al.*, 2020).

Peripheral nerve injury is accompanied by immune system activation, the infiltration of immune cells to injured tissue and often neuroinflammation. Various miRNAs have been found to regulate inflammatory pathways contributing to the onset and development of NeuP. The extracellular signal-regulated kinase (ERK) pathway is activated in response to inflammation and can lead to neuronal sensitisation. MiR-144, which targets the RAS P21 protein activator 1, a member of the ERK signalling cascade, is downregulated in NeuP and its overexpression reduces mechanical hypersensitivity and thermal hyperalgesia as well as the

release of pro-inflammatory cytokines in the DRG (Zhang *et al.*, 2020). A single miRNA can have several targets, as demonstrated by miR-183. In addition to regulating the expression of ion channels, high levels of miR-183 can inhibit inflammatory signalling axes such as that of transforming growth factor-α/C-C motif chemokine ligand 2/C-C chemokine receptor type 2 which in turn inhibits the expression of pro-inflammatory cytokines (Tao *et al.*, 2021). Other inflammatory targets regulated by miRNAs include the nuclear factor kappa B and p38 mitogen-activated protein kinase pathways as well as receptors, such as the C-X-C chemokine receptor type 4 (Pan *et al.*, 2018; Liu *et al.*, 2021; Zhao *et al.*, 2023). Lastly, changes in miRNA expression can alter glial and immune cell activation and proliferation, ultimately affecting inflammation, neuronal excitability and nerve regeneration (Viader *et al.*, 2011; Heyn *et al.*, 2016; Zhao *et al.*, 2023).

The miRNAs and targets discussed here represent only a proportion of the changes that take place in the DRG following nerve injury (Figure 1.2) (Zhao *et al.*, 2023). The human genome encodes more than 2000 miRNAs and further characterisation of their expression in healthy DRG as well as in different models of NeuP is required to fully understand their role in disease development and identify novel therapeutic targets (Gao *et al.*, 2025).

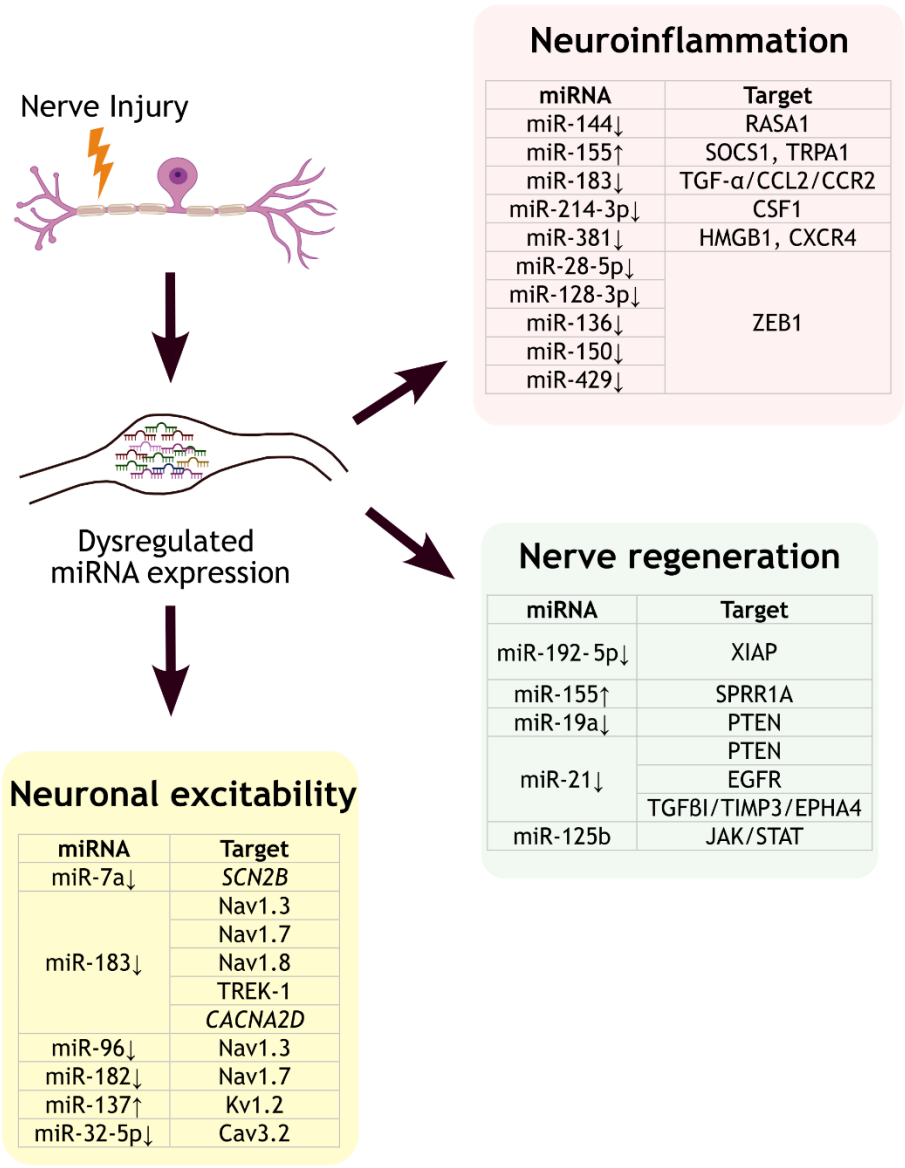


Figure 1.2 Changes in miRNA expression within the DRG following peripheral nerve injury. *Dysregulated miRNA expression impacts neuronal excitability, nerve regeneration and neuroinflammation, contributing to the development and maintenance of neuropathic pain (Zhao et al., 2023). Arrows indicate whether specific miRNAs are up- or downregulated. CCL2; C-C motif ligand 2, CCR2; C-C chemokine receptor 2, CSF1; colony stimulating factor 1, CXCR4; C-X-C chemokine receptor type 4, EGFR; epidermal growth factor receptor, EPHA4; erythropoietin-producing human hepatocellular receptor A4, HMGB1; high mobility group box 1, JAK; Janus kinase gene, PTEN; phosphatase and tensin homolog, RASA1; RAS P21 protein activator 1, SCN2B; sodium voltage-gated channel subunit 2, SPRR1A; small proline-rich protein 1A, SOCS1; suppressor of cytokine signalling 1, STAT; signal transducer and activator of transcription, TGF-a; transforming growth factor a, TGFBI; transforming growth factor beta induced protein, TIMP3; tissue inhibitor of metalloproteinases 3, TREK-1; TWIK-*

related K channel 1, TRPA1; transient receptor potential ankyrin 1, XIAP; X-linked inhibitor of apoptosis protein, ZEB1; Zinc finger E-box-binding homeobox 1

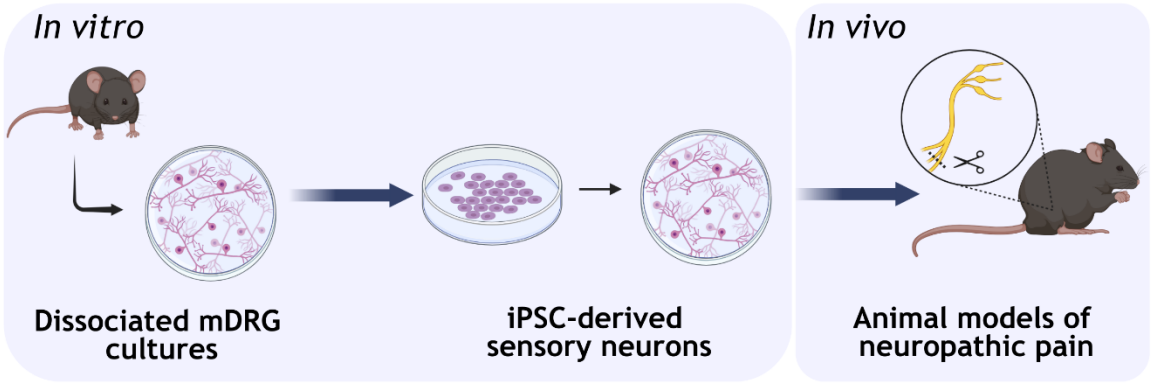
1.2.5 Modelling neuropathic pain

The use of representative preclinical NeuP models is essential for advancing our understanding of disease mechanisms and identifying novel therapeutic targets. Cell-based models have a considerable number of attractive qualities, including practical advantages such as the reduction of expenses, ethical concerns, and experimental labour. As a result, *in vitro* models offer a platform capable of facilitating medium-high throughput processes, such as evaluating the efficacy of potential therapeutic compounds, and are used throughout this work as initial screens for our AAV candidates.

DRG neurons can be derived from a number of sources and the study of dissociated rodent sensory neurons has been critical to our understanding of nociceptive signalling pathways and drug target identification. These cells are readily available and protocols for their culture have been optimised over decades, rendering them a valuable tool in preclinical sensory neuron research. However, comparisons of mouse and human DRG tissue have unveiled species-specific differences (Rostock *et al.*, 2018; Shiers, Klein and Price, 2020). Human sensory neurons can also be cultured in the lab and their use has increased over the last years owing to their improved availability (Chrysostomidou, Cooper and Weir, 2021). They are undoubtedly an exciting model, but while accessibility is improving, it is still challenging to obtain live hDRG neurons in the UK. One confound common to both rodent and human DRG neuron cultures is that these neurons are injured since they have been axotomized and enzymatically and mechanically dissociated. Transcriptomic comparisons of cultured and intact human and rodent DRG neurons reveal an upregulation of neuronal injury and inflammation markers, such as ATF3, galanine, and matrix metalloproteinase-9, in cultured neurons (Wangzhou *et al.*, 2020).

Recent advances in stem cell technologies have led to the production of sensory neuron-like cells from induced pluripotent stem cells (iPSCs). Donor cells are usually skin fibroblasts and iPSCs are particularly amenable to genome engineering and can provide a theoretically limitless supply of neurons.

Currently available protocols can generate approximations of many, but not all, sensory neuron subtypes, from non-peptidergic nociceptors to Aß-LTMRs (Chrysostomidou, Cooper and Weir, 2021). These neurons offer a more accessible alternative to hDRGs while also being more amenable to experimental manipulation (e.g. genetic modifications) owing to their longer times in culture. Sensory neurons derived from iPSCs have been successfully used to model injury *in vitro* in the past (Baskozos *et al.*, 2020; M. Wang *et al.*, 2021; Schinke *et al.*, 2021; Cunningham *et al.*, 2022; Mortensen *et al.*, 2023). Keeping species differences in mind and using a combination of human and animal tissue-based systems can help bridge the gap between preclinical and clinical research and improve translatability (Figure 1.3).



Advantages

- Representation of all endogenous sensory neuron subtypes
- Low culture and maintenance costs
- Capacity to create complex multicellular cultures
- Compatible with molecular tools for labelling, gene editing and manipulation of neuronal activity
- Suitable for larger scale production
- Can be maintained long-term
- Cryopreservation possible
- Opportunity to investigate cell types using transgenic animals
- More accurate physiological representation

Limitations

- Injury phenotype induced by dissociation
- Limited capacity for genetic manipulation
- Expensive and laborious cultures
- Unlikely to fully replicate endogenous human sensory neuron subtypes
- Immature phenotype
- Currently unable to generate all subtypes in one culture
- Loss of environmental impact on chronic pain
- Ethical considerations
- High cost
- Complex biological context
- Physiological differences between humans and model organisms

Figure 1.3 Advantages and limitations of human and rodent in vitro and in vivo modelling systems for neuropathic pain.

1.2.6 Animal models of NeuP

NeuP is a complex condition with diverse aetiologies. There are numerous animal models of NeuP, reflecting the different types of peripheral nerve injury that can lead to human disease. Several systemic models have been established to investigate neuropathy caused by disease, including diabetes, cancer and HIV, or

neurotoxic agents. These have had varying degrees of success, highlighting the complexity of mechanisms giving rise to NeuP (Boehmerle *et al.*, 2014; Burdo and Miller, 2014; Rani *et al.*, 2017; Pham *et al.*, 2019; Ou *et al.*, 2023; Chen *et al.*, 2024). The most commonly used NeuP models mimic traumatic peripheral nerve injury and arguably represent the most consistent and reproducible of the NeuP animal models. In the rodent spared nerve injury (SNI) model, the tibial and common peroneal branches of the sciatic nerve are transected while leaving the sural nerve intact (Decosterd and Woolf, 2000). This results in partial denervation of the paw and the behavioural effects can be seen almost immediately; animals display mechanical hypersensitivity and hyperalgesia in the lateral paw innervated by the sural nerve which can still be seen 6 months later. Cold hypersensitivity and heat hyperalgesia have also been reported; however, those findings are inconsistent when performed by different groups, likely due to equipment discrepancies (Cooper *et al.*, unpublished observations). Spontaneous pain is a defining characteristic of NeuP and one of the most commonly reported clinical symptoms, having a detrimental impact on patients' quality of life (Baron *et al.*, 2009). Detecting spontaneous pain in rodents can be challenging, leading researchers to rely on the use of behavioural assays such as CPP, a procedure assessing the amount of time animals spend seeking pain relief, and facial grimace scoring (King *et al.*, 2009; Tuttle *et al.*, 2018). Ongoing pain in rodents has been detected following SNI by these methods. Behavioural cues such as grooming, eating, nesting and burrowing can also be indicators of spontaneous pain. These changes in pain sensitivity closely resemble clinical NeuP symptoms. In addition, the partial nature of the injury allows for the study of both injured and intact primary afferents, making SNI the most widely used animal model of NeuP and the one we employed throughout this work (Decosterd and Woolf, 2000).

1.3 Advances in gene therapy

Gene therapy is defined as the use of biological products mediating their effects by the transcription or translation of transferred genetic material or by altering human genetic sequence to treat or prevent disease. Gene therapies have emerged as particularly attractive therapeutic approaches due to their potential to offer both long-term treatment and refined control, thus minimising side effects. Advances in our understanding of the human genome and the molecular

basis of disease as well as the availability of novel materials and techniques ultimately led to the first successful viral mediated gene transfer (Rogers and Pfuderer, 1968). Using tobacco mosaic virus, Rogers and Pfuderer (1968) introduced a polyadenylate stretch to viral RNA, providing proof-of-concept for viral gene therapy. Following the development of protocols for the introduction of foreign genes into humans and the first clinical trials in the 1980s and 1990s, the first gene therapy for the treatment of head and neck squamous cell carcinoma was approved for clinical use in China in 2003. This biological therapy, known as Gendicine, is a recombinant human p53 adenovirus delivered by intratumoral injection and intracavity or intravascular infusion (Rosenberg *et al.*, 1988; Peng, 2005; Wirth, Parker and Ylä-Herttuala, 2013). Transduced cells express the wild-type form of p53, helping them restore normal cell cycle control and induce processes such as apoptosis, senescence and autophagy depending on cellular stress state (Zhang *et al.*, 2018). In Europe and the US, the first gene therapies were not approved until 2012 and 2017 respectively (Büning, 2013; Braendstrup, Levine and Ruella, 2020). This delay was largely due to the death of Jesse Gelsinger, an 18-year-old participating in a gene therapy clinical trial in 1999. The trial aimed to treat ornithine transcarbamylase (OTC) deficiency, an inherited disease leading to dangerous levels of ammonia accumulating in the blood, using an adeno associated viral vector to deliver a functional version of the *OTC* gene. The high viral dose, however, caused a strong immune response and multiple organ failure, leading to death four days after the therapy was administered (Lehrman, 1999; Sibbald, 2001; Wilson, 2009). As a result, other gene therapy trials were halted and protocols and regulations surrounding gene therapies were reevaluated. Since then, research into the development of safer vectors has become a priority and is facilitated by advances in our understanding of viral vector basic biology and the risks associated with their use.

Gene therapy can be distinguished into *in vivo*, mediated via the transfer or editing of genetic material, and *ex vivo* in the form of gene-modified cell therapies. The later account for more than half of disclosed gene therapies currently being developed, followed closely by therapies involving the transfer of gene material (35%) and *in vivo* gene editing (10%) (Chancellor *et al.*, 2023)(Chancellor *et al.*, 2023)(Chancellor *et al.*, 2023). Gene therapies have

various methods of delivery, including viral vectors (accounting for 90% of all disclosed gene therapies), nonviral vectors and physical delivery. Adeno associated viruses (AAVs) are the most commonly used viral vector (49% of viral gene therapies currently under development) owing to their reduced likelihood of integration into the host genome (Chancellor *et al.*, 2023; Wang *et al.*, 2024). Glybera is an AAV serotype 1 (AAV1)-based therapy used to treat severe lipoprotein lipase deficiency and the first gene therapy to be to be approved in the European Union in 2012 (Wirth, Parker and Ylä-Herttuala, 2013). Since then, an additional five AAV-mediated therapies have been approved for the treatment of a wide range of disorders, including spinal muscular atrophy and haemophilia A/B. As of 2023, there are now 111 gene, cell, and RNA-based therapies which are approved in at least one country (Chancellor *et al.*, 2023; Wang *et al.*, 2024).

Advances in tissue targeting mechanisms and genome manipulation make gene therapies an attractive option for the treatment of chronic pain. Most preclinical gene therapy approaches for pain target neurons in the DRG and TG, which reside in the PNS and are therefore more easily accessible (Li and Ji, 2024). There is a broad range of actuators being tested for their translational potential. Optogenetic techniques offer the ability to manipulate sensory neuron function in a controlled manner and are actively being trialled preclinically as a potential treatment modality for NeuP. Zhang *et al.* (2019) created a wireless, fully implantable device capable of modulating primary afferent activity by delivering localised optical and/or pharmacological stimuli. The device consisted of an inorganic light-emitting diode and an ultralow-power electrochemical micropump system, required for fluid delivery through microfluidic channels located on optofluidic caps surrounding target nerves. No effects on overall health and nerve condition were observed 10 weeks after implantation and positive or negative manipulation of the device modulated sensory neuron activity accordingly (Zhang *et al.*, 2019). Viral vectors have also been used to deliver optogenetic and chemogenetic tools to sensory neurons, allowing for manipulation of their activity *in vivo*. Using a chemogenetic-based approach, Weir *et al.* (2017) introduced a modified form of the glutamate-gated chloride channel (GluCl) to a wide range of sensory neurons *in vivo* via intrathecal AAV injections. Channel activation resulted in significant silencing of DRG neurons

and reversed thermal and mechanical hypersensitivity after SNI. A recent study improved on this approach by replacing GluCl with a designer pharmacologically selective actuator module (PSAM), creating a humanised system with improved translational potential. This receptor was based on the human α7 nicotinic acetylcholine receptor combined with the glycine receptor pore domain, resulting in the chloride channel PSAM^4-GlyR. This modulatory system was delivered to primary afferents and its activation resulted in the silencing of neurons and the amelioration of acute, inflammatory, and NeuP-related behavioural readouts. Reduced hyperexcitability was also observed in human iPSC-derived sensory neurons (iSNs), underscoring its promise as a gene therapy (Perez-Sanchez *et al.*, 2023). Combined, these results highlight the potential of using AAVs to facilitate the use of neuromodulatory techniques to manipulate sensory neuron activity.

In addition to delivering exogenous transgenes, the capacity of gene therapies to restore dysregulated endogenous signalling pathways has also been explored. Hereditary loss-of-function mutation in Nav1.7 leads to congenital insensitivity to pain without further neurodevelopmental changes while gain-of-function mutations lead to the development of pain syndromes (Cox *et al.*, 2006; Bennett and Woods, 2014). This highlights the channel's importance in nociception as well as its attractiveness as a therapeutic target. The high degree of structural similarity between Na^+ voltage-gated channels and their large sequences, however, complicate the development of selective pharmacological inhibitors. Moreno *et al.* (2021) overcame these limitations by developing an epigenetic tool known as long-lasting analgesia via targeted *in vivo* epigenetic repression, or LATER. This tool combined clustered regularly interspaced short palindromic repeats (CRISPR)-dCas9 technology with zinc finger proteins and was delivered via AAVs to lumbar DRG to repress Nav1.7 expression. This system was trialled in chemotherapy-induced neuropathy, triethylammonium salt (BzATP)-induced pain and carrageenan induced inflammatory pain models and resulted in long-lasting analgesia without affecting normal motor function (Moreno *et al.*, 2021).

Numerous miRNAs, as well as proteins involved in miRNA processing pathways, are significantly dysregulated in primary afferents after nerve injury (Figure 1.2) (Andersen, Duroux and Gazerani, 2014). For instance, miR-7a is downregulated following neuropathic injuries. Intraganglionic injection of AAV6-miR-7a has

been shown to abolish mechanical allodynia and thermal hyperalgesia caused by nerve injury (Sakai *et al.*, 2013). These treatment exemplars highlight the potential of gene therapy for chronic pain.

In sum, there is a broad range of candidate transgene actuators that show therapeutic promise for treating pain, at least in preclinical models. However, technical challenges remain namely issues with specific delivery and off-target activity, biocompatibility and toxicity (Mullard, 2021; Ertl, 2022). Perhaps the main of these issues is the ability to target therapeutic transgenes to the correct neurons, with minimal transduction in other populations.

1.4 Targeting AAV delivery

Treatments, both pharmaceutical and gene based, often fail in late-stage clinical trials due to efficacy and safety concerns. These are caused largely by drug accumulation or expression in off-target organs, highlighting the pressing need for improved drug selectivity (Hordeaux *et al.*, 2018; Buss *et al.*, 2022; Kohn, Chen and Spencer, 2023). AAVs are at the forefront of gene therapy research and are one of the safest and most effective methods of gene delivery. These viruses are small, non-enveloped and have a single-stranded DNA genome of approximately 4.7kb (Wu, Yang and Colosi, 2010). This includes the genes *rep*, *cap*, and *aap*, encoding for replication, capsid and assembly proteins respectively, flanked by two inverted terminal repeats (ITRs) required for genome replication and packaging. AAVs require co-infection with other viruses to replicate and recombinant AAVs (rAAVs) do not carry the same protein coding sequences as wild type AAVs. These are instead replaced with therapeutic gene expression cassettes. As a result, AAVs are not thought to cause disease in humans (Naso *et al.*, 2017). A summary of commonly used gene therapy approaches and methods for targeting their delivery is provided in Figure 1.2.

1.4.1 Capsids

AAVs are able to enter cells by interacting with carbohydrates, as well as secondary receptors on the cell's surface. Depending on the AAV serotype, capsids have different sequences conferring for binding preferences and influencing cell type transduction. Thirteen distinct human and nonhuman

primate (NHP) AAVs have been identified to date (AAV1-13) through natural discovery. AAV1 interacts with sialic acid and has tropism for skeletal and cardiac tissue, as demonstrated by its use as a gene therapy for the treatment of lipoprotein lipase deficiency (Gaudet *et al.*, 2013). In contrast, AAV9 preferentially binds galactose and therefore has the unique ability to cross the blood-brain barrier, making it a leading candidate for the transduction of CNS cells (Schuster *et al.*, 2014). AAV immunogenicity is a primary concern associated with AAV-mediated gene therapy. It is estimated that between 40-80% if the population is seropositive for neutralising antibodies against AAVs, dramatically reducing their efficacy (Calcedo *et al.*, 2011). Capsids isolated from NHPs and other vertebrates show lower immunoreactivity, although they are also likely to have reduced transduction efficiency.

Significant efforts have been made towards the development of new AAV capsids through rational design and directed evolution. Capsid modifications can be made to alter the tropism of AAVs. For instance, the introduction of modified peptide sequences in AAV2 capsids improved the vector's brain tropism while inclusion of integrin-binding sequences targets AAV2 to infection resistant cells (Girod *et al.*, 1999; Chen, Chang and Davidson, 2009). Other methods of engineering tropism include the introduction of larger proteins, such as designed ankyrin repeat proteins, and the fusion of peptide sequences to the amino terminus of certain capsid proteins (Warrington *et al.*, 2004; Münch *et al.*, 2015). The tropism and immunogenicity of capsids is largely shaped by 3-fold protrusions, rendering them ideal candidates for modification. Insertion of peptide sequences to these protrusions has been shown to improve targeted binding and reduce immunogenicity (Asokan *et al.*, 2010).

Gaps in our understanding of the molecular mechanisms behind AAV binding, trafficking and expression considerably limit progress made using rational design. Directed evolution, on the other hand, mimics natural evolution and relies on placing capsids under selective pressure to obtain variants with the desirable properties. Cre recombination-based AAV targeted evolution (CREATE) is a powerful technology developed for evolving AAVs that can target specific cell types in a Cre-dependent manner (Deverman *et al.*, 2016). The process begins with the creation of an AAV capsid library wherein mutations in the form of randomised peptide sequences are introduced in the 3-fold protrusions. The

library is then injected into transgenic animals expressing Cre recombinase in specific cell types. Only variants which successfully infect Cre-expressing cells will undergo recombination. These are then recovered, amplified by PCR and used for the next round of selection. Multiple repeats of the process ensure the final AAV variant has enhanced specificity and transduction efficiency in the cell or tissue of interest (Deverman *et al.*, 2016). CREATE has been used to generate the AAV-PHP.B variant which has at least 40-fold greater efficiency at carrying genes to the CNS than AAV9 (Deverman *et al.*, 2016). A considerable viral load is required to achieve transduction, however, leading to attempts to use CREATE to further evolve the AAV-PHP.B variant. These resulted in AAV-PHP.eB, which requires a lower viral load to transduce neurons across the CNS, and the AAV-PHP.S variant which successfully targets 86% of DRG neurons (Chan *et al.*, 2017).

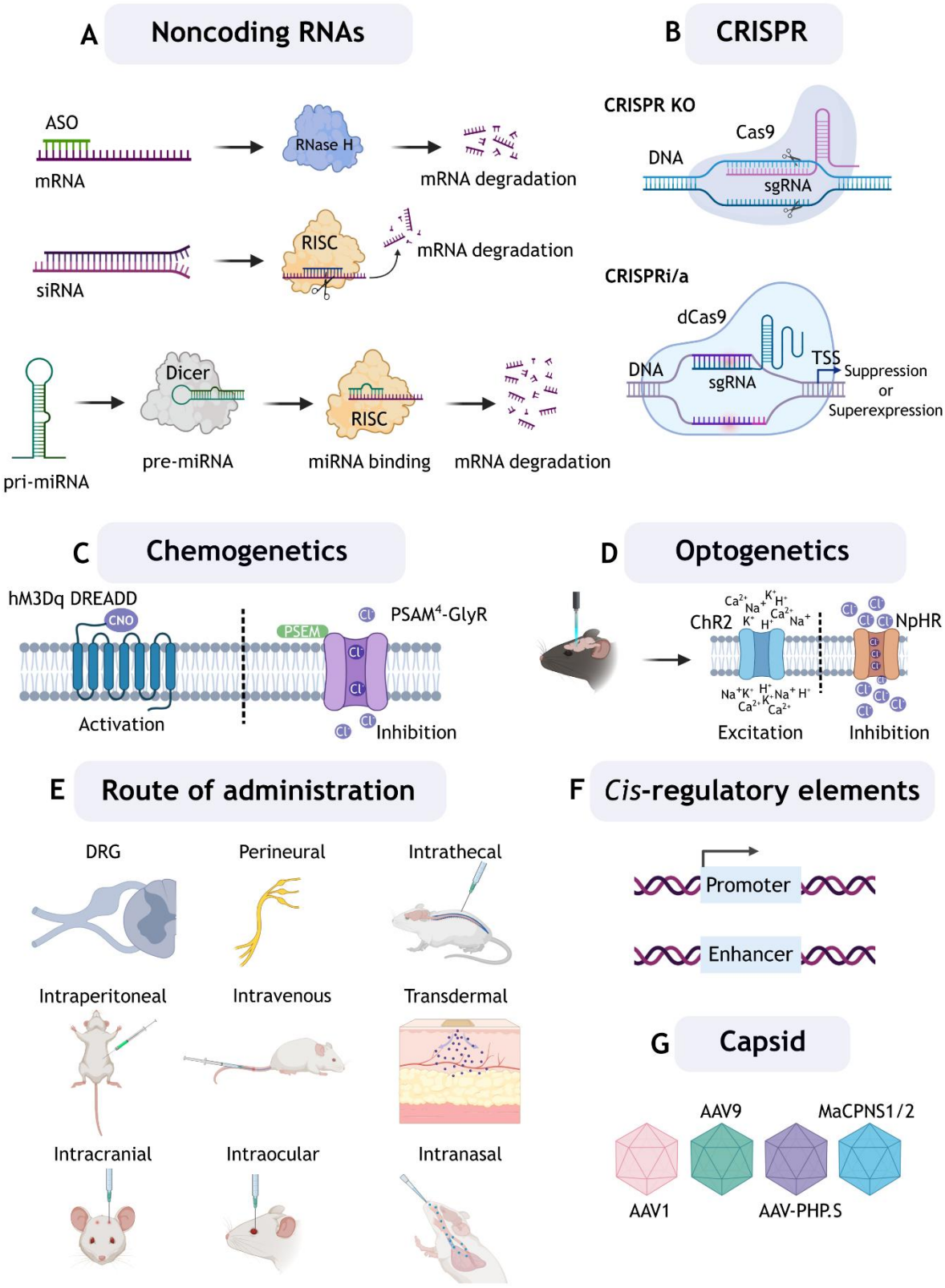


Figure 1.4 Common preclinical gene therapy and targeted vector delivery approaches. (A) *Noncoding RNAs are small can induce mRNA degradation in several ways. Antisense oligonucleotides (ASOs) are short, single-stranded nucleotide sequences capable of binding mRNA, resulting in the recruitment of RNase H and mRNA degradation. Small interfering RNAs (siRNAs) and microRNAs (miRNAs) bind to target*

sequences, leading to mRNA degradation mediated by the RNA-induced silencing (RISC) complex. ***(B)*** *CRISPR, a powerful gene editing tool, can be used to knock out genes by inducing double strand breaks (CRISPR KO) via the Cas9 protein. CRISPR can also be used to reversibly suppress gene expression by blocking transcription at the transcription start site (TSS) or to induce super-expression through the introduction of activators (CRISPRi/a).* ***(C)*** *Chemogenetic techniques introduce genetically engineered proteins responding to otherwise biologically inert molecules. The chemogenetic hM3Dq receptor is an example of an excitatory designer receptor exclusively activated by designer drugs, or DREADD, used to modulate neuronal activity. It is derived from muscarinic receptors and binding of its agonist clozapine-N-oxide (CNO) leads to neuronal depolarisation. Inhibitory receptors are also available, such as the pharmacologically selective actuator module (PSAM) chloride channel consisting of human a7 nicotinic acetylcholine receptor combined with the glycine receptor pore domain (PSAM^4-GlyR) shown here. Activation of the channel by pharmacologically selective effector molecules (PSEM) leads to chloride influx and reduces neuronal activity.* ***(D)*** *Optogenetic techniques use light to control cell activity. Channelrhodopsin-2 (ChR2) is cation channel which when activated causes neuron depolarisation. Activation of Natronomonas halorhodopsin (NpHR) by yellow light, on the other hand, causes hyperpolarisation and inhibits firing.* ***(E)*** *There are many available routes of administration for gene therapies. In this thesis, AAVs were delivered either intrathecally or via intraperitoneal injection.* ***(F)*** *Cell type specific cis-regulatory elements, such as enhancers and promoters, can be introduced in viral vectors to improve tissue targeting.* ***(G)*** *In the case of AAVs, different serotypes exhibit different cell tropisms depending on capsid sequence. Capsids developed via Cre recombination-based AAV targeted evolution (CREATE), such as AAV-PHP.S, tend to have higher transduction efficiency than naturally occurring AAVs, such as AAV9. Adapted from Li and Ji (2024). dCas9; dead Cas9, DRG; dorsal root ganglia, pre-miRNA; precursor miRNA, pri-miRNA; primary miRNA, sgRNA; single guide RNA*

1.4.2 Routes of administration

In addition to capsid modifications, the route of administration can also have an impact on the biodistribution, efficacy and safety of AAV-mediated gene therapies and needs to be taken into consideration. AAVs are quite robust and can be administered via many routes. Systemic administrations have been, until recently, preferred when targeting the liver. Their use in clinical trials, however, has decreased dramatically following the discovery and engineering of novel capsids (Burdett and Nuseibeh, 2023). The greatest challenge with

systemic administrations is the increased risk of immune response due to circulating antibodies which also limits the potential for re-administration. Additionally, systemic deliveries require higher viral loads to be effective, leading to neurotoxicity in the DRG and spinal cord (Burdett and Nuseibeh, 2023; Stone, Aubert and Jerome, 2023). In contrast, more localised routes of administration, such as subretinal, intracranial, and intravitreal routes, require lower viral doses and are associated with less adverse effects. Many of these routes, however, are invasive and can cause injury, often leading to changes in routes of administration from preclinical studies to clinical trials (Burdett and Nuseibeh, 2023). In chronic pain conditions, the DRG are considered a primary target for gene therapies, however the distribution of symptoms can vary depending on the underlying cause of disease. If the neuropathy is focal, such as in the case of trigeminal neuralgia or carpal tunnel syndrome, a more targeted AAV delivery (e.g. intraneural) would be appropriate. Alternatively, if the damage is more widespread and the neuropathy affects the entire torso and/or extremities, a systemic or intrathecal administration may be preferred, depending on the therapeutic target. Thus, the selection of the administration route is primarily dictated by the disease and affected tissue and needs to be carefully considered.

1.4.3 *Cis*-regulatory elements

Several serotypes have overlapping tropisms due to their capsids' shared homology and can transduce more than one cell type (Drouin and Agbandje-McKenna, 2013). As a result, it is necessary to further enhance AAV target specificity by using cell type specific regulatory elements. These include, but are not limited to, promoters, enhancers, miRNA target sites, and internal ribosome entry sites and can restrict transgene expression by modulating RNA processing, stability and translation. AAVs are restricted in their packaging capacity, limiting the search to short DNA sequences. Nevertheless, regulatory elements have been successfully used in preclinical studies to restrict AAV gene expression. The calmodulin/calcium dependent kinase II and human synapsin 1 promoters have been used to target neurons, achieving expression levels similar to when using a ubiquitous promoter (Finneran *et al.*, 2021). In the context of pain, Mouchbahani-Constance *et al.* (2023) inserted a portion of the *SCN10A* gene promoter into AAV2/8 vectors in an attempt to target nociceptors.

Rapid advances in single cell transcriptomics and epigenomics have helped focus the search for suitable regulatory elements. Vormstein-Schneider *et al.* (2020) used single cell ATAC sequencing (scATAC-seq) to investigate the epigenetic landscape of the *Scn1a* gene expressed in distinct neuronal populations and implicated in severe epilepsy. Combining ATAC data with sequence conservation across species, they identified three enhancers which successfully targeted vectors to Scn1a-expressing neuron populations. One element in particular was able to restrict expression to parvalbumin (PV)-expressing cortical interneurons across rodents, NHPs and humans (Vormstein-Schneider *et al.*, 2020). Enhancers have also been shown to be effective in guiding gene expression when combined with one another, as well as with transgenic animals (Graybuck *et al.*, 2021). Gene regulation is complex, however, and it is difficult to predict whether regulatory elements will retain their properties once inserted into AAVs. These exemplar studies provide proof of principle about the approaches that could be employed to target DRG neurons.

1.4.4 Post transcriptional regulatory elements

Post-transcriptional regulatory elements can also be used to help regulate and target transgene expression. The tissue specific expression of many miRNAs coupled with their rapid onset of action and dysregulation following disease renders them excellent tools for the spatiotemporal control of AAV activity (de Rie *et al.*, 2017; Bali *et al.*, 2021). Normally, miRNAs regulate gene expression by binding to target sequences on the 3' UTR of mRNA with imperfect complementarity, leading to translational inhibition via target deadenylation or degradation (Jonas and Izaurralde, 2015). The engineering of synthetic target sites for tissue-specific miRNAs in the 3' UTR of AAV-delivered genes can successfully restrict transgene expression. AAV9 has wide tropism and is able to transduce both cardiac and liver cells, posing issues of unwanted side effects in potential cardiac gene therapies. Geisler *et al.* (2011) was able to restrict AAV9 expression to cardiac cells by introducing target sites for the liver-specific miR-122. MiRNA target sites have also been used to improve AAV-associated immunity and toxicity (Domenger and Grimm, 2019; Xiao *et al.*, 2019). Delivery of AAV gene therapies to the CNS is associated with DRG toxicity, likely due to transgene overexpression causing cellular stress (Hordeaux *et al.*, 2018; Buss *et al.*, 2022). Hordeaux *et al.* (2020) identified miR-183 as a DRG specific miRNA

and included target sites for it in an AAV which was then injected into NHPs. They observed reduced transgene expression and toxicity in the DRG, while expression elsewhere in the CNS remained unaffected (Hordeaux *et al.*, 2020). Other post-transcriptional regulatory elements include mRNA stability elements, the inclusion of the woodchuck hepatitis virus post-transcriptional regulatory elements (WPRE), and introns.

The elements described above can be used in a complementary manner to achieve greater specificity (Gleichman *et al.*, 2023). This, however, has not yet been accomplished in the PNS.

1.5 Aims

This work aims to use a complementary approach to design novel viral vectors capable of distinguishing between injured and intact primary afferents and limiting transgene expression only to damaged fibres. To achieve this, this work will:

1. Develop a pipeline for testing AAV capacity to restrict transgene expression to injured primary afferents *in vitro* and *in vivo*.

2. Characterise the activity of injury gene promoters and assess their ability to restrict transgene expression to injured primary afferents.

3. Identify regulatory elements active in injured but not intact primary afferents by profiling their chromatin accessibility using snATAC-seq.

4. Identify miRNAs downregulated in the DRG following injury and assess the ability of AAVs with target sites to limit transgene expression to injured, but not intact, primary afferents.

Chapter 2 General Methods

Detailed information on techniques pertaining to specific experiments can be found in the method sections of the appropriate chapters. Diagrams outlining the experimental process for each chapter are presented in Figure 2.1.

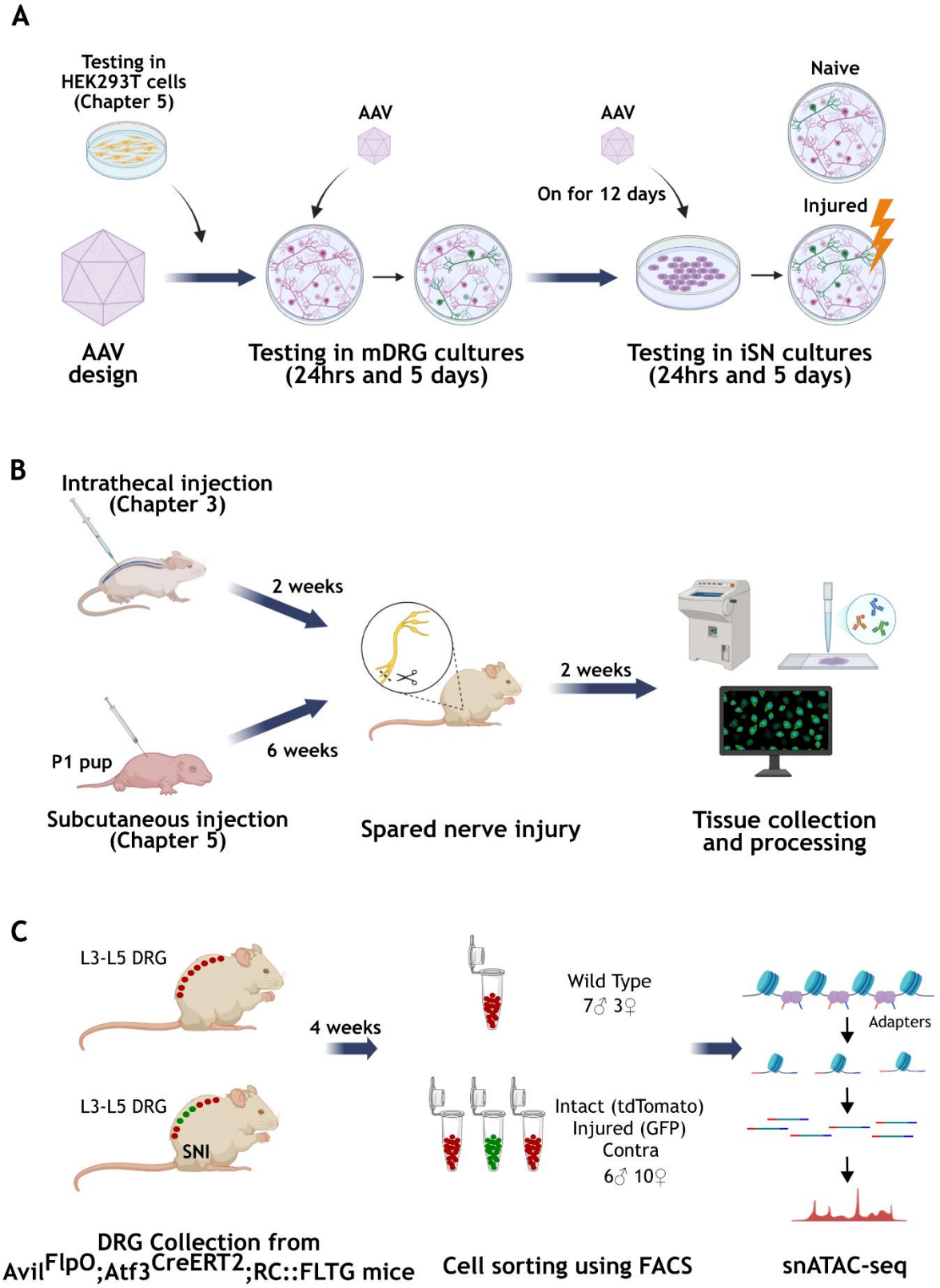


Figure 2.1 Flow diagrams of experiments presented in Chapters 3, 4, and 5. *(A) Schematic of AAV in vitro testing process for Chapters 3 and 5. Following their design and production, AAVs were tested in mouse DRG cultures, where transgene (GFP) expression was assessed 24 hours and 5 days after AAV addition. (B) Schematic of AAV testing in vivo. In Chapter 3, AAVs were delivered to adult animals via intrathecal*

injection and spared nerve injury (SNI) was performed two weeks later. In Chapter 5, P1 pups received subcutaneous AAV injections and underwent SNI once they reached adulthood (≥ 6 weeks). Tissue was collected and processed two weeks after SNI in both chapters. (C) Schematic of sample collection, sorting and snATAC-seq using $Avil^{Flpo}$;$Atf3^{CreERT2}$;*RC::FLTG mice presented in Chapter 4. FACS; fluorescence activated cell sorting, mDRG; mouse DRG, snATAC-seq; single nucleus ATAC-seq, SNI; spared nerve injury*

2.1 Animals

All procedures were performed in accordance with the UK Home Office regulations and in line with the Animals Scientific Procedures Act (1986). All studies were carried out at a licensed facility within the University of Glasgow, following approval by the Ethical Review Process Applications Panel, and adhere to the ARRIVE guidelines. All experiments were performed under the UK Home Office approved project license PP3488859.

Animals were group housed in temperature and humidity-controlled rooms on a 12-hour light/dark cycle with food and water available *ad libitum*. Neonate (P1) and adult (≥ 6 weeks of age) male and female mice were used in all studies. Specific details on transgenic animals and tamoxifen dosing are provided in each chapter.

2.2 Spared nerve injury

Mice (14-30g) were anaesthetised with 2% inhaled isoflurane and received preoperative analgesia with Vetergesic (0.05 mg/kg) and Rimadyl (5 mg/kg) to manage acute post-surgical pain. Under aseptic conditions, the sciatic nerve was exposed, and the tibial and common peroneal branches were ligated and transected, leaving the sural nerve intact (Decosterd and Woolf, 2000). The muscle was closed using 6-0 Vicryl sutures (Ethicon, US) and the skin was sealed with Michel clips. Animals were monitored daily for 7 days for self-mutilation and weight loss. Tissue was collected 14 days post-SNI, unless otherwise specified, and no animals were excluded from analysis.

2.3 Perfusion & fixation

Adult mice were anaesthetised with 2% isoflurane, followed by an overdose of Dolethal (200mg/ml, Vetoquinol, UK) administered by intraperitoneal (i.p.) injection. Following the loss of corneal and tail pinch withdrawal reflexes, animals were pinned to a cork board by the forepaws and tail. The heart was exposed by cutting the anterior and lateral aspects of the diaphragm and the lateral extremity of the ribcage up to the sternum. The ribcage was moved to expose the heart and vena cava, and a 25-gauge butterfly was inserted into the left ventricle while the right atrium was cut. A gravity-based perfusion system was opened at the same time, perfusing each animal with approximately 25 mL of Ringer's solution, followed directly by 100 mL of 4% paraformaldehyde (PFA). Laminectomy was performed to expose the spinal cord. Cord and DRG tissue were collected and post-fixed for a minimum of 1 hour before being transferred to 30% sucrose with 0.03% sodium azide at 4°C for long-term storage or use after 48 hours.

2.4 Tissue processing and immunohistochemistry

DRGs were placed on a microscope slide to remove any remaining nerve roots prior to being embedded in OCT medium (Tissue-Tek, Sakura Finetek, Japan). Samples were then cut into 14 µm thick sections using a cryostat (Leica CM1950, Leica Microsystems, UK), mounted on to slides and stored at -20°C. Cryostat sections were first washed with phosphate buffered saline (PBS) (Thermofisher Scientific, US) for 10 minutes in room temperature to remove OCT. They were then incubated at 4°C overnight with primary antibodies in PBS with 0.3% Triton™ X-100 and 10% normal donkey serum. Finally, samples were washed 3 times with PBS before being incubated at room temperature for 2 hours with the appropriate fluorophore conjugated secondary antibodies diluted in blocking solution. After 3 further rinses, antifade medium (Vectashield; 2B Scientific, UK) was pipetted onto the sections which were covered with a glass coverslip and left to harden at room temperature for 1 hour. Stained slides were stored long term at -20°C.

A list of primary antibodies used throughout this work is provided in Table 2.1. All species-specific secondary antibodies were raised in donkey and conjugated

to either Alexa Fluor 488, Alexa Fluor 647 or Rhodamine Red. Secondary antibodies were purchased from Jackson ImmunoResearch, US.

Antibody	Species	Source	Catalog No.	Dilution
ATF3	Rabbit	Novus Biologicals (Biotechne)	NBP1-85816	1:500
α-BRN3A	Rabbit	Merck	AB5945	1:100
GFP	Chicken	Abcam	Ab13970	1:1000
GFP	Rabbit	Frontier Institute Co. Ltd	GFP-Rb-Af2020	1:1000
IB4-biotin	N/A	Sigma-Aldrich	L2140	1:500
NeuN	Guinea pig	Synaptic Systems	266004	1:500
NF200	Mouse	Sigma-Aldrich	N5389	1:1000
ß-tubulin III (TUJI)	Mouse	Abcam	Ab78078	1:1000

Table 2.1 Summary of primary antibodies used throughout this work.

2.5 Confocal microscopy and image analysis

All sectioned and processed DRG tissue was scanned with a Zeiss LSM900 confocal microscope equipped with diode lasers with wavelengths 405nm, 488nm, 560nm and 640nm. Dry lenses (10x, 20x) were used to scan tissue with the aperture set to 1 Airy unit to reduce out of focus light. Confocal z-stacks were analysed using ImageJ (National Institutes of Health, US). More details on image analysis are provided in each chapter.

2.6 Primary DRG cultures

Adult C57BL/6 female and male mice were sacrificed by rising CO_2 levels and the spinal column was removed and stored in ice-cold Hank's balanced salt solution (HBSS) without Ca^{2+}/Mg^{2+} (Thermofisher Scientific, US). DRGs from all levels were dissected into 500µl of HBSS without Ca^{2+}/Mg^{2+} stored on ice. DRGs were enzymatically digested with Collagenase type II (4 mg/mL; Gibco, Thermofisher Scientific, US) and Dispase type II (4.6 mg/mL; Sigma-Aldrich, US) in 37°C for 1

hour and 15 minutes, agitating every 10 minutes. Cells were centrifuged at 300 x *g* for 5 minutes, washed with mature N2 medium (Neurobasal® media supplemented with 2% (v/v) B-27™ Plus Supplement and 1% antibiotic/antimycotic (A/A; Thermofisher Scientific, US) to remove residual enzymes and mechanically dissociated using fire-polished glass pipettes of two different sizes. Following mechanical dissociation, cells were plated onto laminin drops (5 ng/ml, Merck, US) located on poly-D-lysine (Thermofisher Scientific, US) treated coverslips in mature Neurobasal® medium. Mouse nerve growth factor (50 ng/ml; NGF; PeproTech, US) and glial derived neurotrophic factor (10 ng/ml; GDNF; PeproTech, US) were added to the media. Culture media was replaced every 3-4 days over the course of experiments.

To investigate AAV activity *in vitro*, control and experimental viruses were added to cultures at a multiplicity of infection (MOI) of 100,000 VG/cell one day after plating. Neurons were fixed and transgene expression was assessed 24 hours and 5 days post AAV exposure.

2.7 Induced pluripotent stem cell-derive sensory neuron cultures

2.7.1 Culture and maintenance of iPSCs

The iPSC line AD2 used here for differentiation was derived from fibroblasts collected from a healthy, 51-year-old male and reprogrammed using a commercially available reprogramming kit (McDermott *et al.*, 2019). A list of all the reagents used during iPSC maintenance, differentiation and cryopreservation is provided in Table 2.2. iPSCs were plated on to six-well plates and maintained in mTeSR medium supplemented with chroman 1, emricasan, polyamines and trans-ISRIB (CEPT) for the first 24 hours. Cells were passaged once prior to differentiation.

2.7.2 Differentiation of iPSCs

Cells were allowed to reach 50% confluency prior to differentiation according to Chambers *et al.* (2012). Medium was exchanged with KSR medium containing KnockOut™ DMEM, 15% knockout serum replacement, 1% nonessential amino acids, 1% GlutaMAX™, 1% A/A, and 100μM 2-mercaptoethanol supplemented with

500nM LDN-193189 and 10µM SB431542, both SMAD inhibitors. On the second day *in vitro* (DIV 2), KSR media was supplemented with 500nM LDN-193189, 10µM SB431542, 3µM CHIR99021, 10µM SU5402 and 10µM DAPT. From DIV 4, KSR medium was gradually replaced by N2 medium (Neurobasal® medium, 1% N2 supplement, 2% B-27™ supplement, 1% GlutaMAX™, 1% A/A). SMAD inhibitors were removed from media on DIV 6. After DIV 11, immature neurons were dissociated using StemPro™ Accutase™ and replated onto Matrigel®-coated coverslips and glass-bottomed ibidi chambered coverslips at a density of 35,000 cells/cm^2 in 100% N2 media supplemented with NGF, GDNF, BDNF, NT-3 (all 25 ng/ml), 3µM CHIR99021 and CEPT. Matrigel® was supplemented once a week and CHIR99021 was removed from the media from DIV 16 onwards. Cytosine-ß-arabinouranoside (AraC, 1µM) was added on DIV 14 for 24 hours to remove any remaining proliferating progenitor cells. N2 medium was replaced by mature N2 medium (Neurobasal™ Plus medium, 1% N2 supplement, 2% B-27™ Plus supplement, 1% GlutaMAX, 1% A/A) from DIV 25 onwards. The differentiation resulted in a pure neuronal culture and iPSC-SNs were used once aged ≥ 45 days.

2.7.3 iPSC cryopreservation

Undifferentiated iPSCs and immature neurons were cryopreserved using the previously described CryoPause method (Wong *et al.*, 2017; Saito-Diaz *et al.*, 2021). During passaging on DIV 11, neurons not plated for further differentiation were resuspended in 1mL of CryoStor® CS10 at a concentration of 1×10^6 cells/mL. Cryovials were placed in a Mr Frosty controlled rate freezer to ensure cooling at a rate of -1°C/minute and stored in -80°C overnight before being moved to -150°C for long-term storage. For thawing, vials were placed in a 37°C water bath and gently swirled until only a pea-sized frozen pellet remained. Pre-warmed media was then slowly added to the cells before they were transferred to a 15mL tube containing the appropriate culture media. Cells were centrifuged and plated in media supplemented with CEPT for the first 24 hours.

Reagent	Source	Catalog No.
mTeSR	Sigma-Aldrich	T2194
Chroman 1	Sigma-Aldrich	59222C
Emricasan	Sigma-Aldrich	M1028
Polyamines	Sigma-Aldrich	P8483
Trans-ISRIB	Sigma-Aldrich	I8896
KnockOut™ DMEM	Thermofisher Scientific	BN2006
KnockOut™ serum replacement	Sigma-Aldrich	A8806-1G
Non-essential amino acids	Thermofisher Scientific	11140035
GlutaMAX™	Thermofisher Scientific	35050038
Antibiotic/Antimycotic	Thermofisher Scientific	15240096
2-mercaptoethanol	Thermofisher Scientific	1350010
LDN-193189	Stratech	S2618-SEL
SB431542	Stratech	S1067-SEL
CHIR99021	Stratech	S2924-SEL
SU5402	Stratech	S7667-SEL
DAPT	Stratech	S2215-SEL
Neurobasal® medium	Thermofisher Scientific	21103049
N2 supplement	Thermofisher Scientific	17502048
B-27™ supplement	Thermofisher Scientific	12587010
StemPro™ Accutase™	Thermofisher Scientific	A1110501
Matrigel®	Corning	354234
NGF	PeproTech	450-01
GDNF	PeproTech	450-10
BDNF	PeproTech	450-02
NT-3	PeproTech	450-03
AraC	Merck	C6645
Neurobasal™ Plus medium	Thermofisher Scientific	A3582901
B-27™ Plus supplement	Thermofisher Scientific	A3582801
CryoStor® CS10	Thermofisher Scientific	07959

Table 2.2 Reagents used for iPSC culture, maintenance, differentiation and cryopreservation.

2.7.4 Modelling axotomy and transducing iSNs

Cells to be used for modelling injury *in vitro* where cultured on ibidi chambered coverslips. A scalpel was used to cut across the iSNs and generate smaller clusters which were then transferred into 300µl of mature N2 media. Fire-polished glass pipettes of progressively smaller sizes were used to mechanically dissociate iSNs. Cells in suspension were moved to separate tubes before adding 300µl of mature N2 media and moving to the next pipette size. Upon complete dissociation, iSNs were centrifuged at 1,000 x *g* for 5 minutes, resuspended in the desired volume using mature N2 media supplemented with human growth factors and CEPT and plated onto a Matrigel drop on a coverslip. The injured neurons were allowed to attach overnight before flooding the wells with mature N2 media.

To investigate AAV activity *in vitro*, control and experimental viruses were added to cultures at an MOI of 100,000 VG/cell for a total of 12 days. Neurons were fixed and transgene expression assessed 24 hours and 5 days post-injury.

2.8 Immunocytochemistry

DRG neurons, iSNs and HEK293T cells were fixed with 4% PFA for 15 minutes at room temperature. Coverslips were washed once with PBS before incubating with primary antibodies diluted in PBS with 0.3% Triton™ X-100 and 10% normal donkey serum for 3 hours at room temperature. After three PBS washes, cells were incubated with secondary antibodies for 2 hours at room temperature. Coverslips were mounted onto antifade medium drops and allowed to harden for 1 hour at room temperature.

2.9 Bacterial transformation

Stable *E.coli* competent bacteria were thawed on ice for approximately 30 minutes prior to the addition of plasmid DNA at a final concentration of 10ng/ml. The cells were then incubated on ice for 30 minutes, heat-shocked at

42°C for 30 seconds and returned on ice for 2 minutes. Following the addition of stable outgrowth media, the cells were grown in a shaking incubator at 37°C for 45 minutes to 1 hour. Part of the transformed bacteria was then added onto LB agar plates with ampicillin and allowed to grow overnight at 37°C. The remaining transformed competent cells were stored in 30% glycerol at -80°C.

Starter cultures were grown overnight, and plasmid DNA was extracted from cell pellets using Qiagen plasmid midi/maxi kits according to manufacturer's instructions.

2.10 Restriction enzyme digest

Plasmids and oligos were digested with NdeI (Chapter 3) or EcoRI and HindIII (Chapter 5) for 3 hours at the recommended incubation temperature and proprietary buffer (New England Biolabs, US). Digested samples were heat inactivated before either being run on a 1.5% agarose gel at 100V for 2 hours or being used for ligation.

2.11 Data analysis and visualisation

All statistical analyses were performed using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, US). Two-way ANOVA analyses were followed by Tukey's *post hoc* multiple comparisons tests unless otherwise specified. P values of ≤ 0.05 were considered significant and all data are presented as mean ± standard error mean (SEM), unless otherwise stated. Figures were created with Inkscape and BioRender.com.

Chapter 3 Developing a pipeline for testing AAV constructs and investigating the efficiency of an ATF3 promoter in injured sensory neurons

3.1 Introduction

The overarching aim of the work discussed in this chapter was to develop a viral vector containing a promoter of the immediate early gene ATF3 and test its ability to restrict transgene expression to injured sensory neurons. We first present our pipeline for testing constructs, used also for alternative approaches described in future chapters and developed by optimising *in vitro* and *in vivo* models of NeuP. This is followed by an investigation of ATF3 promoter activity, the development of viral constructs, and their assessment using our AAV-testing pipeline.

3.1.1 The ATF3 gene

Following injury, DRG neurons undergo transcriptional changes to facilitate axonal regeneration and the development of neuronal hyperexcitability. This transcriptional reprogramming is largely mediated by ATF3 (Renthal *et al.*, 2020). ATF3 belongs to the ATF/cyclic AMP responsive element binding (CREB) family of TFs and has a basic region leucin zipper motif. The basic region is used for binding to the consensus ATF/CRE site while the leucine zipper is used to form hetero- or homodimers with other proteins and activate or repress transcription respectively (Chen *et al.*, 1994). ATF3 is an immediate early gene facilitating cellular responses to stress and injury and has very low to no expression in healthy sensory neurons. ATF3 expression is induced *de novo* in approximately 98% of injured sensory neurons after SNI and has therefore been used extensively to mark damaged sensory neurons (Tsujino *et al.*, 2000; Renthal *et al.*, 2020). ATF3 mRNA expression has been seen in mDRG neurons of various sizes 12 hours, 3 days, and 3 weeks after axotomy, before decreasing at 6 weeks and has been correlated with regenerative responses (Tsujino *et al.*, 2000; Seijffers, Mills and Woolf, 2007; Bráz and Basbaum, 2010; Lindå, Sköld and Ochsmann, 2011).

The ATF3 gene is found on chromosome 1q32.3, spans approximately 56kb and is comprised of six exons (Liang *et al.*, 1996; Hunt, Raivich and Anderson, 2012). Using two different 5' primers, Miyazaki *et al.* (2009) amplified two separate products from human and mouse cells. One matched previous reports and corresponded to the putative P2 promoter while the other contained novel 5' UTRs and corresponded to the alternate P1 promoter located roughly 43.5kb upstream of P2. This variation in the 5' UTR accounts for the differential regulation of ATF3 expression in response to various stimuli (Miyazaki *et al.*, 2009). The P2 promoter is predominantly active in cells expressing oncogenic hRAS and TGF-ß and consists of a canonical TATA box, inducible TF binding sites and cell-cycle dependent sites. Conversely, P1 is strongly activated by serum in cancer cells expressing ATF3 and comprises of a non-canonical TATA box, inducible TF binding sites and has a lower GC content (~70% for P2 vs 50% for P1). The P1 promoter is also constitutively active in prostate and Hodgkin Reed-Sternberg cancer cells, as indicated by its open (active) chromatin structure (Miyazaki *et al.*, 2009; Hunt, Raivich and Anderson, 2012).

Ubiquitous promoters, such as chicken ß-actin (CAG), have been used extensively in viral vectors to drive potent transgene expression. When used in AAVs, they take up considerable space and do not confer cell type specificity. Endogenous promoters can help improve AAV targeting but often lack the ability to drive strong expression and replicate their endogenous specificity due to being isolated from their regulatory network. Their large size also limits their packaging potential. Both ATF3 promoters, however, are relatively small (~2kb) and ATF3 expression is seen in virtually all injured DRG neurons and is limited in intact neurons (Tsujino *et al.*, 2000; Renthal *et al.*, 2020). We therefore sought to test whether ATF3 promoters packaged into an AAV can drive gene expression only in injured primary afferents.

3.1.2 Aims

This chapter aims to develop a construct-testing pipeline of *in vitro* and *in vivo* models of NeuP and assess the ability of ATF3 promoters to restrict AAV-mediated transgene expression to injured but not intact primary afferents. More specifically, the objectives of the chapter are the following:

1. Develop and characterise *in vitro* models of nerve injury using iSNs and dissociated mDRG neurons.
2. Optimise fluorescent labelling of damaged afferents in ATF3Cre:Ai9 mice.
3. Investigate the ability of ATF3 promoters to restrict transgene expression to injured primary afferents.

3.2 Methods

3.2.1 Animals

Adult ATF3CreERT2:Ai9 mice (> 8 weeks of age), heterozygous for both ATF3Cre and Ai9, were used for the work presented in this chapter. The knock-in mouse line ATF3CreERT2 expresses Cre fused to a mutated oestrogen receptor under the control of the native ATF3 promoter. Cre-recombination occurs in cells that have undergone axotomy in a tamoxifen-dependent manner (Denk *et al.*, 2015). Ai9 mice (Cre-dependent tdTomato expression) have also been previously described (Madisen *et al.*, 2010). Mice are dosed with 7.5 mg/kg tamoxifen (Sigma-Aldrich, US) on day 7 post-SNI via i.p. injection. Tamoxifen was prepared by being dissolved in wheat germ oil in sterile conditions to a concentration of 20 mg/mL.

3.2.2 Intrathecal injections

All intrathecal deliveries were performed by Dr Greg Weir, as previously described (Weir *et al.*, 2017). Eight-week-old mice (20-26g) were put under 2% inhaled isoflurane anaesthesia and received preoperative analgesia with Vetergesic (0.05 mg/kg) and Rimadyl (5 mg/kg) to manage acute post-surgical pain. The back was shaved, and the dura was exposed by carefully dissecting the soft tissue between the T12 and T13 vertebrae and punctured using a 30 G needle. A small cannula was then inserted 1.2 cm caudally into the subarachnoid space and 8µl of AAV, either AAV9-ATF3P1-GFP or AAV9-sCAG-GFP, were infused at a rate of 1 µl/min. The cannula was finally removed following a rest period of 1 minute to limit backflow. Animals underwent SNI 2 weeks after intrathecal injections and DRG were collected 2 weeks later following perfusion with fixatives, as described in the *General Methods* chapter (Chapter 2).

3.2.3 Plasmids and AAVs

ATF3P1-GFP, ATF3P2-GFP, and short CAG (sCAG)-GFP (control) DNA plasmids were cloned and gifted by Dr Greg Weir. Packaging plasmids contained the inverted terminal repeats of AAV2 flanking either the 2kb ATF3 promoters or sCAG promoter, part of a CMV enhancer, transgene and WPRE (Figure 3.1A and B). The sequence of P1- and sCAG-containing constructs was confirmed via sequencing and restriction enzyme digest (Figure 3.1B) before they were commercially packaged and serotyped with the AAV9 capsid protein by the Viral Vector Facility VVF at the University of Zurich to the final titres of $7.9x10^{13}$ GC/mL and $7x10^{13}$ GC/mL respectively.

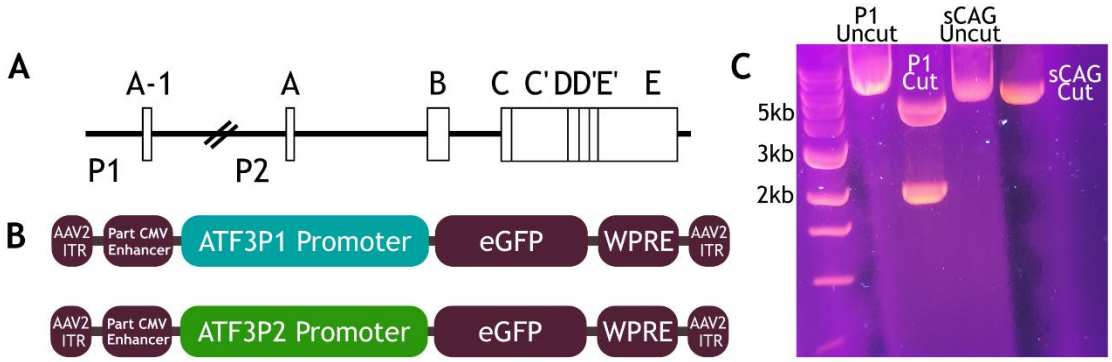


***Figure 3.1 Plasmid structure**. **(A)** Structure of the ATF3 gene adapted from Hunt, Raivich and Anderson (2012). **(B)** Schematics of ATF3P1 and ATF3P2 plasmids. The transgene sequence is flanked by AAV2 ITR sites and consists of an ATF3 promoter, part of a CMV enhancer, GFP gene and WPRE, a viral enhancer included to increase gene expression. **(C)** Restriction digest of ATF3P1 and sCAG AAVs digested with NdeI. The 2kb band present in the digested ATF3P1 but not the sCAG plasmid, contains the majority of the ATF3P1 promoter sequence. ITR; inverted terminal repeats, WPRE; woodchuck hepatitis virus post-transcriptional regulatory elements*

3.2.4 DRG electroporation

Following dissection and enzymatic and mechanical dissociation (Chapter 2), DRG neurons were electroporated with P1, P2 or sCAG plasmid DNA. Neurons (10^4 cells/well) were resuspended in 100µl Nucleofector solution (Lonza, Switzerland) with 2µg DNA and electroporated using the Lonza Nucleofector II (Lonza, Switzerland) device, program O-003. After transfection, cells were allowed to recover in pre-warmed magnesium- and calcium-free HBSS

(Thermofisher Scientific, US) for 10 minutes before being plated and used 48 hours later.

3.2.5 Paclitaxel in iSNs

The process for differentiating and maintaining iSNs is described in Chapter 2. Mature iSNs were treated with 0.1-3 μM paclitaxel (Sigma-Aldrich, US) or matched vehicle control (DMSO) corresponding to the amount of DMSO in each dose for 24 hours, 48 hours and 5 days.

3.2.6 Image analysis

Image analysis was carried out using ImageJ. Background noise from Alexa Fluor 488 interfered with the identification of GFP-expressing cells when assessing ATF3P1 AAV activity *in vivo*. To ensure cells were confidently labelled, we established both low and high thresholds for GFP signal intensity. We began by identifying five cells with no GFP expression and calculating the average GFP signal intensity and standard deviation (SD) among these values. The low threshold was set at the average signal intensity plus 3 times the SD, while the high threshold was set at the average signal value plus 5 times the SD. Cells were considered GFP-expressing only if their signal intensities exceeded the established thresholds.

3.2.7 Statistical analysis

The Kolmogorov-Smirnov test was used to compare the cumulative distribution between all neurons and tdTomato-expressing neurons for the optimisation of ATF3Cre:Ai9 mouse line in Figure 3.6. The two-way ANOVA comparison for data presented in Figure 3.9 was followed by Šidák's multiple comparisons test.

3.3 Results

3.3.1 ATF3 expression in dissociated mouse DRG neurons

Primary cultures closely represent their tissue of origin, offering a physiologically relevant platform for pre-screening targets while maintaining low costs. The first step we established in our AAV screen was dissociated mDRG cultures. ATF3 expression is robustly up-regulated following injury in DRG

neurons *in vivo* (Tsujino *et al.*, 2000). It is not known, however, whether expression is maintained *in vitro* and for how long, either of which may not be consistent with the *in vivo* scenario due to the treatment of cultures with exogenous growth factors. Assessment of ATF3 expression in enzymatically and mechanically dissociated mDRG neurons found expression after 48 hours in culture, which still persisted at 6 days (Figure 3.2A). In fact, a significantly higher proportion of neurons expressed ATF3 after 6 days in culture compared to 48 hours (92.4 ± 2.64% vs 79.4 ± 2.99% (Figure 3.2B). These results confirm that ATF3 expression is upregulated and sustained in cultured mDRG neurons. This suggests that dissociated mDRG neurons represent a model system for assessing vector expression in injured neurons but are not capable of testing expression in uninjured neurons.

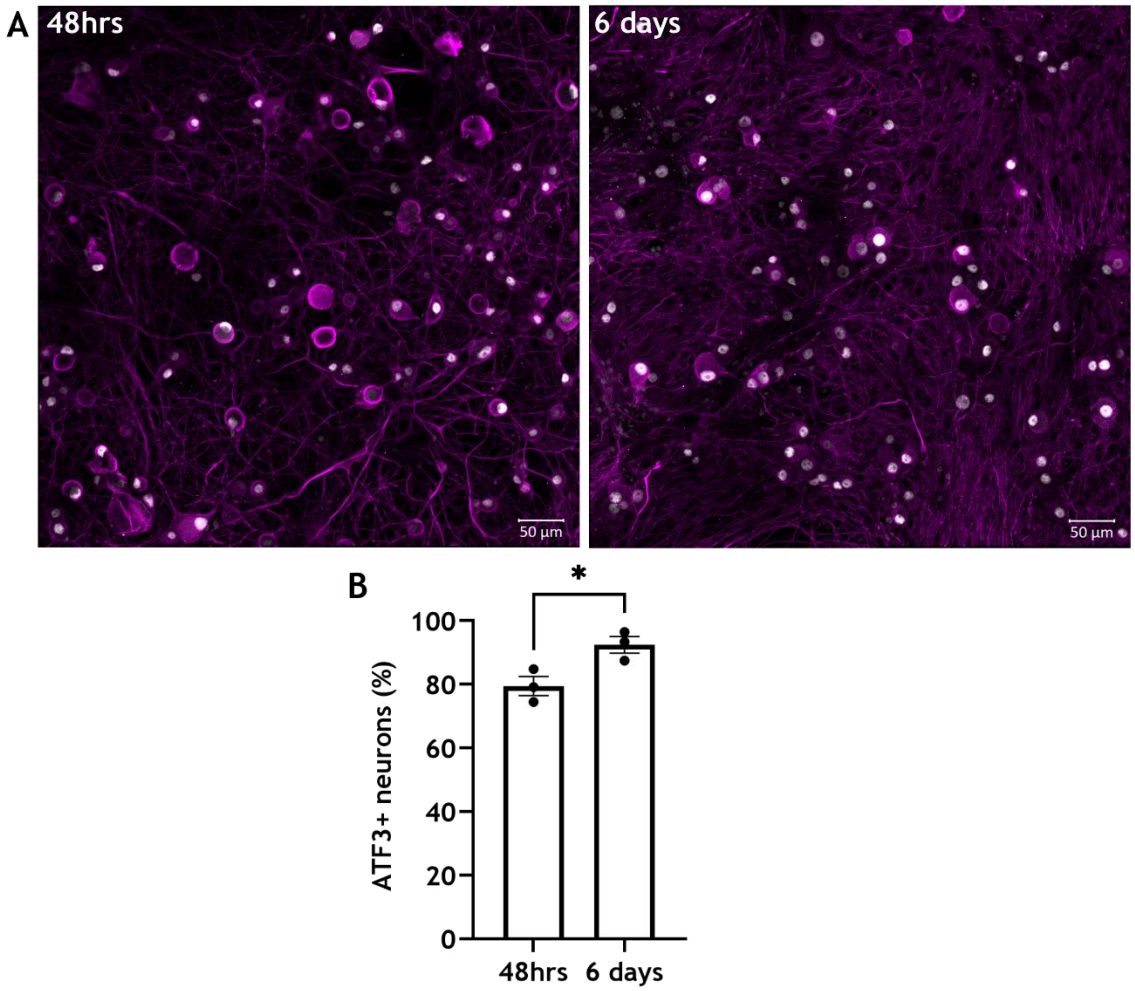


Figure 3.2 ATF3 expression in dissociated mDRG neurons. (A) *Representative images of ATF3 expression in mDRG neurons cultured for 48 hours and 6 days. Neurons stain positive for the neuronal marker TUJI (magenta) and ATF3 expression is shown in*

white. ***(B)*** *Percentage of ATF3-expressing neurons after 48 hours and 6 days in culture. Data is shown as mean ± SEM, with individual values plotted. Unpaired t-test; t = 3.25, df = 4, *p < 0.05, n = 3 mice. Experimental unit is defined as cultures derived from one mouse. Hrs; hours.*

3.3.2 Differentiation of sensory neurons from iPSC

Mouse DRG neurons closely resemble their human counterparts, yet differences between the two have also been discovered (Rostock *et al.*, 2018; Shiers, Klein and Price, 2020). Using sensory neurons derived from human iPSCs can help account for these differences while also providing a large supply of sensory neurons. There is a plethora of protocols available for the production of different sensory neuron subtype approximations (Chrysostomidou, Cooper and Weir, 2021). Sensory neurons were differentiated from the human iPSC line AD2 using a protocol known to generate a homogenous culture of neurons with transcriptional and functional properties consistent with endogenous nociceptors (Chambers *et al.*, 2012). The neuralisation of iPSCs is induced using dual-SMAD inhibition. This is achieved by the addition of SB431542, a small molecule inhibiting the Lefty/Activin/TGFβ signalling pathways by blocking anaplastic lymphoma kinase (ALK) receptor (ALK4, ALK5, and ALK7) phosphorylation, and LDN-193189, a bone morphogenic protein inhibitor (Figure 3.3A) (Chambers *et al.*, 2009, 2012). On DIV 2, nociceptor induction is initiated using the small molecule inhibitors, SU5402, CHIR99021, and DAPT (Figure 3.3A). SU5402 is a potent vascular epithelial growth factor, fibroblast growth factor and platelet-derived growth factor tyrosine kinase signalling inhibitor while DAPT is a γ-secretase inhibitor clocking Notch signalling (Sun *et al.*, 1999; Dovey *et al.*, 2001). CHIR99021 is a glycogen synthase kinase 3 inhibitor capable of mimicking WNT signalling and stabilising cytosolic β-catenin and the key component of this small molecule inhibitor cocktail (Bennett *et al.*, 2002; Chambers *et al.*, 2012).

Neurons become postmitotic by DIV 10, at which point PAX6, Ki67 and phosphorylated histone H3 expression is lost, and TUJI expression is induced (Gerdes *et al.*, 1983; Hendzel *et al.*, 1997; Chambers *et al.*, 2012). On DIV 12, neurons are passaged and plated on to Matrigel® at the desired cell density and maintained in media supplemented with human growth factors (Figure 3.3A). It was recently shown that differentiating iPSCs can be frozen at the neural crest

stage on DIV 12 using CryoPause, a method allowing immediate differentiation of cryopreserved cells (Wong *et al.*, 2017; Saito-Diaz *et al.*, 2021). This does not affect differentiation efficiencies post thawing and allows for the creation of large banks of cells. This method reduces the labour, cost, and variability of performing multiple rounds of differentiation (Saito-Diaz *et al.*, 2021). On DIV 14, cultures can be treated with the anti-mitotic agent AraC to eliminate any dividing cells. By DIV 21, iSNs express the neuronal marker TUJI as well as the sensory neuron specific TF BRN3A and by DIV 45, cultures have extensive neurites, organise in cell body clusters and have reached suitable maturity (Figure 3.3B). The Chambers *et al.* (2012) protocol has been adopted by many groups and its neurons have been extensively characterised (Chambers *et al.*, 2012; Eberhardt *et al.*, 2015; Schwartzentruber *et al.*, 2018; McDermott *et al.*, 2019; Mis *et al.*, 2019; Pettingill *et al.*, 2019).

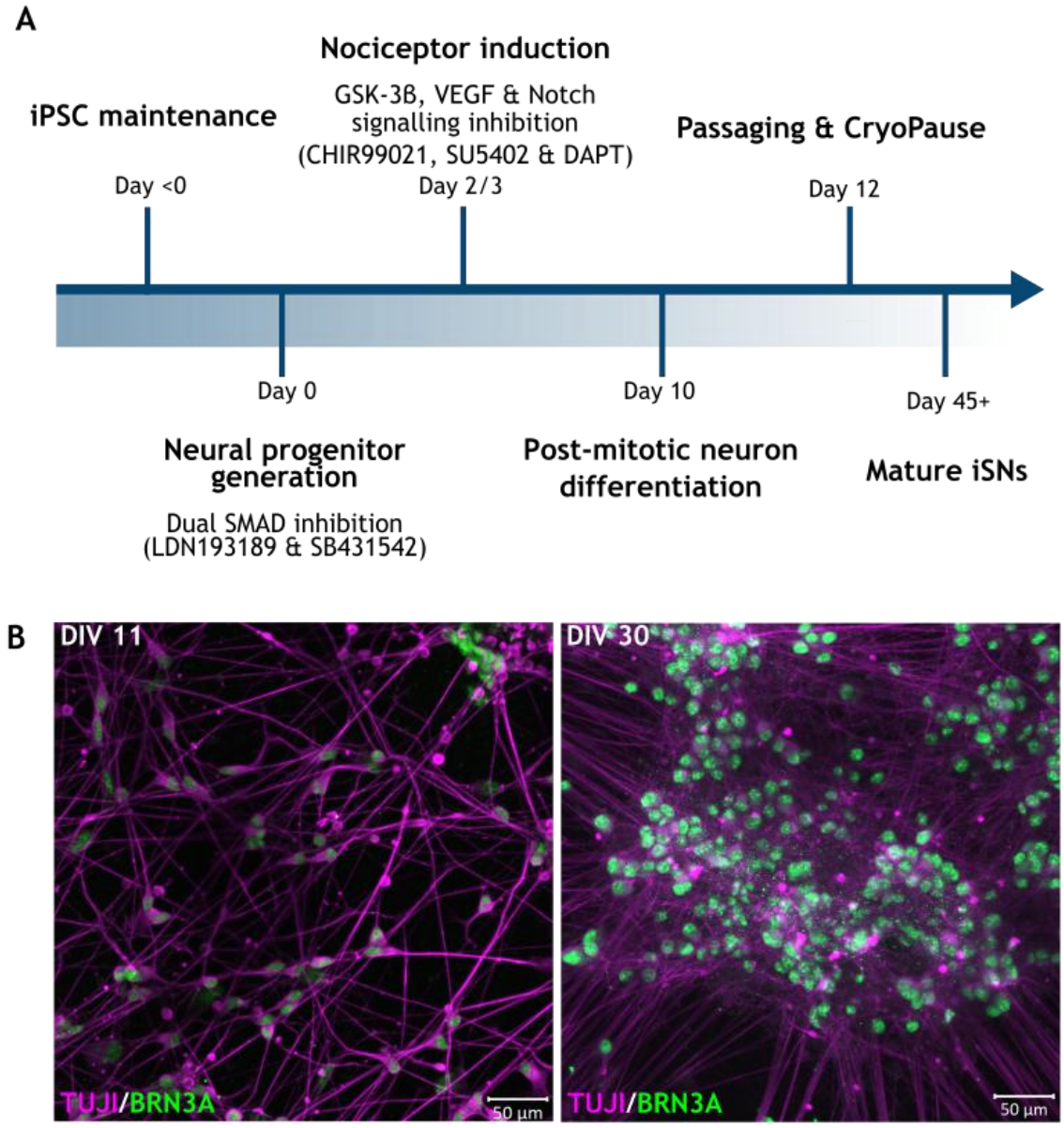


Figure 3.3 Differentiation of human iPSC-derived sensory neurons. ***(A)*** *Timeline of sensory neuron differentiation from human iPSCs.* ***(B)*** *Neurons stain positive for the neuronal marker β-III tubulin (TUJI) (magenta) and the sensory neuron transcription factor BRN3A (green) at days 11 and 30 of differentiation. Once mature, neurons start organising themselves into clusters. DIV; days in vitro, iPSC; induced pluripotent stem cell, GSK-3β; glycogen synthase kinase-3β, VEGF; vascular endothelial growth factor, iSNs; iPSC-derived sensory neurons.*

3.3.3 Modelling peripheral nerve injury in iSNs

Next, we established the second stage of our AAV-testing pipeline and attempted to model traumatic and systemic injury *in vitro* using iSNs. In our traumatic injury model, iSNs were fully axotomized before being mechanically

dissociated and replated to allow their neurites to regenerate. ATF3 expression was assessed 24 hours and 5 days post-replating. Surprisingly, we detected ATF3 in both naïve and replated iSNs (Figure 3.4). More than half of naïve neurons expressed ATF3 (57.6 ± 10.1%), although the proportion of positive neurons varied considerably between experimental units (coverslips) (Figure 3.4B). A modestly significant increase ($p = 0.046$) in the proportion of ATF3+ iSNs occurred after 24 hours (84.8 ± 4.36%) before returning to naïve levels after 5 days in culture (61.1 ± 5.53%). Despite the increase, the background signal in naïve neurons suggests that ATF3 expression is not as reliable a marker to differentiate axotomized and intact iSNs as it is *in vivo*. We proceeded with our iSN model of traumatic injury and considered all naïve neurons to be healthy, and all replated neurons injured, with the caveat that naïve neurons may be under a tonic level of cellular stress owing to their prolonged periods of culture and/or the use of the CryoPause method.

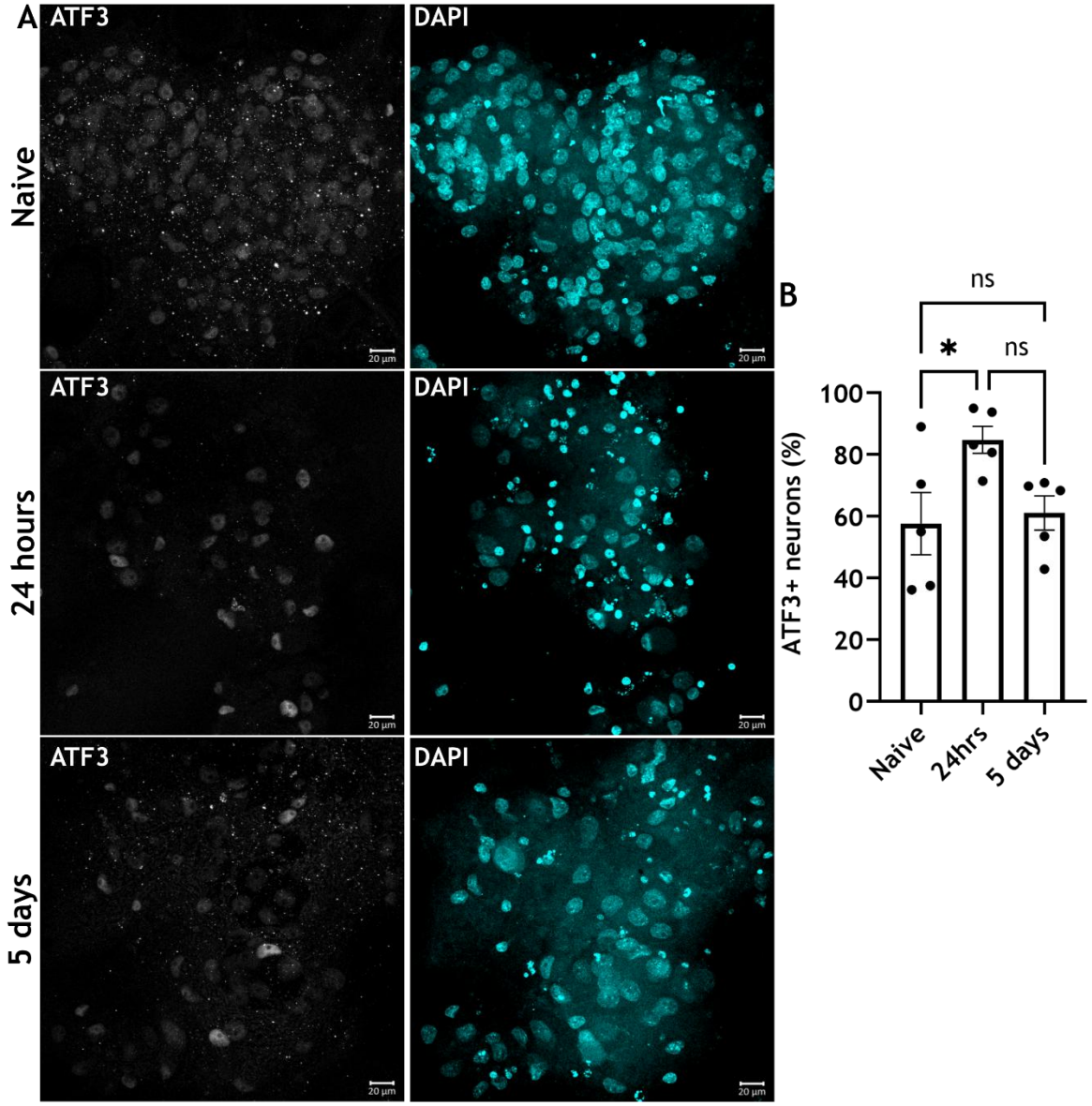


Figure 3.4 ATF3 expression in iSNs. ***(A)*** *ATF3 expression in naïve and injured iSNs 24 hours and 5 days post-replating. Varying degrees of expression were detected in all conditions. DAPI profiles are shown to identify all neurons in each condition and small intense profiles were discarded as dead cells.* ***(B)*** *Percentage of iSNs expressing ATF3. One-way ANOVA followed by Tukey's post hoc multiple comparisons test; $F(2, 12) = 4.36$, $p = 0.03$, $n = 5$ coverslips, $*p < 0.05$. Experimental unit defined as a coverslip (well), derived from two independent differentiations. Data is shown as mean ± SEM, with individual values plotted.*

Most patients reporting to the clinic with NeuP symptoms develop them following systemic rather than traumatic injury. Chemotherapy-induced peripheral neuropathy is a common side effect of cytotoxic chemotherapeutics, such as paclitaxel, and develops in approximately two thirds of treated patients

(Seretny *et al.*, 2014). Signs of paclitaxel induced peripheral neuropathy may occur as early as 24-72 hours following single high-dose administrations and its addition to primary DRG cultures causes profound changes in electrophysiology, gene expression, neuron morphology and neuropeptide release (Lipton *et al.*, 1989; Cavaletti *et al.*, 2000; Staff *et al.*, 2020). In iSN cultures, exposure to paclitaxel leads to decreases in cell viability and neurite outgrowth as well as changes in cell excitability in a time- and concentration-dependent manner (Wheeler *et al.*, 2015; Vojnits *et al.*, 2019; M. Wang *et al.*, 2021; Schinke *et al.*, 2021; Xiong *et al.*, 2021; Cunningham *et al.*, 2022; Cantor *et al.*, 2024). An increase in ATF3 mRNA has also been shown following iSN treatment with paclitaxel for 48 hours (Mortensen *et al.*, 2024). We sought to supplement our traumatic injury model with a taxane treatment model and so exposed iSNs to physiologically relevant paclitaxel concentrations, ranging from 100nM to 3μM, to assess whether they would upregulate ATF3 expression (Hertz *et al.*, 2018; Stage, Bergmann and Kroetz, 2018). Different treatment times were also explored, and representative results are shown here for 48 hours (Figure 3.5). While ATF3 expression increased in some cases following prolonged exposure to paclitaxel and higher concentrations, this was inconsistent between differentiations and could not be reliably replicated. As a result, this model was not developed further.

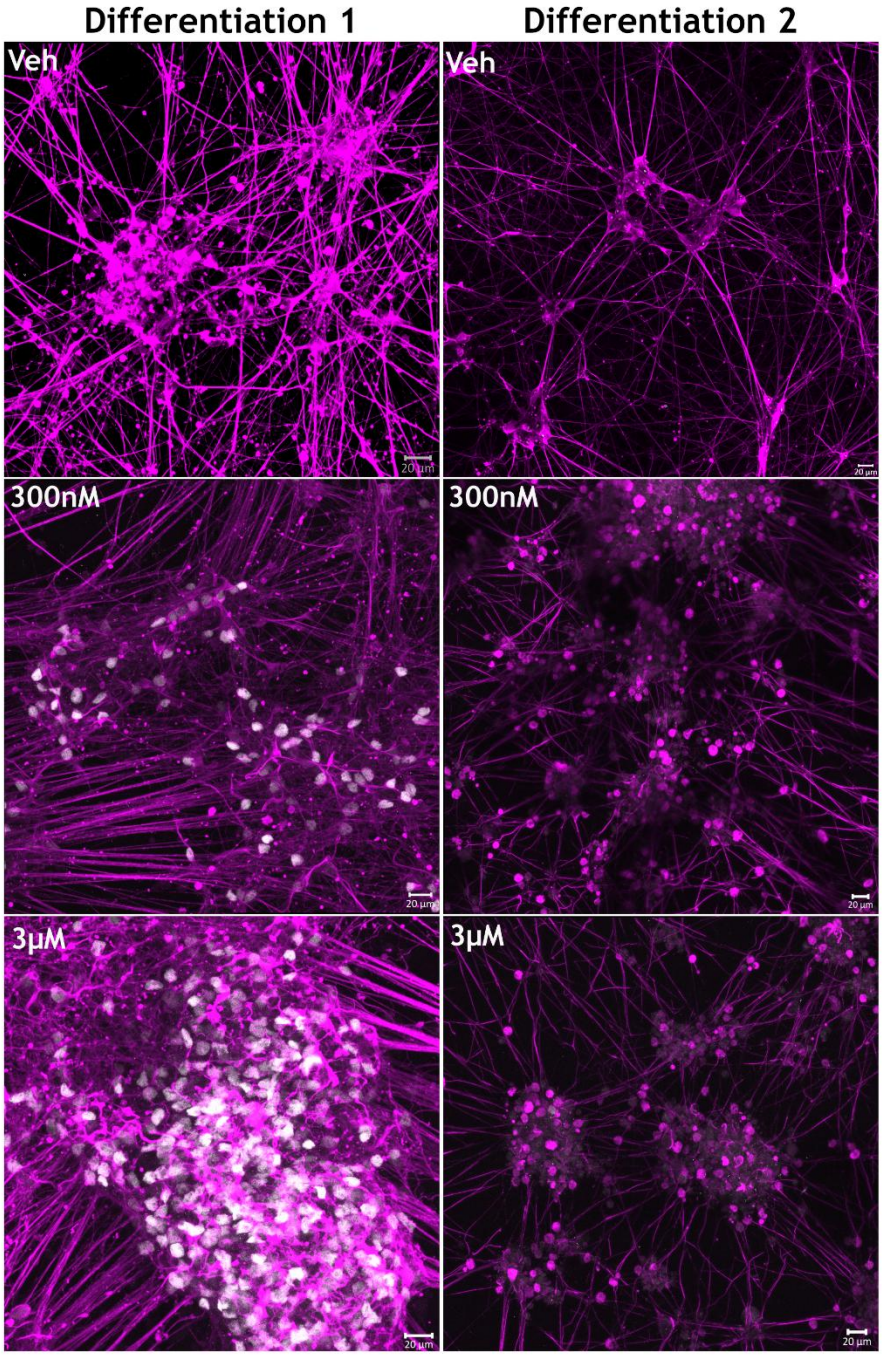


Figure 3.5 Pilot trials of modelling chemotherapy-induced peripheral neuropathy in iSNs. *Neurons from two separate differentiations were cultured in media with either vehicle (DMSO) or 300nM or 3µM paclitaxel for 48 hours. Cells were stained for TUJI (magenta) and ATF3 (white). Exposure of iSNs to paclitaxel resulted in a concentration-dependent increase in ATF3 expression by neurons from differentiation 1 but had no effect on neurons from differentiation 2.*

3.3.4 Optimising a mouse model to fluorescently label axotomised afferents

Cellular models provide only an approximation of the physiological environment of the body. For example, the cultures above do not include the full diversity of interacting cell types which are present in the DRG and contribute to the response to nerve injury. It is therefore essential to employ *in vivo* models in parallel to comprehensively test whether our candidate vectors are able to restrict expression to injured primary afferents. To achieve this, we used the ATF3-CreERT2:Ai9 transgenic mouse line to identify axotomized sensory neurons following injury (Denk *et al.*, 2015; Holland *et al.*, 2019). There are two important considerations when using this mouse line. Firstly, standard dose tamoxifen treatment (single dose of 75 mg/kg) itself has been shown to induce ATF3-mediated recombination independent of axotomy (Denk *et al.*, 2015). This occurs in 20% of DRG neurons and predominantly affects large diameter neurons. Secondly, recombination has been reported to occur in 65-80% of axotomised afferents in the absence of tamoxifen treatment, likely due to high levels of CreERT2 overwhelming its cytoplasmic anchor (Holland *et al.*, 2019). We assessed whether low-dose tamoxifen could be used to increase recombination efficiency without inducing expression in intact afferents. Seven animals underwent SNI, four of which received 7.5 mg/kg of tamoxifen. Four weeks post-SNI, 25.6 ± 2.69% of ipsilateral L4 DRG neurons were tdTomato+ in animals that did not receive tamoxifen compared to 71.1 ± 3.33% in those that were treated with 7.5 mg/kg tamoxifen ($p < 0.0001$) (Figure 3.6A and B). Tamoxifen treatment did not significantly increase recombination in contralateral L4 DRG ($p = 0.525$), indicating that fidelity of expression was preserved at this dose of tamoxifen. Cell size analysis showed recombination in the absence of tamoxifen was lacking in a considerable proportion of small diameter neurons (Figure 3.6C). This cell-sized bias in recombination was not present in the tamoxifen group. These results show that low-dose tamoxifen is an effective strategy of targeting and identifying axotomized afferents without leading to aberrant recombination in spared afferents.

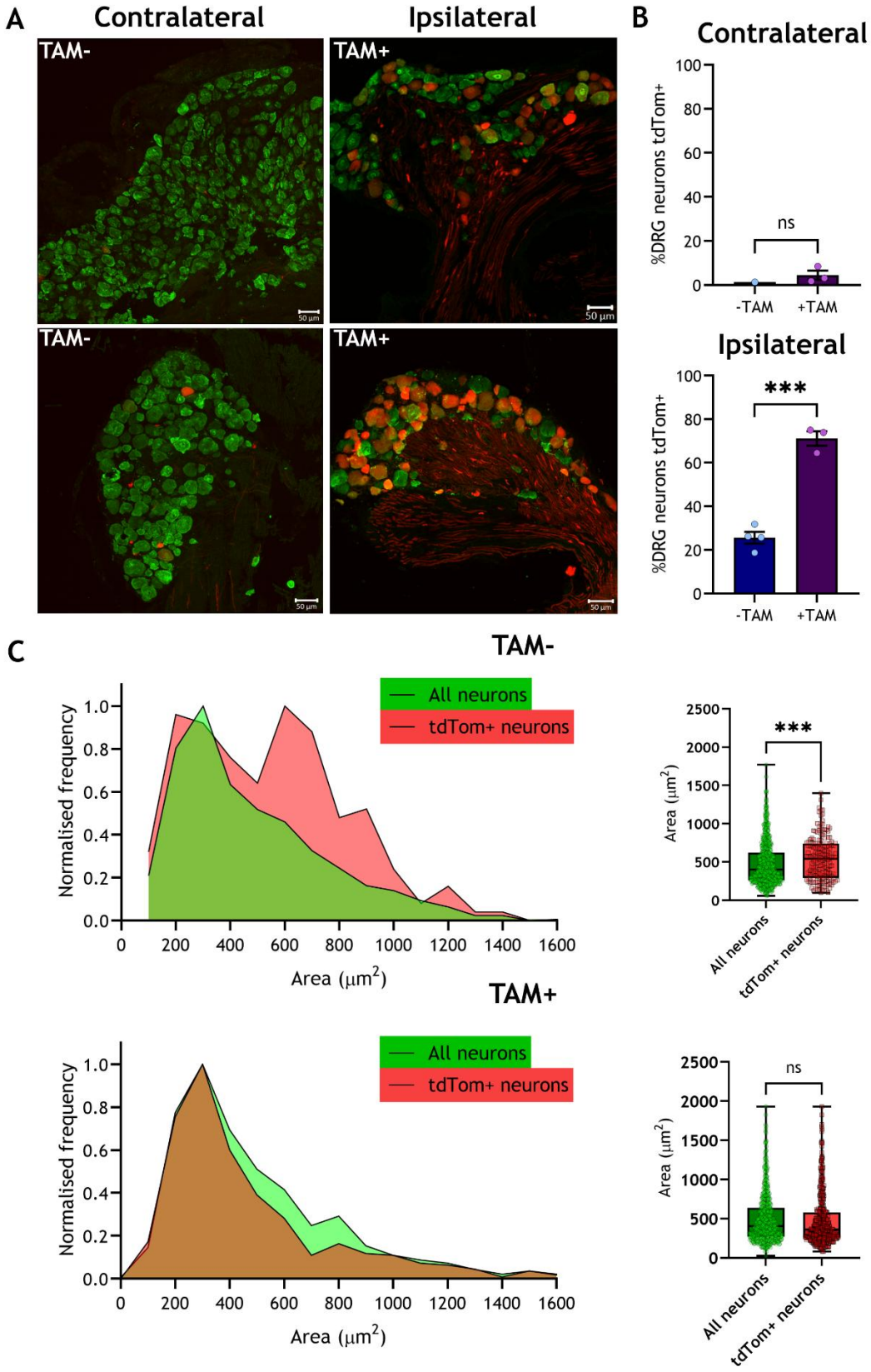


Figure 3.6 Effects of tamoxifen on recombination in ATF3CreERT2:Ai9 mouse DRG neurons. *(**A**) Contralateral and ipsilateral DRG sections four weeks post-SNI stained for NeuN (green) and tdTomato (red).* ***(B)*** *A higher proportion of cells were tdTom+ following administration of 7.5 mg/kg tamoxifen seven days post-SNI, indicating a higher degree of recombination. Unpaired t-test; Contralateral: t = 0.76,*

*df = 2, p = 0.52. Ipsilateral; t = 10.75, df = 5, p <0.0001, n = 3 mice, ***p < 0.0001.* ***(C)*** *Tamoxifen administration significantly reduced cell size bias in tdTom+ neurons. Kolmogorov-Smirnov tests with cumulative distributions; TAM-: D = 0.17, p < 0.0005; TAM+: D = 0.08, p = 0.06, n = 3, ***p < 0.0005. Experimental unit is defined as the averaged values from three DRG sections from one mouse. All grouped data represent the mean ± SEM.*

3.3.5 Endogenous ATF3 promoter activity in dissociated mouse DRG neurons

The ATF3 gene is located on chromosome 1q32.3 and has two defined promoters, P1 and P2 (Figure 3.1A) (Miyazaki *et al.*, 2009; Hunt, Raivich and Anderson, 2012). We cloned each promoter in plasmid vectors carrying GFP (Figure 3.1B) and delivered them to cultured mDRG neurons via electroporation. Both promoters were successful in driving GFP expression 48 hours later, confirming their activity in injured sensory neurons (Figure 3.7A). A distinction based on efficiency could not be made as only cells with high GFP expression could be confidently identified as positive. Due to the high species conservation between human and mouse sequences reported for ATF3P1, activation by DNA damage and oxidoreductive stress, binding motifs for TFs involved in stress responses and low GC content, we decided to clone ATF3P1 and not ATF3P2 into an AAV vector (Miyazaki *et al.*, 2009). To ensure that our newly packaged vector was functional, we transduced dissociated mDRG neurons with the novel AAV. GFP expression was detected in neurons 5 days after transduction, providing proof of concept for the AAVs activity (Figure 3.7B).

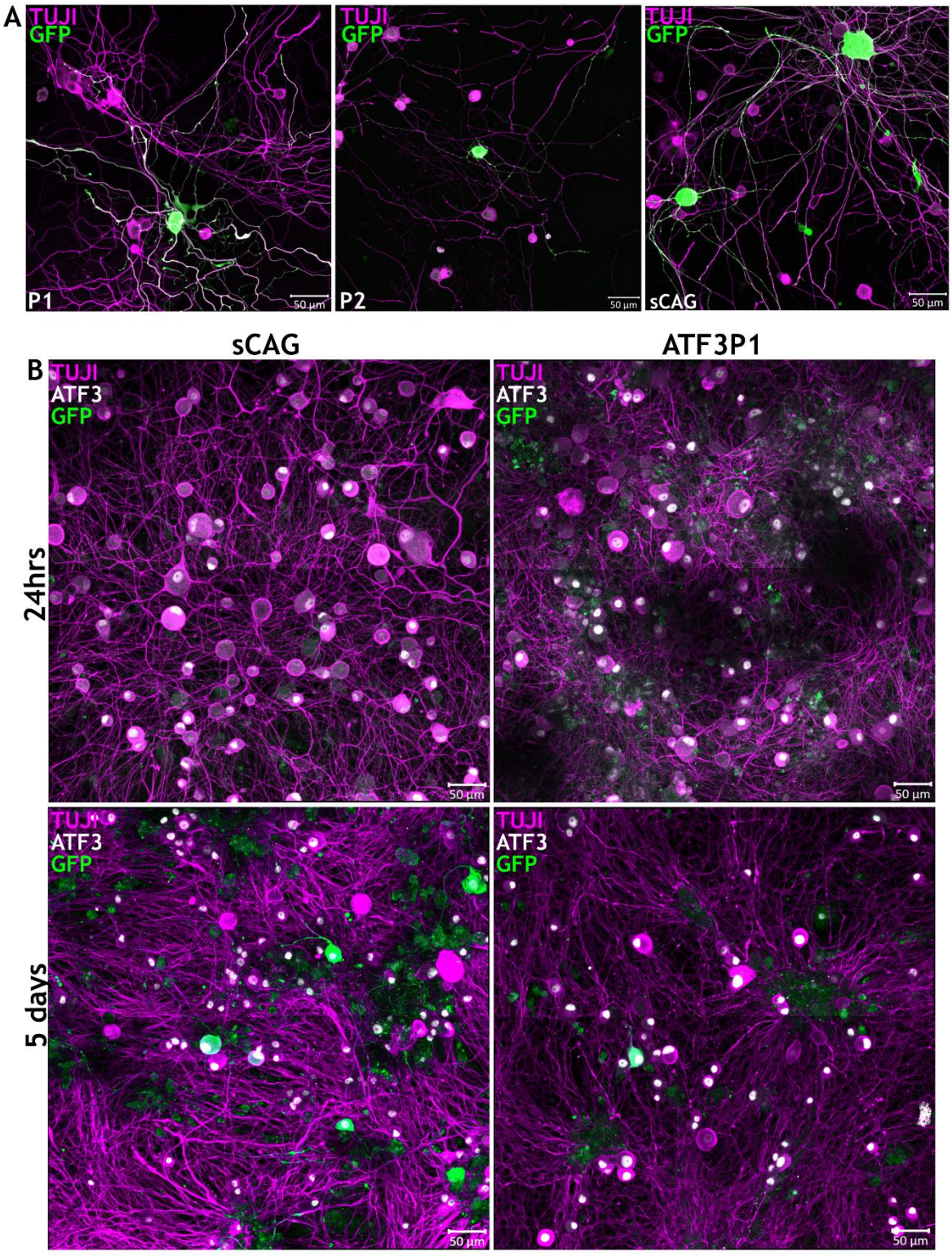


Figure 3.7 Endogenous ATF3 promoter activity in mDRG neurons. ***(A)*** *Mouse DRG neurons transfected with plasmids containing GFP under the control of the P1 and P2 ATF3 promoters or the ubiquitous sCAG promoter. GFP expression was assessed 48 hours later, and no discernible differences could be seen between the two ATF3 promoters.* ***(B)*** *ATF3P1 AAV activity in dissociated mDRG neurons. Mouse DRG cultures were incubated with the sCAG (control) or ATF3P1 AAVs for 24 hours or 5 days. ATF3P1*

AAV successfully transduced neurons and higher GFP expression was observed 5 days post AAV addition. n = 1 mouse.

3.3.6 ATF3P1 AAV activity in iPSC-derived sensory neurons

We initially assessed the ability of the ATF3P1 promoter to restrict transgene expression to injured sensory neurons in our *in vitro* model of peripheral injury. AAVs containing the ATF3P1 or ubiquitous sCAG (control) promoter were added to iSN cultures for 12 days in total and GFP expression was evaluated 24 hours and 5 days post-injury (Figure 3.8A). Transduction with either AAV resulted in higher GFP expression in iSNs both 24 hours and 5 days post-axotomy compared to naïve neurons (**sCAG**: 39.9 ± 9.5% in naïve vs 72.2 ± 9.5% and 61.3 ± 7.03% after 24 hours and 5 days; **ATF3P1**: 9.1 ± 4.5% in naïve vs 50.9 ± 29.2% and 51.0 ± 22.5% after 24 hours and 5 days) (Figure 3.8B). We noticed higher iSN transduction dependent on both the injury timepoint ($F(2, 24) = 9.48$, $p = 0.0009$) and promoter ($F(1, 24) = 7.72$, $p = 0.01$), however the interaction between the two factors was not significant ($F(2, 24) = 0.62$, $p = 0.54$) (Figure 3.8). Tukey's *post hoc* multiple comparisons tests indicated a significant increase in the proportion of GFP+ iSNs 24 hours and 5 days after axotomy (9.1 ± 4.5% in naïve vs 50.9 ± 29.2% and 51.0 ± 22.5% after 24 hours and 5 days respectively) following transduction with ATF3P1 AAV (Figure 3.8B). A similar effect was not observed with our control vector, suggesting the bias in expression was most likely mediated by the P1 promoter.

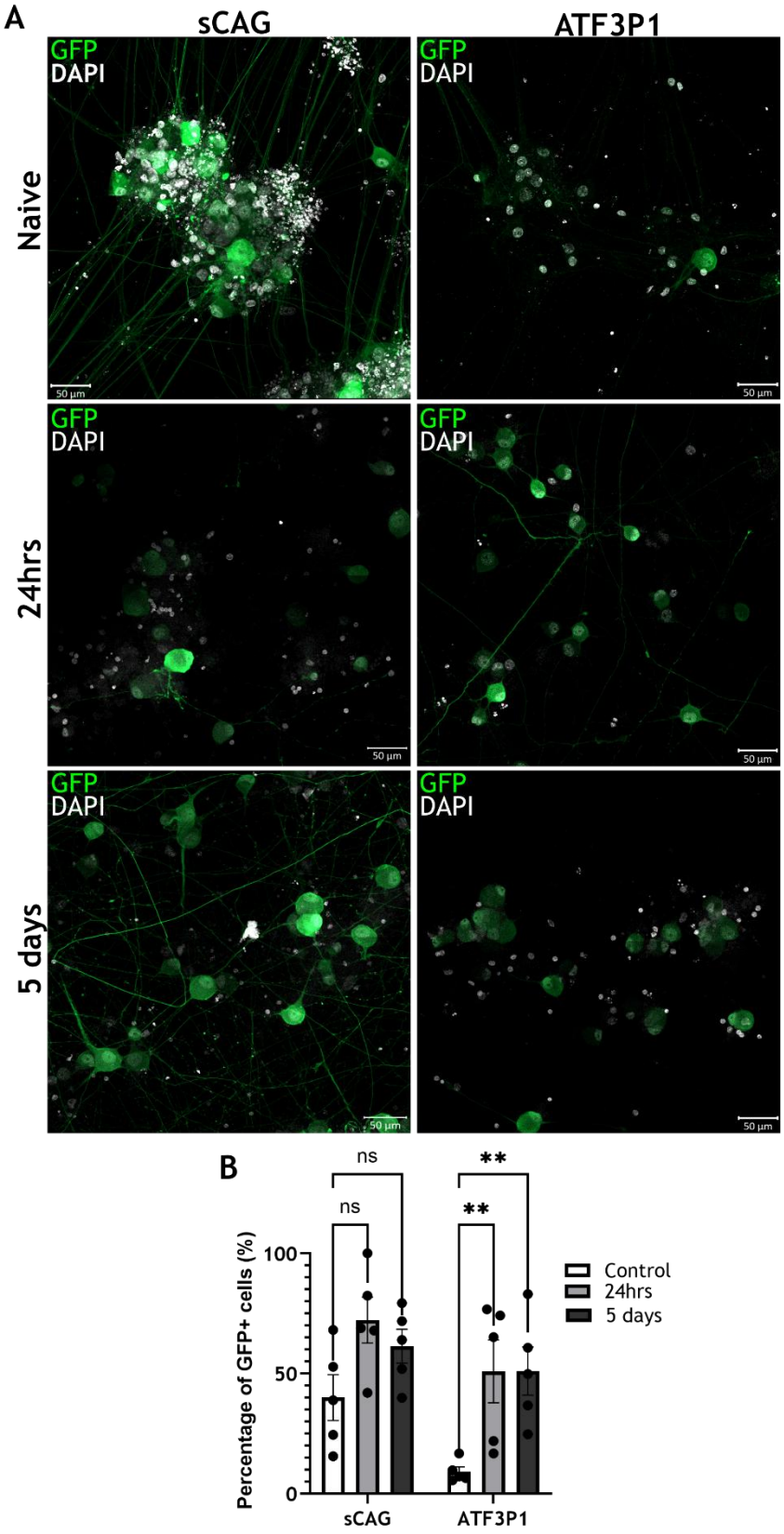


Figure 3.8 ATF3P1 activity in iPSC-derived sensory neurons. ***(A)*** *Sensory neurons transduced with either sCAG (control) or ATF3P1 AAVs for a total of 12 days. GFP expression was assessed in the naïve state as well as 24 hours and 5 days post-injury.* ***(B)*** *Percentage of GFP+ iSNs after transduction with either sCAG or ATF3P1 AAVs. Two-way ANOVA; Interaction (promoter x injury timepoint): F(2, 24) = 0.62, p = 0.54, n = 5*

*coverslips; Tukey's post hoc multiple comparisons tests: **p < 0.01 vs control. Experimental unit is defined as a coverslip and all data was derived from two independent differentiations. All data are presented as mean ± SEM.*

3.3.7 ATF3P1 AAV activity in primary afferents *in vivo*

While a significant interaction between promoter and injury was not noted *in vitro*, *post hoc* tests suggested a greater proportion of injured compared to naïve iSNs were transduced with our ATF3P1 AAV, encouraging us to test the virus *in vivo*. Intrathecal injections have been previously shown to limit transgene expression to lumbar DRG, thus minimising off-target expression, and were therefore our preferred route of AAV administration (Weir *et al.*, 2017). AAVs were delivered to ATF3CreERT2:Ai9 mice which then underwent SNI two weeks later. GFP expression was assessed two weeks after SNI and both promoters successfully drove expression in DRG neurons (Figure 3.9A). To ensure GFP+ cells were confidently identified, we selected five definitively GFP- cells per section, calculated their average GFP signal intensity and set an individualised "background signal" for each section. We then decided on a low threshold, set as the average background signal intensity plus 3 times the background SD (Figure 3.9B), and a high, stricter threshold, set as the average signal plus 5 times the SD (Figure 3.9C). Neurons were considered GFP+ only if their GFP signal intensity surpassed the established threshold. Administration of either AAV resulted in the transduction of both injured (tdTom+) and intact (tdTom-) DRG neurons (Figure 3.9) and two-way repeated measures ANOVA revealed no significant interaction between promoter and neuron injury state (tdTom expression) (Table 3.1), similar to what we observed in iSNs (Figure 3.9B and C). A significant main effect of injury, however, was noted when determining GFP expression by high threshold (Table 3.1). The overall rate of transduction was also comparable between the two AAVs (Figure 3.9B and C).

These results suggest the ATF3P1 AAV was not capable of biasing or restricting transgene expression to injured primary afferents *in vivo*.

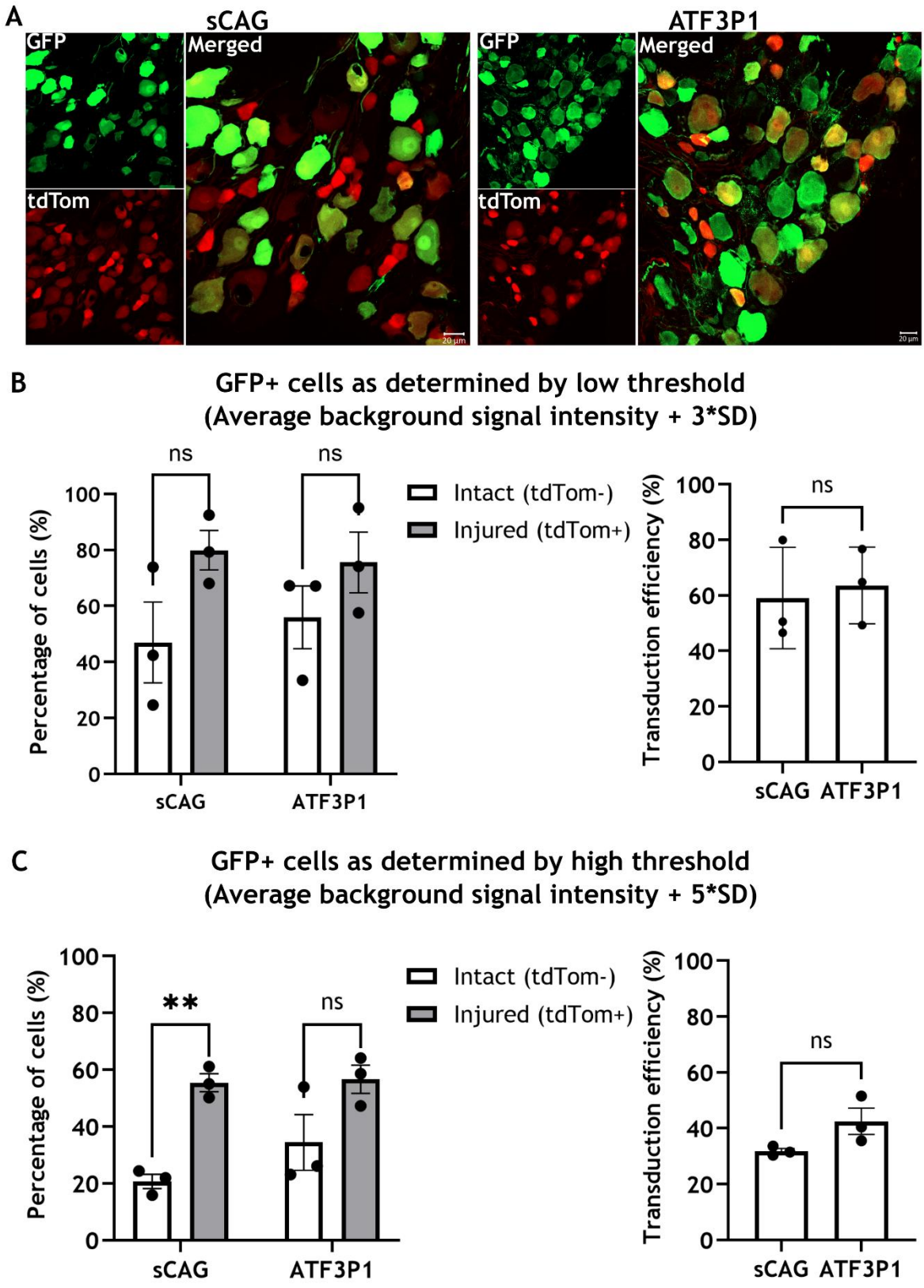


Figure 3.9 ATF3P1 activity in mDRG neurons* in vivo.** ***(A) *Transduction of mDRG neurons with sCAG (control) or ATF3P1 AAV. Vectors were delivered to ATF3Cre:Ai9 mice via intrathecal injection and GFP expression was assessed two weeks post-SNI. Both injured (tdTom+) and intact (tdTom-) primary afferents were transduced by the ATF3P1 AAV.* ***(B)*** *Percentage of GFP+ injured and intact cells and overall neurons transduced with either sCAG or ATF3P1 AAV. To confidently call GFP+ cells, a low threshold was set as the average signal intensity of five GFP- neurons added to 3 times their standard deviation.* ***(C)*** *Percentage of GFP+ injured and intact cells and overall neurons transduced with either sCAG or ATF3P1 AAV. A higher threshold was set as the average signal intensity of five GFP- neurons added to 5 times their standard deviation. Both AAVs had comparable transduction efficiencies in injured and intact primary afferents, as well as neurons overall. Two-way ANOVA followed by Šídák's post hoc multiple comparisons test: **p < 0.01; Unpaired t-test for overall expression; 3*SD: t = 0.35, df = 4, p = 0.75; 5*SD: t = 2.21, df = 4, p = 0.09, n = 3 mice. Experimental unit is defined as the averaged values from three DRG sections from one animal. All data are presented as mean ± SEM. SD; standard deviation*

Threshold	Promoter	Injury	Interaction (Promoter x Injury)
Low (3*SD)	F(1, 8) = 0.04, p = 0.84	F(1, 8) = 5.53, p = 0.046	F(1, 8) = 0.35, p = 0.57
High (5*SD)	F(1, 8) = 1.63, p = 0.24	F(1, 8) = 23.67, p = 0.001	F(1, 8) = 1.14, p = 0.32

Table 3.1 Two-way repeated measures ANOVA degrees of freedom and p values for analyses of the effects of promoter and/or injury state on the AAV-mediated transduction of DRG neurons following SNI*. n = 3 mice.*

3.4 Discussion

Cell-type specificity is a highly desirable quality for viral vectors delivering gene therapies as it can help increase efficacy and minimise off-target effects. *Cis*-regulatory elements, such as promoters and enhancers, have been successfully used in viral vectors to restrict transgene expression to specific cells or tissues in the nervous system (von Jonquieres *et al.*, 2013; Vormstein-Schneider *et al.*, 2020; Graybuck *et al.*, 2021; Mouchbahani-Constance *et al.*, 2023). In this chapter, we developed a pipeline for testing AAV constructs and assessed the ability of an ATF3 promoter to limit transgene expression to injured primary

afferents. We found inclusion of the ATF3 promoter did not adequately restrict transgene expression *in vivo*.

3.4.1 Development and characterisation of *in vitro* NeuP models

ATF3 is expressed in DRG neurons following axotomy and we confirmed this expression persists in culture, even in the presence of growth factors (Figure 3.2) (Tsujino *et al.*, 2000; Seijffers, Allchorne and Woolf, 2006; Renthal *et al.*, 2020). We were, therefore, able to use dissociated mDRG neuron cultures as a first screening platform for testing our vectors' activity. In addition to mDRG neurons, we successfully differentiated sensory neurons from iPSCs to develop a humanised testing platform. The resulting iSNs are transcriptionally and functionally similar to DRG nociceptors and have been previously characterised elsewhere (Chambers *et al.*, 2012; Clark *et al.*, 2017; Weir *et al.*, 2017). Following an initial upregulation of TRKA, iSNs express the sodium channels Nav1.7, Nav1.8, and Nav1.9 and the $P2X_3$, TRPV1 and TRPM8 receptors by DIV 15, stain positive for Ret, the neuronal marker TUJI, and the sensory neuron-specific TF BRN3A and ultimately reach maturity by DIV 35-40 (Figure 3.3). These iSNs also exhibit a shoulder on the falling phase of the action potential waveform and tetrodotoxin-resistant (TTX-R) currents along with responsiveness to α,β-methylene-ATP, all hallmarks of endogenous nociceptors (Chambers *et al.*, 2012). Despite expressing the TRPV1 protein, only few neurons (1-2%) respond to its agonist capsaicin, indicating iSNs do not perfectly replicate human nociceptors. In addition, iSNs become myelinated when co-cultured with Schwann cells, suggesting the neurons produced are a combination of Aδ- and C-nociceptors (Clark *et al.*, 2017). Similar findings have been reported by multiple groups using the Chambers protocol (Eberhardt *et al.*, 2015; McDermott *et al.*, 2019; Pettingill *et al.*, 2019), while others claim to have produced neurons resembling mechanoreceptors (Guimarães *et al.*, 2018), pruriceptors (Umehara *et al.*, 2020) and proprioceptors (Dionisi *et al.*, 2020). This highlights the sensitivity of differentiation protocols and the importance of final cell type validation.

Humanised cellular systems are valuable tools for modelling disease *in vitro* and improving translation. We used iSNs to establish *in vitro* models of peripheral nerve injury since, unlike dissociated mDRG cultures, they represent naïve,

uninjured neurons (Jones *et al.*, 2018). Traumatic injury is one of the simplest forms of nerve injury to model and was achieved by creating tears on the cell monolayer and mechanically dissociating the neurons. Injury was determined by ATF3 expression and while a significant increase was noted 24 hours after injury, expression was also detected in naïve neurons (Figure 3.4). Expression varied considerably between coverslips, with 36-89% of naïve neurons staining positive for ATF3. The presence of ATF3 in naïve iSNs could be an indication of cell stress. Many of our iSNs were stored using the CryoPause method which has been previously shown to not impact further differentiation upon thawing (Saito-Diaz *et al.*, 2021). The effects of cryopreservation on iSN recovery and viability have not been specifically investigated but the process is expected to cause cellular stress, especially if done improperly. To improve cell survival post-thawing, our cultures were supplemented with CEPT, a small molecule cocktail promoting cell viability and fitness (Chen *et al.*, 2021). Further optimisation of the CryoPause protocol is likely required, such as the addition of ice-recrystallisation inhibitors, as is a characterisation of ATF3 expression post-thawing and testing of other injury markers. Since the process of replating iSNs mimics axotomy, we decided to go ahead with our *in vitro* model of traumatic injury operating under the assumption that all replated iSNs are injured and all naïve ones are intact.

In most cases, patients complain of NeuP caused by systemic, rather than traumatic, damage. Modelling chemically induced injury *in vitro* using iSNs has been previously achieved using neurotoxic agents (Wheeler *et al.*, 2015; Li *et al.*, 2021; M. Wang *et al.*, 2021; Schinke *et al.*, 2021; Cantor *et al.*, 2024; Mortensen *et al.*, 2024). We assessed the capacity of paclitaxel to induce nerve injury in iSNs and observed inconsistent effects which could not be reliably reproduced across different timepoints and concentrations (Figure 3.5). Similarly to our traumatic model, ATF3 was used to determine whether neurons were injured. It is possible different viability and cytotoxic assays need to be employed, particularly if ATF3 is not an ideal injury marker in iSN cultures. Since it was not possible to easily determine which neurons were injured, unlike when replating, we decided to not develop this model any further.

3.4.2 Optimisation of an *in vivo* NeuP model

Cellular models, while greatly improved, are still not able to fully replicate the conditions of living organisms. It is necessary, therefore, to complement their use with that of *in vivo* models. Viral vectors developed throughout this work were tested for their ability to restrict transgene expression to injured primary afferents using ATF3CreERT2:Ai9 mice following SNI. Use of this mouse line is complicated by the administration of tamoxifen which is necessary for increasing labelling efficiency but can also cause nerve injury and inappropriate recombination (Denk *et al.*, 2015). In the absence of tamoxifen, between 65-80% of injured L4 DRG neurons have been previously captured, a proportion which could be improved using an appropriately low tamoxifen dose (Holland *et al.*, 2019). We found that administering 7.5 mg/kg tamoxifen on day 7 post-SNI significantly increased recombination, capturing 71.1% of all L4 DRG neurons (Figure 3.6A and B). Our data suggests a greater proportion of the L4 DRG is injured following SNI than previously reported (71.1% vs 34%) (Laedermann *et al.*, 2014) but is consistent with comprehensive stereological efforts in the lab using a separate transgenic cross (Cooper *et al.*, 2024). In the untreated group, we observed recombination was lacking in a considerable portion of small diameter neurons, but this cell-size bias was not present in the tamoxifen-treated group (Figure 3.6C), suggesting that tamoxifen-independent recombination preferentially occurs in medium-large neurons. Overall, we were able to improve fluorescent labelling in ATF3CreERT2:Ai9 mice using a low dose of tamoxifen while maintaining expression fidelity. These efforts validate the transgenic line as one able to reliably and rapidly identify axotomized neurons.

3.4.3 Endogenous ATF3 promoter activity

We successfully introduced the endogenous ATF3 promoters P1 and P2 into plasmids (Figure 3.1) both of which were similarly efficient in driving gene expression in injured murine sensory neurons *in vitro* (Figure 3.7A). P1 was chosen as a candidate for further development and its addition to iSNs resulted in higher percentages of GFP+ neurons after axotomy, suggesting the vector biases transgene expression to injured sensory neurons *in vitro* (Figure 3.8). GFP is still detected in naïve iSNs, although in less neurons compared to the sCAG

AAV, indicating the vector is not capable of restricting expression only to injured neurons (Figure 3.8).

According to our *in vivo* results, ATF3P1 AAV transduces both healthy and injured primary afferents in ATF3CreERT2:Ai9 mice 14 days post-SNI (Figure 3.9). Transduced neurons could not be confidently identified, leading to two different counting strategies being employed. Briefly, the average signal intensity of five GFP- cells was added to either three (Figure 3.9B) or five (Figure 3.9C) times their standard deviation, providing a baseline signal intensity for identifying GFP expressing neurons. Both strategies yielded the same result, demonstrating the ATF3P1 promoter does not sufficiently restrict transgene expression in injured primary afferents *in vivo* (Figure 3.9). Despite their comparable efficiencies in dissociated mDRG (Figure 3.7A), the P2 promoter should also be investigated for its ability to restrict transgene expression due to its closer proximity to the TSS and the CpG islands covering the exon downstream of the promoter and activation by injury stimuli (Miyazaki *et al.*, 2009). We also used the human sequence for both promoters which, while highly conserved, could have negatively impacted the specificity of injured neuron targeting *in vivo*. It could also explain the significant effect observed in iSNs. It is likely neither promoter would have been sufficient in controlling transgene expression on its own. Endogenous, tissue-specific promoters usually return lower expression levels compared to their exogenous counterparts but can be modified through the addition of elements, such as enhancers, to improve transgene expression while maintaining selectivity (Domenger and Grimm, 2019). The discovery of novel regulatory elements (e.g. enhancers and/or promoters) capable of biasing AAV-mediated transgene expression to injured sensory neurons is explored in the next chapter (Chapter 4).

In summary, data presented in this chapter demonstrates the characterisation and/or optimisation of *in vitro* and *in vivo* nerve injury models for testing constructs and the development of an AAV9 containing the ATF3P1 promoter. We found that the ATF3P1 AAV, while capable of offering selectivity *in vitro*, did not successfully restrict expression to injured sensory neurons *in vivo*. This suggests the promoter cannot be used to target AAV gene expression on its own but could

be potentially combined in a vector with other elements regulating transgene expression.

Chapter 4 Epigenomic signatures of intact and injured primary afferents

4.1 Introduction

Since the ATF3P1 promoter did not offer selectivity *in vivo*, we set out to discover novel regulatory elements which could successfully target expression to injured primary afferents. The work presented in this chapter aimed to generate an epigenomic atlas of naïve mouse DRG neurons and provide insight into the epigenomic signatures of injured and intact primary afferents, with the ultimate purpose of identifying regions of DNA containing putative regulatory elements active in injured, but not intact, DRG neurons. In turn, future studies could introduce these elements into viral vectors and explore their capacity to restrict transgene expression to injured neurons, thus reducing off-target effects.

4.1.1 Chromatin accessibility and implications for gene expression

In eukaryotes, the genome is packaged into chromatin and further organised in nucleosomes consisting of approximately 147bp of DNA wrapped around a histone octamer (Luger *et al.*, 1997). Chromatin accessibility refers to the degree to which chromatinised DNA can be contacted by nuclear macromolecules, such as TFs, and therefore plays a critical role in gene regulation (Klemm, Shipony and Greenleaf, 2019). Chromatin accessibility is determined by nucleosome occupancy and location as well as other chromatin binding factors obstructing DNA access (Klemm, Shipony and Greenleaf, 2019). Nucleosome occupancy, or the fraction of time a particular DNA sequence is bound by the histone octamer, varies across cell and tissue types. Nucleosomes typically populate areas of facultative and constitutive heterochromatin while being depleted within regulatory loci for elements such as enhancers and promoters (Lee *et al.*, 2004; Klemm, Shipony and Greenleaf, 2019). Hence, low nucleosome occupancy is an indicator of high chromatin accessibility and vice versa. In addition to physical packaging, chromatin can be epigenetically modified via processes such as DNA methylation, nucleosome positioning, histone composition and modification, non-coding RNAs, and TFs. As a result, these

epigenomic mechanisms play a critical role in regulating gene expression by controlling the accessibility of gene promoters and enhancers to TFs.

4.1.2 Assays for chromatin accessibility

Chromatin accessibility profiling methods have considerably improved in the last few years. Methods such as micrococcal nuclease sequencing (MNase-seq), ChIP-seq and deoxyribonuclease I -hypersensitive site sequencing (DNase-seq) assess accessibility via the use of enzymatic methylation or DNA cleavage and provide information about chromatin accessibility at regulatory loci, TF binding and nucleosome modification and position (Zaret, 2005; Song and Crawford, 2010; Landt *et al.*, 2012; Thurman *et al.*, 2012). These methods, however, have considerable drawbacks. They have complex, time-consuming library preparation protocols and require a large input of cellular material, often amounting to millions of cells, leading to rarer cellular subtypes being underrepresented or even impossible to study sufficiently. In addition, none of these assays are capable of simultaneously investigating the interplay between chromatin accessibility, nucleosome positioning and TF binding. ATAC-seq is currently one of the most commonly used methods of investigating chromatin accessibility developed to overcome these limitations (Yan *et al.*, 2020). In this assay, a genetically engineered hyperactive DNA transposase, Tn5, is modified to carry monovalent mosaic end adaptors. Transposons are capable of being integrated into active regulatory elements, and therefore transposition with the hyperactive Tn5 results in the cleavage and integration of adaptors in areas of accessible chromatin. Contrary to other protocols, ATAC-seq requires a lower cell input (500-50,000 cells) and is a two-step process involving Tn5 insertion followed by PCR (Buenrostro *et al.*, 2013).

4.1.3 Using ATAC-seq data to design AAVs

ATAC-seq data has been harnessed to develop viral vectors with improved cell type specificity in the CNS. Using already available transcriptomic and epigenomic information, Vormstein-Schneider *et al.* (2020) focused on the regulatory landscape of *Scn1a*, a gene expressed in distinct neuronal populations and associated with epilepsy. Examination of intergenic and intronic regions of *Scn1a* led to the discovery of ten candidate sequences with good species

conservation. Following validation via insertion into an rAAV backbone, one enhancer was selected for targeting PV-expressing cortical interneurons with ~90% specificity across mice, non-human primates and humans (Vormstein-Schneider *et al.*, 2020). Similarly, other groups have performed ATAC-seq in mouse and primate cortical neurons and incorporated identified enhancer sequences into viral vectors, either alone or combined with multiple enhancers, to achieve cell type specific transgene expression (Graybuck *et al.*, 2021; Mich *et al.*, 2021). These studies demonstrate how chromatin accessibility data can be used to create viral tools for the investigation and manipulation of specific cell types and potentially improve the specificity of therapeutic interventions in the future. While there is substantial information regarding the transcriptional, molecular and anatomical changes occurring in DRG neurons following injury, this has not been sufficient for the design of vectors capable of selectively targeting injured primary afferents. Finally, the epigenomic landscape of naïve mDRG neurons and changes in chromatin accessibility in specific neuronal subtypes after injury remain largely unexplored.

4.1.4 Aim

This chapter aims to produce an epigenomic atlas of naïve DRG neurons and investigate the epigenomic signatures of injured and intact primary afferents with the purpose of discovering differentially accessible regulatory elements suitable for AAV inclusion.

4.2 Methods

4.2.1 Animals

DRG were collected 4 weeks after SNI from adult Avil^{FlpO};Atf3^{CreERT2};RC::FLTG mice, described previously by Cooper *et al.* (2024). The line crosses FlpO recombinase controlled by the *Advillin* promoter with tamoxifen-inducible Cre recombinase controlled by an *Atf3* promoter and the dual-reporter allele RC::FLTG (Madisen *et al.*, 2010; Denk *et al.*, 2015; Plummer *et al.*, 2015; Choi *et al.*, 2020). *Advillin* is expressed in all peripheral sensory neurons while *Atf3* is expressed *de novo* in primary afferents following injury. The dual-reporter allele contains both *tdTomato* and *GFP* under the control of FlpO and Cre respectively,

allowing the labelling of both intact (tdTomato) and injured (GFP) sensory neurons. Animals received 50 mg/kg tamoxifen via i.p. injection on days 0, 3 and 7 after SNI. Tamoxifen was prepared by being dissolved in wheat germ oil under sterile conditions at a concentration of 20 mg/mL.

4.2.2 Sample collection and FACS

Ipsilateral and contralateral L3-L5 DRG were extracted following perfusion as described in Chapter 2. Animals were not perfused with fixative following ice-cold PBS perfusion. DRG were collected in HBSS stored on ice and were enzymatically digested using Collagenase type II (4 mg/mL) and Dispase type II (4.6 mg/mL) in 37°C for 1 hour and 30 minutes, agitating every 15 minutes. Cells were centrifuged at 300 x *g* for 5 minutes, before being washed with mature N2 medium and being mechanically dissociated using two fire-polished glass pipettes of different sizes. Single cell suspensions were then passed through a 70µm reversible strainer (STEMCELL Technologies, Canada) and centrifuged at 300 x *g* for 5 minutes. Prior to fluorescence activated cell sorting (FACS), parts of each sample were examined under a fluorescent microscope to confirm sample composition.

Pelleted cells were transported in mature N2 medium on ice to the Flow Core Facility at the University of Glasgow for sorting using a BD FACSAria III. A sorter with a 100µm nozzle was used to sort fluorescent cells into a 1.5mL microcentrifuge tube containing 100µl mature N2 medium under pressure at 10 psi. Draq7 (Thermofisher Scientific, US) was used to exclude dead cells and sorting criteria monitoring the distribution of tdTomato and/or GFP signals and forward and side scatter were selected. Following sorting, cells were centrifuged for 5 minutes at 600 x *g*, resuspended in 200µl of Cryostor media (Sigma-Aldrich, US) and frozen gradually in Mr Frosty isopropyl alcohol chamber at -80°C for 24 hours before being moved to -150°C for long term storage. In total, 4 separate collections were performed from SNI mice (6 male, 10 female) and 2 collections were from wild type (WT) mice (7 male, 3 female). WT mice refer to age-matched $Atf3^{CreERT2}$ negative, $Avil^{FlpO}$ positive (heterozygous) and RC::FLTG positive (heterozygous) animals which did not undergo SNI. Time from extraction to freezing was approximately 6 hours during which cells were kept on ice as much as possible.

4.2.3 snATAC-seq library preparation and sequencing

Nuclei were isolated from frozen samples according to the protocol provided by 10X Genomics. Briefly, cells were washed 3 times with pre-warmed PBS supplemented with 0.04% bovine serum albumin (BSA) (Sigma-Aldrich, US) and were centrifuged at 500 x *g* for 10 minutes at 4˚C. Following the last wash, the supernatant was removed, leaving 5µl in the tube. Samples were incubated with 2X chilled lysis buffer (20mM Tris-HCl, 20mM NaCl, 6mM MgCl2, 0.2% Tween-20, 0.2% IGEPAL CA-360, 0.02% Digitonin, 2% BSA) for 10 minutes on ice, gently pipetting every 3 minutes. Cells were washed with wash buffer (10mM Tris-HCl, 10mM NaCl, 3mM MgCl2, 1% BSA, 0.1% Tween-20) and were centrifuged at 500 x *g* for 15 minutes at 4˚C. Cells were then resuspended in chilled diluted nuclei buffer (10X Genomics, US), centrifuged, resuspended in nuclei buffer again and counted to determine cell concentration. Four snATAC-seq libraries were obtained using 10X Genomics Chromium Single Cell ATAC v2.1 chemistry; injured (GFP), intact (tdTomato), contralateral, and WT. Sequencing was performed on the Illumina HiSeq 4000 system using the 10X Genomics pipeline to a minimum of 50,000 reads per nucleus at the Glasgow Polyomics Facility.

Reagent	Source	Catalog No.
Tris-HCl	Sigma-Aldrich	T2194
NaCl	Sigma-Aldrich	59222C
Mg_2Cl	Sigma-Aldrich	M1028
Tween-20	Thermofisher Scientific	P9416
IGEPAL CA-630	Sigma-Aldrich	I8896
Digitonin	Thermofisher Scientific	BN2006
BSA	Sigma-Aldrich	A8806

Table 4.1 Sources of reagents used in lysis and wash buffers for library preparation.

4.2.4 Bioinformatics

Initial analysis was performed using CellRanger-atac and raw sequencing reads were processed and mapped to the mouse (GRCm38) genome by Sam McAllister.

Analysis was carried out using the Signac package (version 1.14.0) in R (version 4.4.2) following the Signac vignette (https://stuartlab.org/signac/articles/pbmc_vignette.html) (Stuart *et al.*, 2021).

Pre-processing, clustering and visualisation

The initial Seurat object was generated from peak/cell matrix and cell metadata files using the Read10X_h5(), CreateChromatinAssay(), and CreateSeuratObject() functions. Quality control was performed according to the vignette's recommendations and samples were subset to exclude nuclei with peak region fragment counts below the 2nd percentile and above the 98th percentile, percentage of reads in peaks and transcription start site (TSS) enrichment below the 2nd percentile and nucleosome signal and blacklist ratio above the 98th percentile. Normalisation was performed using term frequency-inverse document frequency (TF-IDF) transformation, features were selected using the FindTopFeatures (min.cutoff = 'q0') and dimension reduction was carried out by running singular value decomposition (SVD) on the TD-IDF matrix. This process is known as latent semantic indexing (LSI). This was followed by Uniform Manifold Approximation and Projection (UMAP) applied to dimensions 2 to 30 of the LSI reduction of the dataset using RunUMAP(), neighbour finding using FindNeighbors() on the same dimensions and cell clustering using FindClusters() and the Luvain algorithm (algorithm 3) in Seurat.

Annotation of snATAC-seq data

To annotate our snATAC-seq data, we generated a pseudo gene expression matrix using GeneActivityMatrix() in Seurat. This function counts fragments overlapping with promoters and gene bodies within the 2kb region upstream of the TSS. Using this matrix, we clustered nuclei into neuronal and non-neuronal using the expression of neuronal marker genes *Rbfox3* and *Pou4f1*, Schwann cell markers *Bcas1* and *Prx*, and satellite glial cell (SGC) markers *Kcnj10* and *Fabp7*. Non-neuronal cells were excluded from further downstream analyses. Using FindAllMarkers() in Seurat, we compared nuclei from one cluster to all other nuclei and assigned identities based on the expression of at least two subpopulation markers.

Data integration

The WT and contralateral datasets were merged into a single object using merge(), followed by LSI and dimensionality reduction and visualisation. The merged dataset was then split by sample using SplitObject(), each object undergoing normalisation, TF-IDF transformation and top feature selection. SelectIntegrationFeatures() and FindIntegrationAnchors() were used to identify integration features in each sample and integration anchors respectively. The integrated naïve dataset underwent normalisation, clustering and visualisation as described above. The GFP and tdTomato datasets were individually integrated with the naive dataset following the same process. To ensure condition specific peaks were included in downstream analyses and there was no loss of potentially biologically relevant features, the peak sets from each dataset (GFP and naïve or tdTomato and naïve) were combined by taking the union of their respective peak coordinates.

Differential chromatin accessibility analysis

To identify cell-type specific peaks we used FindAllMarkers(). When looking for differentially accessible chromatin between specific clusters, we used FindMarkers(). Analyses in both cases looked for peaks with minimum expression in at least 10% of cells (min.pct = 0.1) and employed a Wilcox rank-sum test (test.use = 'wilcox'). Peak_region_fragments was included as a latent variable to control for differences in sequencing depth across cells. Peak accessibility at different genomic loci was visualised using CoveragePlot().

GO analysis

GO enrichment analysis was carried out using the enrichGO() function from the clusterProfiler package (version 4.12.6) in R (G. Yu *et al.*, 2012). A list of differentially accessible peaks (False Discovery Rate (FDR) < 0.01, Log2FC > 0.5) was input for analysis. The input gene list was annotated using the R package org.Mm.eg.db (version 3.19.1) as the genome wide database for the organism *Mus Musculus*. The pvalueCutoff and qvalueCutoff parameter values were 0.01 and 0.05 respectively.

4.3 Results

4.3.1 Cell collection and library preparation

Next generation sequencing technologies have improved to reduce the amount of DNA or RNA, and thus number of cells, needed for sequencing. Nevertheless, thousands of cells are still required, especially when taking into account the considerable proportion of cells lost at each step of the library preparation process. We originally collected DRG neurons from ATF3Cre:Ai9 mice, described in Chapter 3, sorting neurons into injured and intact based on tdTomato expression. Cre recombination in presumed Schwann cells, however, led to considerable glial contamination and lack of suitable neuron numbers in our sorted samples (data not shown). To overcome this, we used $Avil^{FlpO}$;$Atf3^{CreERT2}$;RC::FLTG mice, originally described in Cooper *et al.* (2024). In this mouse line, all naive sensory neurons express tdTomato (Flp-dependent) and change to expressing GFP following axotomy combined with tamoxifen treatment (Flp- and Cre-dependent) (Figure 4.1). GFP expression, assessed 14 days after injury, is found in 99.1 ± 0.6% of ATF3-expressing ipsilateral L4 DRG neurons, while contralateral expression is limited (4.6 ± 0.7%) (Cooper *et al.*, 2024).

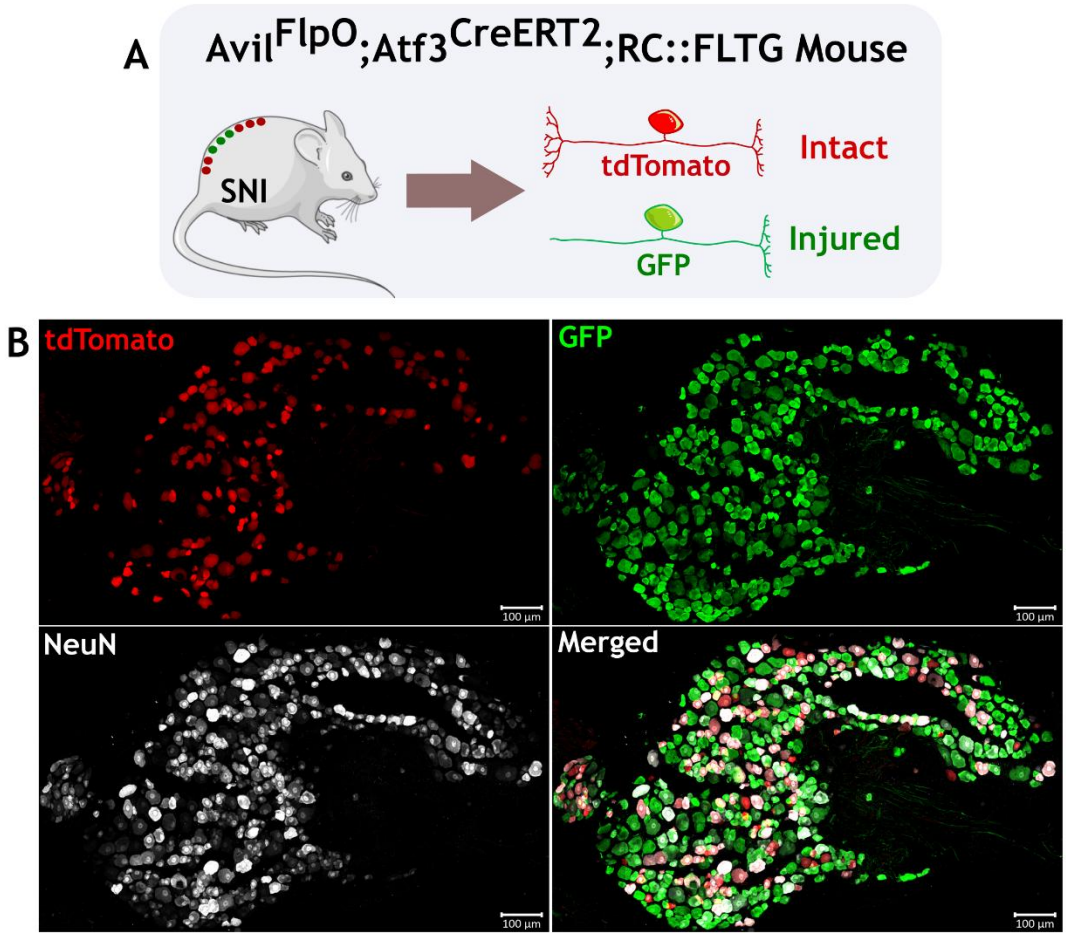


Figure 4.1 Avil^{FlpO};Atf3^{CreERT2};RC::FLTG mice express tdTomato in intact and GFP in injured neurons. ***(A)*** *Schematic of approach used to fluorescently label injured (GFP) and intact (tdTomato) primary afferents using the Avil^{FlpO};Atf3^{CreERT2};RC::FLTG mouse line.* ***(B)*** *Representative L4 DRG section of NeuN, GFP and tdTomato expression 2 weeks post-SNI.*

We collected L3-L5 DRG from naïve and injured Avil^{FlpO};Atf3^{CreERT2};RC::FLTG mice that had undergone SNI. In total, we performed two naïve and four injured collections four weeks after SNI. We sorted neurons based on their differential fluorescence using FACS and cryostored purified samples in batches prior to library preparation and sequencing. Draq7, a far-red fluorescent dye, was used to stain nuclei in dead and permeabilised cells, and Draq7 negative samples were run first to determine gate placement. Draq7 was then added to neurons which were gated for scatter, doublets, live/dead, and fluorescence (Figure 4.2A-H). Samples purified from uninjured animals lacking Cre (Atf3^{Cre} negative, Avil^{FlpO} heterozygous and RC::FLTG heterozygous), referred to as **WT** going forward, contained only tdTomato+ neurons, as expected (Figure 4.2D). In

injured samples, neurons could be easily differentiated based on their expression of GFP and tdTomato (Figure 4.2E) and both populations were collected separately, yielding our samples **GFP+ ipsi** and **tdTom+ ipsi**. The presence of dual GFP+ and tdTomato+ neurons was also detected (Figure 4.2E and F), likely due to uninjured cells switching on GFP during the sample preparation process. Finally, we collected DRG contralateral to SNI and this sample is herein termed **Contra**. Some GFP+ cells were also present in this sample, likely representing neurons with inappropriate Cre recombination (approximately 5% of neurons) (Figure 4.2F). These cells, as well as dual GFP+ and tdTomato+ neurons, were not collected. Appropriate sample composition was verified by taking images before and after sorting (Figure 4.2G and H).

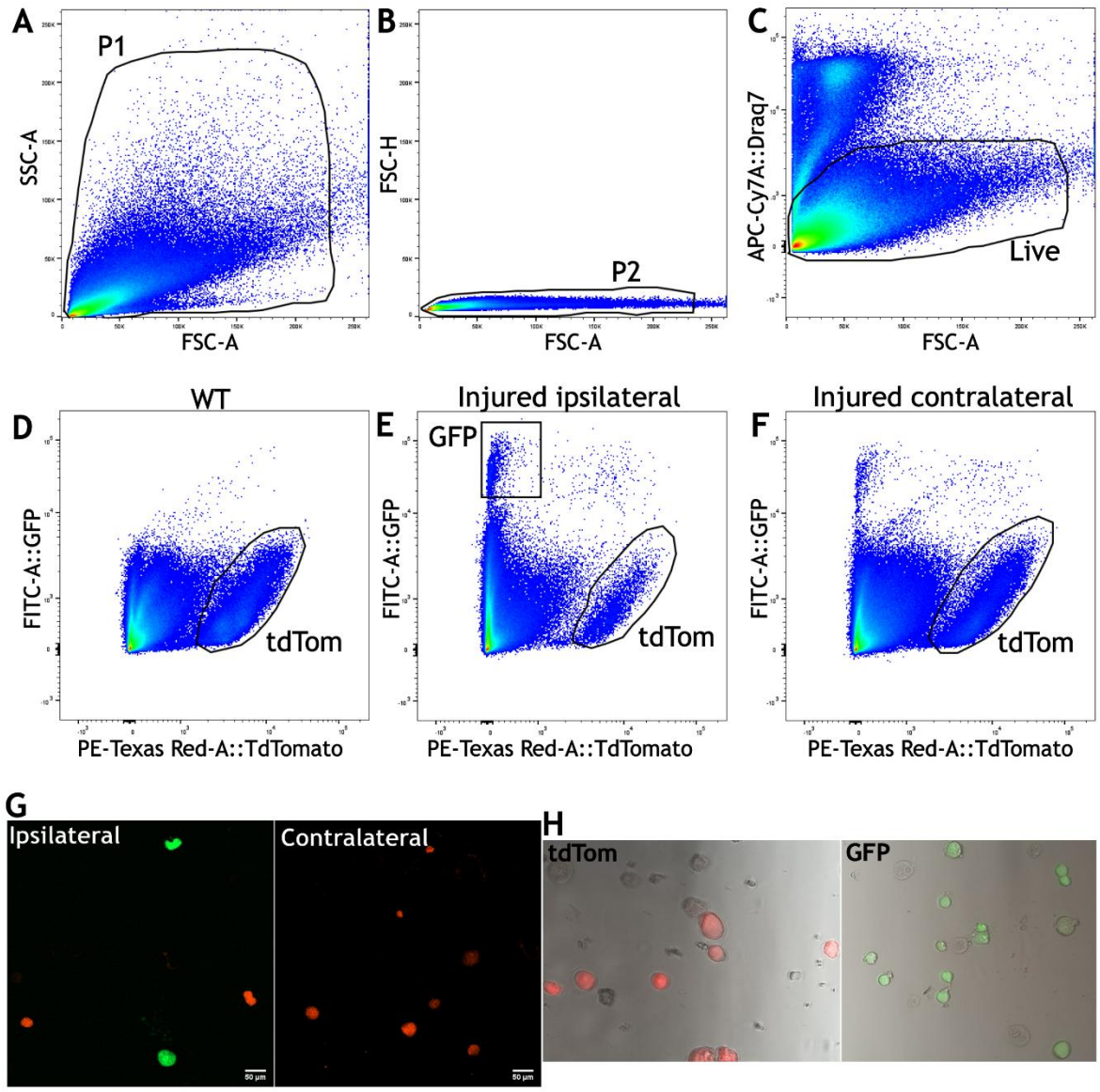


Figure 4.2 Sample collection for snATAC-seq*. All samples were gated for scatter* ***(A)****, doublets* ***(B)*** *and live/dead* ***(C)****, shown here for the WT sample. WT samples were gated for tdTomato fluorescence* ***(D)****, ipsilateral for GFP and tdTomato* ***(E)*** *and contralateral for tdTomato* ***(F)****. Images of ipsilateral and contralateral samples were taken prior to sorting* ***(G)****. Images of ipsilateral samples were also taken after sorting* ***(H)*** *to ensure correct neuronal populations were collected. tdTom; tdTomato, WT; wild type*

4.3.2 snATAC-seq quality control

High quality samples are required to ensure good quality sequencing results. To achieve this, we attempted to optimise the process of nuclei isolation for snATAC-seq. However, adjustments in lysis times and concentrations yielded inconsistent results depending on the status of source tissue (e.g. batch, frozen,

fresh, etc), prompting us to instead visually assess nuclei quality on the day of library preparation using brightfield microscopy. Our final samples were sequenced at sufficient read depth and contained large numbers of peaks (Table 4.2). Median high-quality fragments per cell, used to evaluate overall depth and quality of sequencing, were >10,000 for all samples and the proportion of sequenced fragments mapping to regions of open chromatin, defined as the fraction of high-quality fragments overlapping peaks, was over 15% (Table 4.2). These metrics suggest robust coverage across cells and good capture of open chromatin across regions. All samples were accompanied by a report summarising sample performance using different metrics relating to cell detection, fragment and insert size distribution, TSS enrichment, and single cell targeting (Figure 4.3). These also indicated good sample quality and sequencing and correlated with further quality control metrics which were computed for all libraries (Figure 4.4). Samples were filtered based on these metrics to remove low quality cells as indicated by sequencing depth (Figure 4.4A) and proportion of fragments falling within ATAC-seq peaks (Figure 4.4B). Cells with low TSS enrichment, high nucleosome signal and with reads mapping onto blacklist regions, areas often associated with artefactual signal, were also removed from downstream analyses (Figure 4.4C-E). In all samples, most peaks mapped to distal intergenic regions and introns, suggesting distal non-coding *cis*-regulatory elements could be accessed to regulate gene expression (Figure 4.4F).

Sample	Mean raw read pairs per cell	Number of peaks	Median high-quality fragments per cell	Fraction of high-quality fragments overlapping peaks
WT	45,230	163,864	12,996	51.1%
Intact (tdTomato)	38,429	285,185	10,064	59.5%
Injured (GFP)	46,128	188,281	16,260	50.7%
Contra	38,561	277,078	9,212	60.3%

Table 4.2 Summary metrics for snATAC-seq samples.

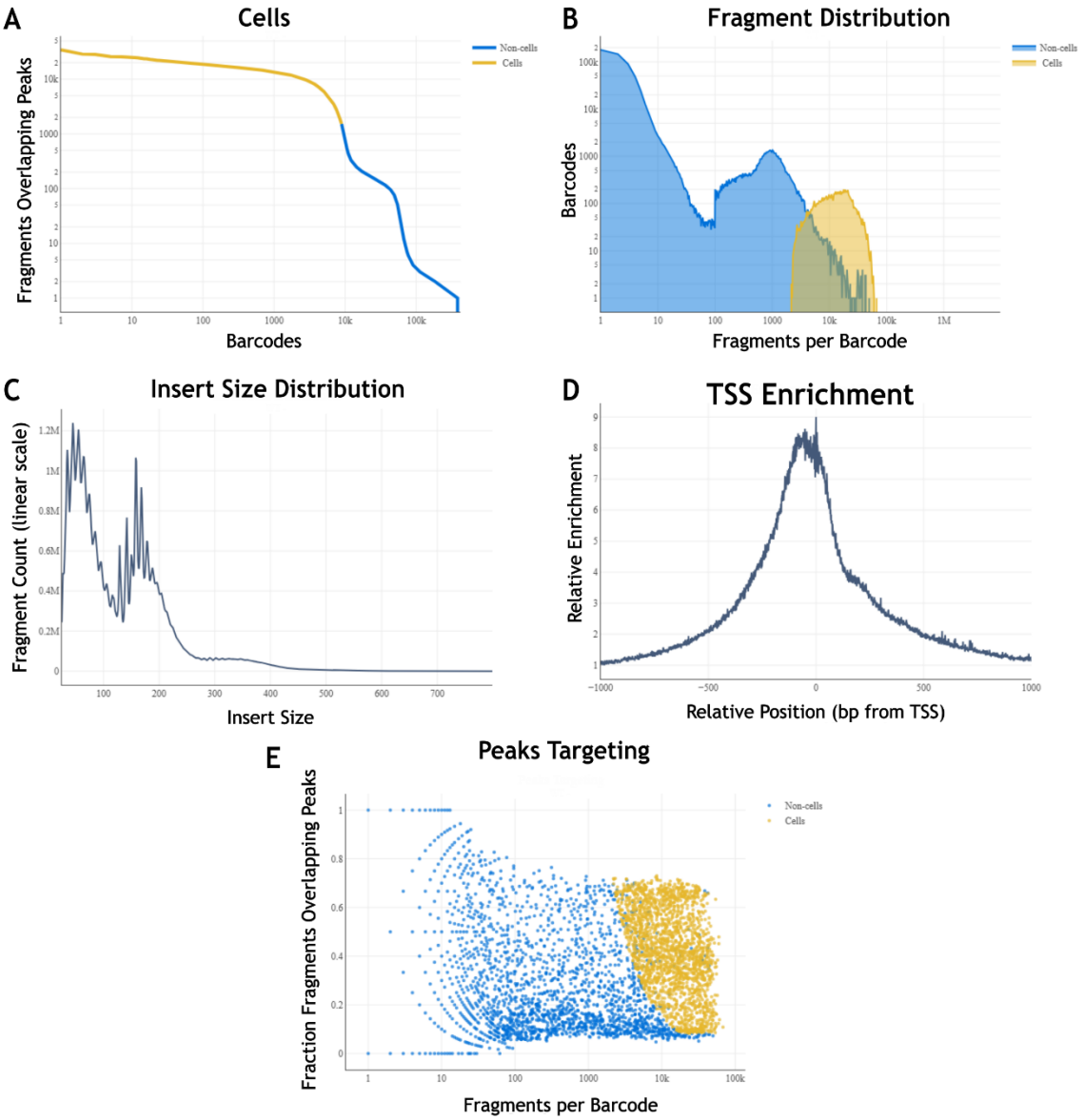


Figure 4.3 10X web summary plots for the WT sample. *Plots included in 10X web summary file supplied to provide a reference for determining sample performance in snATAC-seq assay.* ***(A)*** *The Barcode Rank plot provides information about barcodes determined to be cells by plotting fragments overlapping peaks. A steep drop-off*

indicates good separation between empty barcodes and barcodes associated with cells. ***(B)*** *Fragment distribution plot showing the number of fragments per barcode for cells and non-cells. Good separation between cells and non-cells indicates appropriate distinction between the two groups.* ***(C)*** *Insert size distribution plot demonstrating the distribution of transposase accessible fragments sequenced. Sawtooth pattern indicates a periodicity of approximately 150-200bp.* ***(D)*** *Transcription start site (TSS) plot indicating enrichment around the TSS. This is determined by calculating the number of cut sites per base of all barcodes in a 2kb window around the full set of annotated TSSs.* ***(E)*** *Scatterplot showcasing the number of fragments per barcode and the fraction of fragment overlapping peaks. Barcodes associated with cells are expected to have a large number of fragments per barcode and a high fraction of fragments overlapping peaks. TSS; transcription start site*

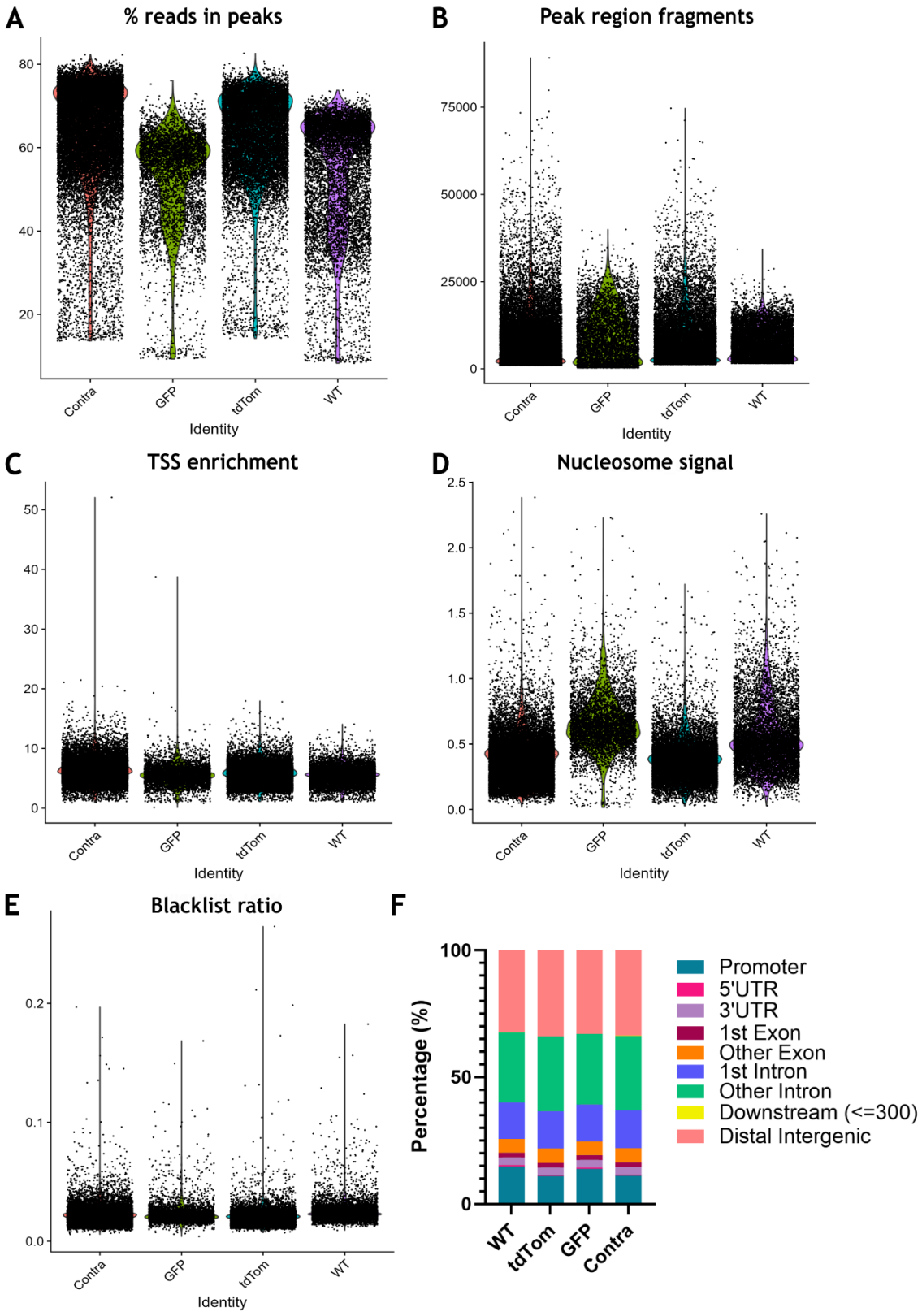


Figure 4.4 Quality control metrics for snATAC-seq. ***(A)*** *Percentage of reads in peaks refers to the proportion of reads falling within regions of accessible chromatin (peaks). A high percentage is indicative of genuine regions being captured while low proportions suggest issues with sample quality.* ***(B)*** *Peak region fragments refer to the DNA*

fragments located within identified peaks. ***(C)*** *TSS enrichment measures read density around TSS regions compared to background regions. A high TSS enrichment score (> 4) denotes high sample quality.* ***(D)*** *Nucleosome signal reveals the position of nucleosomes in chromatin. High signals indicate good nucleosome preservation and transposase accessibility.* ***(E)*** *Blacklist ratio refers to the proportion of reads falling within artifact-prone regions due to high background signal.* ***(F)*** *Proportion of peaks linked to different genomic annotations in each sample. TSS; transcription stat site, UTR; untranslated region*

Annotating clusters in ATAC-seq data can be challenging, especially in the absence of RNA-seq information, since less is known about the role of noncoding than protein coding genomic regions. Peaks found in promoters or in gene bodies are assumed to be good predictors of gene expression (Ackerman *et al.,* 2016; Starks *et al.,* 2019; Granja *et al.,* 2021; Yang *et al.,* 2022). Using chromatin accessibility as an approximation of gene expression, we created gene activity assays, allowing us to distinguish between neuronal and non-neuronal clusters and further filter our samples. Representative data are shown here for the WT sample and remaining samples were subset similarly (Figure 4.5). We initially identified 12 distinct clusters, five of which expressed the markers *Rbfox3* (*NeuN*) and *Pou4f1* (*Brn3a*) (Figure 4.5A and B, E). Non-neuronal clusters were investigated for the expression of Schwann and SGC markers (Figure 4.5C and D) since we hypothesised these cell types were the most likely to also have been captured during FACS. Based on the expression of *Bcas1* and *Prx*, clusters 0-2 were deemed to be Schwann cells (Figure 4.5C and E). Cluster 3 highly expressed SGC markers *Kcnj10* and *Fabp7* while cell types in clusters 5, 10 and 11 could not be confidently identified (Figure 4.5D and E) (Avraham *et al.*, 2020; Jager *et al.*, 2022). SGCs and Schwann cells were likely sequenced due to their proximity to DRG neurons. All non-neuronal clusters were removed from downstream analyses in each sample. Overall, approximately 2.3% of all cells submitted for sequencing were analysed. The total number of cells collected, sequenced, filtered and analysed can be found in Table 4.3.

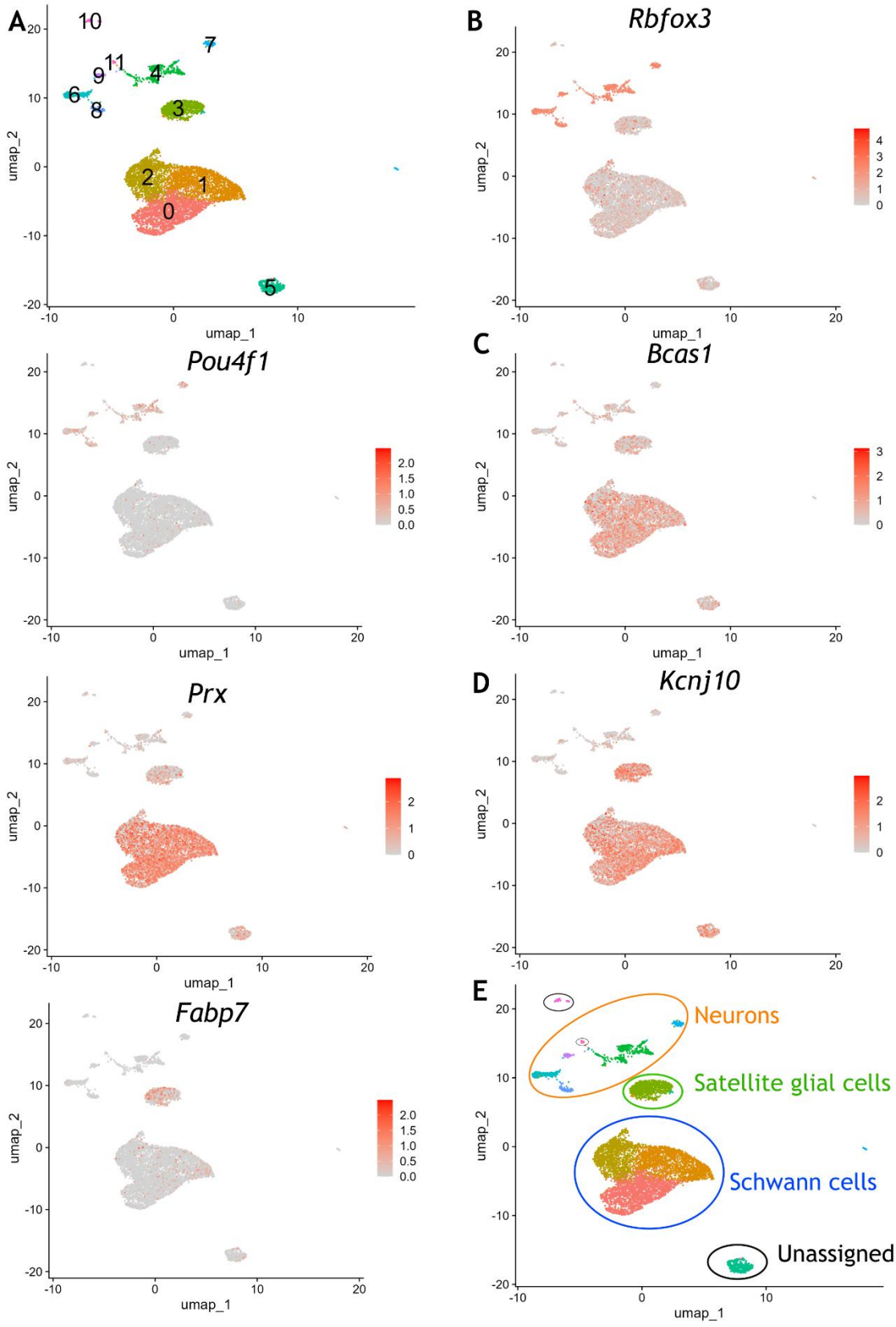


Figure 4.5 WT sample filtering. ***(A)*** *UMAP projection of unfiltered WT data.* ***(B)*** *Feature plots displaying NeuN (Rbfox3) and Brn3a (Pou4f1) expression across individual cells in the WT sample. High expression can be seen in Seurat clusters 4, 6, 7, 8 and 9.* ***(C)*** *Feature plots of Schwann cell markers brain enriched myelin associated protein (Bcas1) and periaxin (Prx) in WT sample, showing enrichment in Seurat clusters 0, 1 and*

2. ***(D)*** *Feature plots of satellite glial cell markers Kcnj10 (encoding the Kir4.1 channel subunit) and fatty acid binding protein 7 (Fabp7) showing enrichment in cluster 3.* ***(E)*** *UMAP projection of WT sample with annotated Seurat clusters.*

Sample	Collected (FACS events)	Sequenced	After QC	Neurons Analysed
WT	62,913	8,906	8,169	1,355
Intact (tdTomato)	94,508	13,607	12,335	2,986
Injured (GFP)	35,012	5,134	4,700	643
Contralateral	189,773	20,002	18,149	3,965

Table 4.3 Number of cells collected, sequenced and analysed for each sample*. QC; quality control*

4.3.3 Chromatin accessibility in naïve DRG neurons

Analysis was first performed on our WT and contralateral, or naïve, samples. We identified 8 distinct clusters in our WT sample, annotated using the Usoskin *et al.* (2015) nomenclature; PEP1, NP1, NP2, NP3, TH, NF1, NF4 and Trpm8 and defined by accessibility of canonical subpopulation marker genes (Figure 4.6A). Our contralateral sample contained similar populations but lacked Aδ-LTMRs (NF1) and proprioceptors (NF4). Instead, a cluster containing Aß-field and Aß-RA-LTMRs, marked by *Kcnj14* expression, was observed (Figure 4.6B). Given the similarity of the two samples and under the assumption that WT and contralateral DRG neurons would be biologically equivalent, we decided to integrate the two datasets, resulting in a combined dataset containing all of the aforementioned populations except Aδ-LTMRs (Figure 4.6C and D). Instead, Aδ-LTMR markers, such as *Ntrk2* and *Scn5a* shown here (Figure 4.6E), appeared to lack specificity i.e. their accessibility was not selective to discrete clusters. Our naïve dataset captures the majority of sensory neuron populations identified with RNA-seq but lacks the granularity to distinguish certain closely related subtypes, such as peptidergic neurons, into more than one class. Some cluster markers, such as *Mrgpra3* and *Nppb*, were accessible only in specific populations compared to other markers, such as *Cacna1i* and *Trpm8* (Figure 4.6E). This is not unexpected, since the gene activity matrix is only an approximation of gene expression. Identities were assigned by computing differentially accessible genes

using the gene activity matrix and checking for the expression of known cluster markers. A manual search was also performed, and clusters were called based on the expression of at least two distinct markers (Figure 4.6E). All further comparisons were carried out using our integrated, **naïve** dataset. These results illustrate that, despite the reduced cell number caused by glial contamination, we could still create a representative naïve mDRG atlas.

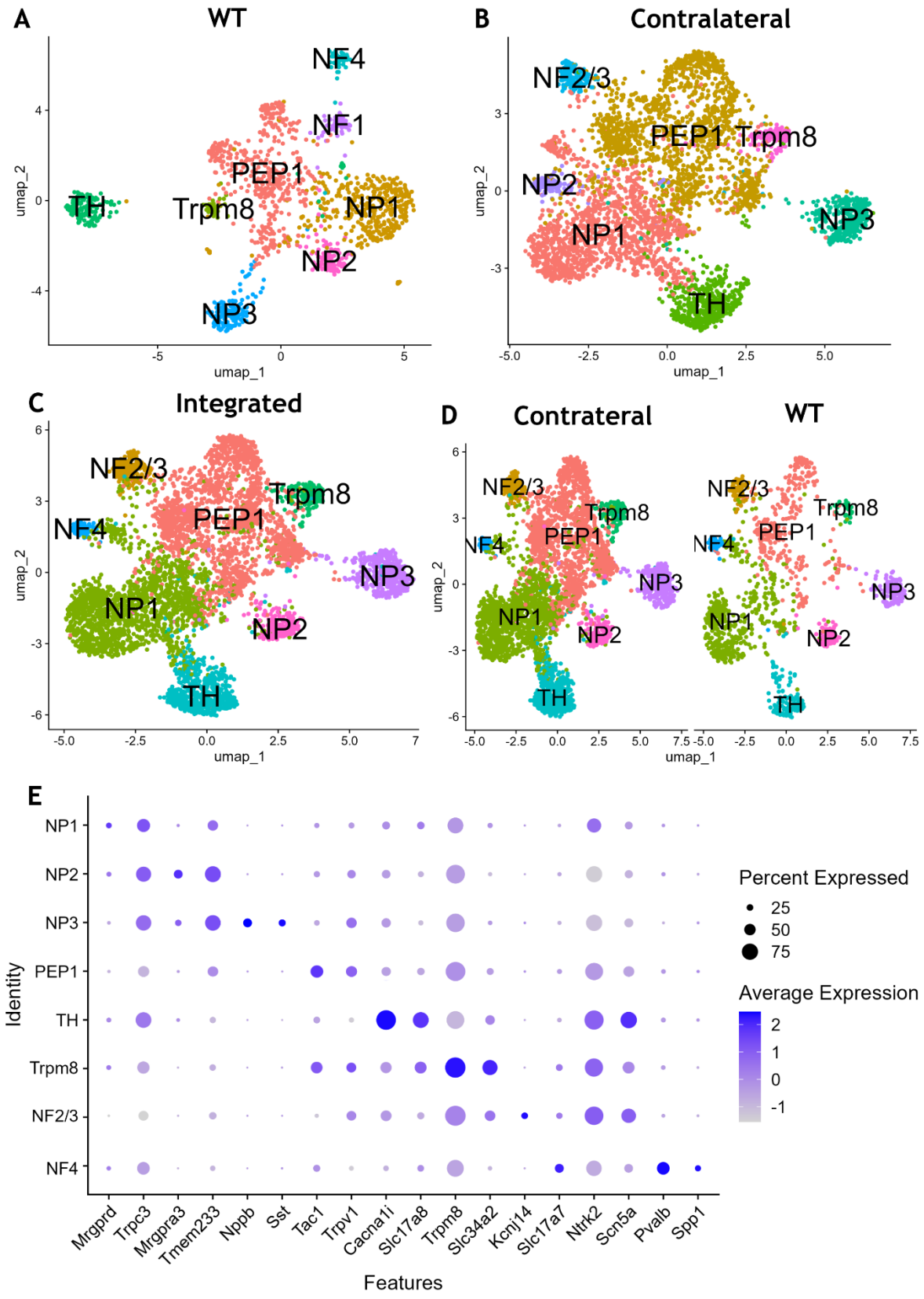


Figure 4.6 Epigenomic atlas of naïve mouse DRG neurons. *UMAP projections of 1,355 WT* ***(A)****, 3,965 contralateral* ***(B)*** *and 5,320 integrated* ***(C)*** *nuclei, annotated by cell type.* ***(D)*** *Integrated WT and contralateral (naïve) UMAP split by condition.* ***(E)*** *Dot plot of average expression for two markers for each cell subtype. WT; wild type*

4.3.4 Comparison of chromatin accessibility in injured DRG neurons

It is known that following peripheral nerve injury, loss of subtype specific expression and cell death occur in the DRG (Renthal *et al.*, 2020; K. Wang *et al.*, 2021; Cooper *et al.*, 2024). Consistent with these observations, our injured (GFP) sample was comprised of fewer populations than all other samples (Figure 4.7A). Although this may be due to the smaller sample size (Table 4.3), the absent populations, namely nonpeptidergic and cold-sensing neurons (Figure 4.7A), align with those suggested to die following SNI (Cooper *et al.*, 2024). When integrated with our naïve dataset, the GFP sample clustered separately from naïve neurons, suggesting major changes in cell identity after injury are reflected in chromatin accessibility (Figure 4.7B and C). *Sprr1a*, encoding small proline-rich repeat protein 1A (Sprr1a), was found to be differentially accessible in GFP nuclei while other known injury markers, such as *Atf3* shown here, were more widely expressed (Figure 4.7D). This could be due to the rapid upregulation of *Atf3* mRNA during the process of DRG extraction, dissociation, FACS sorting and cryostorage and is a phenomenon also observed with RNAseq (Zhang *et al.*, 2022). GO analysis found GFP nuclei were enriched for pathways associated with membrane potential regulation, neurogenesis and synaptic structure and organisation (Figure 4.7B), consistent with transcriptomic and bulk ATAC-seq findings (Stephens *et al.*, 2021, 2024; Zhang *et al.*, 2022; Barry *et al.*, 2023).

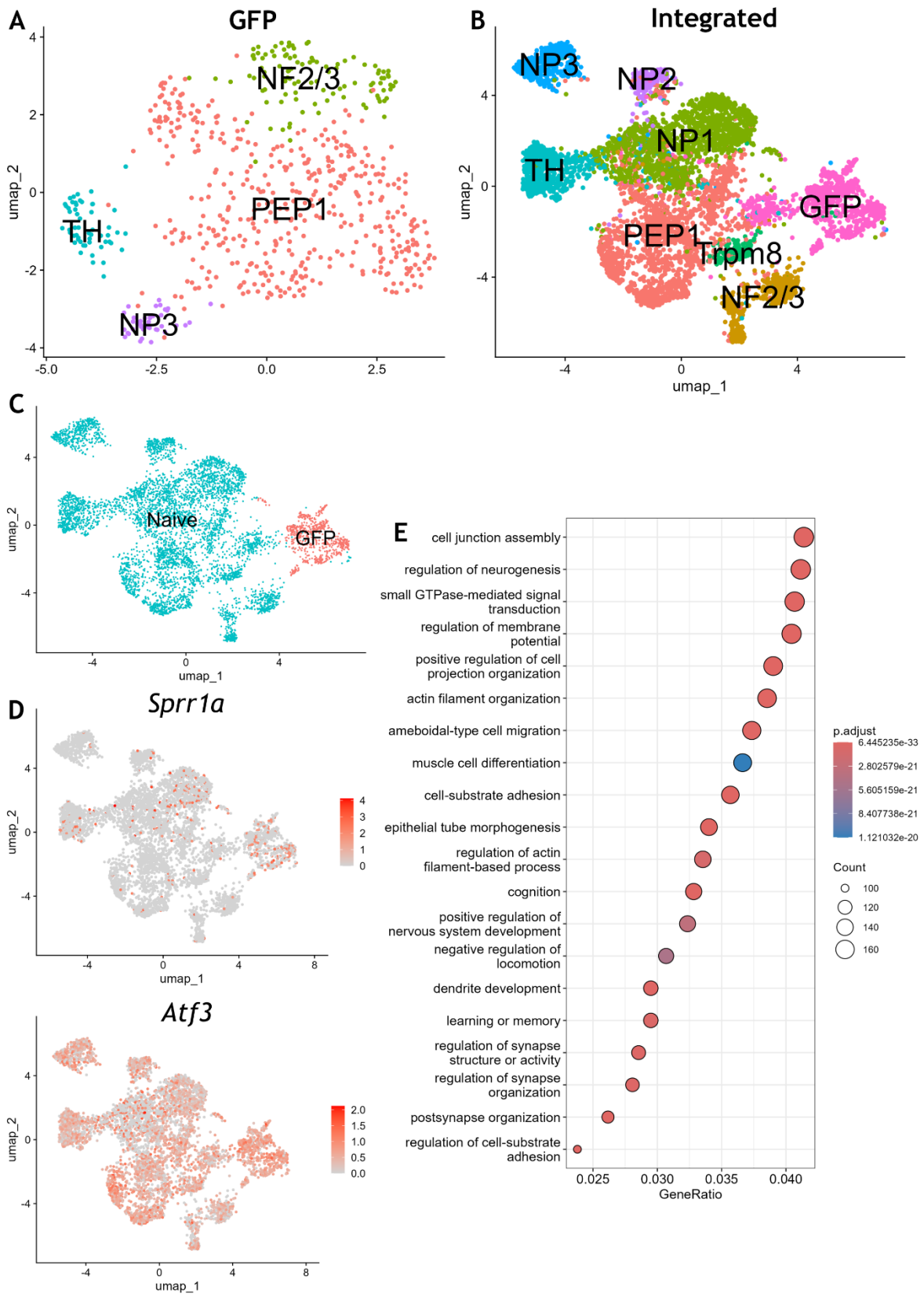


Figure 4.7 Injured sample analysis. *UMAP projection of 643 injured (GFP) nuclei alone* ***(A)*** *and integrated with naïve data* ***(B)*** *also shown grouped by condition* ***(C)****.* ***(D)*** *UMAP plots showing Sprr1a and Atf3 expression in the integrated dataset.* ***(E)*** *Top 20 GO analysis results for biological processed enriched in the GFP cluster. GO terms indicate the involvement of processes related to tissue organisation, growth and response to injury. Input values were filtered based on an FDR < 0.01 and Log2FC > 0.5.*

We plotted chromatin accessibility at the loci of known RAGs, extending 100kb upstream and downstream of the TSS to include both potential proximal and distal enhancers (Figure 4.8) (Graybuck *et al.*, 2021). While many peaks appear to be shared between clusters, particularly at the genomic regions of *Atf3*, this does not necessarily equate to gene expression. Closer inspection and differential accessibility analysis revealed peaks enriched in the GFP cluster.

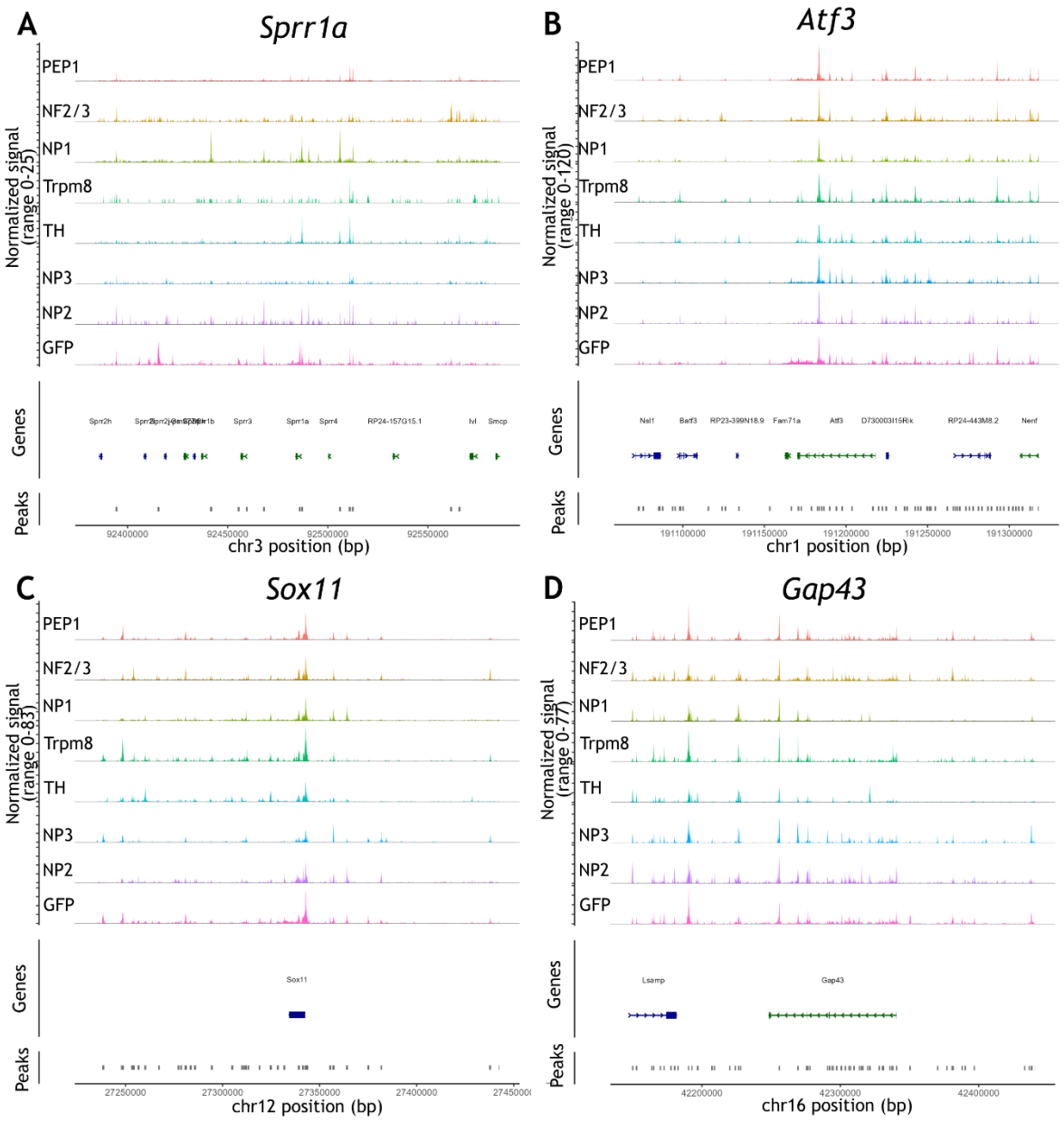


Figure 4.8 Coverage plots for genes associated with peripheral nerve injury. *Chromatin accessibility plotted at the genomic loci (TSS ± 100kb) of Sprr1a* ***(A)****, Atf3* ***(B)****, Sox11* ***(C)*** *and Gap43* ***(D)*** *in the injured GFP cluster of the integrated injured (GFP) and naïve datasets. The direction of transcription is designated by the arrows in the gene annotation.*

Ideal candidate elements for inclusion in viral vectors are small (a few hundred base pairs long), well conserved across species and preferentially accessible in the desired population compared to others (Graybuck *et al.*, 2021). *Sprr1a* was differentially accessible in nuclei from the GFP cluster and an investigation of chromatin accessibility 2kb upstream and downstream of its TSS already revealed peaks specific to the GFP cluster (Figure 4.9A). To assess the sequence conservation of the genomic regions or elements of interest, we used the UCSC genome browser on Mouse (GRCm38/mm10). The "Placental Mammal Basewise Conservation by PhyloP" track (Figure 4.9B) illustrates sequence conservation scores across placental mammals, with positive values indicating conserved elements, while the "Mulitz Alignment of 60 Vertebrates" track provides information on alignment depth across multiple species. The peak located at approximately 92,486,000bp likely corresponds to a promoter, given its proximity to the *Sprr1a* gene (Figure 4.9A) and conservation across mammals (Figure 4.9B). The sequence of the peak at roughly 92,487,000bp, however, does not appear to be conserved and or align with the human sequence (Figure 4.9B). Several criteria therefore need to be taken into consideration when identifying peaks of interest for further characterisation and functional testing. The initial peak analysis described here illustrates the presence of differentially accessible peaks in GFP+ neurons and supports the need for further detailed investigation of their potential to meet our aims.

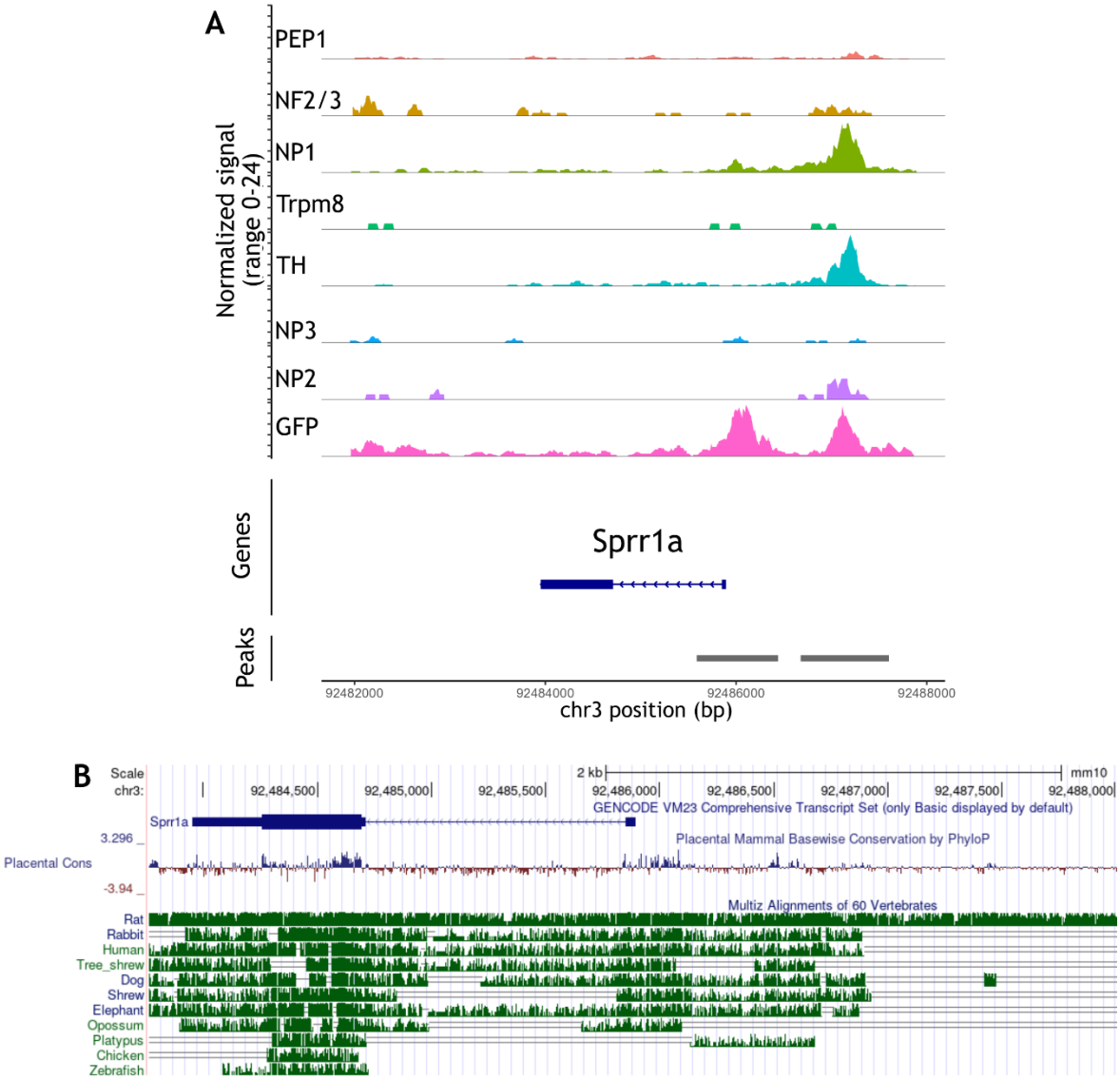


Figure 4.9 Chromatin accessibility and sequence conservation across species at the locus of Sprr1a. ***(A)*** *Coverage plot illustrating chromatin accessibility at the locus of Sprr1a (TSS ± 2kb) in nuclei from the integrated naïve and injured (GFP) samples.* ***(B)*** *Genomic region surrounding the Sprr1a locus, including gene annotations from GENCODE VM23. The "Placental Mammal Basewise Conservation by PhyloP" track illustrates sequences conservation scores among placental mammals, with positive values indicating conserved elements. The "Mulitz Alignment of 60 Vertebrates" track shows alignment depth across multiple species. Certain regions within and upstream of Sprr1a appear to be highly conserved.*

4.3.5 Chromatin accessibility in intact DRG neurons

Neurons adjacent to injured primary afferents were sorted separately based on tdTomato expression and largely resembled DRG neurons from our naïve

integrated dataset. A population defined by increased *Sprr1a* accessibility, however, was also identified (Figure 4.10A). Upon integration with our naïve dataset, good overlap was observed across neuron subtypes except for those displaying an "*Sprr1a* accessible" phenotype (Figure 4.10B and C). This suggests that at least a proportion of sensory neurons near injured primary afferents also undergo changes in their chromatin accessibility following injury.

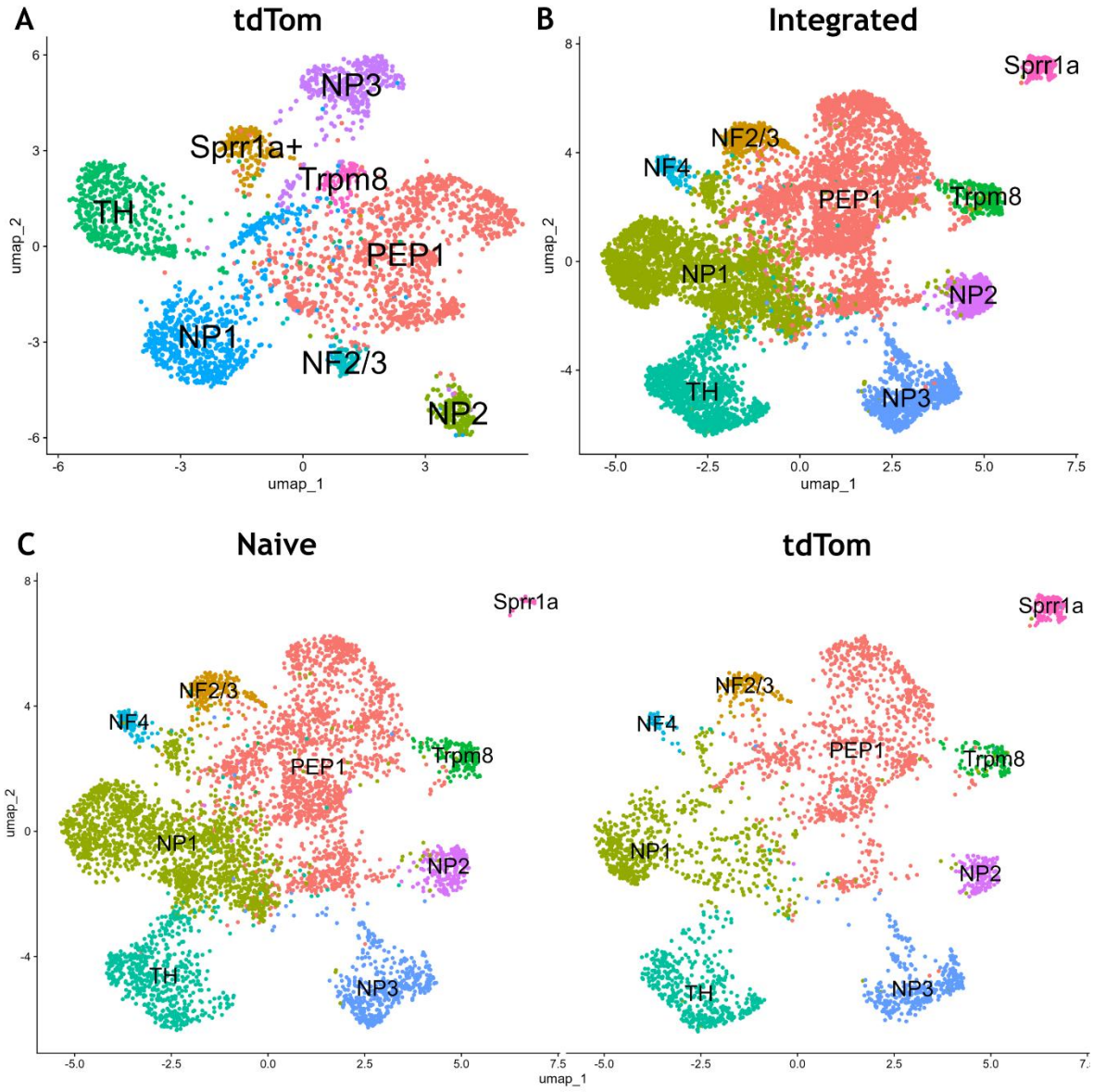


***Figure 4.10 Intact sample analysis**. UMAP projections of 2,986 intact (tdTomato) nuclei **(A)** and intact nuclei integrated with the naive dataset **(B)** and split by condition **(C)**. An injured cluster missing from the naïve dataset can be seen in the intact sample. tdTom; tdTomato*

4.4 Discussion

Chromatin structure plays a critical role in the regulation of gene expression by controlling the access of regulatory factors to DNA and can provide insight into cell status. We generated an atlas of chromatin accessibility in naïve mouse DRG neurons and examined epigenomic signatures in injured and intact neurons with the aim of identifying regulatory elements active only in damaged neurons. These data provide a resource for understanding epigenomic features governing cell-type specific expression patterns, studying DRG functions and investigating epigenomic mechanisms implicated in NeuP.

4.4.1 Library preparation

The DRG contain large numbers of non-neuronal cells, including Schwann cells, SGCs and immune cells, which are often captured and sequenced alongside neurons. Sample collection and library preparation processes can be optimised to reduce contamination, however this does not entirely prevent these cell types from being collected. We optimised our sample collection process by using a transgenic mouse line fluorescently labelling both injured and intact neurons to minimise non-neuronal cell sorting (Figures 4.1 and 4.2). Nevertheless, we discovered the presence of substantial numbers of both SCGs and Schwann cells in our samples (Figure 4.5). Some rarer non-neuronal clusters were also identified but the identities of their cells could not be confidently determined (Figure 4.5E). Non-neuronal contamination can interfere with library preparation and increases the number of input cells required for sequencing to ensure neurons are well represented in the final sample (Table 4.3). It also, however, presents the opportunity to study these non-neuronal populations in the context of chronic pain. For instance, both Schwann cells and SGCs are activated and undergo changes in response to peripheral nerve injury, contributing to chronic pain development (Liu *et al.*, 2012; Hartlehnert *et al.*, 2017; De Logu *et al.*, 2019; Avraham *et al.*, 2020; Jager *et al.*, 2020, 2022; Rinwa *et al.*, 2021; Lovatt *et al.*, 2022). Studying the epigenomic profiles of these cell types in healthy and diseased states can therefore help improve our understanding of their role in chronic pain and identify new therapeutic pathways.

4.4.2 Epigenomic atlas of naïve DRG neurons

Our integrated, epigenomic atlas of naïve DRG neurons contains 8 distinct neuronal subtypes (Figure 4.6). These were identified in the absence of transcriptomic information and correspond to the populations identified in Usoskin *et al.* (2015), indicating that chromatin accessibility is a good predictor of gene expression. Furthermore, expression patterns for marker genes in each cluster align well with transcriptomic data (Zeisel *et al.*, 2018). Despite our ability to capture the major neuronal populations, our data does not have the granularity to distinguish certain closely related subtypes, such as peptidergic neurons, into more than one class. Markers for these subpopulations were detected following differential expression analysis using our gene expression matrix, however, suggesting that they could potentially be identified if a higher number of neurons was supplied. Overall, our epigenomic atlas provides insight into the regulatory landscape of sensory neurons, showing a strong correlation between chromatin accessibility and gene expression. This is a valuable resource which can be used to discover regulatory elements driving gene expression in sensory neuron subtypes as well as elements controlling the expression of pain-associated genes. More specifically, identifying enriched TF motifs within distinct cell populations and conditions (e.g. injured vs intact) can provide insight into key regulatory drivers of different cell types and cell states. The atlas can also serve as a benchmark for comparative analyses and when integrated with transcriptomic data, it can be used to highlight pathways and construct gene regulatory networks. It is also possible to link peaks to specific genes using a variety of tools, such as ArchR, further enhancing our understanding of functional enhancer-promoter relationships and the regulation of chromatin accessibility and subsequently gene expression (Granja *et al.*, 2021).

While populations in this atlas were confidently identified, integrating our dataset with transcriptomic data from DRG neurons could more directly map how chromatin accessibility translates into gene expression. Additionally, integration with human snATAC-seq datasets could provide insight into the evolutionary conservation or divergence of regulatory elements in sensory neurons across species, potentially improving the translatability of future findings. Integration with epigenetic data from TG neurons, on the other hand,

would enable the comparison of regulatory elements between the two cell types while also providing information on shared and distinct pain mechanisms and drug targets. Finally, it is possible to intersect accessible chromatin regions with disease-associated variants, which could shed light on how genetic risk can influence chromatin architecture and gene expression in chronic pain.

4.4.3 Chromatin accessibility in injured and intact primary afferents

It is known that following injury, sensory neurons enter a common transcriptional programme, experiencing loss of identity and undergoing transcriptional changes to facilitate processes such as axonal regeneration and the development of hyperexcitability (Nguyen, Le Pichon and Ryba, 2019; Renthal *et al.*, 2020; K. Wang *et al.*, 2021; Zhang *et al.*, 2022; Barry *et al.*, 2023). Neurons from our injured dataset were distinguished into four clusters; peptidergic neurons (PEP1), itch-sensing neurons (NP3), C-LTMRs (TH) and Aß-fibres (Figure 4.7A). Cell loss after injury impacts primarily non-peptidergic nociceptors marked by *Mrgprd* expression, which were absent from our sample (Cooper *et al.*, 2024). It is difficult, however, to confidently determine whether the reduced number of subpopulations we identified was due to the downregulation of subtype specific gene expression, death of neurons, or both. When integrated with our naïve dataset, injured nuclei clustered separately and were enriched for pathways associated with membrane potential regulation, neurogenesis and synaptic organisation and activity (Figure 4.7B and C,E), consistent with observations from both transcriptomic and prior epigenomic data (Nguyen *et al.*, 2017; Palmisano *et al.*, 2019; Renthal *et al.*, 2020; K. Wang *et al.*, 2021; Stephens *et al.*, 2021, 2024; Zhang *et al.*, 2022). The injury marker *Sprr1a* was found to be differentially expressed in the GFP compared to naïve clusters while other RAGs, such as *Atf3*, were more widely expressed as determined using the gene activity matrix (Figure 4.7D). *Atf3* is rapidly induced following injury and might therefore not be an ideal marker for injured neurons within our datasets (Renthal *et al.*, 2020).

Despite RAGs such as *Atf3*, *Sox11* and *Gap43* showing similar degrees of accessibility in naïve and injured neurons, differentially accessible peaks between the GFP and naïve clusters can be identified (Figure 4.8). By looking for

peaks 100kb upstream and downstream of the TSS we are hoping to identify both proximal and distal regulatory elements that would be capable of distinguishing injured from naïve primary afferents while also being suitable for vector packaging. In addition to looking for regulatory elements with injury-specific activity in neurons, we aim to pinpoint peaks that are well conserved between species, as exemplified here by the peaks identified within 2kb upstream of the TSS for *Sprr1a* (Figure 4.9). Furthermore, we plan to expand our search beyond injury associated genes to discover novel regulatory elements which could be used to differentiate between injured and intact DRG neurons.

Sensory neurons populations identified in our intact sample largely matched those seen in our naive dataset (Figure 4.10A). One cluster, however, was present in the intact but missing from the naïve samples and was marked by increased chromatin accessibility near the *Sprr1a* locus (Figure 4.10B and C). Intact, injured-adjacent DRG neurons undergo transcriptomic, neurochemical and functional changes and there is increasing evidence of their contribution to NeuP, especially evoked hypersensitivities (Pertin *et al.*, 2005; Shim *et al.*, 2005; Djouhri *et al.*, 2006; Chen *et al.*, 2018; Yang *et al.*, 2018; Tran and Crawford, 2020; Kalpachidou *et al.*, 2022; Zhang *et al.*, 2022). While it is possible such neurons contributed to the injured cluster, a more likely explanation is that our intact sample was contaminated with GFP+ neurons accidentally sorted during FACS. Comparisons of Sprr1a+ and GFP (injured) nuclei are necessary to determine the degree of similarity between the two populations and uncover conserved as well as unique features in each population.

In this chapter, we have provided a comprehensive epigenomic atlas of naïve DRG neurons, giving valuable insights into the regulatory landscape underlying sensory neuron diversity. By profiling chromatin accessibility in injured and intact primary afferents we hope to improve our understanding of epigenetic changes in response to axonal injury and identify regulatory elements capable of restricting transgene expression to injured primary afferents. The datasets presented here offer numerous opportunities for further comparisons, including transcriptomic and cross-species analyses. Such comparisons have the potential

to uncover conserved regulatory mechanisms and novel targets for the treatment of NeuP.

Chapter 5 MicroRNA-based approach for targeting injured primary afferents

5.1 Introduction

MicroRNAs are a type of non-coding RNA and play an important role in regulating gene expression by binding to target mRNAs and interfering with their translation. Their expression is often tissue specific and becomes dysregulated in disease. Combined with their rapid onset of action, these characteristics render endogenous miRNA systems excellent candidates for controlling AAV-mediated transgene expression. In this chapter, we set out to identify miRNAs downregulated in the DRG after axotomy and incorporate their target sites in AAV vectors in order to restrict transgene expression to injured primary afferents.

5.1.1 MicroRNA-regulated viral vectors

MiRNAs often have cell- or tissue-specific expression, with approximately 50% of miRNAs in a cell being cell type specific, one quarter being widely expressed, and the remaining being lowly expressed across all cell types (de Rie *et al.*, 2017). This specificity combined with the RNA interference (RNAi) mechanism of miRNAs can be harnessed to develop viral vectors with improved target specificity. In addition, despite their slightly longer half-lives compared to other non-coding RNAs, ranging from a few days to a few weeks, miRNAs are able to degrade their mRNA targets quickly and efficiently, thus not oversaturating the cellular RNAi machinery (Grimm *et al.*, 2006; Kingston and Bartel, 2019).

There are several miRNA-based strategies for controlling AAV gene expression. The most common approach involves the introduction of miRNA target sites in the 3' UTR of the transgene carried by the vector to reduce transgene expression (miR-OFF system) (Figure 5.1). These sites can have perfect complementary with the miRNA of interest, resulting in complete transgene degradation upon miRNA binding, or can contain mismatches, mimicking endogenous mechanisms and offering the option to fine-tune transgene expression levels through mRNA destabilisation. Brown *et al.* (2006) was the first to employ this approach and design a vector containing target sequences for miR-142, a miRNA enriched in

haematopoietic cells, effectively preventing transgene expression in haematopoietic lineages and maintaining it in non-haematopoietic cells. Since then, this approach has been exploited to develop vectors for targeted gene delivery across a range of systems and diseases, including the CNS (Geisler and Fechner, 2016; Gleichman *et al.*, 2023). Additionally, inclusion of miRNA target sequences can help reduce immunity and toxicity associated with viral vector gene therapies by causing transgene degradation in tissues susceptible to toxicity, particularly the DRG (Domenger and Grimm, 2019; Xiao *et al.*, 2019; Hordeaux *et al.*, 2020).

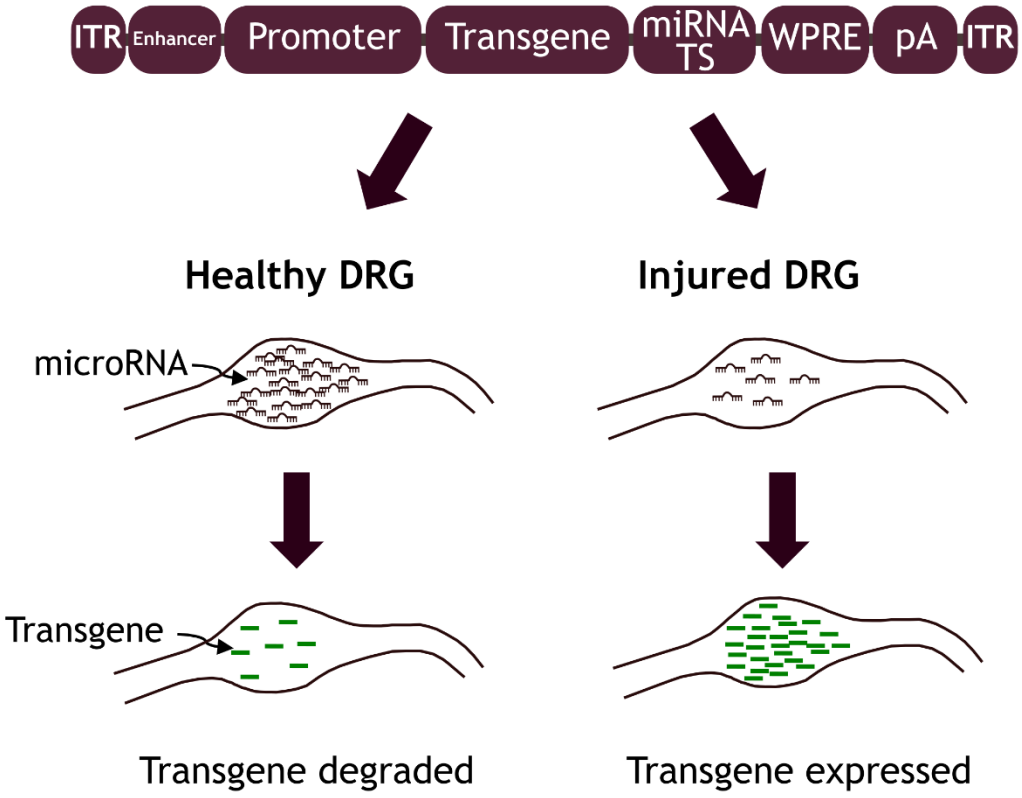


Figure 5.1 Schematic of miRNA-mediated regulation of AAV gene expression in the DRG. *The miRNA portion of the vector, located in the 3' UTR of the transgene, contains binding sites which are perfectly complementary to miRNA(s) downregulated in the DRG following injury. In healthy DRG, the highly expressed miRNA binds these sites, leading to transgene degradation. In injured DRG, however, where miRNA expression is lower, the transgene is expressed. The degree of complementarity between the binding sites and miRNA can be modified to fine-tune transgene expression levels. ITR; inverted terminal repeat, pA; polyadenylation signal, TS; target sites, WPRE; woodchuck hepatitis virus post-transcriptional regulatory element*

Depending on their miRNA signatures, de-targeting certain cells and tissues using the miR-OFF approach is not always possible. An alternative is to use the miR-ON system whereby miRNA binding downregulates the expression of bacterial repressor proteins binding to operator sequences in the plasmid. As a result, gene expression is activated in tissues expressing the miRNA of interest. MiRNAs are normally used in tandem with other strategies for regulating and targeting AAV-mediated gene expression, such as synthetic promoters/enhancers and modified capsids. It is possible to combine these into one vector to maximise specificity (Gleichman *et al.*, 2023).

Several factors need to be taken into consideration when designing vectors containing miRNA target sites. Candidate miRNAs need to have cell- and tissue-specific expression that surpasses the expression threshold of 100 copies/pg of small RNA (Brown *et al.*, 2007). It has been suggested that higher miRNA expression levels lead to faster and greater suppression of gene expression, however, this is not a linear relationship and exceptions have been observed (Geisler *et al.*, 2011; Kozomara *et al.*, 2014). Finally, the configuration of the target sites dictates the degree of transgene degradation, with perfect complementarity resulting in stronger repression of gene expression. Multiple repeats of miRNA target sequences are often included in AAV vectors. While greater degradation is observed with an increasing number of target sites, this relationship is also not linear and maximal repression can be achieved with 3-4 repeats (Geisler and Fechner, 2016).

5.1.2 The miR-183 family

A third of miRNAs can be found in genomic clusters, where members originate from the same hairpin (Baskerville and Bartel, 2005). The highly conserved miR-183 cluster is comprised of miR-96, -182, and -183 and plays a key role in sensory organ development, with its expression being largely restricted in tissues such as the retina, olfactory epithelium, ear, and the DRG (Pierce *et al.*, 2008; Aldrich *et al.*, 2009; Peng *et al.*, 2017; Hordeaux *et al.*, 2020). Despite their sequence homology, small differences in the sequences of these miRNAs result in common and distinct mRNA targets, often within the same pathways. In addition to being implicated in a range of autoimmune, cancer, psychiatric and neurological disorders, the miR-183 cluster is essential for modulating noxious

mechanosensitivity. Deletion of the cluster in sensory neurons results in mechanical hypersensitivity and hyperalgesia by supressing *Cacna2d1/2* expression and increasing Aδ-LTMR sensitisation (Dambal *et al.*, 2015; Peng *et al.*, 2017). Transcriptomic analysis of DRG from wild type and miR-183 cluster-deficient mice found more genes are upregulated in the later after neuropathy, suggesting the cluster normally suppresses some injury related changes in gene expression. In addition, the cluster potentially targets nerve-injury induced TFs such as *Atf3*, *Jun* and *Sox11* (Peng *et al.*, 2017). Hordeaux *et al.* (2020) took advantage of the miR-183 cluster expression in the DRG to create vectors containing miR-183 target sites capable of inhibiting transgene expression in sensory neurons, thus reducing AAV-associated toxicity in nonhuman primates. This demonstrates members of the miR-183 family are sufficiently expressed in healthy DRG to regulate transgene expression. Nerve injury leads to downregulation of the cluster in the DRG while expression in most other tissues remains unaffected, rendering the miRNAs attractive candidates in theory for guiding transgene repression in intact afferents, while allowing for expression in injured neurons (Aldrich *et al.*, 2009; Peng *et al.*, 2017; Asadi *et al.*, 2021).

5.1.3 Aim

This chapter aims to develop a viral vector containing miRNA target sites that is capable of restricting transgene expression to injured but not intact primary afferents.

5.2 Methods

5.2.1 Animals

ATF3CreERT2:Ai9 mice were used irrespective of their alleles for Cre and Ai9. Neonate mice (P1 pups) were subcutaneously injected with AAV using a Hamilton syringe. Mice underwent SNI once they reached adulthood and L3-L5 ipsilateral and contralateral DRG were collected 2 weeks later. Spinal cords and muscle from the operated area were also collected. The muscle was treated and stored similarly to the DRG while spinal cords were fixed in PFA overnight at 4°C before being stored in 30% sucrose.

5.2.2 Plasmids and AAVs

The control plasmid contained GFP under the control of the ubiquitous sCAG promoter. The experimental plasmid included the addition of target sites for miR-182 in the 3' UTR of the transgene and before WPRE (Figure 5.2).

Designing and annealing oligos

The miRNA sequence was obtained from the public database www.mirbase.org (hsa-miR-182, MI0000272/MIMAT0000259). Complementary oligos consisting of four repeats of the reverse complementary miR-182-5p sequence flanked by EcoRI and HindIII binding sites on either side were purchased from Integrated DNA Technologies (US). The oligos were resuspended and mixed in equimolar concentrations. A total of 2µg of each oligo was added to 50µl composed of r2.1 buffer (New England Biolabs, US) and nuclease-free water and was placed on a thermal block heated to 95°C for 5 minutes. Annealed oligos were allowed to cool down to room temperature by switching off the block.

Plasmid modification by annealed oligo cloning

The sCAG-GFP vector described in Chapter 3 was digested using the restriction enzymes EcoRI and HindIII and run on a 1.5% agarose gel. The digested DNA band was cut out and purified using a gel purification kit from Qiagen (Germany) and following the manufacturer's instructions. For the ligation, annealed oligos were diluted in nuclease-free water to a final concentration of approximately 8 ng/µl. This was mixed with the cut vector in 1:1, 3:1 and 5:1 molar ratios (vector: insert) and incubated with T4 ligase (New England Biolabs, US) and ligase buffer (New England Biolabs, US) at 16°C overnight. Competent *E.coli* cells were transformed with the final ligated product. Colonies were selected from the ratio yielding the highest colony count, with preference given to smaller, isolated colonies. DNA was extracted using a mini-prep kit (Qiagen, Germany) according to the manufacturer's instructions and sequenced to verify insert. This experimental miR-182 construct and the sCAG-GFP control plasmid were commercially packaged and serotyped with the AAV-PHP.S capsid protein by the Viral Vector Facility VVF at the University of Zurich to the final titres of 7.9 x 10^{13} GC/ml and 7 x 10^{13} GC/ml respectively.

5.2.3 Tissue processing and immunohistochemistry

DRG sectioning and staining were performed according to the protocol described in Chapter 2. GFP signal was not amplified in studies for this chapter since this would interfere with intrinsic signal intensity, a measure used to quantify our AAV's effectiveness in restricting transgene expression.

Spinal cord segments were notched on the contralateral ventral horn and sectioned using a Leica VT1200 vibrating blade microtome (Leica Biosystems Ltd, Germany) into 60µm sections in the transverse plane. Sections were incubated with primary antibodies (Table 2.1) diluted in PBS with 0.3% TX-100 and 5% normal donkey serum for 3 days at 4°C. After 3 PBS washes, sections were incubated with secondary antibodies overnight at 4°C, rinsed three times and mounted on glass slides with antifade medium.

5.2.4 HEK293T culture and transfection

HEK293T cells were cultured on glass coverslips in Dulbecco's Modified Eagle's Medium (Thermofisher Scientific, US) supplemented with 1% A/A and 10% foetal bovine serum and kept at 37°C, 5% CO_2. Cells were split at least once before transfecting. Once cultures had reached 50-70% confluency, cells were transfected using Lipofectamine™ 3000 reagent (Invitrogen, Thermofisher Scientific, US) previously incubated with 1µg plasmid DNA for 15 minutes at room temperature. Cells were fixed 24 hours later and mounted on a glass slide with antifade medium.

5.3 Results

5.3.1 Plasmid modification and production

The three members of the miR-183 family have been previously compared for their ability to prevent DRG toxicity by causing transgene degradation in DRG neurons. MiR-183 was chosen as the most effective candidate since it substantially reduced transgene expression while maintaining its expression in other tissues, including the liver, heart, muscle and brain cortex (Hordeaux *et al.*, 2020). In the same study, animals treated with miR-182 AAV experienced a significant reduction in cortical transgene expression concomitant to reduced

DRG expression. Since we wanted our vector to have as little off-target expression as possible, particularly in the nervous system, we chose miR-182 as our candidate system to employ. To construct our plasmid, we introduced four target site repeats in the 3' UTR of a plasmid containing GFP under the control of a sCAG promoter (Figure 5.2A). The human miR-182-5p sequence was used due to the high conservation between human and mouse (Figure 5.2B) (Xu *et al.*, 2007).

To ensure we created a functional plasmid, we tested its activity in HEK293T cultures (Figure 5.2C). HEK293T cells are a convenient and inexpensive initial testing platform since they express a wide array of miRNAs, including miR-182, and are easy to maintain and manipulate (Tian *et al.*, 2012). GFP expression was observed 24 hours post-transfection in comparable levels to those achieved with an identical control vector lacking miRNA target sites (Figure 5.2C and D). Although this could be an indication that the miR-182 plasmid was not functioning as expected, the levels and patterns of miR-182 expression in HEK293T cells are not well defined and could therefore be too low to have an observable effect on GFP expression (owing to high transgene levels following DNA transfection). Prior to packaging our plasmid into a vector, its sequence was confirmed by restriction enzyme digest and sequencing.

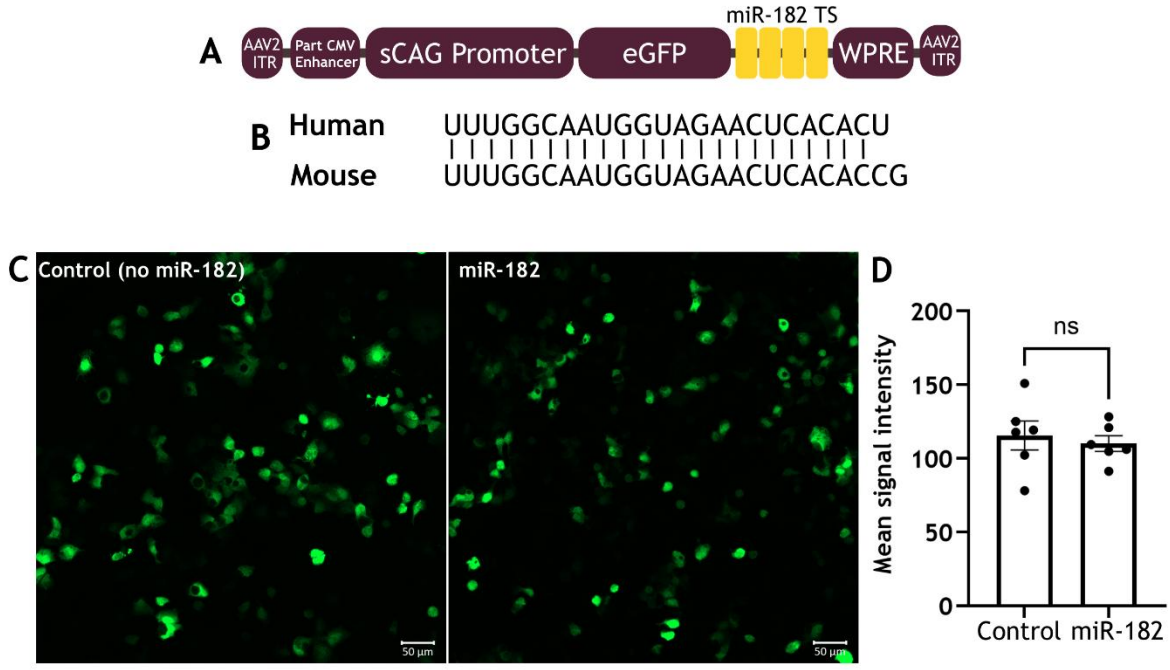


Figure 5.2 MiR-182 plasmid vector and activity in HEK293T cells. ***(A)*** *Map of miR-182 construct.* ***(B)*** *Human and mouse sequences of miR-182-5p, showing high degree of conservation.* ***(C)*** *Plasmid activity in HEK293T cells. HEK293T cells were transfected with either control (no miR-182) or experimental plasmids using Lipofectamine 3000.* ***(D)*** *Mean signal intensity of GFP+ cells 24 hours post-transfection. All data are presented as mean ± SEM. Unpaired t-test; t = 0.49, df = 10, p = 0.64, n = 6 coverslips pooled from 3 transfections. TS; target sites*

5.3.2 MiR-182 AAV activity in dissociated mDRG neurons

Despite the addition of our miR-182 plasmid in HEK293T cultures not providing any evidence of the target sites' functionality, we packaged both plasmids into AAV-PHP.S vectors known to preferentially target the PNS (Chan *et al.*, 2017). We hypothesised that since dissociated DRG neurons are injured, their miR-182 levels may be lower, resulting in higher GFP expression achieved with our miR-182 AAV. A lack of GFP-expressing neurons was observed after 24 hours for both control and miR-182 AAVs, suggesting this timepoint was not sufficient for the transgene to be expressed (Figure 5.3A). After 5 days, the control vector had successfully transduced 35.5 ± 5.53% of neurons (Figure 5.3B). Surprisingly, our miR-182 vector did not transduce any neurons, and we only observed GFP expression in glial cells (Figure 5.3A). This suggests that the miR-182 target sites

have a functional effect. However, it challenges our assumption that miR-182 expression is downregulated in DRG neurons in culture.

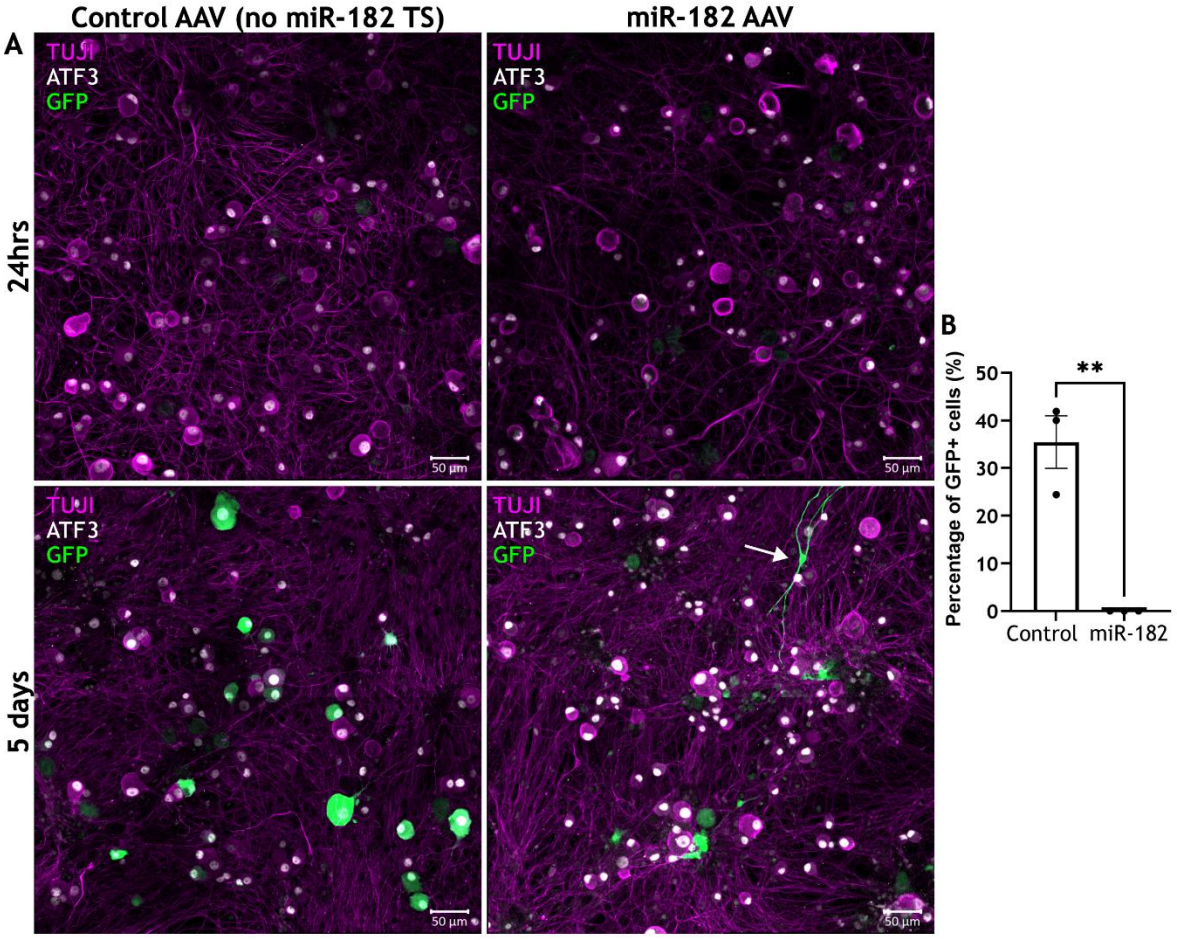


Figure 5.3 MiR-182 AAV activity in dissociated mDRG cultures. *(A) Mouse DRG neurons were transduced with either control or miR-182 AAVs for 24 hours or 5 days. GFP expression was detected 5 days post-transduction but was restricted to glial cells in the case of miR-182 AAV.* ***(B)*** *Quantification of AAV transduction efficiency after 5 days. Data is shown as mean ± SEM, with individual values plotted. Unpaired t-test; t = 6.41, df = 4, p = 0.003, **p < 0.005, n = 3 mice. TS; target sites.*

5.3.3 MiR-182 AAV activity in iPSC-derived sensory neurons

Following the unexpected results from mDRG cultures, we still did not have evidence that our miR-182 vector was active in naïve sensory neurons. We therefore introduced both vectors to iSNs for a total of 12 days before evaluating GFP expression 24 hours and 5 days after mechanical injury. We found a significant interaction between promoter and injury timepoint, suggesting the effect of the promoter differed depending on the time after injury (Table 5.1).

More specifically, our control vector resulted in significantly higher percentages of GFP+ cells in the injured conditions (55.6 ± 5.29% in naïve vs 94.3 ± 3.05% and 96.5 ± 1.30% after 24 hours and 5 days respectively, $p < 0.0001$) (Figure 5.4A and B). The mean GFP signal intensity also increased significantly between naïve iSNs and iSNs 5 days post-injury (Figure 5.4C/Table 5.1). While iSNs have been suggested to express miR-182, it remains unclear whether injury can sufficiently reduce its expression and how long those effects would persist (Zeidler *et al.*, 2021, 2023). Our miR-182 AAV achieved GFP expression in similar percentages of iSNs across all conditions (34.1 ± 4.49% naïve vs 25.3 ± 3.56% and 35.9 ± 3.27% at 24 hours and 5 days respectively) (Figure 5.4A and B). However, a lower percentage of transduced neurons was recorded when compared to that seen with the control AAV, suggesting the miR-182 AAV resulted in an overall repression of transgene expression (Figure 5.4B). We further evaluated miR-182 AAV activity by measuring the mean GFP signal intensity of all GFP+ neurons. This approach accounts for the possibility our AAV does not induce binary transgene repression and instead influences overall levels of transgene expression. These results did not provide evidence of our miR-182 vector restricting GFP expression to injured afferents in this model system. It was, however, not possible to determine whether this resulted from deficiencies in the vector itself or the levels of miR-182 in iSNs before and after injury.

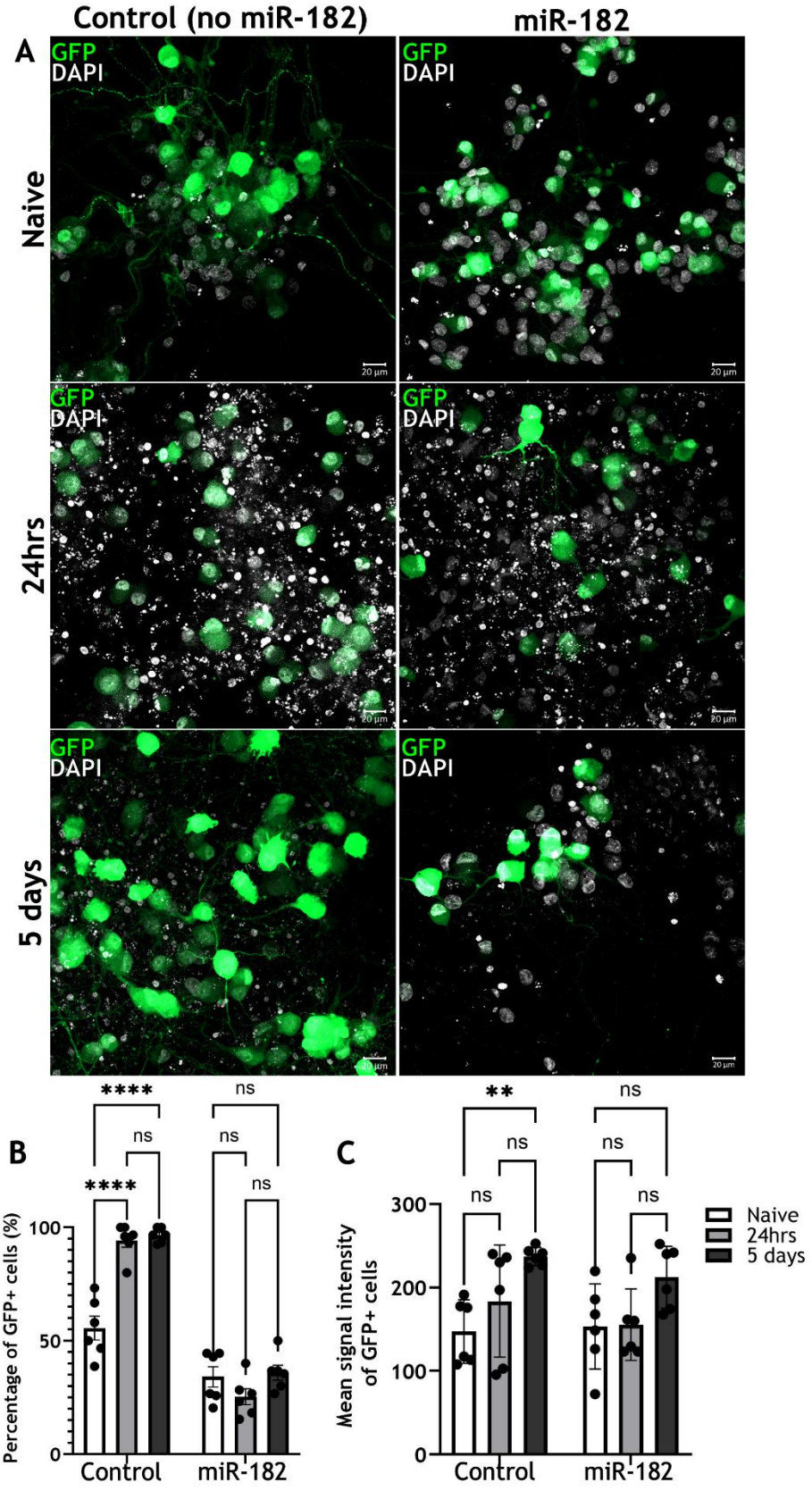


***Figure** 5.4 **MiR-182 AAV activity in iPSC-derived sensory neurons**. **(A)** Sensory neurons transduced with control (no miR-182) or miR-182 AAVs for a total of 12 days. GFP expression was assessed in the naïve state and 24 hours and 5 days after injury. **(B)** Percentage of GFP+ iSNs transduced with control or miR-182 AAV. Two-way ANOVA; Promoter x Timepoint interaction $F(2,30) = 23.45$, $p < 0.0001$, $n = 6$ coverslips; Tukey's*

*post hoc multiple comparisons test: ****p < 0.0001 vs naïve* ***(C)*** *Mean signal intensity of GFP+ iSNs transduced with control or miR-182 AAV. Two-way ANOVA; Promoter x Timepoint interaction F(2, 30) = 0.54, p = 0.59, n = 6 coverslips; Tukey's post hoc multiple comparisons test: **p < 0.005 vs naïve. Experimental unit defined as a coverslip (well), derived from two independent differentiations. All data are presented as mean ± SEM.*

Threshold	Promoter	Timepoint	Interaction (Promoter x Timepoint)
Transduction efficiency	F(1, 30) = 277.0, p < 0.0001	F(2, 30) = 17.55, p < 0.0001	F(2, 30) = 23.45, p < 0.0001
Signal intensity	F(1, 30) = 1.10, p = 0.30	F(2, 30) = 9.04, p = 0.0008	F(2, 30) = 0.54, p = 0.59

Table 5.1 Two-way repeated measures ANOVA degrees of freedom and p values for analyses of the effects of promoter and/or injury timepoint on the AAV-mediated transduction or transgene signal intensity of iSNs*. n = 6 coverslips.*

5.3.4 MiR-182 AAV in mDRG neurons *in vivo*

Due to their location outside the CNS and blood brain barrier and their high density of porous, fenestrated capillaries, DRG are a tractable target for AAV transduction (Hordeaux *et al.*, 2020). Hence, DRG neurons can be accessed by viral vectors delivered systemically, removing the need for invasive procedures. In Chapter 3, AAVs were delivered via intrathecal injection to help restrict transgene expression to the DRG. In this chapter, however, we wanted to test whether our miR-182 AAV had off-target expression and decided to deliver AAVs systemically. AAV-PHP.S vectors administered intravenously have been previously shown to successfully transduce DRG neurons to a greater extent than AAV9 (Chan *et al.*, 2017; Chen *et al.*, 2022). Furthermore, developmentally immature neurons are thought to be more amenable to transduction, leading us to test subcutaneous injections into P1 pups as a new, quicker route of administration. Mice were injected with the control AAV lacking miR-182 target sites a day after they were born, followed by SNI six weeks later and tissue collection 14 days after that. Since our vector lacked any additional elements conferring specificity except for its capsid, we expected GFP to be unbiasedly expressed in PNS neurons. Overall, we achieved higher transduction in the DRG

with the systemically delivered AAV-PHP.S vector compared to the intrathecally delivered AAV9 vector in Chapter 3. This was based on both a higher GFP signal intensity, detected in the absence of further amplification, and a higher percentage of transduced neurons (75.5 ± 6.86% for AAV-PHP.S vs 31.8 ± 0.95% for AAV9 based on stringent thresholding, $p = 0.0034$, paired t-test). AAV-PHP.S had similar transduction rates in ipsilateral and contralateral neurons (75.5 ± 6.86% vs 67.7 ± 8.27%, $p = 0.06$), confirming that our control AAV did not distinguish between injured and intact primary afferents (Figure 5.5A and B). The percentage of GFP-expressing neurons was slightly lower than that reported for intravenous injections (~82%) but appeared to be restricted to the PNS when evaluating expression in the spinal cord, since we found no evidence of GFP+ spinal neurons (Figure 5.5C) (Chan *et al.*, 2017). These results confirm the usability of systemic AAV-PHP.S injections and demonstrate that our control vector is not biased to either DRG neuron population.

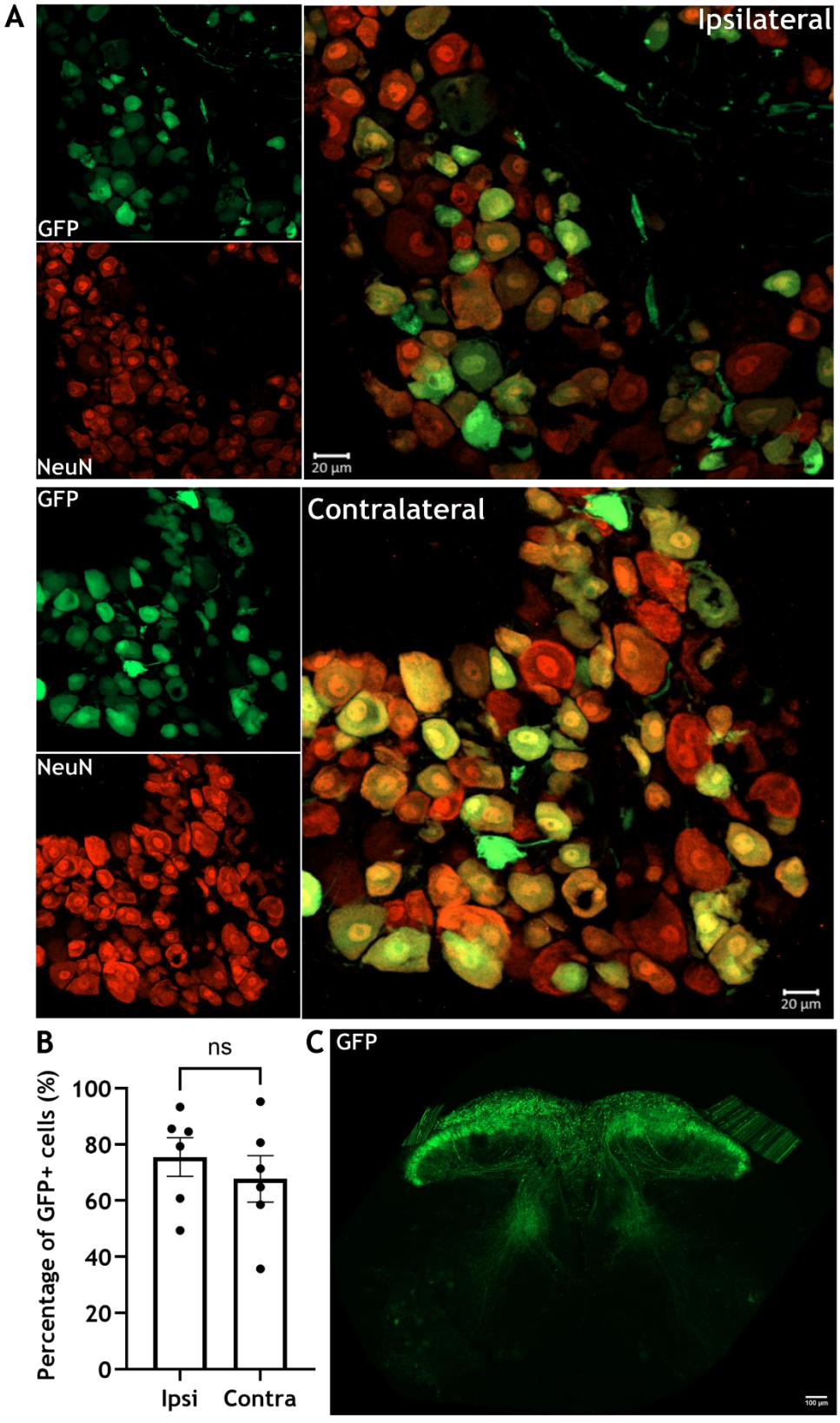


Figure 5.5 Evaluation of subcutaneous injections in neonate mice as a route of administration for AAV9-PHP.S AAVs. ***(A)*** *Transduction of mDRG neurons with control (no miR-182) AAV. GFP expression was evaluated two weeks post-SNI and was confirmed in both ipsilateral and contralateral neurons.* ***(B)*** *Percentage of GFP+ ipsilateral and contralateral neurons. Paired t-test; t = 2.42, df = 5, p = 0.06, n = 6 mice. All data are*

presented as mean ± SEM. ***(C)*** *Transverse section of L4 dorsal horn showing spinal GFP expression in mice treated with control AAV after SNI. GFP was not expressed by spinal neurons and can instead be seen in primary afferent central terminals. Experimental unit is defined as the averaged values from three DRG sections from one mouse. Ipsi; ipsilateral, contra; contralateral*

We next sought to test whether our miR-182 AAV restricted GFP expression to injured primary afferents. MiR-182 AAV had generally lower transduction compared to the control vector (Figure 5.6), suggesting a tonic level of repression in the DRG. More specifically, miR-182 AAV transduced significantly more ipsilateral than contralateral neurons (37.4 ± 2.15% vs 19.9 ± 1.98%, p = 0.009) (Figure 5.6A and B). Ipsilateral neurons were further divided into injured and intact based on expression of the injury marker ATF3, and GFP expression was found to be enriched in injured neurons (53.9 ± 2.73% injured ipsilateral vs 19.9 ± 1.98% contralateral and 29.1 ± 2.24% intact ipsilateral neurons) (Figure 5.6A and C). Interestingly, a modest increase in transduction (p = 0.0395) was also observed between contralateral and intact ipsilateral neurons, suggesting neurons adjacent to injured primary afferents also dynamically regulate miR-182 expression (Figure 5.6C). This shows miR-182 AAV was successful in biasing expression to injured primary afferents *in vivo*. This restriction, however, did not alter levels of transgene expression of GFP+ cells, as indicated by GFP signal intensity (Figure 5.6D). When assessing expression in the spinal cord, no GFP was found in spinal neurons and a clear distinction between ipsilateral and contralateral sides could be made based on greater GFP expression in zones with injured primary afferent terminals (IB4 negative regions) (Figure 5.6E).

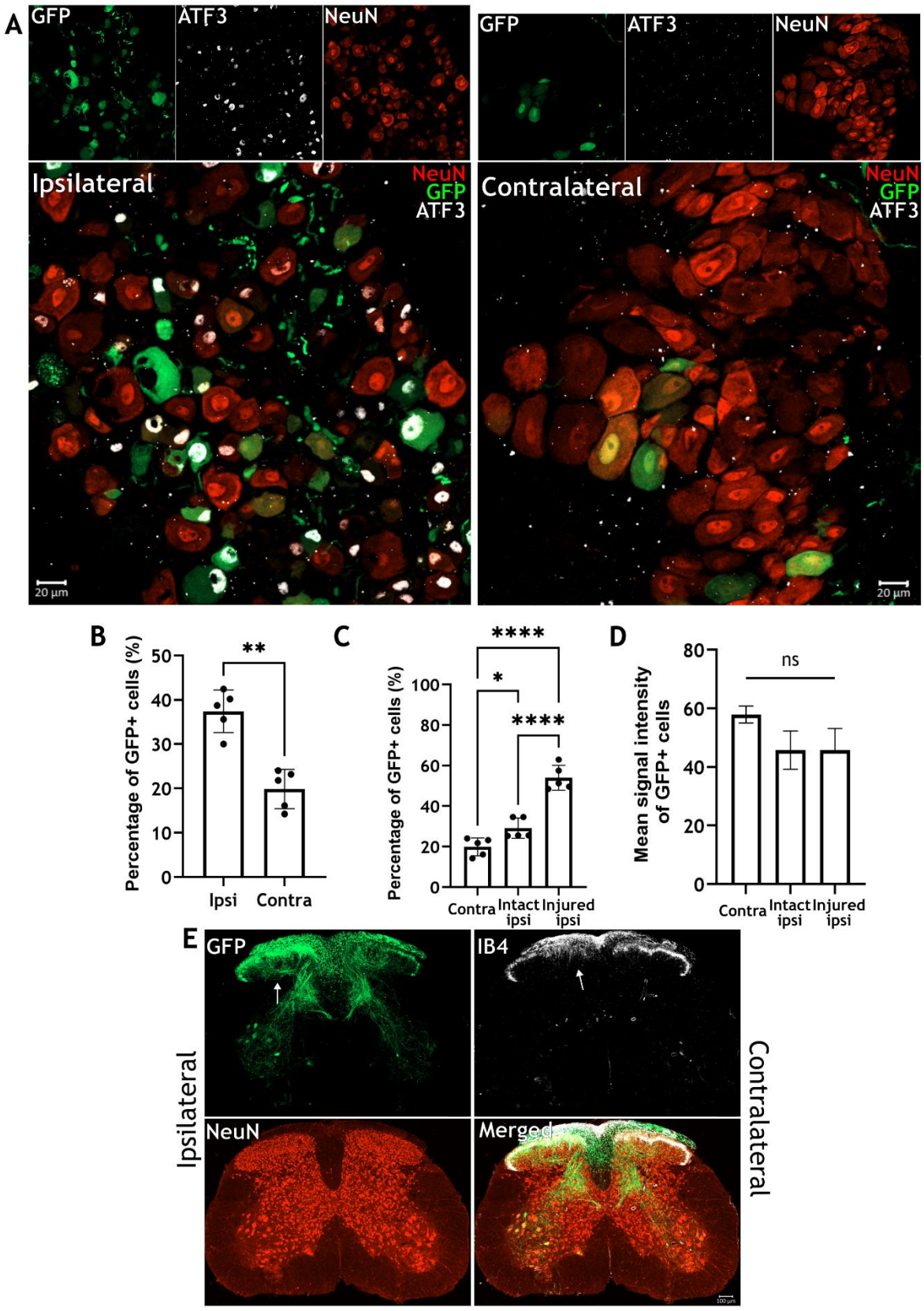


Figure 5.6 MiR-182 AAV in mDRG neurons in vivo. ***(A)*** *Primary afferent neurons transduced with miR-182 AAV and stained for the neuronal marker NeuN, GFP and ATF3. More GFP+ neurons can be seen on the ipsilateral side.* ***(B)*** *Percentage of GFP+ neurons after transduction with miR-182 AAV. Paired t-test; t = 4.62, df = 4, p = 0.009, n = 5, **p < 0.005.* ***(C)*** *Percentage of GFP+ contralateral, ipsilateral intact and*

*ipsilateral injured primary afferents. One-way ANOVA followed by Tukey's post hoc multiple comparisons test; F(2, 12) = 56.83, p < 0.0001, n = 5 mice. *p <0.05, ****p < 0.0001.* ***(D)*** *Quantification of GFP signal intensity in ipsilateral injured and intact and contralateral primary afferents. One-way ANOVA followed by Tukey's post hoc multiple comparisons test; F(2, 9) = 1.39, p = 0.29, n = 5 mice. Experimental unit is defined as the averaged values from three DRG sections from one mouse. All data are presented as mean ± SEM.* ***(E)*** *Transverse section of L4 dorsal horn showing spinal GFP expression. GFP is detected in motor neurons but no spinal neurons in the dorsal horn. The injured (ipsilateral) side can be distinguished by the stronger GFP signal in primary afferent central terminals. Arrows indicate injured side of dorsal horn. The diffused IB4 signal observed in the denervated region is the result of background, non-specific signal. Ipsi; ipsilateral, contra; contralateral*

The miR-183 family plays a role in a variety of biological processes, including cell proliferation, development, differentiation and apoptosis. While it is mainly expressed in sensory organs, low levels of this cluster can also be found in some non-sensory tissues (Hordeaux *et al.*, 2020). During DRG dissections, we noticed a greenish hue in muscle tissue, prompting us to take samples from the operated area and check for native GFP fluorescence. AAVs were delivered systemically and some GFP expression in off-target tissues is to be expected (Chan *et al.*, 2017). GFP was detected in animals treated with either AAV. However, the GFP signal was starkly increased in animals injected with miR-182 *vs* control AAV (Figure 5.7). This contrasts with the generalised lower expression we observed in neuronal systems throughout this chapter (Figure 5.5A and C), suggesting our finding is due to the miR-182 target sites in the vector and is not an artefact due to vector packaging issues.

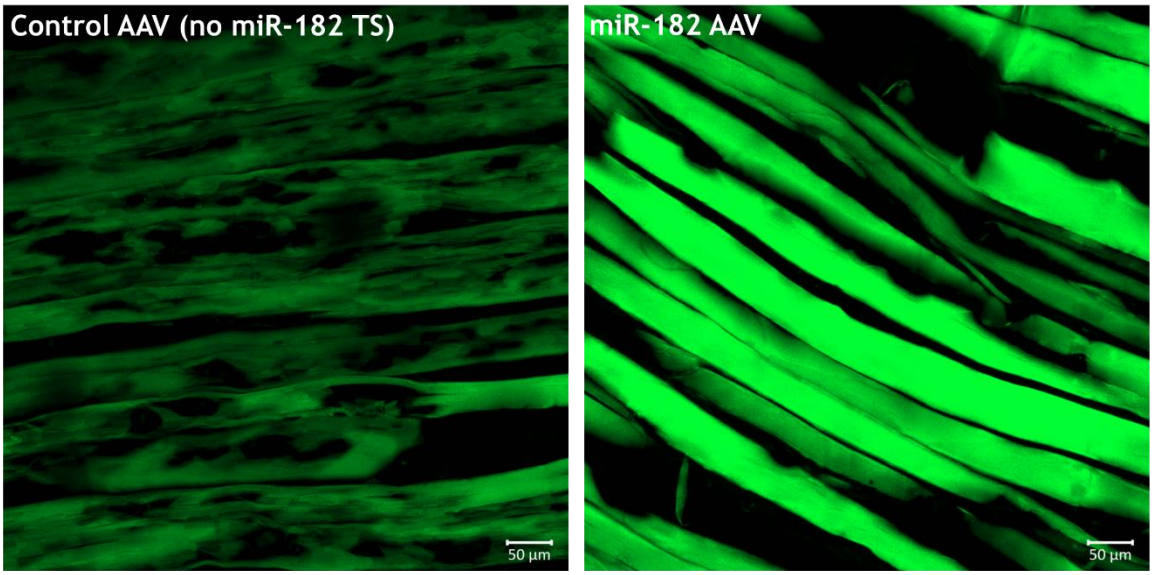


Figure 5.7 GFP expression in muscle from mice injected with control or miR-182 AAV*. Transduction was achieved with both AAVs, however, markedly higher levels of GFP expression were observed with the miR-182 AAV.*

5.4 Discussion

Endogenous miRNAs can be leveraged to improve the specificity of transgene expression and develop new gene therapies, with one AAV-based therapy currently in clinical trials for the treatment of Huntington's disease (Diener, Keller and Meese, 2022). We identified a miRNA cluster that is highly expressed in sensory organs and downregulated in the DRG following injury and introduced target sites for one of its members into a vector. The resulting AAV was successful in biasing transgene expression to injured primary afferents *in vivo*, however even in this scenario its selectivity was not absolute.

5.4.1 miR-182 AAV activity *in vitro*

Several factors need to be taken into consideration when designing a viral vector for miRNA-mediated post-transcriptional transgene silencing, including miRNA expression levels and target site configuration (Geisler and Fechner, 2016). In rodents and non-human primates, miR-182 is highly expressed in the DRG and is downregulated following injury while maintaining low levels of expression in parts of the CNS, such as the cortex and spinal cord (Aldrich *et al.*, 2009; Zhou *et al.*, 2017; Cai *et al.*, 2018; Hordeaux *et al.*, 2020; Gleichman *et al.*, 2023).

We incorporated four perfectly complementary target sequences in our vector (Figure 5.2A), a number which has been previously shown to suffice for effective transgene repression. The plasmid's viability was confirmed in HEK293T cultures (Figure 5.2C) before being packaged into an AAV-PHP.S vector despite the lack of an observable effect on GFP expression in this immortalised cell line (Geisler *et al.*, 2011; Hordeaux *et al.*, 2020).

Interestingly, addition of the miR-182 AAV in mDRG neuron cultures resulted in the transduction of glial cells instead of neurons, in contrast to the control AAV which led to expression in both cell types (Figure 5.3). The assumption therefore would be that mDRG neurons in culture maintain a sufficient level of miR-182 to induce transgene repression, in contrast to our original hypothesis that it would decrease due to the axotomy induced by dissociation. There is limited research on miR-182 expression in glial cells, although some studies have suggested that this miRNA is expressed by non-neuronal cells in the sensory nerve and plays a role in these cells' response to injury (B. Yu *et al.*, 2012; Fei *et al.*, 2021). Our results suggest that at least some non-neuronal cells in culture lack sufficient miR-182 to repress transgene expression. The miR-183 cluster appears to play a conserved role in sensory organ development and differentiation, and miR-182 is highly expressed in mature iSNs (Zeidler *et al.*, 2021, 2023). Incubation of iSNs with miR-182 AAV resulted in similar proportions of naïve and injured GFP+ neurons (Figure 5.4). This result could be attributed to insufficient downregulation of miR-182 expression, potentially due to the use of enriched culture media supplemented with growth factors. An increase in GFP signal intensity correlating with time after injury was noted, however that trend did not reach significance.

A common challenge faced when investigating miR-182 AAV activity in different *in vitro* systems concerned the lack of comprehensive characterisation of miR-182 expression. Currently, we are aware of miR-182 being expressed in mDRG neurons and iSNs *in vitro,* but further profiling would be valuable to fully elucidate expression patterns pre- and post-injury and how these might be affected by culture conditions. For instance, it is possible the miR-182 AAV could not distinguish injured from intact iSNs due to insufficient changes in miR-182 levels resulting from the use of enriched culture media supplemented with

growth factors. We carried out preliminary experiments to assess miR-182 expression in mDRG neurons and iSNs using fluorescence *in situ* hybridisation (FISH) but found results to be inconsistent across trials. This was likely due to FISH not being the best suited approach given the small target size of miRNAs. Instead, more commonly used profiling techniques, such as qRT-PCR, miRNA microarray and RNA-seq, could be employed in future studies to provide insight into miR-182 expression *in vitro*, although these would lack single cell resolution and the ability to correlate miR-182 levels with transgene expression (Pritchard, Cheng and Tewari, 2012).

5.4.2 miR-182 AAV activity *in vivo*

AAV-PHP variants have been previously delivered systemically to neonate mice via intravenous, intramuscular and intraventricular injections and achieved good transduction (Chan *et al.*, 2017; Wang, Misgeld and Brill, 2022). We evaluated subcutaneous injections as a route of administration in neonate (P1) mice and found it to be a highly effective AAV delivery method. Transgene expression was observed in a high proportion of DRG neurons, which is more efficient that other means of AAV-mediated gene transfer to the DRG (e.g. intrathecal AAV9 injection of Chapter 3). Expression showed no bias for ipsilateral or contralateral DRG neurons (Figure 5.5) using our control (no miR-182) AAV. Although we used a higher AAV titre compared to previously published work, transduction was of a similar order, albeit slightly lower than that achieved with intravenous injections (Chan *et al.*, 2017). Overall transduction efficiency with our miR-182 AAV was reduced compared to control, suggesting tonic transgene repression in DRG neurons. Positively, miR-182 AAV resulted in higher GFP expression in ipsilateral than contralateral neurons and further investigation revealed a bias for transgene expression in injured ipsilateral neurons (Figure 5.6). Intact ipsilateral neurons were also transduced, however, indicating that the AAV did not fully restrict GFP expression exclusively to damaged afferents. In addition, transduction was slightly higher in intact ipsilateral compared to contralateral neurons (Figure 5.6C). While we considered ipsilateral neurons lacking ATF3 expression as intact, uninjured primary afferents close to injured DRG have been shown to exhibit hyperexcitability and spontaneous activity, suggesting they undergo some molecular changes in their properties likely to set them apart from intact contralateral neurons (Noguchi *et al.*, 1995; Tsuzuki *et al.*, 2001; Wu

et al., 2001; Obata *et al.*, 2004; Chen *et al.*, 2017, 2019; Tran and Crawford, 2020; Zhang *et al.*, 2022). This notion is further supported by epigenetic evidence presented in Chapter 4. In addition to controlling whether the transgene is expressed, the inclusion of miRNA target sites can impact the overall level of transgene expression. We did not detect a significant difference in mean GFP signal intensity (of the cells classed as positive) between intact, injured and contralateral neurons (Figure 5.6D), contrary to what has been previously observed in non-human primate DRG transduced with a miR-183 AAV (Hordeaux *et al.*, 2020). Similarly to the *in vitro* work, our results suggest investigation of the sensory neuron types that downregulate miR-182 and the kinetics of this, would be valuable.

Since AAVs were delivered systemically, we evaluated gene expression in tissues other than the DRG. Both AAVs resulted in GFP expression in motor neurons, but not spinal cord neurons, confirming the assumption that this serotype is restricted to cells present in the periphery (Chan *et al.*, 2017). AAV-PHP.S led to high GFP expression in muscle (Figure 5.7), with our miR-182 AAV resulting in such high expression that fluorescence could be observed by the naked eye and without specific fluorophore excitation. One valuable aspect of this result was that it suggests our miR-182 AAV was not simply less infective compared to the control, which could be an interpretation of our reduced levels of neuronal transduction across *in vitro* and *in vivo* models. This result was, however, in contrast with findings from other studies showing similar levels of GFP expression in muscle from mice injected with miR-182 or control AAV (Hordeaux *et al.*, 2020). Future studies will have to address issues with transgene expression in tissues with naturally low miR-182 expression, such as the liver and heart, particularly since these tissues are also highly transduced by AAV-PHP.S vectors (Chan *et al.*, 2017; Hordeaux *et al.*, 2020). AAV targeting specificity can be improved by employing a complementary approach and introducing multiple regulatory elements in a viral vector. For instance, Gleichman *et al.* (2023) developed a viral vector with improved astrocytic specificity by combining a GFAP-based promoter with target sites for six distinct miRNAs. This approach, however, is limited by the small AAV packaging capacity. Specificity can be further improved by adjusting the viral titre and carefully choosing the appropriate AAV capsid and route of administration. Gleichman *et al.* (2023)

observed the highest specificity and transduction efficiency when using AAV2/5 capsids coupled with a higher titre and localised intracortical, rather than systemic, injections.

In summary, data presented in this chapter demonstrates inclusion of miR-182 target sites is an effective way of biasing transgene expression to injured primary afferents, without providing total selectivity. Further characterisation of miR-182 expression in sensory neurons as well as other tissues is required to help understand and predict off-target expression. Combining miRNA regulation with other methods of increasing AAV specificity is necessary to prevent transgene expression in intact DRG neurons and tissues with low miR-182 expression.

Chapter 6 General discussion

There are currently no viral tools allowing for the selective targeting of injured primary afferents. In this thesis, we explored different approaches of developing viral vectors capable of delivering therapeutic transgenes and restricting their expression to injured, but not intact, primary afferents. Our main findings are summarised in the following points:

- We optimised the ATF3CreERT2:Ai9 transgenic mouse line to improve targeting and identification of axotomised afferents in an *in vivo* model of peripheral nerve injury.

- The endogenous ATF3P1 promoter does not selectively restrict expression to injured primary afferents *in vivo* when used in an AAV in the absence of other regulatory elements.

- Injured and intact primary afferents have distinct epigenomic signatures, allowing for the identification of regulatory elements active only in the damaged neuronal population.

- We created the first single cell epigenomic atlas of naïve mouse DRG neurons, providing insight into the regulatory landscape underlying sensory neuron identity.

- We developed an AAV containing miR-182 target sites which is capable of biasing transgene expression to injured primary afferents, albeit without providing complete selectivity.

The intricacies of each finding are discussed in detail below.

6.1 Endogenous ATF3 promoters

Endogenous promoters are usually large in size, discouraging their use in AAVs which have a smaller packaging capacity compared to other vectors. There is however evidence of their success in targeting AAV gene expression, such as in the case of the astrocytic GFAP promoter (Griffin *et al.*, 2019). We identified

the endogenous ATF3P1 promoter as a suitable candidate for guiding transgene expression and incorporated it into an AAV9 vector. While transducing iSNs with our ATF3P1 vector did result in higher percentages of GFP+ neurons after injury, this finding was not reproduced *in vivo*. The lack of *in vivo* specificity could be attributed to the use of the human promoter sequence despite its high degree of homology with the mouse sequence (Miyazaki *et al.*, 2009).

Initially, the activity of the P2 promoter was also investigated in mDRG cultures but was not chosen for further development. This was due to the two promoters displaying comparable efficiencies in cultured mDRG neurons and evidence from previous studies of P1 being activated by stimuli such as DNA damage and oxidative stress, as well as having a lower GC content and higher species conservation (Miyazaki *et al.*, 2009). Furthermore, the A2 exon, located downstream of the P2 promoter, contains potential CpG islands which are highly conserved between human and mouse (~72% sequence homology) (Hai, Wolford and Chang, 2010). CpG islands are short sections of DNA, usually located in promoter regions, which play a crucial role in gene regulation by acting as sites for transcription initiation. As indicated by their name, these sequences have a high GC content and contain large numbers of CpG dinucleotides that are primarily unmethylated. CpG islands are susceptible to histone and other epigenetic modifications which can alter chromatin accessibility and gene expression, thus affecting expression strength, long-term stability and tissue specificity (Ghosh *et al.*, 2010; Deaton and Bird, 2011). Based on these characteristics, future studies should investigate the capacity for the P2 promoter to target AAV gene expression to just injured afferents.

ATF3 is only one of the many regeneration-associated genes expressed in sensory neurons in response to injury (Nguyen, Le Pichon and Ryba, 2019; Renthal *et al.*, 2020; K. Wang *et al.*, 2021; Zhang *et al.*, 2022). Most of these genes, such as *GAP43* and *SOX11*, have promoters that are too large (> 1kb) to be considered for AAV inclusion (Udvadia, Köster and Skene, 2001; Udvadia, 2008; Gustavsson *et al.*, 2010). SPRR1A is a marker of differentiating keratinocytes and squamous epithelial cells that is absent from healthy DRG. Its expression is upregulated in nearly all DRG neurons following injury, reaching peak levels one week post axotomy, and is associated with axonal regeneration (Bonilla, Tanabe and

Strittmatter, 2002; Starkey *et al.*, 2009; Zhang *et al.*, 2022). *SPRR1A* has a proximal (-0.2kb from TSS) and distal (+73.5kb from TSS) promoter which are approximately 500bp and 200bp long respectively (Sark *et al.*, 1998). In addition, data presented in Chapter 4 of this thesis suggests the proximal promoter sequence is differentially accessible in nuclei from injured sensory neurons. SPRR1A promoters therefore present a promising alternative for targeting AAV-mediated transgene expression.

6.2 Epigenomic profiling of mouse DRG neurons

The epigenomic atlas of mouse DRG neurons we generated confidently identified major neuronal subtypes in the absence of transcriptomic data, reinforcing the strong correlation between chromatin accessibility and gene expression. Our data, derived from both male and female mice, addresses limitations in existing studies and provides a view of the sensory neuron epigenomic landscape at single cell resolution. This allows for the identification of regulatory elements governing cell-type specific gene expression in sensory neuron subtypes as well as elements associated with the expression of pain-associated genes. Integration of our data with published RNA-seq and human ATAC-seq datasets, as has been done previously for the TG, would add confidence to our results while also facilitating cross-species comparisons (Yang *et al.*, 2022). In addition to providing insight into epigenomic mechanisms of gene regulation, this atlas can be used for the discovery of pathways and regulatory networks implicated in chronic pain. A recent study profiling chromatin accessibility in TG neurons found non-coding genomic variants associated with migraine are located in accessible regions predicted to regulate genes expressed in TG nociceptors and SGCs, highlighting another use for epigenomic data (Yang *et al.*, 2022). TG and DRG exhibit considerable transcriptional similarities (Yang *et al.*, 2022). Comparison of their epigenomic profiles could therefore improve our understanding of different pain mechanisms and provide valuable information for designing targeted, tissue-specific gene therapies.

Epigenomic data from CNS neurons has been leveraged to design viral vectors containing elements (e.g. enhancers) capable of restricting gene expression to specific cell types (Vormstein-Schneider *et al.*, 2020; Graybuck *et al.*, 2021; Mich *et al.*, 2021). We used our epigenomic atlas of naïve DRG to compare

healthy and injured primary afferents aiming to ultimately identify elements that could selectively restrict transgene expression to injured neurons. Injured neurons had considerably different chromatin accessibility profiles compared to the naive and ipsilateral intact counterparts. We detected fewer populations in our injured sample, consistent with transcriptomic and anatomical data which describes a loss of subtype-specific gene expression profiles (Renthal *et al.*, 2020; Zhang *et al.*, 2022; Barry *et al.*, 2023; Cooper *et al.*, 2024). It is difficult, however, to determine from single cell omics data whether this was due to cell loss or loss of identity. We found nuclei from injured neurons to be enriched for processes associated with neurogenesis and the regulation of membrane potential and display differences in chromatin accessibility at the loci of known RAGs. This data could be built upon in the short term to define candidate DNA sequences for novel AAV constructs.

6.3 MicroRNA-mediated targeting of AAV gene expression to injured primary afferents

We employed the miR-OFF strategy of controlling AAV gene expression by introducing target sites for miR-182 into an AAV-PHP.S vector. We found that while our miR-182 AAV does not differentiate between injured and intact sensory neurons *in vitro*, it is capable of biasing expression to injured primary afferents *in vivo*. Transgene expression was also observed in intact primary afferents, indicating our vector does not exclusively restrict expression to injured neurons. The abundant expression of the miR-183 family in healthy sensory neurons has been previously exploited to inhibit transgene expression in the whole DRG and reduce toxicity associated with AAVs (Hordeaux *et al.*, 2020). This is the first time, however, that miRNA-mediated regulation has been used to restrict expression to specific DRG populations.

Intact neurons appear to be transduced differently, as indicated by the detection of a slightly higher percentage of transgene-expressing ipsilateral intact compared to contralateral neurons. This is in line with evidence demonstrating uninjured neurons adjacent to damaged fibres undergo changes in their neurochemical and electrophysiological properties which distinguish them from naive sensory neurons (Noguchi *et al.*, 1995; Obata *et al.*, 2004; Chen *et al.*, 2017, 2019; Tran and Crawford, 2020; Zhang *et al.*, 2022). Finally, a certain

degree of transgene expression in intact primary afferents could potentially be attributed to the particularly strong activity of the sCAG promoter, which might overwhelm the miR-182 system, and the AAV-PHP.S serotype, which produces very high transgene expression under normal conditions.

The use of miR-182 target sites in viral vectors is complicated by the limited information regarding its expression across different cell types, tissues and conditions. For instance, while miR-182 is suggested to be expressed by HEK293T cells and iSNs, little is known about the absolute level of expression and how this is impacted by injury (Tian *et al.*, 2012; Zeidler *et al.*, 2021, 2023). Furthermore, there is no information on expression in mDRG neurons *in vitro*. Further profiling is required to accurately determine miR-182 expression levels both *in vitro* and *in* vivo and how these vary across sensory neurons subtypes as well as different timepoints after injury. While we did trial detecting miR-182 across our testing systems using FISH, the results were inconsistent, and we did not have confidence in undertaking analysis. Mature miRNAs are single stranded and short (~21-23 nucleotides long), making their visualisation with *in situ* techniques extremely challenging.

In contrast to the ATF3P1 vector, we delivered our miR-182 AAV systemically via subcutaneous injection in neonate mice, thereby validating a new route of administration for AAV-PHP.S viruses. This method produced very high levels of peripherally restricted transgene, and as such is a useful means to transduce the DRG, so long as the experiment is not confounded by expression in non-target peripheral tissues. Notably, this systemic delivery resulted in high transgene expression in muscle tissue, observed with both the control and experimental vectors. Curiously, much higher muscle expression was achieved with the miR-182 AAV. There is evidence of miRNA-mediated activation of gene expression, however it is unclear how those mechanisms can apply to our vector (Vasudevan and Steitz, 2007; Truesdell *et al.*, 2012; Chaluvally-Raghavan *et al.*, 2016; Ramchandran and Chaluvally-Raghavan, 2017; O'Brien *et al.*, 2018). This coincidental finding could be of interest to follow-up due to the importance of transducing skeletal muscle for certain gene therapies (Zhou *et al.*, 2024). In this work, we only assessed off-target expression in muscle due to the unusually high level of transgene expression which was visible to the naked eye. Expression

in other tissues, particularly those known to have naturally low miR-182 levels and to be transduced by AAV-PHP.S, such as the heart, will need to be further investigated.

6.4 Limitations

We carried out initial screens to evaluate our AAVs' activity in iSNs produced according to the protocol by Chambers *et al.* (2012). This produces neurons with features consistent with both Aδ- and C-non-peptidergic nociceptors, which form only a subset of primary afferent neuron types. Protocols are now available for generating almost a full panoply of sensory neurons (Guimarães *et al.*, 2018; Schwartzentruber *et al.*, 2018; Dionisi *et al.*, 2020; Umehara *et al.*, 2020; Saito-Diaz *et al.*, 2021). These should be leveraged in future studies to comprehensively assess AAV activity *in vitro* before progressing to *in vivo* studies. This approach could also help determine whether vectors behave differently across distinct sensory neurons subtypes.

Our diverse testing platform leads to some challenges for interpreting the success of our targeting approaches. In both Chapters 3 and 5, our AAV vectors offered biased targeting of injured sensory neurons in one system (e.g. iSNs *in vitro*) that failed to replicate in another (e.g. *in vivo* mouse). Which, therefore, is the reliable result? We have taken the gold standard as being effectiveness *in vivo* with a desired replication *in vitro* in human neurons. Furthermore, we investigated the cell-type specificity of our AAVs by assessing GFP expression in injured and intact primary afferents using immunostaining. In Chapter 3, determining GFP expression in a binary fashion was challenging, leading us to adopt a thresholding technique to confidently distinguish GFP+ neurons from background signal. Additionally, immunostaining is limited in the number of markers that can be used to co-stain a single section. While we categorised DRG neurons based on the expression of tdTom (Chapter 3) or ATF3 (Chapter 5), it would be beneficial to include markers for specific sensory neuron subtypes. Employing techniques such as FISH would allow the simultaneous spatial visualisation of injury markers, and primary afferent subtype probes, thus providing insight into whether certain primary afferent subtypes are more amenable to AAV transduction. Alternatively, neurons could be purified and their contents detected or sequenced using qPCR or RNA-seq. These methods would

provide more quantitative information regarding GFP expression, although they lack spatial resolution and qPCR does not permit distinction between cell types. Overall, our immunostaining approach can confidently distinguish damaged from intact cells, provided controls are included to account for injury-independent recombination and injury marker expression. Differences in GFP expression levels between cells can also be gleaned, however that is only possible if GFP is driven by a strong promoter and does not require additional antibody amplification (Chapter 5).

All our vectors were tested in traumatic models of NeuP. While we attempted to establish an *in vitro* model of chemotherapy-induced NeuP, this was unsuccessful, likely due to the unsuitability of ATF3 as a marker of injury in iSN cultures. Interestingly, there are conflicting results in the literature as to whether paclitaxel treatment induces ATF3 expression in mouse DRG neurons in *vivo*, with some studies suggesting gene upregulation and others observing limited expression changes (Flatters and Bennett, 2006; Jamieson *et al.*, 2007; Peters *et al.*, 2007). This might suggest that the response of sensory neurons to paclitaxel is nuanced and sensitive to time and dosing regimen. Similarly, nuclei for snATAC-seq were derived from animals that had undergone SNI. Transcriptomic and epigenomic differences have been observed in DRG neurons derived from different pain models, such as CCI, inflammatory and chemotherapy-induced models (Palmisano *et al.*, 2019; Renthal *et al.*, 2020; Stephens *et al.*, 2021). Future studies should therefore aim to explore the epigenomic differences across various types of NeuP, as this could facilitate the development of more targeted and effective pain treatments. Of note, some of the RAGs investigated in this thesis have been demonstrated to be upregulated across nerve insults (Tsujino *et al.*, 2000; Peters *et al.*, 2007; Renthal *et al.*, 2020; Kan *et al.*, 2021). Different timepoints should also be investigated. We assessed AAV activity and chromatin accessibility in sensory neurons two and four weeks after SNI, respectively, and used two timepoints *in vitro*. Apart from our dataset, there is a lack of information surrounding epigenetic changes beyond the 14-day timepoint despite evidence from RNA-seq studies hinting at constant changes in gene expression even at four weeks post injury (Nguyen, Le Pichon and Ryba, 2019; Renthal *et al.*, 2020; Barry *et al.*, 2023). Given the

chronic nature of NeuP, it would also be of interest to explore AAV activity at different stages of disease.

It is often assumed that injury leads to directly to pain. However, only a subset of patients with damage to their somatosensory system will go on to develop NeuP. Nerve injury induces a transcriptional response that encompasses both pain- and regeneration-associated programmes (Renthal *et al.*, 2020). The degree to which these programmes overlap is unclear. ATF3, a regeneration associated gene, labels all injured neurons following axotomy (Tsujino *et al.*, 2000; Renthal *et al*,. 2020). ATF3 knockout mice exhibit impaired axon regrowth following nerve injury, and targeted knockdown of RAGs like *ATF3* and *GAP43* produces a reduction in both nerve regeneration and pain behaviours (Gey *et al.*, 2016; Xie, Strong and Zhang, 2017; Kan *et al.*, 2021). Similarly, inhibition of axon regrowth with semaphorin 3A in a regenerating spinal nerve results in a reduction in both regeneration and pain responses (Xie, Strong and Zhang, 2017). Data from our lab further supports the role of injured afferents in pain, since chemogenetic silencing of ATF3-expressing fibres attenuates ongoing pain as determined by CPP (Dr Andrew Cooper, unpublished data). This evidence is suggestive of a potential mechanistic overlap between pain and regeneration. It is possible, however, that ATF3 expression is normalised in later timepoints, as indicated by a considerable reduction in expression noted six weeks after injury, and especially if regeneration is complete (Tsujino *et al.*, 2000; Lindå, Sköld and Ochsmann, 2011; Renthal *et al.*, 2020). In addition, regeneration and pain gene programmes can differ across DRG subpopulations and types of nerve injury (Renthal *et al.*, 2020; Bolivar *et al.*, 2023). It is therefore reasonable to assume that not all ATF3-expressing neurons will drive chronic pain. This has important implications on our AAV design since vectors using regulatory elements from RAGs may target a broader population than those driving pain. This highlights the importance of fully delineating the molecular relationship between pain and regeneration to ensure our vectors capture pain-driving neuron populations while sparing regeneration.

6.5 Future directions

6.5.1 Identifying differentially accessible peaks in injured primary afferents

We have generated a list of differentially accessible peaks by comparing chromatin accessibility in nuclei from injured and naïve sensory neurons. Next steps will focus on selecting peaks located on or near known injury markers, such as *ATF3* and *SPRR1A*, identifying their sequences, incorporating them into vectors and testing their capacity to restrict transgene expression to injured primary afferents. We also aim to look beyond RAGs and identify novel regulatory elements not necessarily associated with known injury genes. Comparing injured and naïve epigenomic profiles is only one of the many analyses that can be carried out using these datasets. For instance, given the neurochemical and electrophysiological changes taking place in intact, injured-adjacent primary afferents, it would be of interest to compare them to neurons from injured and naïve samples. This could reveal how these changes are reflected in chromatin accessibility and whether they can be used to inform viral vector design.

Despite our best efforts to promote neuronal enrichment in our samples by fluorescently labelling both injured and intact neurons, SGCs and Schwann cells were still the main cell type sequenced. While further optimisation of the library preparation process could reduce the cell input required, these non-neuronal cells contribute to normal nociception as well as the development and maintenance of NeuP (Hartlehnert *et al.*, 2017; Abdo *et al.*, 2019; Avraham *et al.*, 2020; Rinwa *et al.*, 2021; Jager *et al.*, 2022; Lovatt *et al.*, 2022; Ojeda-Alonso *et al.*, 2024). Analysis of their epigenetic signatures in naïve and injured states could therefore improve our understanding of their role in pain and aid the identification of novel therapeutic targets.

6.5.2 Combining multiple approaches in one vector

There are several methods of targeting AAV gene expression. To minimise off-target expression without introducing elements besides the ones being investigated in our vectors, we packaged our plasmids in AAV9 and AAV-PHP.S vectors known to effectively transduce DRG neurons (Schuster *et al.*, 2014; Chan

et al., 2017). We also delivered AAV9 vectors intrathecally to further aid targeting and DRG neuron transduction (Schuster *et al.*, 2014). In addition to capsid optimisation and careful consideration of the route of administration, it is also possible to combine multiple elements into one vector. Gleichman *et al.* (2023) demonstrated the feasibility of this approach in a study combining a GFAP-based promoter and target sites for multiple miRNAs to improve astrocyte specificity across different serotypes in mice. Hence, the ability of our miR-182 AAV to target injured primary afferents could be enhanced by incorporating an injury specific promoter within the same vector. Furthermore, while we included four repeats of miR-182 target sites, it may be the case that increasing that number would lead to greater suppression of transgene expression (Geisler *et al.*, 2011; Geisler and Fechner, 2016). Adding target sites for multiple miRNAs that are downregulated in injured primary afferents as well as miRNAs from tissues we wish to de-target can further improve vector specificity (Keaveney *et al.*, 2018; Gleichman *et al.*, 2023). Novel regulatory elements identified through our snATAC-seq data can also be incorporated into these vectors to enhance their precision. Enhancers and miRNA target sites tend to be small, however, endogenous promoters are usually too large for AAVs. Using computational biology and phylogenetic conservation, it is possible to further develop these elements into mini promoters retaining the original cell type specificity (de Leeuw *et al.*, 2014).

6.6 Gene therapy for chronic pain

There are currently more than 100 approved gene, cell and RNA-based therapies worldwide and more than 2,500 active clinical trials (Chancellor *et al.*, 2023). Chronic pain affects millions of people around the world and has numerous therapeutic targets within the PNS and CNS, making gene therapies an attractive, long-term treatment option (Ovsepian and Waxman, 2023; Li and Ji, 2024). So why are clinical trials with pain as the primary outcome so rare and why are there no approved pain gene therapies? Traditionally, gene therapies have been delivered using viral vectors, such as AAVs. These however are associated with increased immunogenicity, limiting the potential for re-administration, and DRG toxicity (Hordeaux *et al.*, 2020; Ertl, 2022). The inability to target specific cell types and the requirement for sustained or repeated treatment have therefore limited the success of gene therapies in the

field of pain. Additionally, gene therapies are generally complicated by time-consuming manufacturing processes, high costs and regulatory and ethical challenges, further contributing to delays in development and approval.

Recent breakthroughs in pain biology, delivery systems and genetic tools are transforming gene therapies for chronic pain into a viable therapeutic option (Ovsepian and Waxman, 2023; Li and Ji, 2024). Advances in single cell technologies are continuously improving our understanding of the molecular and genetic underpinnings of chronic pain, facilitating the discovery of novel therapeutic targets and targeting tools. A technology providing access to cells based on their mRNA markers through RNA sensing by endogenous adenosine deaminases acting on RNA (CellREADR) has been developed and equipped to successfully target and manipulate CNS neurons (Qian *et al.*, 2022). Our snATAC-seq research offers another example of how these technologies can be used to discover epigenetic drivers of chronic pain and identify new therapeutic pathways. Targets currently in clinical development include growth factors, such as the vascular endothelial and human growth factors, and genes or TFs active in non-neuronal cells, such as keratinocytes (Barć *et al.*, 2021; Esrick *et al.*, 2021; Dhillon, 2023; Perin *et al.*, 2023; Li and Ji, 2024). Key components of nociception, such as voltage-gated and transient receptor potential channels, are notably absent from clinical trials. These targets, however, are being extensively investigated in preclinical studies (Chen *et al.*, 2022; Haroun *et al.*, 2023; Kandel *et al.*, 2024; Li and Ji, 2024).

Immunogenicity and DRG toxicity challenges are being actively addressed through the development of novel, targeted delivery tools, a focus that aligns with our research efforts to restrict transgene expression to injured sensory neurons. For instance, new capsids, such as MaCPNS1/2, are being engineered to offer higher transduction in the PNS while minimising liver expression (Chen *et al.*, 2022). Tools used to discover and validate pain targets, such as antisense oligonucleotides, RNAi, CRISPR and optogenetic and chemogenetic techniques, are also feasible gene therapy approaches for the treatment of pain. CRISPR-dCas9 has been successfully used in combination with zinc finger proteins to create an epigenetic tool termed long-lasting analgesia via targeted in vivo epigenetic repression, or LATER, to repress $Na_V1.7$ expression in the DRG. This

resulted in reduced hyperalgesia across different models of chronic pain and required a lower viral load while also reducing the danger of immunogenicity (Moreno *et al.*, 2021). In this thesis, we provide an example of leveraging the endogenous RNAi machinery to restrict transgene expression to injured primary afferents through the addition of miR-182 target sites in AAVs.

Finally, DRG toxicity has been observed in NHPs following the administration of AAVs targeting the CNS. This toxicity manifests in the form of neuronal degeneration, mononuclear cell infiltration and axonopathy, affecting both central and peripheral axons, and is associated with transgene overexpression and accumulation in the DRG (Hordeaux *et al.*, 2018, 2020; Buss *et al.*, 2022). While this complicates the use of AAV vectors for gene therapies in the DRG, our work addresses these concerns by focusing on creating vectors capable of de-targeting intact primary afferents and restricting transgene expression to already damaged neurons.

In conclusion, our growing understanding of chronic pain pathophysiology coupled with the development of novel targeting technologies point at a promising future for gene therapy in chronic pain. These efforts have the potential to offer long-lasting relief, addressing a life-changing unmet need for chronic pain patients. Findings presented in this thesis highlight the complexity of targeting distinct neuronal populations within the DRG. While we were able to successfully bias transgene expression to injured primary afferents via miRNA-mediated regulation, the endogenous ATF3P1 promoter did not offer the same selectivity. The snATAC-seq datasets we have generated provide insight into epigenetic mechanisms governing gene expression in the DRG and will be used to identify regulatory elements capable of restricting AAV gene expression to injured primary afferents. We aim to identify regulatory elements using our snATAC-seq data and combine them with approaches discussed here to develop a vector capable of carrying therapeutic transgenes and limiting their expression to injured primary afferents.

Chapter 7 References

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