Assessing the Excitability Changes of DRG Neurons in Models of Diabetic Neuropathy

Katerina Kaloyanova Kaloyanova

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The University of Sheffield Faculty of Science

Department of Biomedical Science

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ABSTRACT

Diabetic neuropathy (DN) is the most common type of neuropathy, with 25-50% of patients experiencing pain at any stage of its progression. However, the understanding of the pathophysiology of DN is still incomplete. No disease-modifying treatments have yet been developed and pain is only treated symptomatically with drugs targeting the central nervous system (CNS). These have limited efficacy and can produce serious side effects. Research into novel analgesics is turning away from the CNS and now focusing on the peripheral nervous system (PNS) and more specifically the pain-sensing neurons (nociceptors) to produce better-targeted and safer treatments. However, to this end, a suitable medium-to-high throughput screening tool is needed, able to discriminate nociceptors from non-nociceptors in a heterogeneous cell population, such as the dorsal root ganglia (DRG). Previously, our lab showed a link between the veratridine (VTD)-induced oscillatory (OS) and slow decay (SD) calcium response profiles with nociceptors and non-nociceptors, respectively. Here, we validated the VTD-response profiles as broad functional markers of these DRG subpopulations using mice with genetically ablated nociceptors (1.8-DTA). Then, by using voltage-gated sodium channel (VGSC) blockers with different specificities, we demonstrated that the VTD-Ca^{2+} imaging assay can be used as a drug screening platform for drugs, individually or in novel combinations. The VTD-Ca^{2+} imaging assay was then applied in combination with nociceptive agonists to investigate the excitability changes in distinct neuronal subpopulations during DN. In *db/db* mice, during the early metabolic phase, small- to medium-diameter nociceptors showed 1.4 -fold increased sensitivity to CAP and 1.2-fold increased VGSC excitability. In the late, NEU phase, small-diameter nociceptors showed increased sensitivity to CAP (1.6-fold) and increased VGSC excitability (1.4-fold), whereas medium nociceptors show decreased sensitivity to AITC (1.8-fold) . This is the first study to characterise phase- and subpopulation-specific excitability changes in the well-established *db/db* mouse model. Collectively, these results point to specific subpopulations of DRG neurons affected during the early and late stages of DN. These findings could aid in the better targeting of novel therapies for the treatment of DN and pain. Furthermore, we have demonstrated that the VTD-Ca^{2+} imaging assay can be applied as a tool for the characterisation of excitability changes in distinct DRG subpopulations during neuropathological conditions such as DN.

DECLARATION

I declare that the work presented in this thesis is my own original research and has been composed entirely by myself. All supporting literature, resources, and collaborative contributions have been acknowledged clearly.

Part of this work has been published prior to submission of this thesis under the title:

"An unbiased and efficient assessment of excitability of sensory neurons for analgesic drug discovery"

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Katerina Kaloyanova Kaloyanova,

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LIST OF ABBREVIATIONS

•OH	Superhydroxyl
AGE	Advanced glycation end products
AITC	Allyl isothiocyanate
AMH	Aδ-mechano-heat
AP	Action potential
AR	Aldose reductase
ATP	Adenosine 5'-triphosphate
AUC	Area under curve
CAP	Capsaicin
CGRP	Calcitonin gene-related peptide
CIP	Channelopathy-associated insensitivity to pain
CNS	Central nervous system
DAG	Diacylglycerol
DN	Diabetic neuropathy
DoA	Days of age
DRG	Dorsal root ganglia
eGFP	Enhanced green fluorescent protein
FADH2	Flavin dinucleotide + hydrogen
FEPS	Familial episodic pain syndrome
FFAs	Free fatty acids

GECI	Genetically encoded calcium indicator
GlcNac	5-diphosphate-N-acetylglucosamine
GLUT	Glucose transporter
GoF	Gain of function
GP	Glutathione peroxidase
H$_2$O$_2$	Hydrogen peroxide
HFD	High fat diet
IB4	Isolectin B4
ID	Intermediate decay
IEM	Inherited erythromelalgia
IENF	Intraepidermal nerve fibre
iPSC	Induced pluripotent stem cell
IR	Insulin resistance
KO	Knock-out
LDL	Low density lipoproteins
LoF	Loss of function
LOX1	Oxidized-LDL-receptor 1
LTMR	Low-threshold mechanoreceptors
NADH	Nicotinamide dinucleotide + hydrogen
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCV	Nerve conduction velocity

NF200	Neurofilament 200
NOD	Non-obese diabetic
OS	Oscillatory
P2X3	P2X purinoceptor 3
PARP	Poly(ADP-ribose) polymerase
PC12	Pheochromocytoma cells
PDN	Painful diabetic neuropathy
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
RAGE	Receptor for advanced glycation end products
RD	Rapid decay
RMP	Resting membrane potential
ROS	Reactive oxygen species
SC	Schwann cell
scRNA-seq	Single-cell PCR and RNA sequencing
SD	Slow decay
SNRI	Serotonin and noradrenaline reuptake inhibitors
STZ	Streptozotocin
T1D	Type 1 diabetes
T1DN	Type 1 diabetic neuropathy

T2D	Type 2 diabetes
T2DN	Type 2 diabetic neuropathy
TCA	Tricyclic antidepressants
TH	Tyrosine hydroxylase
TLR4	Toll-like receptor 4
TRP	Transient receptor potential-activated
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin resistant
TTX-S	Tetrodotoxin sensitive
VGCC	Voltage-gated calcium channels
VGKC	Voltage-gated potassium channels
VGSC	Voltage-gated sodium channel
VTD	Veratridine
WoA	Weeks of age

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CHAPTER 1

INTRODUCTION

1.1. The nervous system and dorsal root ganglia (DRG) neurons

The nervous system is the most complex body system with a highly organised structure. It is composed of two major parts: the central nervous system (CNS), constituting the brain and spinal cord; and the peripheral nervous system (PNS), comprising all the nerves connecting the CNS to all organs, tissues and skin. The PNS contains sensory (afferent) neurons responsible for conveying sensory stimuli to the CNS, where the information is processed and a response to the stimuli is generated. The response signal is then relayed through efferent neurons and expressed in the form of movement[1].

The sensory branch of the PNS comprises an intricate network of sensory neurons and pathways responsible for transducing and transmitting information that organisms can "feel". The cell bodies of sensory neurons are contained within small, ball-like structures called ganglia. These can be located at the base of the skull, innervating the head and face, called trigeminal ganglia (TG). The cell bodies of the neurons innervating the rest of the body reside

Figure 1.1. Cross section of a spinal cord illustrating the relative position of the dorsal root ganglion containing the cell bodies of sensory neurons. Source: CNX OpenStax. (2012, August 22). Biology [Cross section of Spinal Cord], available at https://commons.wikimedia.org/wiki/File:Figure_35_04_04.jpg
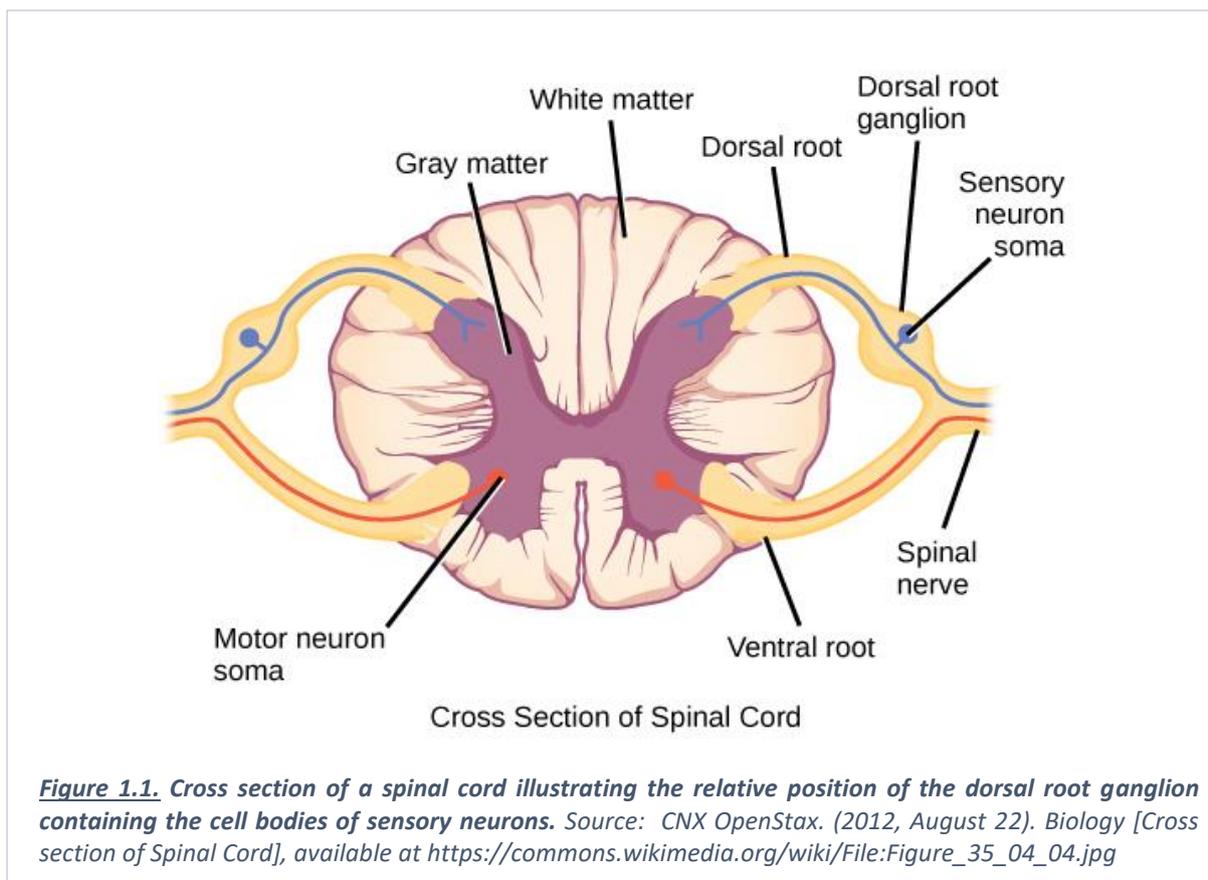
in ganglia along the spinal column called dorsal root ganglia (DRG) – the focus of this thesis (Figure 1.1). The pseudounipolar nature of DRG neurons somata means a single afferent fibre (axon), extends briefly from the cell body and then bifurcates into: a short central branch,

projecting into the CNS through the spinal cord; and a longer peripheral branch, innervating peripheral tissues and terminating in the skin, viscera, tendons, bones and muscles[1,2].

1.1.1. DRG neurons: heterogeneity and classification

Sensory neurons relay complex information from an abundant variety of innocuous (harmless) and noxious (painful) stimuli to the CNS. This richness of sensory potential is supported by the complex heterogeneity of DRG neurons. The cell bodies of sensory neurons are intermingled together within the DRG, however they are organized into a number of types and subtypes, each specialised for the detection of distinct stimuli. These include neurons sensing body movement and position (proprioceptors), touch and pressure (mechanoreceptors), itch (pruriceptors), temperature (thermoreceptors) and pain (nociceptors). Although a certain level of distinct categorisation exists, sensory neurons can also be "polymodal", or able to integrate multiple modalities, e.g., thermal and mechanical stimuli. Additionally, up to 25% of DRG nociceptors are normally dormant and only activated upon injury and are thus called "silent nociceptors"[3,4].

The basis for the classification of DRG neurons was formed as early as the 1920s by Gasser and Erlanger – the first to show a relationship between DRG neurons soma diameter, axon myelination degree, signal conduction velocity and fibre projection targets within the spinal cord lamina. They established that the smaller the soma diameter, the less the myelin sheathing and the slower the conduction velocity but the higher the excitation threshold is, and vice versa (Figure 1.2)[5,6]. From their morphological and electrophysiological observations arose the conventional DRG sensory neurons classification as it is known today: mammalian sensory neurons can be categorised into three general types: Aβ-fibres, Aδ-fibres and C-fibres. Further behavioural studies correlated certain modalities with each fibre class[5].

Table 1.1. Table summarising the relationship between neuronal properties and features. CV, conduction velocity; S, small; M, medium; L, large sizes of mouse DRG neurons. Based on Basbaum et al. (2009) and Li et al.

Myelin	CV	Excitation threshold	Soma size	Fiber class	Modality	Dorsal horn termination lamina
			L ≥ 30 µm	Aα Aβ-LTMR	Proprioception Mechanoreception	VII, VIII III - V
			M 20 - 30 µm	Aδ-LTMR	Mechanoreception	III
				Aδ myelinated fibers	Nociception Thermoception Pruriception	I, V
			S ≤ 20 µm	C (non-peptidergic) C (peptidergic) C-LTMR	Mechanoreception	I, II I, II II
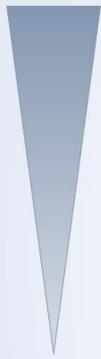
Aα-fibres are the largest in diameter, with conduction velocities in the range of 80 – 120 m/s. Defined by Gasser and Erlanger as motor nerves, the sensory neurons of the proprioceptive modality are usually denoted by the Aα-fibre type. They project into the deeper laminae of the spinal cord [5].

Aβ-fibres have large soma diameter, heavily myelinated and with fast conduction velocities (30 – 70 m/s). The majority have low thresholds of activation and are dedicated to the detection of innocuous mechanical stimuli, such as touch and vibration, stretch and hair deflection [7]. Of note, a significant proportion (e.g. ~20% of A-fibres in rat) of Aβ-fibres are Aβ-nociceptors, conducting in the Aβ conduction velocity range [8]. In the spinal cord lamina, Aβ-fibres project into laminae III-V [9].

Aδ-fibres have thin myelination and hence slower signal conduction velocity (5 – 30 m/s) than Aβ-fibres. Their size distribution in DRG is skewed, with populations of small- and medium-sized cells. In the body, Aδ-fibres innervate mostly superficial organs (e.g. the skin). Aδ-fibres are associated with the detection of innocuous as well as noxious stimuli. Therefore, they include Aδ low-threshold mechanoreceptors (LTMRs) and Aδ nociceptors, responding to noxious mechanical and heat stimuli [10]. Aδ nociceptors can be further divided into Type I and

Type II Aδ-mechano-heat (AMH) units according to the degree of sensitivity to either stimulus. Type I AMH are sensitive to chemical stimuli and have lower threshold to mechanical and higher threshold to heat (>53°C) stimuli. Hence, they are suggested to mediate first pain to noxious mechanical stimuli. In contrast, Type II AMH have lower heat (<46°C) and higher mechanical threshold and are thus suggested to serve the first pain sensation to noxious heat[11]. Activation of Aδ-nociceptors by noxious thermal or mechanical stimuli results in short-lasting, prickling type of pain. In the spinal cord, Aδ-fibres terminate in laminae I, V (Aδ nociceptors) and III (Aδ-LTMRs) [10].

C-fibres constitute over 50% of all DRG neurons and are of the smallest soma diameter. Their axons are unmyelinated and the signal conduction velocity ranges between 0.5 – 2 m/s. They are activated by one or a combination of two or more modalities, including temperature shifts, pruritogens, chemical irritants and mechanical pressure. Their high activation thresholds to these stimuli render the majority, but not all, of them nociceptors. Nociceptive C-fibres innervate deep somatic structures, such as the muscles and joints [12]. While Aδ-fibre nociceptors convey acute and localized nociception, called 'first' or fast pain, nociceptive C-fibres propagate 'second pain' which is more diffuse, dull and longer lasting [13]. A portion of C-fibre neurons, termed C-LTMRs, are responsible for propagating innocuous touch stimuli [9]. Centrally, C-fibres project into the superficial laminae I-III.

Albeit still used today, fibre classification of DRG neurons does not reflect the full heterogeneous spectrum of neuronal function and hence is not an adequate functionality predictor on its own. Neurochemical markers and molecular classifications of DRG neurons have become key in building a more complete picture. A comprehensive summary of somatosensory cell type markers is presented in Table 1.2.

More recently, transcriptomic techniques such as microarray, single-cell PCR and RNA sequencing (scRNA-seq) have produced their own elaborate DRG classifications. Usoskin et al. used scRNA-seq to analyse 622 DRG neurons, detecting 3900 ± 1880 genes per neuron and distributing the neurons into 11 molecularly distinct subtypes with *in vivo* validation of the predicted subpopulations [14]. Not long after them, Li et al. performed an even more in-depth sequencing of 197 DRG sensory neurons detecting 10950 ± 1218 genes per neuron and generating 17 molecularly distinct neuronal subtypes (however, not *in vivo* validated) [15]. Their work was later reanalysed and their predicted number of classes reduced to 9 subtypes which largely overlapped with the predicted subtypes by Usoskin [16]. Most recently, Zeisel et al. published a comprehensive scRNA-seq analysis of 1580 DRG neurons, which, merged with

that   ***Table 1.2. Summary of the most commonly used markers for neuronal***   of
subpopulations. *Source: Pichon and Chesler 2014*

Marker	Sensory cell type
Parvalbumin	Proprioceptors and Aβ-fibres
CGRP	Peptidergic C fibres, subpopulation of Aδ-fibres
Substance P	Peptidergic C fibres
NF200	Myelinated Aδ-fibres, Aβ-fibres and proprioceptors
IB4	Non-peptidergic C-fibres
Trpv1	Small diameter C-fibres (heat & pain)
Trpm8	Small diameter C-fibres (cold & pain)
MrgprD	Small diameter C-fibres (noxious mechanical,pain)
MrgprA3	Small diameter C-fibres (itch)
MrgprB4	Small diameter C-fibres (innocuous mechanical)
VGlut3	Non-peptidergic C-fibres (innocuous mechanical, cooling)
TH	Non-peptidergic C-fibres (innocuous mechanical, cooling)
TrkB	Aδ-fibres (innocuous mechanical, cooling)
Npy2r	Aβ-fibres (innocuous mechanical)
Chondrolectin	Aβ-fibres (innocuous mechanical)
DOR	Subpopulations of non-peptidergic C fibres and myelinated NF200+ fibres

Usoskin's, delivers the most elaborate molecular classification of DRG sensory neurons to date, with a total of 18 neuronal subtypes identified [17].

1.1.2. DRG neurons excitability

A non-firing, or 'resting' neuron maintains an electrochemical gradient across its membrane called a resting membrane potential (RMP). It is determined by the uneven distribution of sodium (Na^+), potassium (K^+), chloride (Cl^-), calcium (Ca^{2+}) and organic anions across the membrane. The RMP is established by the difference in ion concentrations inside and outside the neuron and the relative permeabilities of the membrane to different ions. At rest, there are more extracellular Na^+ ions than intracellular K^+ ions. This concentration difference is maintained by Na^+/K^+ pump cycles, exchanging 3 Na^+ out for 2 K^+ into the cell.

At rest, the membrane is more permeable to K^+ than Na^+, ions, letting them diffuse down their concentration gradient to the outside of the cell. Eventually, the free movement of K^+ ions,

unbalanced by the movement of Na$^+$ to the inside, will cause the neuron to have negative charge inside and positive charge outside. Due to the established extracellular/intracellular charge difference, the excess negatively charged ions on the inside are attracted to the outside and vice-versa, accumulating along the inside and outside surfaces of the membrane and establishing a negative membrane potential. The membrane is 'polarised', causing the RMP of an average neuron to be around -70mV (Figure 1.2). Stimulus detection causes membrane depolarisation, which even small can activate some VGSCs and increase the Na+ permeability of the membrane, producing even greater depolarisation. The 'threshold' potential (~ -55mV) of a neuron is the critical point at which the membrane is depolarised to elicit an action potential (AP). During its depolarised state, the membrane shifts its permeability from K$^+$ to Na$^+$ ions, influencing the reversal of the charge difference across it. A critical component of the AP is the influx of Ca^{2+} ions through voltage-gated Ca^{2+} channels (VGCCs), important for the activation of K$^+$ channels (key during repolarisation) and transmitter release [2,18] . Following AP generation, the neuron needs to engage cellular mechanisms for Ca^{2+} ion clearance to avoid Ca^{2+} overflow in the cell and initiate repolarisation. Most of the intracellular Ca^{2+} is cleared through extrusion via the transmembrane Na$^+$/Ca^{2+} exchanger or Ca^{2+} ATPase. Alternatively, Ca^{2+} ions can also be sequestered through uptake by the neuronal endoplasmic reticulum via its own transmembrane Ca^{2+} ATPases or into mitochondria [19]. All Ca^{2+} clearance mechanisms require energy in the form of ATP to shuttle Ca^{2+} ions out of the neuronal cytoplasm and play an important protective role and influence the shape of the Ca^{2+} signal and thus AP [19,20].

The pathway of the signal propagation follows three main steps: transduction, transmission and perception. Each of them is carried out through the highly coordinated activation and deactivation of all the different ion channels across the membrane, allowing the external stimulus to travel through the PNS and be perceived and processed in the CNS. This process will be reviewed in more details in the context of pain signalling in the next section of this chapter along with some of the key channels and receptor regulators in the signalling pathway.

Figure 1.2. *A simplified visualisation of the action potential generation in neurons and phases of depolarisation and repolarisation and thresholds.* *Adapted from: Yam et al. 2018*
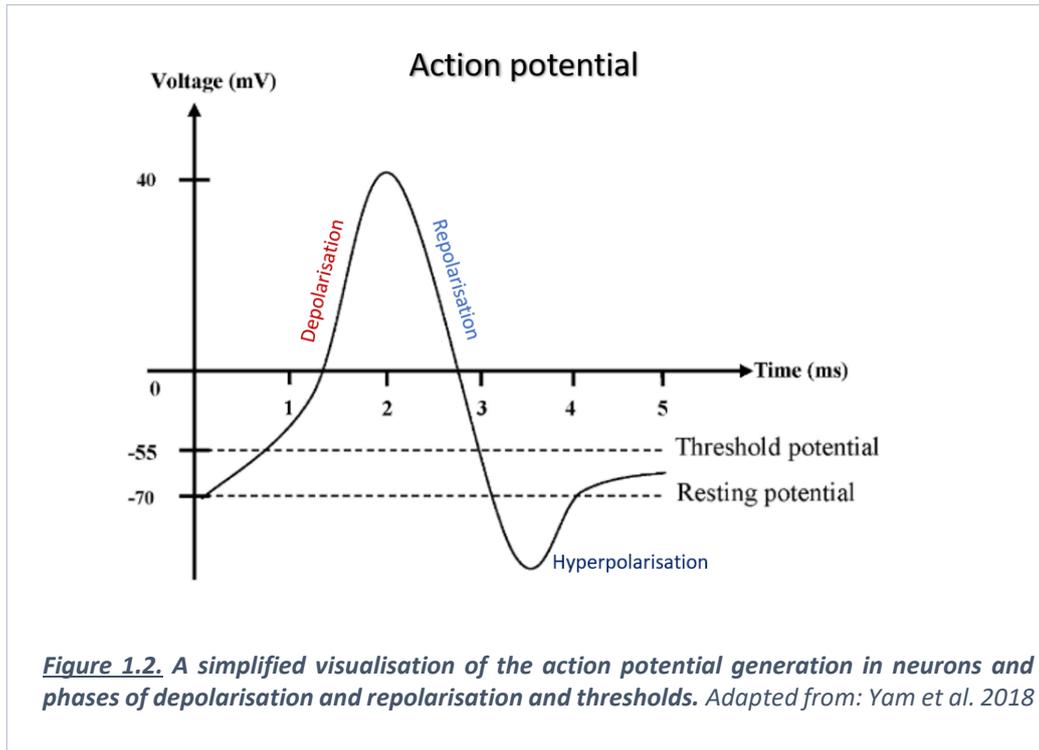
1.2. Pain: the good and the bad

1.2.1. The pain signalling pathway

The ability to detect, transmit and perceive pain is a key protective mechanism essential for one's survival. The process of pain signalling is called nociception. It starts with a noxious stimulus (chemical, mechanical or thermal) being detected at the terminals of nociceptors. The detection of the stimulus activates ion channels and receptors such as transient receptor potential activated (TRP) channels. This leads to the influx of Na^+ which starts depolarizing the membrane, bringing it closer to the activation threshold of voltage-gated sodium channels (VGSCs) which act to amplify the signal. Once the threshold potential is reached, local VGSCs open simultaneously resulting in complete membrane depolarization and peak potential. The external stimulus is transduced into an action potential (AP)[21]. VGSCs then begin inactivating and voltage-gated potassium channels (VGKCs) and K+ leakage channels open allowing K^+ efflux. This initiates membrane repolarization and subsequently hyperpolarization. Finally, the Na+/K+ pump takes over to restore and maintain the RMP (Figure 1.2). In the meantime, the AP generated is propagated up the axon driven by the same sequence of ion channels opening and closing akin to a chain reaction, going through the DRG cell body and eventually reaching the dorsal horn of the spinal cord. Once at the nociceptor's presynaptic terminal, the AP

causes VGSCs there to open and Na$^+$ to flood in, which in turn activates local voltage-gated calcium channels (VGCCs). This results in calcium influx, bringing on a release of neurotransmitters such as substance P, CGRP or glutamate. The AP is thus passed over to the postsynaptic terminal of a second-order neuron (interneuron), completing the synaptic transmission. Finally, from there the signal can be propagated to the brain, where the site of pain perception and processing lies [21].

1.2.2. Pain as a disease

Dysregulation in any of the signalling steps outlined above can lead to abnormal pain signalling producing neuropathic pain. Unlike nociceptive pain, which is transient in nature and benefits the individual, neuropathic pain is sustained, usually chronic and evolving throughout the neuropathy duration. In its pathological state, pain becomes a serious disease affecting 7-10% of people worldwide[22,23]. Neuropathic pain can arise from a direct injury to the sensory nerves or as a complication of a disease such as diabetes mellitus and other metabolic diseases[24]; cancer and chemotherapy[25]; HIV-infection[26], leprosy[27] and immune and inflammatory responses[28] amongst others[29]. Damage to different pathway components can produce different signalling impairments such as decreased or increased AP firing thresholds, causing hyper- and hypoexcitability, respectively; dysregulated firing duration and/or degree and hence pain intensity (hyper- or hypoalgesia) or inappropriate nociceptive firing in the presence of harmless stimuli (allodynia) or in the absence of a stimulus altogether (spontaneous pain episodes)[30].

1.2.3. DRG-expressed channels and receptors involved in pain

From noxious stimuli detection to pain perception, pain signalling is a highly organised and controlled process. It relies on the appropriate expression and function of different voltage-gated sodium, potassium and calcium channels, leak channels and ligand-gated channels

Figure 1.3. The ion channels involved in the pain signaling pathway.
Site 1, Transduction: Noxious stimuli are sensed by DRG neuron terminals which activates local VGSCs and nonselective cation channels (e.g. TRP channels); Site 2, Propagation: the action potential is propagated along the axon driven by the activation of VGSCs, VGKCs. Site 3, Synaptic transmission: action potential reaches DRG cell bodies and activates local ion channels which mediate the transmission to the synaptic terminals in the dorsal horn. There, VGSCs and VGCCs open to enable neurotransmitter release necessary for the transmission of the signal to the CNS where it is processed. TRP, transient receptor potential; VGSC, voltage-gated sodium channel; VGKC, voltage-gated potassium channel; VGCC, voltage-gated calcium channel; Source: Waxman et al., (2014)

(TRPs and acid-sensing ion channels). Among the vast diversity of ion channels implicated in pain signalling, several specific families and subtypes have been demonstrated as key regulators of sensory neurons excitability (Figure 1.3). Channel subtype-specific changes in distribution, density and kinetics are the main drivers of abnormal activity in the afferent neurons [21,31,32]. Genetic and pharmacological studies have validated several of them as promising drug targets in human conditions of pain signalling malfunction [21,32–34]. These include DRG-expressed voltage-gated ion channels (VGSCs, VGCCs, VGKCs), channels of the TRP family and ligand-gated ion channels and leak channels. Those of them relevant to the experiments in this thesis will be discussed in more detail next.

1.2.3.1. Voltage-gated sodium channels

VGSCs regulate AP firing in all electrically excitable cells, such as skeletal and cardiac muscle cells and neurons. Their main role in neurons is to initiate, amplify and propagate the AP. The major functional Na⁺-conducting component of VGSCs is formed by their α-subunit [35], while their kinetics and biophysical properties are regulated by their β-subunits [36]. In mammals, there are nine VGSC α-subunits and thus nine different VGSC isoforms (Na$_v$1.1-Na$_v$1.9) sharing overall structural and gating motifs but differing in their expression patterns, pharmacological and functional signatures (table 1.3). A 10th VGSC isoform has also been identified (Nax), however it does not share the voltage-gating properties of the rest[37].

Table 1.3. Voltage-gated sodium channel isoforms discovered to date with respective gene, tissue distribution and sensitivity to tetrodotoxin (TTX). TTX-S, tetrodotoxin-sensitive; TTX-R, tetrodotoxin-resistant;TG, trigeminal ganglia; SCG, superior cervical ganglia; ND – not determined. Source: Cummins et al. (2020)

Channel	Gene	Tissue distribution	TTX sensitivity
Na$_v$1.1	SCN1A	DRG, TG, CNS	TTX-S
Na$_v$1.2	SCN2A	CNS	TTX-S
Na$_v$1.3	SCN3A	Foetal DRG, CNS	TTX-S
Na$_v$1.4	SCN4A	Skeletal muscle	TTX-S
Na$_v$1.5	SCN5A	Heart muscle	TTX-R
Na$_v$1.6	SCN8A	DRG, TG, CNS	TTX-S
Na$_v$1.7	SCN9A	DRG, TG, SCG	TTX-S
Na$_v$1.8	SCN10A	DRG, TG	TTX-R
Na$_v$1.9	SCN11A	DRG, TG	TTX-R
Nax	SCN7A	Enteric, lung, nerve	ND

VGSCs have different sensitivity to pore-blocking toxins, the most extensively used of which is tetrodotoxin (TTX). Accordingly, it is used to differentiate TTX-sensitive (TTX-S) from TTX-resistant (TTX-R) VGSCs. Sodium channels of the PNS can be both TTX-S and TTX-R [38–40]. Distinct TTX sensitivity has been used to distinguish VGSC biophysical properties and

contributions, particularly in nociceptive neurons. By and large, TTX-S VGSCs are characteristic with fast activation and inactivation, whereas TTX-R channels are slowly activating and inactivating[41] .

Five VGSC isoforms are expressed in the DRG (Na$_v$1.1, Na$_v$1.6-1.9) and of these, three (Na$_v$1.7-Na$_v$1.9) are expressed relatively specifically in nociceptors. Although virtually all isoforms have been implicated in pain, the contribution to nociception of Na$_v$1.3, Na$_v$1.6, Na$_v$1.7, Na$_v$1.8, and Na$_v$1.9 has been the most extensively evidenced[42,43]. The next part of this chapter will explore their role in nociception and pain disorders in more detail by discussing the expression and biophysical properties of each as well as evidence from clinical and animal studies.

- *Na$_v$1.3*

Albeit not as strongly implicated in pain as the rest of the channels discussed in this section, Na$_v$1.3 is of particular interest as a pain target due to its circumstantial expression pattern. Namely, this channel is usually not detectable in the adult, fully developed nervous system but its expression is upregulated in DRG neurons as well as second- and third-order neurons of the dorsal horn and thalamus under certain pathological conditions such as peripheral nerve inflammation and transection [44,45]. Na$_v$1.3 has fast activation and inactivation and rapid repriming kinetics as well as persistent current that contribute to spontaneous ectopic discharges and sustained repetitive firing in injured neurons [31]. Due to the absence of isoform-selective and safe blockers, evidence of its involvement in neuropathic pain stems from genetic experiments. Antisense-driven Na$_v$1.3 knockdown studies show both attenuation of pain [46] and no effect [47]. More recently, knockdown based on adeno-associated virus delivery of Na$_v$1.3 short hairpin RNA has been demonstrated to produce amelioration of pain behaviour in rodent models of neuropathic pain [48] as well as diabetic neuropathy [49].

- *Na$_v$1.6*

The TTX-S channel Na$_v$1.6 is the most abundantly expressed isoform in the CNS. In the PNS, it is present in all parts of the peripheral nerves, but it is especially abundant in the nodes of Ranvier, the microscopic myelination gaps along an axon, in myelinated DRG fibres. It is expressed constitutively in all DRG sensory neurons, with higher concentration in large, NF200+ neurons [50]. In fact, Na$_v$1.6 has been shown to contribute to up to 60% of Na$^+$ currents in large DRG compared to 34% in small DRG neurons [51]. It has a more hyperpolarised activation voltage compared with other VGSC isoforms and mediates persistent and resurgent Na$^+$ currents contributing to repetitive AP firing [52,53]. The role of Na$_v$1.6 in pain signalling has been established mainly through genetic studies. While gain of function mutations are linked to trigeminal neuralgia in humans [54], Na$_v$1.6-KO attenuates neuropathic pain in mice [51] and

Na$_v$1.6-knockdown decreases TTX-S resurgent DRG currents and mechanical allodynia [55,56]. Emerging evidence has pointed to the differing temporal upregulated expression of Na$_v$1.6 in different stages of neuropathic pain. Different models of neuropathic pain, including nerve injury and diabetic neuropathy, show decreased or unchanged levels of expression of Na$_v$1.6 in DRG in the first 4 weeks of pain [57,58], whereas at later stages there appears to be a consensus over the upregulated expression of Na$_v$1.6 in DRG neurons [59,60].

- *Na$_v$1.7*

Na$_v$1.7 is highly expressed in the PNS, predominantly in DRG, TG and sympathetic neurons. In DRG neurons, it is detected mainly in Aβ-fibres and C-fibres (nociceptors), along the entirety of the neuron [61,62]. Other excitable cells expressing Na$_v$1.7 include myenteric neurons, visceral sensory neurons and neurons of the olfactory sensory system [63,64] as well as in the hypothalamus of rodents [65]. In non-excitable cells, it has been demonstrated to have a functional role in the pancreatic islet β-cells of some species [66].

Na$_v$1.7 is TTX-S and, like the rest of the channels from this category, is characteristic with rapid activation and inactivation kinetics. However, it has a distinct slow recovery rate from inactivation, making it unlikely to contribute to repetitive firing [53,67]. Na$_v$1.7 also inactivates slowly at negative membrane potentials, which means it can remain open for longer at potentials close to RMP. Thus, it contributes to Na$^+$ currents at RMP, generating "ramp currents", important for recruiting other VGSCs (such as Nav1.8) to elicit an AP [53]. These specific biophysical properties, together with its high expression in fibre terminals, render Na$_v$1.7 an AP generator or 'threshold' channel, setting the gain in nociceptors.

The first clues for Na$_v$1.7 implications in pain signalling were provided by genetic and functional profiling studies of mutations in the gene encoding it – *SCN9A*. Missense mutations in *SCN9A* produce inherited erythromelalgia (IEM), a disorder presenting with severe burning pain in the distal extremities in response to mildly warm stimuli (thermal hyperalgesia) [68,69]. The exaggerated thermal pain phenotype is presumed to be the result of a gain of function (GoF) mutations in the SCN9A gene causing hyperpolarized activation of Na$_v$1.7 bringing its activation threshold further down. Being easier to activate, even small depolarizing stimuli are sufficient to trigger Na$_v$1.7 opening in nociceptors causing allodynia and hyperexcitability [70,71]. Another dominant GoF mutation in the *SCN9A* gene, which impairs Na$_v$1.7 rapid inactivation, causes a syndrome known as paroxysmal extreme pain disorder [72]. The depolarising shift in the channel's fast inactivation produces an increase in resurgent Na$^+$ currents leading to enhanced DRG excitability [73,74]. Patients suffering from this disorder experience extreme rectal, ocular and mandibular pain and erythema [72]. Other GoF *SCN9A* mutations have been identified

to occur in up to 30% of patients with diagnosed small-fibre neuropathy, impairing $Na_v1.7$ kinetics in both directions, i.e. hyperpolarising its activation potential and depolarising its inactivation potential in nociceptive Aδ- and C-fibres [75]. GoF $Na_v1.7$ variants have also recently been associated with diabetic neuropathy [76] - discussed in more detail later. The increased $Na_v1.7$ activity in these subjects produces spontaneous firing in DRG neurons causing severe pain.

In contrast, loss of function (LoF) recessive mutations in the *SCN9A* gene have been linked to channelopathy-associated insensitivity to pain (CIP). Affected individuals present with normal sensitivity to innocuous stimuli but display painless wounds, fractures and childbirth [77,78] as well as, curiously, an inability to smell (anosmia) [63]. In this case, the mutations produce a truncated variant of the $Na_v1.7$ channel which is not functional. The function of the small fibres is impaired while that of large fibres is preserved. Further to that, a recent study investigating the role of $Na_v1.7$ in CIP patients in more depth showed that the absence of a functional $Na_v1.7$ leads to structural changes in the affected afferents as well. They reported a significant decrease in intraepidermal nerve fibre (IENF) density of the distal leg and thigh of patients, implying a potential role of $Na_v1.7$ in long-term structural integrity in human nociceptors, particularly in the distal terminals [78]. Additional links between $Na_v1.7$ and human pain disorders have also emerged from studies showing that single nucleotide polymorphisms in the $Na_v1.7$ gene lead to a subtle increase in nociceptor excitability and increased pain sensitivity in pain disorders [79,80]. Albeit extremely rare, collectively, these mutations strongly support the importance of $Na_v1.7$ in the pain signalling pathway and as a target for pain relief.

Clinical case data has been further supported by animal studies. $Na_v1.7$-knock-out (KO) mice have aided significantly in the understanding of the mechanisms of $Na_v1.7$ role in pain. Originally, a $Na_v1.7^{-/-}$ global KO in mice was lethal due to producing pups unable to feed. It was later demonstrated that this was due to the high expression of $Na_v1.7$ in olfactory sensory neurons in rodents [64]. Knocking the channel out completely prevented blind new-born pups from relying on their sense of smell to feed. This insight allowed for the successful generation of a robust global $Na_v1.7$-KO model in 2014 by manually supporting KO mouse pups feeding [81]. The optimised $Na_v1.7$-KO model produced mice with near complete acute, inflammatory and neuropathic pain deficit.

Tissue-specific $Na_v1.7$-KO mutants utilising the Cre-recombinase-loxP system have also been available for the last decade. The first evidence of $Na_v1.7$ function in pain from such studies was supplied by a KO mouse whereby Cre- was driven specifically into $Na_v1.8$-expressing neurons, i.e. small-diameter nociceptors, since Nav1.8 is predominantly expressed there. The

conditional deletion of Na$_v$1.7 led to a dramatic loss of mechano- and inflammatory pain sensitivity [82]. Other tissue-specific Nav1.7-KO models employing alternative DRG-targeting approaches (e.g. DRG-specific advillin, or neural crest-specific protein Wnt1) produced similar phenotypes [83,84].

Of note, not long ago it was demonstrated that there exist Na$_v$1.7 independent pain states. One case study reported a unique CIP patient experiencing chronic neuropathic pain following pelvic fractures and lumbar nerve impingement where Na$_v$1.7 signalling was intact [85]. Similarly, a different study showed that Na$_v$1.7 was not required for the phenotype of chemotherapy-induced or cancer-induced bone pain in a mouse model [86] suggesting that similar pain phenotypes can be driven by different molecular mechanisms.

Overall, Na$_v$1.7 has been strongly established as a key regulator in DRG excitability, specifically in nociceptors. Its dysregulation has been extensively demonstrated to contribute to abnormal pain signalling in animal experiments and patients [78]. Thus, it has remained as, perhaps, the most studied target for analgesic drugs with various Na$_v$1.7 channel blockers being developed (discussed in more detail in section 1.3.3.2.)

- *Na$_v$1.8*

Encoded by the *SCN10A* gene, Na$_v$1.8 is expressed in sensory neurons in the DRG, TG as well as nodose ganglion neurons [87,88]. In the DRG, it is expressed in up to 90% of both non-peptidergic (IB4+) and peptidergic (IB4-) small nociceptive fibres [89], however it has been shown to be expressed in large myelinated neurons, including LTMR C- and Aβ fibres responsible for touch sensation [90]. Elsewhere, it is detected in high quantities in intracardiac neurons where it plays a significant role in cardiac electrophysiology [91]. Na$_v$1.8 is of the TTX-R family of VGSCs, but it is characteristic with its own unique biophysical properties. Compared to Na$_v$1.9 and TTX-S channels, Na$_v$1.8 is distinguished with a more depolarized activation threshold and slow inactivation. What this means is that: 1) Na$_v$1.8 can be activated only following the activation of a TTX-S channel and 2) Na$_v$1.8 channels can remain active long after other VGSCs have been inactivated, contributing to most of the generated Na$^+$ current upon depolarisation [92,93]. Na$_v$1.8 also recovers quickly from inactivation and thus, contributes to high frequency AP firing when membrane depolarisation is sustained [92].

Several GoF mutations have emerged from patient cases. An international coalition from 2012 conducted genetic analyses of 104 small-fibre neuropathy patients. No *SCN9A* (Na$_v$1.7) mutations were identified, but 7 *SCN10A* mutations were described in 9 of the patients. All of them shared similar shifts in the Na$_v$1.8 channel's kinetics, including hyperpolarization of its activation threshold and acceleration in its recovery from inactivation[94–96]. This lowered the AP

generation threshold and increased the rate of AP firing, thus producing high-frequency spontaneous firing even in the absence of stimuli, culminating in DRG hyperexcitability.

Natural *SCN10A* LoF mutations have not yet been identified in humans, therefore knowledge of Na$_v$1.8's role in pain has been derived from genetic studies on animal models. The global Na$_v$1.8KO mouse showed only moderate analgesia to painful stimuli [97]. In a later study, a Na$_v$1.8KO mouse model of neuropathic pain displayed no change in its painful phenotype [98]. These observations contradict those obtained from mice with genetically ablated neurons expressing Na$_v$1.8 channels, where animals showed profound loss of pain [99]; or knocked-down Na$_v$1.8 channel, where TTX-R current was reported to be significantly reduced and spinal nerve ligation-induced neuropathic pain behaviour reversed [100]. This discrepancy was attributed to a key compensatory mechanism being triggered in the global Nav1.8KO, whereby Na$_v$1.8 absence induced upregulation of Na$_v$1.7 expression and hence TTX-S current, thus preserving the neuropathic pain phenotype [87]. However, in a later study, a Na$_v$1.7/Na$_v$1.8 double KO mouse model displayed reduced inflammatory pain and impaired mechanical and thermal acute pain thresholds but developed normal levels of neuropathic pain, implicating Nav1.8 is not necessary for nerve injury-induced pain [101].

Interestingly, Dib-Hajj et al. (1996) observed that Na$_v$1.8 expression and TTX-R current is downregulated in injured DRG neurons (following transection of the peripheral nerve), however they were upregulated in neighbouring uninjured neurons[102]. A similar conclusion was also reached by Gold et al. (2003) who observed a significant redistribution of Na$_v$1.8 channels to uninjured neurons adjacent to injured fibres of the sciatic nerve. This was accompanied by an increase in the TTX-R current, particularly in uninjured C-fibres and partially in thinly myelinated Aδ-fibres following injury, leading to spontaneous activity in these neurons [103]. Remarkably, Coward et al. (2000) established a time-dependent shift in Na$_v$1.8 involvement in such neuropathic pain. Overtime, post-injury Na$_v$1.8 upregulation and hyperexcitability affected not only uninjured but also the injured nociceptors as well. Therefore, considering neuropathic pain in particular, Na$_v$1.8 might be involved via a temporal role shift from intact to injured neurons as the injury progresses [104]. In contrast to neuropathic pain, the contribution of Na$_v$1.8 to inflammatory pain is better established. Different inflammatory mediators, such as NGF, TNFα and other cytokines, have been evidenced to modulate Na$_v$1.8 biophysical properties and causing hyperexcitability in DRG neurons [98,105–108].

- *Na$_v$1.9*

The other DRG-expressed TTX-R channel is Na$_v$1.9, encoded by the *SCN11A* gene. Unlike Na$_v$1.8, however, it is activated at low voltage potentials (approx. -70mV), close to the RMP

and produces a persistent current [109]. Na$_v$1.9 is expressed in the soma of small-diameter DRG and TG neurons. Expression in the PNS is particularly concentrated in non-peptidergic nociceptors [110,111]. The specific biophysical properties of Na$_v$1.9 and its distribution along sensory neurons suggest it may determine the activation threshold of small DRG neurons [109].

The role of Na$_v$1.9 in neuropathic pain is not fully understood and the literature remains controversial. In humans, 7 different mutations have been identified in the *SCN11A* gene in peripheral neuropathy patients which modified the channel properties via various mechanisms. These include reducing the current threshold and increasing the firing frequency thus increasing DRG excitability and causing patients to experience episodic chronic pain [112–114] and, in the case of some – cold pain [115]. Nerve-injury models of Na$_v$1.9-null mice including partial sciatic nerve injury [116], chronic constriction injury [117] and spinal nerve transection [86], demonstrated no change in the pain-thresholds of the animals. In contrast, similar to Na$_v$1.8, Na$_v$1.9 expression and current in sensory neurons has been reported to be significantly lower in different animal models of neuropathic pain, including in DRG [118,119] and TG [120] neurons.

Inflammatory pain, on the other hand, has been strongly linked to the presence of Na$_v$1.9. Several studies assessing the effects of a great diversity of inflammatory agents on Na$_v$1.9KO mice were all consistent in their reports, observing reduction in the pain behaviour of these animals [116,121–123]. It is speculated, that inflammatory mediators act via a G-proteins-dependent mechanism to increase Na$_v$1.9 persistent current and lead to spontaneous activity in affected sensory neurons [124].

From all of the above, DRG-expressed VGSCs contribute greatly to various pain states and represent attractive targets for novel analgesics in development. Characterising the changes in their function in models of neuropathic pain is, therefore, essential for the development of better-targeted and safer therapies. However, they are only one avenue of research into neuropathic pain.

1.2.3.2. Transient receptor potential cation channels: TRPV1 and TRPA1

External stimuli are transduced into APs via the activation of another group of specific cation-conducting channels expressed in the neuronal membrane. These are the superfamily of transient receptor potential (TRP) ion channels which contribute to sensory perception in animals. Most lately, the great diversity within TRP channels was categorised into nine animal subfamilies: TRPA (ankyrin), TRPV (vanilloid), TRPVL (vanilloid-like), TRPC (canonical), TRPN (no mechanoreceptor potential C), TRPM (melastatin), TRPS (soromelastatin), TRPML (mucolipin) and TRPP (polycystin). Each of the subfamilies comprises several subtypes of

channels with distinct structural homology giving rise to specific signal integration and regulation functions. Members of the TRP subfamilies are activated by a great range of stimuli, including photosensation, mechanosensation, thermosensation and nociception, some integrating two or more modalities. Thus, they are key polymodal cellular sensors (for in depth reviews see [125,126]).

ThermoTRP channels represent those members of the families that are particularly sensitive to temperature. Of all TRP channels, nine are characteristic with differing ranges of activation temperatures (Figure 1.4). TRPV1-4, and TRPM2, 4 and 5 are heat-activated, while TRPA1 and TRPM8 are cold-activated [127]. Of these, two have received a great amount of interest and have been implicated in nociception the most [128,129]. Since functional changes in the same two TRP channels are also the focus of part of the upcoming experiment chapters, the rest of this section will cover the properties and role in pain of these two channels, namely TRPV1 and TRPA1.

- *TRPV1*

The TRPV1 (transient receptor potential cation channel subfamily V member 1) channel is the first channel of the TRP family to be cloned and have its structure resolved. To date, it is also the most studied member of the TRP superfamily due to its intriguing polymodality and role in pain [130]. Encoded in humans by the *TRPV1* gene, it is a nonselective cation channel with a ligand- and heat-gated mechanism. In the nervous system, it is mainly expressed in the TG and DRG sensory neurons and cranial nerve cervical ganglia neurons innervating organs in the head [130,131]. In primary afferents, TRPV1 is distributed throughout, from the skin-terminating

Figure 1.4. Diagram depicting the thermal sensitivity spectrum of thermoTRP channels. Source: Latorre et al. (2009)
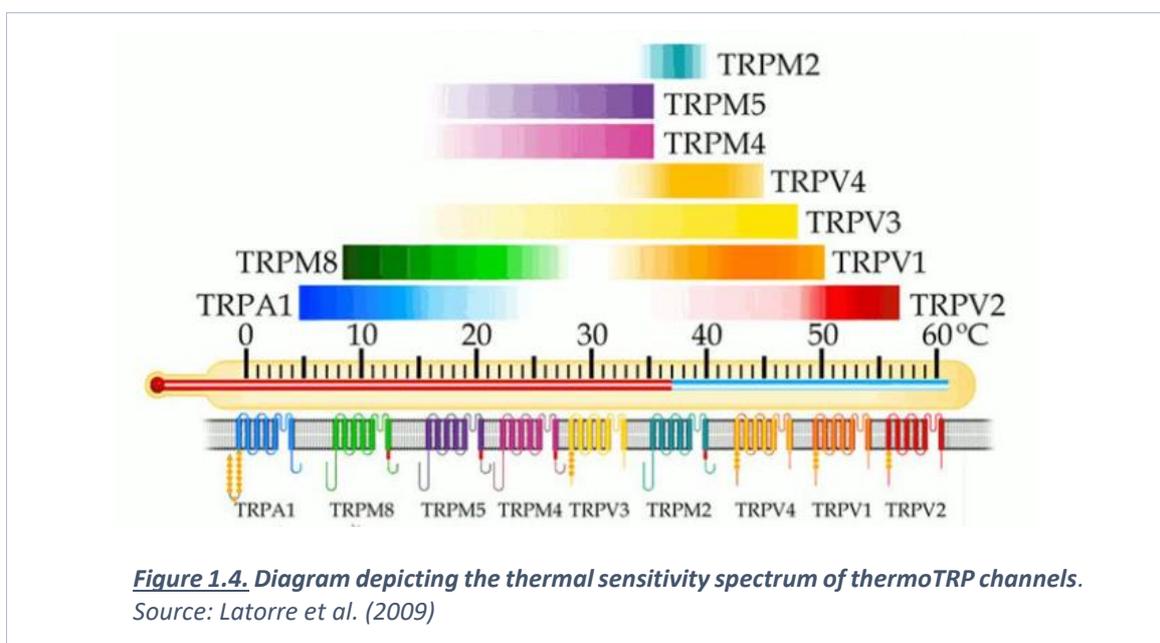
free endings (highest density), along the axons and in the cell bodies, with especially high

expression rates in small-diameter peptidergic nociceptors demonstrated using reporter mice (up to ~82% in the adult mouse DRG) [132].

The structure of TRPV1 has been studied extensively [130] due to its sophisticated gating, regulation and ion selectivity mechanisms. TRPV1 is a cation-permeable channel with a strong preference for Ca^{2+} ions (10-fold more Ca^{2+} conductance than other ions). Upon TRPV1 activation, a strong Ca^{2+} and moderate Na^+ influx is triggered, depolarising the membrane which, if strong enough, can lead to the activation of voltage-gated ion channels (such as $Na_v1.7$) and thus generate an AP [133].

A homotetrameric membrane protein, TRPV1 possesses a distinct structural complexity to which it owes its vulnerability to a range of different stimuli categories. These include vanilloids (capsaicin, resiniferatoxin), lipids, noxious heat (≥43°C) and acidic solutions (protons) (pH< 6.0) [134]. Interestingly, each group of stimuli has its distinct site of action on TRPV1. For example, vanilloids are lipophilic and have been shown to diffuse through the lipid membrane and bind to an intracellular site on TRPV1 [135,136]. Protons, on the other hand, act exclusively on an extracellular acidic site of the channel [137,138], while high temperatures critically change the proximal region of the C-terminus [138]. The segregation of active sites allows for each stimulus to trigger channel gating alone but also for two or more stimuli to act together and cooperate to potentiate the channel. For instance, when pH is low, for example during tissue acidosis following an injury, local TRPV1 channels have lowered temperature activation thresholds so that they can be activated at as low as room temperature [139–141]. This facilitates TRPV1 activation, thus sensitising nociceptors during moderate acidosis in ischemic or inflamed nerve tissues, contributing to allodynia and/or hyperalgesia [140].

TRPV1 has been strongly linked to nociception. Upon an injury, various inflammatory molecules (such as bradykinin and prostaglandin E2) are released which together with cytokines and neurotrophins have been shown to sensitise TRPV1 to its stimuli through phospholipase C, protein kinase A (PKA) or C (PKC) pathways [142–145]; or even directly activate it (such as some lipid mediators) [146] [147]. Evidence for the role of TRPV1 in inflammatory pain comes from experiments with TRPV1-null mice. The mutant mice showed no capsaicin-induced pain behaviour and had attenuated inflammation-induced thermal hyperalgesia and did not respond to noxious heat [148,149]. These observations were confirmed by pharmacological experiments with mice injected with a TRPV1 antagonists (such as capsazepine), also reporting attenuated thermal hyperalgesia under inflammatory conditions [148,150].

In neuropathic pain, the evidence of TRPV1 contribution is still contradictory. In TRPV1-null mice, pain perception does not change following nerve damage [148,151]. However, several animal

models of neuropathic pain exhibit decreased pain following pharmacological inhibition of TRPV1 [152,153]. In addition, development of sustained thermal hyperalgesia has been correlated with the increased expression of TRPV1 [154]. Finally, TRPV1 has been implicated in neuropathic pain arising as a complication from certain conditions. Elevated expression levels of the thermo channel in DRG and dorsal horn have been reported in diabetic neuropathy and cancer-induced chronic pain models, correlating with hyperalgesia behaviour [58,155,156]. Therefore, targeting TRPV1 for pain alleviation may prove promising. In fact several TRPV1-targeting ligands have been in development and focus appears to be equally on agonists as well as antagonists [157].

- *TRPA1*

Another well-studied TRP channel is the TRP ankyrin 1 (TRPA1) channel. TRPA1 is expressed in the sensory neurons of the DRG and TG [158], predominantly in, non-peptidergic (IB4+) nociceptors [159]. It is often co-expressed with TRPV1 in a subset of nociceptive neurons [160] and has been suggested to interact with it and form a heteromeric channel [161]. Being a Ca^{2+}-permeable channel, upon TRPA1 activation intracellular Ca^{2+} levels increase drastically leading to neuronal excitation [158].

Much like TRPV1 and other thermoTRPs, it is activated by a range of stimuli, individually as well as simultaneously. These include mechanical perturbations [162], exogenous irritants such as cinnamaldehyde (compound in cinnamon), cannabinoids, acrolein (present in tear gas), menthol and allyl isothiocyanate (AITC, found in wasabi, radish and mustard) [163–165]; as well as endogenous molecules released upon tissue damage and inflammation [165]. In addition to being a chemosensor, TRPA1 has been implied to be activated by cold temperatures (~17°C), demonstrated clearly *in vitro* [160,166,167]. However others using heterologous expression systems [163,168] as well as TRPA1-null mice [169] report contradicting findings on its ability to be activated by noxious cold, leaving the role of TRPA1 as a cold sensor highly controversial. Of note, TRPM8 is also activated by menthol and cool temperatures (~25°C), shaping a separate population of cells specialised for low temperature sensing [170].

With high expression levels in nociceptors, TRPA1 has been implicated in acute, inflammatory and neuropathic pain. The role of TRPA1 in inflammatory hypersensitivity was first reported in a study demonstrating its contribution to the excitatory effects on nociceptors induced by the inflammatory mediator bradykinin[164]. These results were confirmed by a pivotal study showing decreased formalin-induced nociceptive response in rodents following TRPA1 pharmacological blockade and genetic deletion [171]. Adopting the same methods, TRPA1 was

documented to be involved in mechanical and cold hypersensitivity associated with inflammation as well [172–174].

The involvement of TRPA1 in neuropathic pain was initially suggested by observations made in spinal nerve ligated mice, where TRPA1 was downregulated in injured L5 DRG but upregulated in uninjured L4 DRG (much like the case with Nav1.8 in the same nerve injury model, see section 1.2.3.1, *Nav1.8*) [175]. Similar redistribution of TRPA1 expression was noted in other nerve injury models [176–178]. Several TRPA1 antagonists [179,180] and TRPA1$^{-/-}$ animal models [181–183] have shown attenuated allodynia and hypersensitivity after peripheral nerve injury. TRPA1 has been implicated in diabetic neuropathy: its inhibition reduces mechanical allodynia and hypersensitivity in rat diabetic neuropathy models [184], while methyglyoxal, an abundant glucose metabolite during diabetes, has been shown to activate TRPA1, contributing to hyperalgesia [185].

1.2.3.3. Purinoceptors: P_2X purinoceptor 3 (P_2X_3)

The P_2X purinoreceptor family includes ligand-gated ion channels activated by binding of extracellular adenosine 5'-triphosphate (ATP). Apart from providing energy for cellular processes, ATP also acts as an important intercellular messenger released locally by damaged tissues. It binds P_2X receptors expressed by sensory neuronal terminals and cell bodies in the periphery and interneurons in the dorsal horn [186,187]. There are seven different P_2X subunits (P_2X_{1-7}) expressed in DRG, TG and nodose ganglia. Amongst them, P_2X_3 is the only subtype selectively expressed in small C-fibre nociceptors, particularly the non-peptidergic subpopulation [188,189]. P_2X_3 is a trimeric cation channel contributing to up to 96% of ATP-induced responses in DRG sensory neurons [186]. Upon ATP binding, P_2X_3 channels activate and are desensitized within milliseconds, however ATP dissociation and desensitization recovery can take minutes thus limiting repetitive AP firing [190,191].

Pharmacological studies using P2X3 selective antagonists [192–195] and animal studies using P2X3-deficient [196,197] or P2X3-selective-antisense-treated animals [198–200] have demonstrated that P2X3, along with its heteromeric assembly with P2X2 (forming P2X$_{2/3}$), are responsible for transmitting persistent, inflammatory and neuropathic pain. For example, the intraplantar flinching in P2X3-/- mice was reduced in response to α,β-MeATP and formalin (used to induce inflammatory pain in animals) [197], indicating a strong role of DRG P2X3 receptors in inflammatory pain. Antisense-driven knockdown of P2X3 confirmed these findings and demonstrated reversal of mechanical allodynia in different neuropathic injury models [199,200]. Thus, P2X3 has emerged as a promising target for treating pain conditions and several

antagonists are in development for treating migraine, itch-associated pain and cancer pain [201–204].

To summarise, these sections highlighted the importance of several VGSCs, TRPV1, TRPA1 and P2X3 in nociception and pathological pain promoting further research into their function during chronic pain conditions. These channels form an attractive cohort of targets for efficient molecules that can downregulate their function to alleviate pain and have an important function as markers of sensory subtypes in DRG characterizing experiments. The study presented here focuses on targeting the activation of VGSCs, TRPV1, TRPA1 and P2X3 in order to investigate their contribution, as well as that of the neuronal subpopulations they mark, to neuronal excitability changes during diabetic neuropathy – the topic of the next section of this chapter.

1.3. Diabetic neuropathy and pain

Diabetes mellitus is an umbrella term covering a group of metabolic disorders leading to impaired glucose uptake and metabolism by the body. In a healthy organism, its metabolization involves several steps, simplified and summarised in Figure 1.5. Glucose molecules are obtained from food and metabolised in cells to release energy. Their uptake by the cells from the blood relies on the adequate function of the anabolic hormone insulin, secreted by the pancreatic β-cells. Its role is to bind to its receptor on the cell membrane of hepatocytes, skeletal muscle cells and promote glucose absorption from the blood primarily for energy generation (ATP). If not utilized for energy, excess glucose is stored in the form of glycogen (chains of glucose molecules) in the muscle and liver tissue or triglycerides (fat) in adipose tissue [205,206].
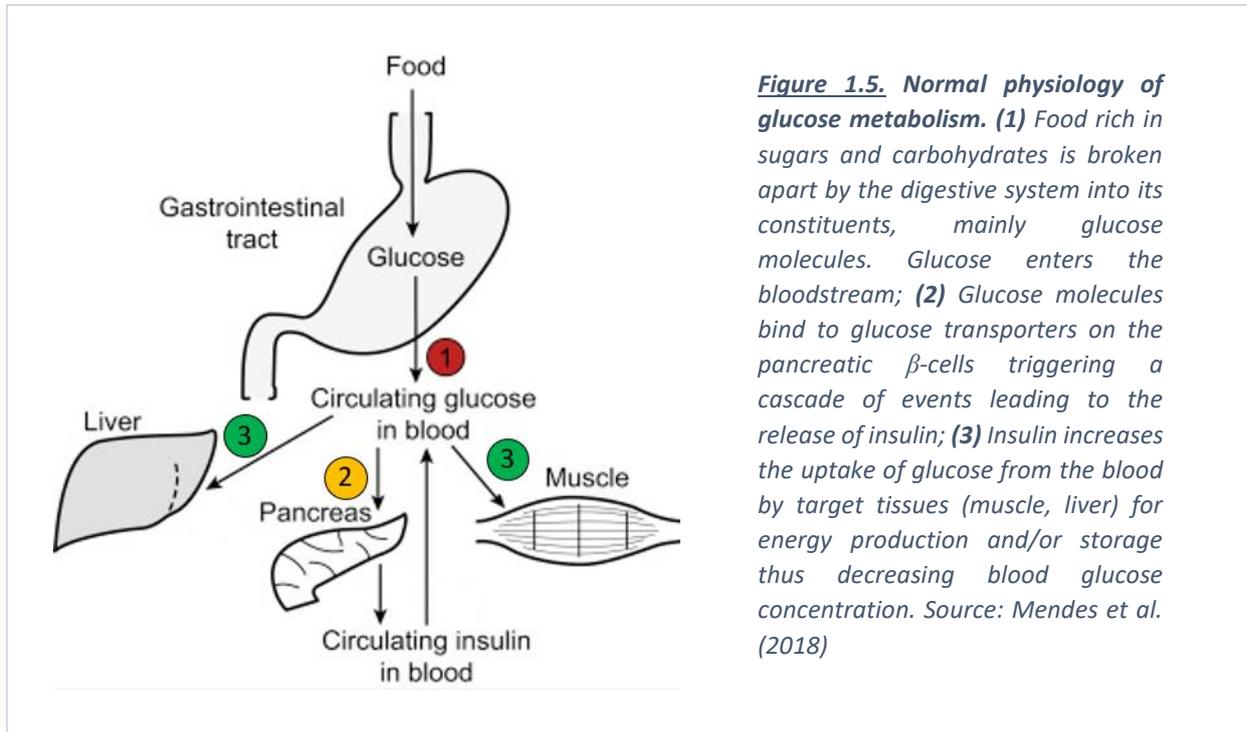
Figure 1.5. ***Normal physiology of*** ***glucose metabolism.*** ***(1)*** *Food rich in* *sugars and carbohydrates is broken* *apart by the digestive system into its* *constituents, mainly glucose* *molecules. Glucose enters the* *bloodstream;* ***(2)*** *Glucose molecules* *bind to glucose transporters on the* *pancreatic β-cells triggering a* *cascade of events leading to the* *release of insulin;* ***(3)*** *Insulin increases* *the uptake of glucose from the blood* *by target tissues (muscle, liver) for* *energy production and/or storage* *thus decreasing blood glucose* *concentration. Source: Mendes et al.* *(2018)*

1.3.1. Diabetes

The main characteristic of all diabetic disorders is impaired insulin signalling rendering the body unable to regulate blood glucose levels. The result is chronic hyperglycaemia, or elevated blood glucose. If uncontrolled, prolonged hyperglycaemia can lead to disabling and even fatal acute and chronic complications including cardiovascular disease, kidney damage (nephropathy), eye disease (retinopathy) and nerve damage (diabetic neuropathy) [207,208].

Although diabetic complications are all predominantly caused by excess blood glucose, the cause of hyperglycaemia can be different, giving rise to the two main distinct forms of diabetes: type 1 (T1D) and type 2 diabetes (T2D). In order to understand the underlying mechanisms of diabetic neuropathy and address its research and treatment adequately, one must first consider T1D and T2D as the distinct, if not opposite in some respects, diseases that they are, which is the aim of this chapter section. A brief outline of the characteristics of each is given in table 1.4

Table 1.4. *Summary of the main characteristics for diabetes type 1 and 2. Adapted from Ozougwu O. (2013).*

	Type 1 Diabetes	Type 2 Diabetes
Prevalence	~ 10 %	~ 90 %
Onset	Sudden	Gradual
Age of diagnosis	Mostly children	Mostly adults
Body mass	Low to normal	High to obese
Blood glucose levels	High	High
Blood insulin levels	Low or absent	High initially, then decreasing
Insulin resistance	No	High
Autoantibodies	Present	No
Symptoms	• Abnormal thirst & frequent urination • Sudden weight loss • Fatigue • Chronic hunger • Blurred vision • impaired wound healing	• Excessive thirst & urination • Weight gain • Fatigue • Blurred vision
Complications	• Ketoacidosis • Neuropathy • Retinopathy • Nephropathy • Cardiovascular disease	• Neuropathy • Retinopathy • Nephropathy • Cardiovascular disease
Treatment	Insulin supplementation	Lifestyle and diet changes, thiazolidinedione, metformin, sulfonylureas, insulin (later stages)

1.3.1.1. Type 1 Diabetes – an autoimmune response

T1D is now well-recognised as a chronic autoimmune disorder, characteristic with immune-mediated destruction of the insulin-producing pancreatic β-cells. Their absence leads to a marked deficiency in insulin secretion (Figure 1.6b). With no circulating insulin to regulate blood glucose levels, hyperglycaemia develops. With insulin unavailable, target cells cannot uptake any of the abundance of glucose and become starved and unable to produce energy leading to rapid weight loss despite an increase in appetite amongst other symptoms (Table 1.3). The underlying causes for this devastating process are believed to include a combination of genetic susceptibility (with multiple genes altered, typically ones encoding components of the immune system) and environmental factors, commonly viral infections, toxins or diet, that trigger an autoimmune response [209]. The prevalence of T1D is approximately 10% of diabetic cases with highest incidence in children and adolescents [209,210]. Diagnosis of both diabetes

types requires fasting blood glucose levels over 7 mmol/L and blood glycated haemoglobin levels of 6.5% or higher. To distinguish it from T2D, T1D diagnosis also requires detection of diabetes-associated autoantibodies, which can be present up to years before symptomatic onset [211]. Daily insulin supplementation begins immediately following diagnosis and is normally efficient at controlling the disease. Patients usually develop chronic T1D complications over approximately 10 years from diagnosis, depending on blood glucose levels management and the degree of blood vessels damage [209]. The prevalence of symptomatic diabetic neuropathy in T1D patients is substantial and consistent. Two large scale studies from 2020 established an average 11-13% prevalence estimate of symptomatic diabetic neuropathy in T1D population, with the proportion approximating 20% in older subgroups [212,213].

1.3.1.2. Type 2 Diabetes – a lifestyle problem

In contrast, T2D does not involve an autoimmune response, but is rather more tightly influenced by environmental factors, with an especially strong link to the global obesity pandemic [214,215]. In T2D, initially, insulin is still being produced and secreted, however it cannot exercise its effect on target cells (muscle and liver). This state of cell insensitivity to the hormone is termed "insulin resistance" (IR) and is the key component distinguishing T2D from T1D pathophysiology. Thus, target cells remain "closed" for the high glucose flow from the blood. Ironically, the body's response to IR is to secrete even more insulin, which over time leads to β-cell failure and inadequate insulin production [216].

While genetics, advancing age and ethnicity have been shown to be potential risk factors in T2D [217], the driving force behind IR is mainly high sugar and processed carbohydrate consumption, sedentary lifestyle and obesity and usually IR develops many years before T2D is diagnosed [215,218–220]. When the sugar intake is chronically excessive and exceeding energy demands, the cells in a healthy body metabolise and store the excess as glycogen (in muscle and liver cells) and triglycerides (in liver and adipocytes). With time, muscle and liver cells reach full glycogen storage capacity and thus become irresponsive to insulin attempts to store more incoming glucose. Still, the body prioritises lowering blood glucose levels, so the excess sugar can now only be metabolised into free fatty acids and deposited into new fat cells, continuing to increase body weight [221]. Thus, in T2D blood levels of glucose as well as insulin (hyperinsulinemia) and triglycerides (dyslipidaemia) are abnormally high [222222–224]. With these two additional pathophysiological elements, T2D already differs significantly in its natural history from T1D. Although symptoms largely overlap between the two types,
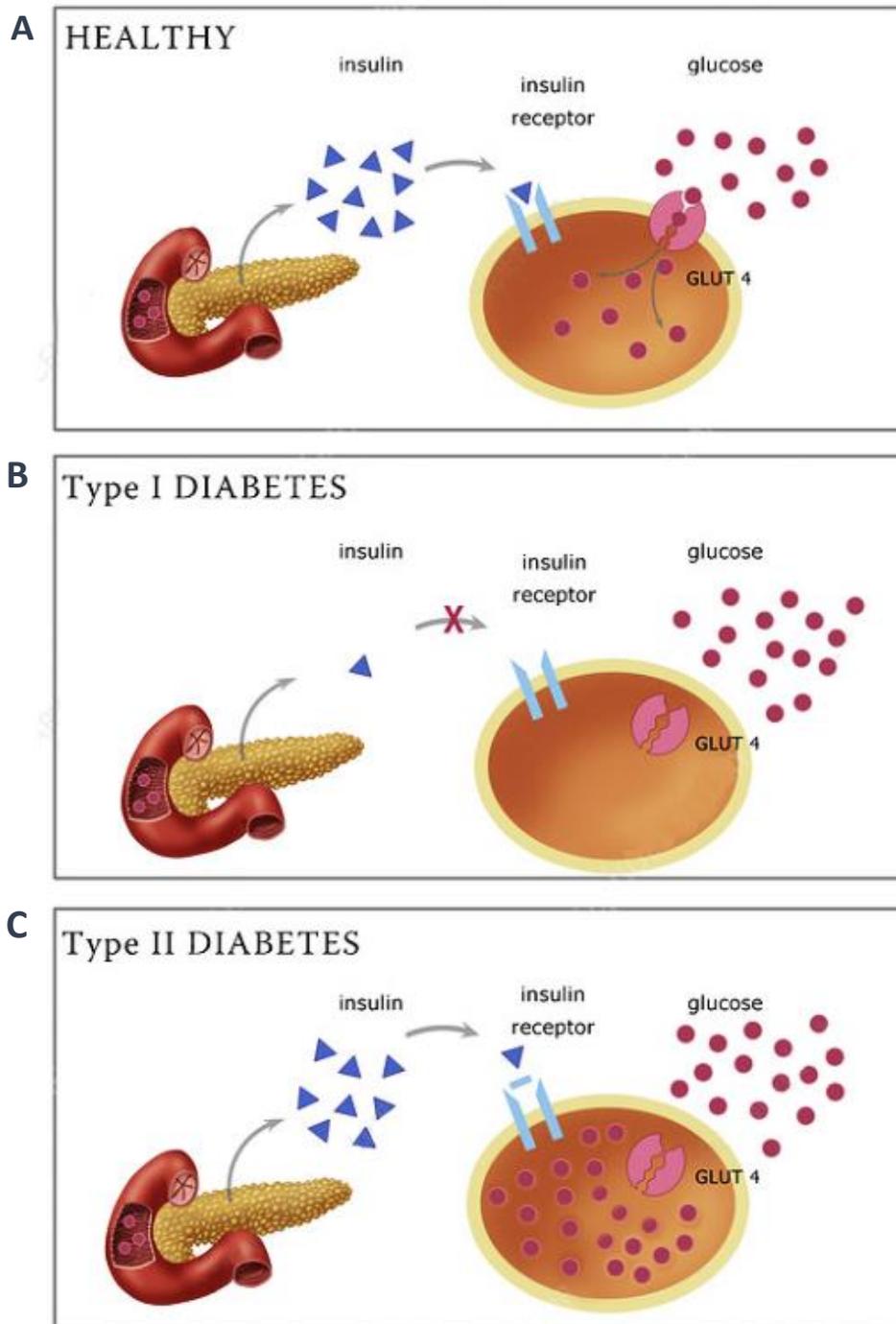
Figure 1.6. **Insulin production compared between healthy, type 1 diabetes and type 2 diabetes setting.** *Insulin (blue triangles) is released by pancreatic β cells to allow glucose (red circles) uptake by target cells through glucose transporters (GLUT 4, pink). In a T1D scenario, insulin production is impaired leading to deficiency in insulin signaling to target tissue and no glucose uptake. In a T2D setting, insulin is still being produced, however target tissues are saturated with glucose, preventing insulin binding to its receptor and inhibiting further glucose uptake. In both T1D and T2D glucose is not cleared from the blood, leading to hyperglycemia. While in T1D cells are starved, leading to overall weight loss. Adapted from: Monica Schroeder, Science photo library, in T2D excess glucose is diverted to de novo adipose cells generation, contributing to weight gain and in severe cases obesity. Source: Monica Schroeder/Science Photo Library, available at: https://www.sciencephoto.com/media/706546/view*

T2D typically has a much less dramatic presentation, occasionally developing

asymptomatically for a prolonged time [215].

Ninety percent of diabetic patients suffer from T2D. It is most common in older adults (age 40+) but has been increasingly more often diagnosed in young adults and children due to the worldwide rise in sedentary lifestyle and energy-dense diets. These circumstances are also propelling the overall T2D high and rising prevalence worldwide [225].

1.3.2. Diabetic neuropathy

Diabetic neuropathy (DN) is the most common complication arising from diabetes. DN is an impairment of the nerves of the body, caused mainly by prolonged hyperglycaemia damaging the microvasculature supplying the nerves (vasa nervorum). This can alter the functions of proximal and distal sensory, motor and autonomic nerves and thus damage virtually any organ system of the body causing a wide variety of symptoms [226]. Worryingly, it is also the most problematic diabetic complication with statistics placing it as the most widespread form of neuropathy in developed countries. Approximately 50% of diabetic patients develop some type of nerve impairment, with neuropathy onset correlating positively with diabetes duration [227,228]. DN accounts for more patients hospitalised than all other diabetic complications combined and is the leading cause of lower limb amputations [229]. It thus represents a great economic burden for any healthcare system as well as the patients themselves [228].

The most common type of DN is a symmetric neuropathy of the peripheral, predominantly sensory neurons or "distal symmetric polyneuropathy". Since this form is the focus of this thesis, from here on and throughout, "DN" shall refer to distal symmetric polyneuropathy. It initially affects the distal nerves, innervating the feet and hands and gradually spreads proximally following the "stocking-glove" distribution model [230]. Symptoms include paraesthesia (prickling sensation), dysesthesia (abnormal sensations), pruritus (itching), sensory loss, numbness and loss of balance. However, one particular symptom is deemed so debilitating and characteristic for patients that has defined a separate neuropathic condition stemming from DN. Pain during DN is experienced by 8 to 50% of DN patients and is clinically termed as painful diabetic neuropathy (PDN) [231]. Patients suffering with PDN describe pain as burning, shooting, stabbing, dull and aching, usually exacerbated at night. Frequently, PDN patients present with allodynia and hyperalgesia [232]. Although symptoms and progression of DN vary from patient to patient, it has been proposed that, overall, positive symptoms (prickling, tingling, pain) are characteristic of the early stages of DN, while negative symptoms (numbness, loss of sensation) are typical of the late stages, with possible extension of pain symptoms into the advanced DN phase [233,234].

T1D long-term complications largely overlap with those seen in T2D in their detriment and intensity. For decades, DN in T1D and T2D has been considered to be one and the same disease brought about by a shared underlying master mechanism – hyperglycaemia. However, accumulating evidence points to differences in the pathophysiology underlying T1DN and T2DN [231,233,235–239]. The next section discusses the common and contrasting features of the two diabetes forms and how they may impact the neuropathy progression in each.

1.3.2.3. T1D and T2D inconsistencies

Diabetes is ranked 5th globally in disease incidence, affecting 1 in 11 people [225]. Albeit the lifetime incidence of neuropathies is estimated to be between 10% and 50% for both T1D and T2D [228], the superior global prevalence of T2D over T1D (90% vs 10%, respectively) [240] makes T2D-associated nerve damage the most common and imperative cause of neuropathies. Logically, therefore, if the focus is on treating DN in T2D patients, this prompts the use of T2DN pre-clinical models over T1DN ones to dissect the underlying mechanisms driving the disease's progression. Yet, our understanding of painful DN pathology has mostly been obtained from streptozotocin (STZ)-induced diabetes in rats, a model of T1D [241,242]. Pre-clinical model selection has been speculated as an important reason for the failure of translating drug efficacy from pre-clinical animal models to patients [242]. For example, the involvement of oxidative stress [243], the polyol pathway [244] and aldose reductase [245] was confirmed largely by studies using the STZ rat (also reviewed in [246]). Subsequently, the STZ rat was also implemented as a pre-clinical DN model for the development of aldose reductase inhibitors as a potential DN-modifying treatment. Indeed, in STZ rats, the novel family of drugs corrected DN-induced nerve structure and nerve conduction velocity (NCV) impairments [247]. Yet, despite the promising pre-clinical data, all clinical trials failed to replicate the drug's pre-clinical efficacy. A meta-analysis of 32 aldose reductase inhibitor clinical trials showed that participants were predominantly T2D patients and diagnostic criteria for diabetes were not even stated in most of the studies included, highlighting the lack of discrimination between the diabetes types [248]. A very similar drug development course was followed by PKC inhibitors. Most of the pre-clinical success noticed with this drug class was obtained from STZ rodents, however, PKC inhibitors were ineffective in the treatment of DN in human patients [249].

Evidence for fundamental differences in the underlying mechanisms of T1DN and T2DN is also provided by clinical research with patients. In 2012, Callaghan et al. published a comprehensive systematic review of 17 clinical studies investigating the effects of glycaemic control on DN development in T1D and T2D. It was concluded that intensive glycaemic control significantly decreases risk and progression of DN in T1D patients but was only moderately

beneficial in T2DN patients [250]. Their analysis shows that hyperglycaemia alone is not the driving force of DN in T2D. What is more, it strongly accentuates on the concept of T1DN and T2DN being separate disease entities [236]. Furthermore, painful symptoms have been shown to have higher prevalence in T2D patients than T1D [231,239,251]. In one of the largest cohort observational clinical studies (n = 15,692) of diabetic UK patients, Abbott et al. demonstrated that the adjusted risk of painful neuropathy in T2D is double that of T1D patients [231].

What is more, these observations confirm those reached in studies conducted by Sima and Kamiya investigating DN in T1D and T2D rat models of each. They demonstrated that T1DN is much more severe than T2DN, with bigger decrease in NCVs and thermal sensitivity latencies as well as more acute IENF damage and loss, all reversible with an insulinomimetic compound [233,252,253]. Others showed that in contrast to T1D animals, limited amelioration to the DN phenotype was seen in *db/db* mice too, even when administered 3 times higher doses of insulin than T1D animals [254]. The contrasting effects of insulin intervention were attributed to the different insulin levels, actions and signalling impairments in the two diabetic types. These conclusions were a strong indicator of different mechanisms driving DN in T1D and T2D. Factors other than hyperglycaemia are now being widely discussed as contributors in the development of T2DN with the main focus falling on metabolic syndrome components including dyslipidaemia [255], inflammation [256] and IR [257].

In the next sections, I will address the pathways, conventional and emerging, known to be dysregulated during DN, some of which are common for both diabetic types, while others are more characteristic of T2DN.

1.3.3. Mechanisms implicated in (painful) diabetic neuropathy

Although DN development varies amongst individuals, it is generally accepted that it follows a specific temporal and spatial progression. The earliest changes during DN are believed to affect the C-fibres leading to an axonal degeneration/regeneration cycle [258]. This causes 'positive' symptoms such as pain, tingling, allodynia and hyperalgesia [259]. Overtime, degeneration prevails resulting in the loss of C-fibres, as well as Aδ-fibres and consequently diminished pain sensation. As DN progresses, myelinated A-fibres undergo a demyelination/remyelination cycle until complete destruction of the myelin sheath [258,260,261]. The loss of protection and nutrient supply by the supporting Schwann cells (SCs) (constituting the myelin) [261] leads to direct axonal degeneration, resulting in impaired proprioception and touch sensation [234].

For a long time, DN was argued to either originate from metabolic abnormalities within the nerve and Schwan cells or develop secondary to diabetic microvascular complications. It is now believed that DN pathogenesis is shaped by an intricate interplay between both vascular and metabolic factors at all stages of the disease. Hyperglycaemia, dyslipidaemia and insulin's abundance (hyperinsulinemia, T2D) or absence (insulinopenia, T1D) all affect both the microvasculature and the nerves themselves. The next part of this chapter will look into these mechanisms in more detail.

1.3.2.1. Damage to the microvasculature supplying the neurons

Blood vessels and nerves rely on one another to upkeep their normal function: nerves depend on adequate blood flow for oxygen and nutrient supply and blood vessels depend on normal nerve function for flow dynamics regulation [262]. Excess blood glucose damages smaller blood vessels (microvasculature) early on in diabetes progressing towards major blood vessels and the heart (macrovasculature) in later stages. The increased number of glucose molecules per millilitre of blood flowing through damages the endothelial cells lining the inside walls of capillaries supplying the neurons. This leads to increased cell proliferation (hyperplasia), thickening of the walls and thus narrowing of the lumen [263,264]. Peripheral perfusion to the nerves is severely reduced leading to neuronal ischemia. Glucose-induced microvascular injuries underlie the development of DN, retinopathy and nephropathy [265]. The critically low oxygen levels (hypoxia) [266] to the neurons results in progressive neurodegeneration [265,267,268].

With the extremities (fingers, toes) and also retina, being supplied by the smallest and thus most vulnerable capillaries, these areas are typically the first to be affected and to present with neuropathic symptoms. The role of vascular factors in DN etiology has been confirmed in experiments with diabetic rats where vasodilators corrected NCV deficits caused by microvascular complications [269,270]. The role of microvascular damage is further supported by clinical trials employing drugs targeting vascular changes in DN patients, reporting slowing of the progression of neuropathy and/or improving nerve function [271–273].

1.3.2.2. Metabolic pathways

Microvascular complications lie in the heart of the pathogenesis of DN. Triggered by hyperglycaemia, a collection of detrimental metabolic pathways is initiated that contribute to the damage of the vasa nervorum. Research suggests that hyperglycaemia drives microvascular damage through multiple cellular mechanisms, including the formation of advanced glycation end products (AGEs) , oxidative stress, polyol pathway, protein kinase C (PKC) pathway and others [267]. Neurons are damaged not only by the dysfunctional blood

supply but also directly by the same hyperglycaemia-triggered pathways that damage endothelial cells. These will be discussed in the next sections in the context of direct neuronal damage, however the mechanisms by which these processes cause damage in neuronal and endothelial cells overlap. Figure 1.7 shows a schematic summary of the main detrimental metabolic pathways involved in neurodegeneration.

Figure 1.7. ***Metabolic mechanisms of diabetic neuropathy.*** *Factors driving diabetic neuropathy in type 1 diabetes (T1D, marked with blue dots), type 2 diabetes (T2D, marked with yellow dots), highlighting the difference in the pathogenesis between the two diabetic neuropathy types. Factors participating in the pathogenesis of both diabetes types (marked with green dots) are or are triggered by hyperglycemia. In the end, all mechanisms culminate in the same neurodegenerative events (orange boxes). Adapted from Feldman et al (2019) and Pop-Busui et al. (2017)*

- *Dyslipidaemia*

DRG neurons and SCs metabolise free fatty acids (FFAs) as well as glucose for energy. FFAs are processed by β-oxidation into nicotinamide (NADH) and flavin (FADH$_2$) adenine dinucleotide + hydrogen, which are then used by the mitochondria to produce ATP through oxidative phosphorylation [274]. Low levels of ROSs are generated as a by-product of this

process, usually rapidly cleared by endogenous antioxidants. However, under T2D conditions, there is an excess of FFA entering this cycle thus leading to ROS overproduction [275], mitochondrial overload and eventually failure, causing metabolic and oxidative damage to SCs and DRG neurons [276]. Levels of cholesterol are also reported to be increased in the plasma of T2D patients [277] (hypercholesterolemia). Similarly, excess cholesterol metabolism leads to the accumulation of oxysterols. Oxidised low-density lipoproteins (LDLs), in particular, are able to bind specific receptors such as oxidized-LDL-receptor 1 (LOX1), toll-like receptor 4 (TLR4) and RAGEs, initiating a cascade of events driving apoptosis such as caspase 3 activation and DNA degradation. This all contributes to inflammation and ROS accumulation and ultimately SC impairment and neurodegeneration (Figure 1.7).

- *Insulin signalling*

The metabolic mechanisms underlying DN that are most investigated are predominantly concerning signalling pathways triggered in response to hyperglycaemia that lead to cellular damage (polyol pathway, glycation of proteins, oxidative stress and others). However, it is important to remember that hyperglycaemia is the result of the primary diabetic insult – impaired insulin signalling. Insulin's complete absence (T1D, late T2D) or abundance (early T2D) has been increasingly reported to contribute to the symptoms of T1DN and T2DN as a main hyperglycaemia driver but also through direct effects on neurons [278].

Neurons do not rely on insulin for glucose absorption but take it up via a concentration gradient-mediated manner through membrane-expressed glucose transporters (GLUT) [279]. However, insulin receptors are still expressed by sensory neurons in DRG cell bodies and axons [280–282]. They are predominantly expressed by small nociceptors with approximately 68% of TRPV1-positive neurons expressing the insulin receptor [283]. Neuronal expression of the receptor for insulin implicates direct signalling dynamics between the hormone and the PNS. Indeed, for a couple of decades insulin has been well-established as a potent neurotrophic factor, essential for supporting normal neuronal health, growth and function as well as increase in neuronal survival [284–286]. *In vitro,* insulin supplementation has been demonstrated to increase the rate of neurite generation, length and area in both embryonic sensory neurons and adult DRG [287]. Similar results were obtained from *in vivo* studies on nerve injury models [288–290]. Experiments with rat DRG neurons also show that insulin sensitises TRPV1 by lowering the activation threshold and increasing its translocation to the membrane. Such TRPV1 alterations are established to contribute to early T2DN positive symptoms, suggesting a direct effect of insulin on neuronal excitability [291–293]. Therefore, disruption in insulin-neuron signalling can be impair neuronal function and contribute to DN just as much as hyperglycaemia itself.

In order to evaluate the damaging effects of dysregulated insulin signalling on the nerves independently from the effects of glucose in a diabetes setting, one must experimentally sever the intimate physiological connection between insulin and blood glucose levels. Achieving this has proven challenging, however several studies succeeded and supply the literature with perhaps the strongest evidence of insulin's role in T1DN. STZ-injected rats develop robust T1D and DN [294,295]. However, the response to STZ can be variable and sometimes STZ injection produces a unique phenotype of normal glucose levels (euglycemic) and significantly decreased serum insulin. Romanovsky et al. benefited from this abnormal STZ reaction and used this cohort of euglycemic rats for their study into the effects of insulin unavailability on neuronal health. Despite not being hyperglycaemic, these rats developed sensory abnormalities just like animals with high blood glucose levels. They presented with mechanical hyperalgesia which was correlated with insulin deficiency and reversed with low dose insulin injections [296,297]. Similar observations were made in another low insulin/normal glucose animal model, the Goto-Kakizaki rat, where severe DN symptoms were apparent after 18 months of T1D development [298]. The findings of these two studies support a dependence of neurons on insulin for normal cellular health. More importantly, this may explain the marked improvement of nerve signalling seen in T1DN animal models and beneficial effects seen in T1DN following insulin supplementation, but not T2DN patients, where insulin levels are usually higher than normal [299]. These studies provide evidence for a strong connection between insulin's direct signalling to neurons and neuropathic complications, but only in the context of T1DN.

T1D and T2D can be viewed as opposing diseases in view of the insulin availability in each in the initial stages of the disease. In T2D, insulin overproduction and skeletal muscle IR is detected up to 10 years before diagnosis and lasts for the earlier stages of the disease [300]. Neurons have also been demonstrated to develop IR *in vitro* [301,302] and in animal models of T2DN [286] but not yet in humans. PNS IR in animal models of T2DN was shown to correlate with reduced neurite outgrowth [303]. Therefore, neuronal IR is another component speculated to contribute to neuronal insulin deficiency and the following neuronal damage in T2DN.

Overall, emerging research implicates insulin signalling as a glucose-independent trigger to the progression of DN in both diabetes types, be it due to insulinopenia or insulin resistance.

- *Polyol pathway*

Neuronal glucose uptake is insulin-independent and neurons rely solely on the direct glucose supply by the microvasculature [279]. Thus, vascular barriers are also their only protection against glucose toxicity in a state of hyperglycaemia. Therefore, during hyperglycaemia, when endothelial cells lining the blood vessels are damaged, neuronal intracellular glucose

concentrations are also abnormally high. Then, instead of undergoing normal metabolism through glycolysis, glucose is diverted to metabolic pathways that can result in neurotoxicity[304].

The polyol pathway is one of the first proposed mechanisms underlying hyperglycaemia-induced neuropathy. Under hyperglycaemic conditions, hexokinase (the first step in the glycolysis pathway) is saturated with glucose and another glucose-converting enzyme, aldose reductase (AR) takes over. AR converts excess glucose to sorbitol which accumulates intracellularly due to its low membrane permeability (Figure 1.7). Sorbitol is an osmolyte and increased intracellular sorbitol concentrations generate intracellular osmotic stress. In response, the neuron begins compensatory efflux of taurine and myoinositol [305]. Myoinositol is an essential component of Na^+/K^+ ATPase and its depletion from neurons leads to impaired neuronal physiology [306–309]. AR activity also depletes cellular stores of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a proton donor for reactions generating nitric oxide and regenerating glutathione (antioxidant). This consequence of the polyol pathway activation defines the "metabolic flux" hypothesis (see [309]). It contributes to the generation and reduced clearance of cytoplasmic reactive oxygen species (ROS) which mediate intracellular injuries, discussed next.

- *Oxidative stress and reactive oxygen species (ROS)*

Oxidative stress is promoted by two glucose-driven events: ROS generation and the impairment of ROS scavenging mechanisms. During hyperglycaemia, mitochondria in neurons are overwhelmed and glucose oxidative metabolism increases. This generates superoxide (O_2^-) which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase and, under normal conditions, H_2O_2 itself is metabolised by glutathione peroxidase (GP) to harmless water. However, as mentioned above, during hyperglycaemia the polyol pathway uses up NADPH, which is needed in the glutathione cycle, and thus prevents GP from converting H_2O_2 to water. H_2O_2 is then diverted to conversion into superhydroxyl free radicals ($\cdot OH$) exerting oxidative stress in the neuron [310,311]. Moreover, H_2O_2 and O_2^- can react with nitrite to produce peroxynitrite which causes nitrosative stress to the cell [312]. Oxidative and nitrosative stress result in triggering DNA-strand breaks which activate poly(ADP-ribose) polymerase (PARP). This protein is involved in DNA repair and its action depends on NAD^+. Overactivation of PARP depletes cellular NAD^+, also augmented by the polyol pathway. This slows down glycolysis, electron transport and ATP-generation rates resulting in neuronal dysfunction and death [311].

- *Disruptions of Schwann cell metabolism*

Schwann cells (SCs) are a type of glial cells supporting neurons and an essential component of the PNS. They help in the formation of the myelin sheathing of myelinated afferents as well

as the "Remak bundles" – an association of a bundle of C-fibre afferents via non-myelinating SCs [313]. However, the role of SCs in neuronal health goes beyond myelination as accumulating research suggest they provide active metabolic support to axons. Thus, dysregulation of SC metabolism and function, such as under diabetic conditions, will ultimately affect neuronal function and health [313]. SCs express GLUT through which glucose uptake is possible via an insulin-dependent manner for metabolism and energy production [314]. For example, in a healthy environment, glucose is metabolised in the SCs to lactate which is transported to the underlying axon for energy production. However, under hyperglycaemic conditions, the excess glucose activates the polyol pathway leading to the AR-dependent accumulation of sorbitol which has been suggested to lead to SC de-differentiation to immature cells, contributing to nerve demyelination and dysfunction [315]. Furthermore, SCs are highly efficient in lipid metabolism [316]. They are able to uptake FFAs, activating a sequence of events leading to ATP production. However, during T2D, where extracellular levels of FFA can be very high, there is a substrate overload, resulting in metabolic reprogramming of the SC and overproduction acylcarnitine molecules. These are then shuttled into the underlying axon, where they exercise their toxic effects, contributing to axonal degeneration [274,313]. These diabetes-induced alterations to SC metabolism, along with evidence for high glucose-induced oxidative damage [317,318] are now shaping a novel idea that the disrupted communication between SCs and their axons contributes to DN pathogenesis.

- *PKC activity*

Excess glucose increases the rate of glycolysis. In a series of biochemical reactions, this eventually leads to the overproduction of complex molecules such as 5-diphosphate-N-acetylglucosamine (GlcNac) and diacylglycerol (DAG) [319]. GlcNac reacts with transcription factors to promote lipid dyshomeostasis, inflammation and peripheral nerve injuries [320]. Similarly, DAG accumulation in the nerves has also been shown to lead to neuronal damage via activating PKC. Its activation can cause a myriad of metabolic complications through increasing neuronal IR and disturbing Na^+/K^+ ATPase function and thus signal propagation. It has also been demonstrated to impair expression of genes associated with vascular function thus contributing to the microvascular complications surrounding neurodegeneration [321].

- *Protein glycation and advanced glycation end products (AGEs)*

Excess glucose levels lead to its reaction with amino acids of proteins to generate potentially detrimental products knows as advanced glycation end-product (AGEs) (Figure 1.7). AGEs have been shown to accumulate in the peripheral nerves of T2D patients with DN [322]. AGEs can cross-link essential proteins thus preventing them from functioning properly. Furthermore,

AGEs react with AGE receptors (RAGEs) on neurons initiating downstream signalling cascades partially mediated by nuclear factor (NF) -κβ activation, leading to neuronal dysfunction and loss of nerve cells. This can eventually result in reduced nociception. Events such as vasoconstriction, inflammation and loss of neurotrophic support have also been reported to occur in rodent PNS as a consequence of AGE-RAGE activation [323,324].

- *Inflammation*

The array of cellular imbalances underlying DN discussed so far are also powerful triggers of systemic inflammation in peripheral tissues, including the nerves. Indeed, the role of the immune system components has been established in DN patients. For example, T2D patients who have DN show higher levels of inflammatory cytokines in their plasma compared to non-diabetic patients and T2D patients without DN [325].

In the last decade, pre-clinical experiments and clinical studies have provided strong evidence for the role of inflammatory processes in DN of both diabetes types [255,256]. The proposed mechanism states that glucose-, insulin- and lipid metabolic abnormalities kick-start cycles of oxidative/nitrosative, endoplasmic and mitochondrial stress that in turn cause cellular damage in the neuron. Neuronal injuries activate multiple downstream kinases (including PKC) and redox-sensitive transcriptional factors, such as NF-κB which is responsible for the regulation of genes associated with the immune response of the body [326]. Activated regulation factors trigger a surge of cytokine and chemokine production which enhance existing inflammatory responses but also feed back to and amplify the whole process by triggering more cellular stress [327]. This cascade of events targets not only neurons but endothelial cells of the blood vessel walls supplying the nerves, thus further feeding into the vicious damage loop [255,326]. This idea is supported by pre-clinical [328–330] and clinical [325,327,331–335] studies. A couple of studies demonstrated not only that DN patients have increased serum levels of inflammatory cytokines and markers of endothelial dysfunction, but that these were even further increased in patients with PDN [325,336], adding to similar evidence from studies with T2DN animal models [337]. One mechanism by which this is believed to occur is the modification of channels and receptors implicated in pain by inflammatory molecules. For instance, the expression density and activation thresholds of TRPV1 and TRPA1 have been demonstrated to be modulated during inflammation by a range of kinases thus sensitizing nociceptors to painful stimuli following injuries [142,338–341]. Such and other dysregulations in ion channel activity during DN will be discussed in the next, final part of this section. Collectively, these observations strongly implicate that inflammatory processes during diabetes contribute greatly to DN and pain directly as well as indirectly through feeding into other neurodamaging pathways.

1.3.2.3. Ion channel alterations

As discussed in more detail earlier, ion channels expressed by sensory neurons set and govern the overall neuronal excitability. The carefully regulated activity and expression of each channel creates a fine balance upon which the normal functioning of the PNS relies. Their plasticity, however, allow for modifications of their expression, trafficking and kinetics that may result in changes in neuronal excitability. Increased excitability of nociceptors due to such ion channel alterations is one of the most widely implicated mechanisms underlying pain during DN [313].

Diabetes triggers multiple signalling pathways that often work together to promote different post-translational modifications of ion channels. One of the best studied reactive metabolites participating in such detrimental cascades is methylglyoxal - a protein-glycating agent and important precursor of AGEs. Its levels increase during diabetes – in fact, one study demonstrated that its concentration is especially high in patients with PDN as opposed to painless DN. Furthermore, in rodents, administration of methylglyoxal renders them hypersensitive to pain [342]. Methylglyoxal's neuron sensitising effect has been linked to post-translational modifications of Nav.1.8, depolarizing its inactivation threshold. Thus, the availability of Nav1.8 at AP threshold is increased and with that, hyperexcitability of the neurons expressing it, usually nociceptors, increases, leading to hyperalgesia [342]. Methylglyoxal's potential to cause modifications stretches beyond VGSCs, as it has been shown to affect TRPA1 too. Like many of TRPA1's agonists, methylglyoxal is an electrophilic metabolite and is able to interact and modify critical cysteine residues on TRPA1 causing a robust activation and hyperexcitability in nociceptive neurons [343]. A strong link between methylglyoxal and the channels TRPA1 and Nav1.8 was demonstrated in experiments by Huang et al. (2016). They showed that subcutaneous administration of methylgyoxal in STZ rats produced nociception, which was reversed by using antagonists for TRPA1 (A967079) or Nav1.8 (A-803467). In their behavioural experiments, they confirmed the association of methylglyoxal in DN with mechanical allodynia and thermal hyperalgesia in the STZ rat [344]. TRPA1 is further implicated in PDN pathogenesis due to its strong activation by ROS – highly abundant during diabetes and further enhancing TRPA1 potentiation by methylglyoxal [345].

There is evidence describing altered expression levels of pronociceptive channels, including P_2X_3 [346], TRPV1 [58,156], certain VGKCs [347] and VGSCs [60,348–351], albeit, historically, the studies have not been consistent in their findings. For instance, in a comprehensive report by Craner et al. (2002), expression levels of VGSCs in a T1D rat model were investigated to reveal a significant upregulation of Nav1.3, Nav1.6 and Nav1.9, downregulation of Nav1.8 and no change in

Nav1.1 and Nav1.7 [348]. These observations are in accordance with others who also report upregulated Nav1.3 expression [58,352] and downregulated Nav1.8 expression [58,353] in diabetic animals. However, they contrast with studies describing significant increase in both TTX-S and TTX-R currents and upregulated levels of both Nav1.7 and Nav1.8 in DRG cell bodies of diabetic rodents [58,349–351,354,355]. Albeit their exact nature is still debated, dysregulated channel expression, particularly of VGSCs, has been agreed to have a key role in the development of neuropathic pain during DN.

Painful manifestations vary vastly between DN patients - some develop painful symptoms as early as pre-diabetes while others never show a painful phenotype [356]. Apart from the differences between the diabetes forms that may contribute to such variance, genetic variances in certain VGSCs have also emerged as possible modulators of an individual's risk of developing pain during DN. Nav1.7 has been of particularly great interest as a key determinant of neuronal excitability. Specifically, GoF mutations in this channel in a diabetes setting have been suggested to produce increased DRG sensitivity. Indeed, multiple variants have been discovered in the past years that were associated with PDN [75,357–359]. Furthermore, a dozen of rare Nav.1.7 variants were recently identified in PDN patients, which correlated with earlier diagnosis of PDN, a more severe painful phenotype and greater mechanosensitivity [76]. Finally, a newly emerging curious hypothesis implicates Nav1.7 along with Nav1.3 in a cause-effect relationship between diabetes and painful neuropathy. The notion arises from the fact that these TTX-S channels are expressed in both DRG as well as pancreatic islet cells. It is therefore postulated as a possible model that diabetes as a metabolic disease and peripheral neuropathy arise as a result of dysregulation in Nav1.3 and Nav1.7 due to genetically inherited mutations. When both channels are dysfunctional, they render both sensory neurons and pancreatic cells more susceptible to damage, making diabetes and neuropathy go hand in hand. Albeit intriguing, this concept is still in its early stages [360–362].

1.3.4. Current diabetic neuropathy treatments

Diabetic neuropathy is the most debilitating complication of diabetes associated with the highest risk of mortality [363]. Yet, despite the advancing understanding of the underlying pathophysiological mechanisms, to date, there are no approved disease-modifying or reversing pharmacological therapies for DN [226]. This section will focus on the established DN-modifying interventions and currently recommended pharmacological therapies targeted at alleviating the symptoms of PDN. Novel analgesic drugs for PDN that are under development will also be briefly discussed. The section will conclude with a short discussion of the possible reasons for the lack of adequate disease-modifying therapies.

1.3.3.1. Diabetic neuropathy management

The main approach evidenced to successfully prevent or delay DN progression is glycaemic control, usually via insulin supplementation. In T1DN patients, blood glucose management has been shown to improve NCVs and vibration thresholds and, when introduced early enough, to prevent the development of clinical neuropathy [235]. In T1DN patients with severe neuropathy, even signs of neuropathy reversal were evident when blood glucose levels were normalised via more invasive interventions such as pancreatic and kidney transplantations.

Albeit a successful approach in T1DN patients, improving glycaemic control in T2DN patients for delaying neuropathy progression is not as effective, even when extended to up to 5 years. In fact, in the Action to Control Cardiovascular risk in Diabetes (ACCORD) study, such prolonged intervention has been shown to be detrimental for T2D subjects as it increases hypoglycaemia and excess mortality amongst intensively treated individuals [364,365]. Glycaemic control may be of minimal benefit for improving T2DN, however its limited efficacy provides the valuable insight that hyperglycaemia is certainly not the sole major process driving neuropathy progression in T2D and other therapy targets need to be explored.

Apart from hyperglycaemia, T2D is characteristic with hyperlipidaemia and hyperinsulinemia, which have also been widely implicated in the progression of DN in T2D (see section *1.3.2.2. Metabolic pathways*). Therapies targeted at lowering cholesterol (such as statins) or triglyceride levels (such as fibrates) have been suggested to slow DN progression in T2D patients, however no randomised clinical trials have evaluated their efficacy clinically [366,367]. Hyperinsulinemia and insulin resistance (IR), on the other hand, are usually improved via weight management through diet and exercise regimes and in extreme cases, weight control surgical interventions (such as bariatric surgery). In fact, weight loss has been established as the safest and most efficient intervention to lead to remission of diabetes and reversal of neuropathy, with noted increase in IENFD, amelioration of small nerve fibre damage, alleviation of pain symptoms and improved microvascular function [368,369]. The most widely prescribed pharmacological therapy targeting IR is metformin. It reduces the amount of glucose released in the blood by the liver, thus improving cellular sensitivity to insulin. However, high doses of metformin have been linked to vitamin B12 deficiency and a recent study demonstrated an inverse correlation between DN severity and vitamin B12 levels, suggesting that metformin might not be suitable for patients with severe DN phenotype [370]. Other therapies employed that target DN progression include supplementation with alpha-lipoic acid (antioxidant) [371] and improving microvascular function via blood-pressure lowering drugs (lisinopril, trandolapril) [271,272]. The most sensible approach remains multifactorial risk factor control combining

treatments   targeting   multiple   pathophysiological   mechanisms   simultaneously (hyperglycaemia, hypertension, dyslipidaemia, insulin resistance).

1.3.3.2. Management of painful diabetic neuropathy (PDN)

Up to a third of diabetes patients develop symptomatic PDN, significantly affecting their quality of life. With the lack of pharmacological interventions modifying DN, symptomatic treatment remains the best-established way for managing pain [372]. The rapid growth in our knowledge of pain-driving pathophysiological processes has yielded a plethora of promising therapeutic targets in these pathways.

- *Approved therapies for managing painful diabetic neuropathy*

A list of the most widely prescribed pharmacological therapies targeting pain during DN is outlined in table 1.5 and the treatment strategy outlined in Figure 1.8. The approved and most recommended therapies target the CNS and modify the processing of the pain signal in the brain. These include anticonvulsants, antidepressants, and opioids [230]. The most widely prescribed anticonvulsant drugs are gabapentin and pregabalin, both acting as inhibitors of VGCCs thus reducing neuronal excitability. Recommended antidepressants are from one of two classes: serotonin and noradrenaline reuptake inhibitors (SNRIs, e.g. duloxetine, venlafaxine) or tricyclic antidepressants (TCAs, e.g. amitriptyline, nortriptyline). Both anticonvulsant [373–376] and antidepressant [377–380] therapies have been demonstrated to be effective at alleviating pain during diabetes and to be relatively well-tolerated. However, they are not without adverse effects, the most common of which include dizziness, confusion, fatigue and somnolence, which tend to be more severe in older patients [381]. Nevertheless, generally the benefits of these drugs outweigh the side effects and anticonvulsants and antidepressants are thus currently recommended as 1st line treatments for PDN by several drug regulatory bodies [376] (Figure 1.8).

Opioid prescription, on the other hand, remains controversial. Albeit pain relief during PDN has been achieved with opioids in clinical trials, this drug class brings serious safety concerns. Due to their nature, opioids prescribed for treatment of chronic pain can lead to addiction, misuse and abuse and are associated with increased mortality due to overdose. Therefore, opioids such as tramadol are often only recommended as a 3rd line intervention for moderate-to-severe pain and only after careful individual benefit-to-risk ratio assessment [382,383].
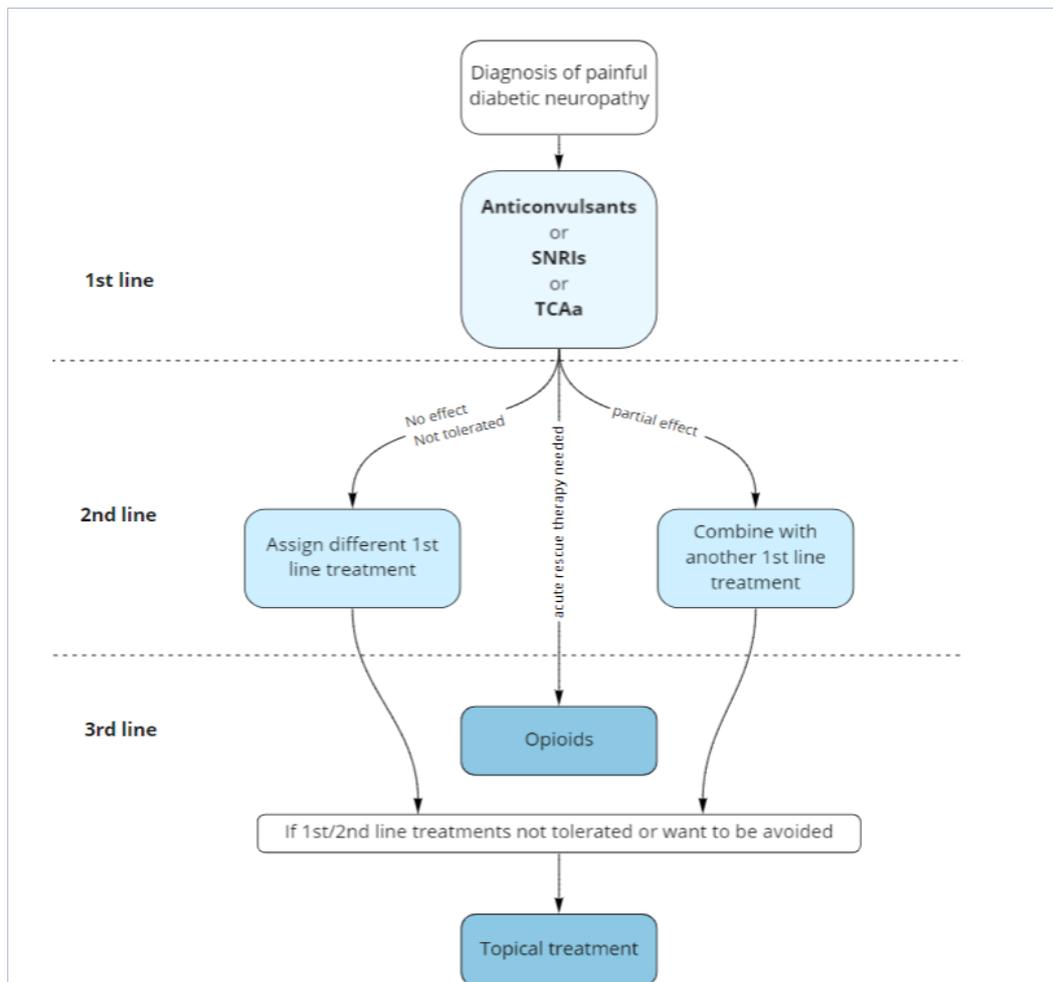
Figure 1.8. *Currently established treatment strategy for painful diabetic neuropathy. Anticonvulsants, serotonin and noradrenaline reuptake inhibitors (SNRIs) and tricyclic antidepressants (TCAs) make up first- and second line treatments for painful DN. Opioids are generally avoided and only prescribed as a third line treatment in extremely severe cases. In the case of first- and second line treatments generate tolerance or generally not well-tolerated, a third line alternative or an add-on are topical treatments. Source: Feldman et al (2019)*

Table 1.5. *Current FDA-approved treatments for painful diabetic neuropathy with their therapeutic targets, most common side effects and serious adverse effects summarised. Adapted from Azmi et al. (2021)*

Drug	Drug class	Therapeutic target/effect	Common side effects	Serious side effects	Reference
Gabapentin	Anticonvulsant	VGCC inhibitor	Fatigue, dizziness, somnolence, headache, dry mouth, peripheral oedema,	hepatotoxicity, ataxia, Seizures following rapid discontinuation	Wiffen et al. (2017) [644]
Pregabalin	Anticonvulsant	VGCC inhibitor	Somnolence, dizziness, ataxia, fatigue,	Seizures (after rapid discontinuation)	Derry et al. (2019) [645]
Duloxetine	Antidepressant (SNRI)	inhibition of serotonin and norepinephrine reuptake in the CNS	Nausea, somnolence, dizziness, constipation, diarrhoea, dry mouth, headache, insomnia, fatigue	Seizures, hepatotoxicity, serotonin syndrome, glaucoma, myocardial infarction, cardiac arrhythmias	Lunn et al (2014) [646]
Amitriptyline	Antidepressant (TCA)	Same as duloxetine but less selective	Dry mouth, fatigue, headache, dizziness, insomnia, orthostatic hypotension, nausea, constipation, blurred vision, somnolence	Hepatotoxicity, heart failure exacerbation, strokes, seizures, serotonin syndrome, cardiac arrhythmias, myocardial infarction	Moore, Derry et al (2015) [647]
Tramadol	Opioid	Inhibition of serotonin and noradrenaline reuptake; μ-opioid receptor agonist	Somnolence, constipation, nausea, vomiting, light-headedness, dizziness,	respiratory depression, serotonin syndrome, seizures, hypertension	Duehmke et al. (2017) [648]
Capsaicin 8% patch	Topical	TRPV1 desensitisation	Burning, stinging, erythema, coughing, sneezing	-	Simpson et al (2017) [386]
Lidocaine 5% patch	Topical	prolongs inactivation of the fast VGSCs	Skin irritation, rashes, itching, or redness, numbness	-	Derry et al. (2014) [649]
Alpha-lipoic acid	Antioxidant	ROS scavenger, insulin-mimetic and anti-inflammatory activity	-	-	Agathos et al. (1999) [650]

The severity of PDN symptoms is often robust enough to justify the implementation of CNS-modifying drugs as 1st line treatments. However, the chronic nature of DN demands long-term dependence on these therapies increasing the risk of developing drug tolerance, requiring higher doses and thus increasing adverse effects severity. Several non-CNS targeted therapies have been proposed that have shown success in relieving pain in DN. Topical treatments are a popular alternative for patients unable to tolerate the conventional systemic treatments. They also present with significantly lower risk of interactions with other medications making them a suitable option for patients with polypharmacy [384]. The most popular topical treatments for DN pain are the capsaicin 8% patch and lidocaine 5% patch. Both have demonstrated significant pain reduction comparable to anticonvulsants and TCAs and have fewer and less serious side effects [385,386]. Capsaicin 8% patches are now FDA-approved for the treatment of neuropathic pain and both are proposed as 2nd or 3rd line treatments [376] (Figure 1.8). Nevertheless, they can cause side effects, e.g. skin irritation, rashes and, especially in the case of capsaicin, loss of IENF and altered thermal sensation, which can compromise patient compliance [387].

- *Novel pharmacotherapeutic agents in development*

Research into novel analgesic treatments for PDN is also focusing away from the CNS and onto the PNS. Table 1.6 presents an inexhaustible list of novel pharmacotherapeutic compounds in development for symptomatic pain relief during DN. Here, I will focus on discussing those targeted at modifying pain-associated ion channels expressed by nociceptors.

Nociceptive ion channels have become an especially attractive target for novel analgesics due to their selective expression in peripheral nociceptive neurons [388]. This facilitates the diversion of conventional therapies towards nociceptors thus limiting detrimental CNS side effects. At the same time, affecting pain signalling at the source by the selective inhibition of specific nociceptive channels could enhance drug efficacy. Furthermore, even in the case of limited individual drug efficacy, ion channel modulators present the opportunity for combination therapies with currently approved treatments (table 1.5), whereby lower doses of both can be implemented to limit side effects while benefiting from both drugs' actions – a concept which is being explored with currently approved therapies in DN [389–392].

Table 1.6. *A range of novel drugs in development for the treatment of painful diabetic neuropathy. Novel analgesic pharmacological interventions are targeted at different molecules and pathways. Some have been through clinical trials and side effects have been notices, whilst others are still in pre-clinical stages of development. Adapted from Rastogi & Jude (2021)*

Drug	Class	Known side effects	Stage of development	Reference
PF-05089771	Nav1.7 blocker	Headache, pneumonia, upper respiratory tract infections	Phase II	McDonnel (2018) [651]
Vixotrigine	VGSC blocker	Headaches, dizziness	Phase II	Hinckley et al. (2021) [396]
VX-150	Nav1.8 blocker	Nausea, headache, vomiting and dizziness	Phase II	Vertex Pharmaceuticals Incorporated. (2018) [398]
A-803467	Nav1.8 blocker	-	Pre-clinical studies	Jarvis et al (2007) [399]
PF-01247324	Nav1.8 blocker	-	Pre-clinical studies	Payne et al. (2015) [400]
Calcitonin	VGSC modulator	-	Pre-clinical studies	Ito et al. (2012) [652]
A-317491	P2X3 blocker	-	Pre-clinical studies	Jarvis et al (2002) [402] McGraughty et al. (2003) [195]
Sinomenine	P2X3 blocker	Injection site flare, pruritus, edema	Pre-clinical studies	Rao et al (2017) [403]
Resiniferatoxin	TRPV1 agonist	Transient burning sensation	Pre-clinical	Bishnoi et al (2011) [405]
Tanezumab	Humanised monoclonal IgG2 antibody	Peripheral edema, paraesthesia	Phase III	Bramson et al (2015) [653]
Pooled human immunoglobulin	Immunomodulatory agent	Allergic reactions	Phase I	Liu et al (2018) [654]
Botulinum Toxin A	Inhibition of sensory neurotransmitters release	Antibody formation and immune-related complications, are reported when a small amount of bont-A enters the circulatory system	Phase II	Lakhan et al. (2015) [655] Salehi et al. (2019) [656] Park, J. & Park H. (2017) [657]
Islet Neogenesis Associated Protein (INGAP)	Enhance nerve growth from sensory ganglia	-	Pre-clinical studies	Tam et al. (2004) [658]
Fidarestat	Polyol pathway inhibitor	-	Phase II	Hamada and Nakamura (2004) [659]
Minalrestat	Polyol pathway inhibitor	-	Pre-clinical studies	Yagihashi et al (2001) [660]
Sulfasalazine	NF-κB inhibitor,	Increase in gas, constipation, and diarrhea	Phase II	Berti-Mattera et al. (2008) [661]; Liedorp et al. (2008) [662]
miR-146a (pro-inflammatory genes suppressant)	Micro RNA supplementation	-	Pre-clinical studies	Liu et al. (2017) [663]
VM202	plasmid containing human growth factor	Infections, diabetic retinopathy, peripheral edema, and skin ulcers	Phase II	Kessler et al. (2015) [664]

Of the DRG-expressed ion channels implicated in pain, Nav1.7 has perhaps been of highest interest as an analgesic target due to its high expression levels in DRG and strongly evidenced role in pathological pain conditions [33]. A number of potential Nav1.7 blockers have been under development, showing promising results in preclinical studies of DN [393,394]. However, none have yet been approved for clinical use due to their failure in clinical trials. For instance, a recent small trial of the Nav1.7 blocker PF-05089771 in PDN failed to show pain alleviation in patients [395]. Another VGSC inhibitor developed by Biogen, Vixotrigine was believed to be Nav1.7-selective but has now been redefined as a non-selective VGSC blocker [396]. Vixotrigine has shown limited efficacy but great tolerability in a phase II clinical trial for trigeminal neuralgia compared to placebo and is currently undergoing phase II trial for small fibre neuropathy in DN (ClinicalTrials.gov Identifier: NCT03339336). Interestingly, a recent study demonstrated that oxcarbazepine (a non-selective VGSC blocker) successfully attenuated pain responses in DN patients with irritable nociceptors compared to those with non-irritable nociceptors. This suggests that non-selective VGSCs might still find place in the clinical treatment of PDN provided that a more detailed patient phenotyping and hence stratification is performed [397]. Blockers of Nav1.8 have also been of interest for the pharma industry: VX-150 has been advanced to phase IIb trials after demonstrating promising efficacy and safety in small fibre neuropathy clinical trials [398]. Other Nav1.8 blockers such as A-803467 and PF-01247324 have shown promising selectivity and antinociceptive effects in preclinical studies [399–401]. Similarly, several P_2X_3 blockers have been explored in animal studies (e.g. A-317491 and sinomenine) and have successfully relieved hyperalgesia by suppressing overexpression and overactivation of P_2X_3, however confirmation in human studies is lacking [195,402,403]. Finally, TRP channels have been demonstrated to be particularly curious pain relief targets as both TRP-channel agonists and antagonists have shown analgesic potential [404–408]. Albeit several promising novel DN therapies are in ongoing clinical trials (table 1.6), numerous therapies targeting various pathogenic molecular mechanisms have shown great promise in preclinical studies only to fail to translate to the clinic [409]. In fact, analgesics in general have only 2% success rate, which is 5 times less than other therapeutic areas. The low development success rate has led to gradually decreasing interest and investment in the area of pain therapeutics further exacerbating the issue of lack of adequate pain treatments [410]. To date, no disease-modifying pharmacotherapy has been developed for DN and the possible reasons behind this will be briefly outlined next.

1.3.3.3. Why are there no disease-modifying therapies for diabetic neuropathy?

The problem with analgesics development for treating PDN extends to DN as a whole with currently no disease-modifying therapies available and largely disappointing clinical trial outcomes [230]. The possible reasons behind this critical issue stretch across all steps in the drug development process from the clinical trials design all the way back to *in vitro* screening of the drug candidates. Clinical trials are recommended to introduce improvements in several aspects:

- ➢ alternative endpoints, particularly focused on monitoring small nerve fibre physiology instead of currently recommended large-fibre-focused readouts [411];
- ➢ detailed DN diagnosis with stress on underlying pathophysiological driver(s) [411–413]
- ➢ patient stratification according to key pathophysiological driver(s) of DN for enhanced, individualised therapies [414,415]
- ➢ following on the previous two points, focus on mechanism-based drug testing approach as opposed to "one size fits all" approach

The complexity of DN poses great challenges in translating potential treatments from animal models to humans [416,417]. Suggested improvements in this stage of the drug development include the implementation of multiple and improved preclinical screening methods with direct clinical equivalents as well as progression through several model systems [411]. However, the most fundamental issue to consider is the choice of the preclinical model: how well does it reflect the DN aspects targeted and how comparable are they to the same aspects in humans? Finally, taking yet another step back in the development process, *in vitro* drug screening would also benefit from advanced techniques with improved readout sensitivity that are able to precisely separate drug action on target from its off-target effects. Such improvement will aid in the advancement of better-targeted and safer drugs towards preclinical and clinical development stages. For more details and an extensive analysis of current drug development practices and areas of improvement, see Jin et al. (2020) [411].

Choosing the appropriate animal model of DN and technique to evaluate its phenotype are key steps in characterising functional changes and identifying potential treatment targets in the neurons. Further considerations for animal and *in vitro* models of DN will be discussed in more details next, followed by the introduction of a novel functional assay developed in our lab which we demonstrate to have potential as a simple but sensitive drug screening tool.

1.4. Modelling diabetic neuropathy

So far, this chapter has delved into the details of DN and the known molecular mechanisms underlying its pathogenesis in order to highlight the enormity as well as depth of the clinical problem that this condition represents. Despite over 50 years of laboratory and clinical research, current treatments are far from achieving satisfactory effectiveness and safety [230].

The development of high-quality therapies depends heavily on the adequate modelling of DN components *in vitro* or in animal models of pre-diabetes, T1D and T2D. To date, several *in vitro* technologies and over 20 rodent models of DN have been introduced offering the opportunity to choose the one most closely reflecting the specific DN aspects being investigated [418]. Although, no model accurately mimics all aspects of DN as manifested in humans, *in vivo* and *in vitro* models arm researchers with the tools needed to keep advancing the understanding of the complex disease that DN is. In this section I will review some of the most common *in vitro* methods for reproducing DN conditions. Then, I will move on to discussing the most widely used T1DN and T2DN animal models. Finally, this part of the chapter will conclude with a comment on important considerations when selecting a model for DN research.

1.4.1. Modelling diabetic neuropathy *in vitro*

As the search for an effective drug to reverse or ameliorate DN progression continues, basic research focuses on unravelling the intricate molecular mechanisms propelling its development. To this end, tissue and cell culture techniques are especially instrumental because they allow for the isolated investigation of complex neuronal responses to different stimuli – a clear benefit over *in vivo* models, where a mixed-cell, global response is taking place. Other advantages of cell cultures to consider are, perhaps above all, that they allow for tight control over the extracellular environment. Thus, they are ideal for the detailed studying of a vast range of cellular processes, including cell death, neurodegeneration, hyper- and hypoexcitability and bioenergetics. With the added benefit of allowing for straightforward real-time imaging and recording, they are also useful for tracking the time kinetics of intracellular molecular events, albeit *in vivo* imaging is now possible too. These advantages render cell cultures the perfect platform for high-throughput drug screens. Finally, *in vitro* techniques usually come with fewer ethical issues than using *in vivo* models of nerve diseases, particularly when investigating pain [419].

The *in vitro* approach to DN research encompasses several different techniques, such as primary and immortalised cultures of dissociated neurons, organotypic explants and, more

recently, generation of neuronal cultures via the induced pluripotent stem cell (iPSC) technology.

Primary neuronal cultures are the most widely used method for investigating DN pathogenesis *in vitro* [318,420–425]. Importantly, primary cultures of DRG neurons from an adult rat or mouse preserve the broad heterogeneity of the neuronal population within the ganglion. DRG neurons can be harvested from healthy, control animals and subjected to DN-inducing stimuli to investigate acute pathogenic responses. Alternatively, neurons can be harvested from a diabetic animal. This is an excellent example of marrying *in vivo* and *in vitro* techniques, benefiting from employing an animal model of diabetes as a DN-stimulus and an *in vitro* approach to assess the specific molecular and physiological events. [419]. Generation of primary neuronal cultures involves the mechanical and/or enzymatic isolation of neurons into a cell monolayer which is then maintained in an optimal extracellular environment designed to mimic physiological conditions [426].

DN entails a network of intersecting pathways driving its pathology and the choice of stimulus depends on the DN aspect under investigation. Most commonly, generating hyperglycaemic conditions is the first consideration, however, the question of the optimal glucose concentration used is still a much-debated issue in diabetes and DN studies. The physiological blood glucose concentration of healthy rodents is 4-8 mM [427], whereas in diabetic ones levels can exceed 20 mM [428–431]. However, the vast majority of published *in vitro* studies using neurons in general (that did not investigate diabetes) maintain neuronal cultures in standard glucose concentrations of approx. 20-30 mM, depending on the culture medium used, which can be considered a hyperglycaemic environment in a diabetes-focused study. It is, therefore, advised that modelling of diabetes *in vitro* requires 7-20 mM additional external glucose in order to not deviate from established neuronal culture protocols and still recreate hyperglycaemic conditions as accurately as possible [419]. Other stimuli that have been successfully used in *in vitro* DN research include pro-oxidants for the assessment of oxidative stress [422], methylglyoxal [432] and lipid oxidation products for the evaluation of hyperglycaemic toxicity [433], oxidized low density lipoproteins for studying hyperlipidaemia [434] or serum from diabetic patients or animals [342,435].

Primary cultures are not without limitations. As neurons are non-proliferative, the yield is low and each culture requires time-consuming collection of neurons. Notably, due to the nature of the process, neuron dissection involves nerve injury, triggering a switch in cultured neurons from a "housekeeping" to a "regenerative" phenotype [436] which should be considered when assessing neuronal excitability overtime in culture. Finally, the simplified neuronal environment

requires caution when interpreting data and demands validation of findings in an *in vivo* setting [419].

An alternative to primary neuronal cultures is the use of immortalised cell lines, such as SH-SY5Y [437,438], NTERA-2 [439] and pheochromocytoma (PC12) [440,441] cells. Most are easy to obtain and maintain and provide an unlimited supply of a homogeneous population of cells. They have been extensively used in electrophysiological, molecular and biochemical neurophysiological research and represent a suitable platform for high-throughput drug screens necessitating vast numbers of cells. In addition, the use of cell lines eliminates the need to sacrifice animals for each culture, bringing a great ethical and time advantage. However, due to cell lines being even a further simplified culture of clonal cells, they do not reflect the heterogeneity of adult rodent neurons nor are they capable of developing processes such as dendrites and axons [442].

A promising novel technology in the research of neuronal diseases are iPSCs. By harvesting fibroblasts from patients, one can genetically reprogram them into pluripotent cells [443] and then stimulate their differentiation into various neuronal cell types, including sensory [444], and SCs[445]. This method holds potential for circumventing a possible problem with interspecies differences that may underlie the poor translation of drugs efficacy from established *in vitro* and *in vivo* techniques to the clinic.

The study of the intricate pathogenesis of DN has greatly benefited from *in vitro* models over the years. However, regardless of the *in vitro* model's advantages and success, findings must always be confirmed in an *in vivo* model to validate the observations when other interfering factors are also present, as they would be in the patient.

1.4.2. Experimental animal models of diabetic neuropathy

In vivo models of diabetes and DN are instrumental for the understanding of behavioural, physiological and structural DN pathology and are key drivers of progress towards successful therapy strategies. Compared to *in vitro* methods, animal models offer the advantage of susceptibility to genetic manipulations and higher system complexity. The diseases they are tailored to represent are thus closer in pathological resemblance to humans allowing for a more holistic interpretation. However, one big limitation remains the relatively short lifespan of rodents, which does not allow for the complete mimicking and investigation of the chronic aspects of human (P)DN which progresses over decades to reach irreversibility [241].

Over the years, a great diversity of rodent models of T1D and T2D have been developed and characterised (for a comprehensive review see [241,446,447]). Although none has yet been perfected to reflect the human disease in its full complexity, virtually all stages of human diabetes and its neuropathic complication have been reproduced by an animal model designed for the purpose [241].

There are three established parameters by which DN is confirmed in a diabetic rodent and the animal model is recognized as useful. These represent major aspects of human DN pathology, namely:

1) *behaviour* – sensory abnormalities are evaluated by assessing thermal and mechanical sensitivity;

2) *nerve conduction velocity (NCV)* – electrophysiological measures of nerve impairment assessing motor and sensory nerve conduction;

3) *nerve structure* – intraepidermal nerve fibre (IENF) density in the animal's footpad is assessed to reveal anatomical evidence of fibre loss.

Two out of three need to be significantly different from control animals for a neuropathic phenotype to be confirmed [448].

There are three main approaches by which rodent models of diabetes are generated – they can be genetically induced, chemically-induced and diet-induced.

1.4.2.1. Genetically induced models of diabetic neuropathy

Transgenic rodent models have been developed for both diabetes types. The most widely used T1D genetically modified models are the non-obese diabetic (NOD) and B6Ins2^{Akita} mice [241]. NOD mice develop a T-cell-mediated autoimmune response against their β-cells caused by a polygenic immunodeficiency that they carry. Thus, they spontaneously develop T1D, consistent with the pathogenesis in T1D patients. However, due to the polygenic nature of their genetic modification, diabetes and DN progression in NOD mice is variable and affected by diet, housing conditions and the sex of the animals and DN in this model is still undercharacterised [449]. Nevertheless, studies using NOD mice have reported hyperalgesia by week 8 [450] and hypoalgesia by week 12 [312]. B6Ins2^{Akita} mice have a point mutation in the *Ins2* insulin gene impairing insulin production [451] resulting in T1D by 7 weeks of age (WoA) and the gradual development of sensorimotor neuropathy [452]. NCV in this model has been reported to decrease at 16 WoA [452], however, in another study, B6Ins2^{Akita} mice showed no NCV

impairments even at 24 WoA [453]. Considering their current limitations, and the relatively controversial literature, DN phenotype in both NOD and B6Ins2[Akita] mice is still unclear and requires further characterisation.

Research of T2DN has benefited from some excellent transgenic animal models. The most popular and best studied are monogenic mouse models carrying a mutation which impairs leptin signalling [447]. Leptin is a hormone secreted after feeding to signal satiety and suppress appetite [454]. Mutation in the leptin hormone or its receptor produce *ob/ob* and *db/db* mice, respectively. Both models develop chronic hyperglycaemia and hyperinsulinemia [428,455]. *Ob/ob* mice are considered as an obesity and mild T2D model and DN in this model is understudied, however, they are reported to present with thermal hypoalgesia and reduced IENF density and sensory and motor NCV at 11 WoA [455].

Db/db mice are the very first mouse model developed for the investigation of DN [428], shown to develop physiologically relevant T2D as early as 4 WoA. Compared to *ob/ob* mice, DN in the *db/db* mouse model has been better characterised with several studies reporting hyperalgesia and allodynia at 8-12 WoA and sensory loss at 12+ WoA accompanied by a significant decrease in motor and sensory NCV and IENF density [453,456–459]. However, the relatively large number of studies using this mouse model has also generated considerable inconsistencies with regards to the DN phenotype with some reporting profound sensory anomalies while others see no significant differences from control animals [460]. Neuropathy severity in the *db/db* mouse is also affected by the choice of mouse strain with hyperglycaemia more stable and DN more severe in the C57BKS *db/db* than C57BL/6 *db/db* mouse [453,461]. Nevertheless, the progression of T2D and DN in the *db/db* mouse is considered to most closely mimic the natural history of these conditions in humans [453]. Part of the experiments in this thesis involve the *db/db* mouse and this model will be discussed in depth in the relevant chapter (Chapter 5).

1.4.2.2. Chemically induced models of diabetic neuropathy

Rodents can also be rendered diabetic via a chemical induction process. The most common model of T1D, the streptozotocin (STZ) model, employs this approach. STZ is an alkylating compound known to selectively destroy β-cells [462]. It is injected in the animal following one of two established protocols: either in a single high dose, producing a robust DN but also bringing a high mortality rate due to severe nonspecific toxicity; or in multiple low doses – a less toxic approach producing gradual T1D but often with no or only moderate neuropathy phenotype [463]. Although an inexpensive, easy to optimise and adequate model for T1DN research and drug tests, the induction's nature subjects animals to severe distress and potential DNA

damage – limitations that might impact measurements of pain responses and other parameters [464]. Furthermore, concerns have been raised over the direct neurotoxicity effects of STZ [465]. More recently, STZ has been shown to directly activate neuronal TRPA1 resulting in acute allodynia [466]. Despite its disadvantages, the diabetic STZ rat is by far the most extensively researched DN model, generating the vast majority of knowledge on the disease and its painful phenotype [242].

1.4.2.3. Diet-induced models of diabetic neuropathy

Drastic alterations in rodents' standard nutrition have also been employed that establish T2DN and neuropathic pain by successfully mimicking human metabolic syndrome development. Several diet modification strategies have been tried but a high fat diet (HFD), has been repeatedly demonstrated to have undeniable success at inducing T2D and neuropathy in mice [467]. Usually, mice on a HFD develop obesity gradually, followed by metabolic imbalances, including hyperglycaemia and moderate hyperinsulinemia until T2D is fully established. DN is close to follow with decreased sensory NCV, IENF density and thermal hypoalgesia often reported in these mice [468]. Although inappropriate diet is a leading cause for human T2D development too, compared to other approaches of DN induction, the diet approach can be time-consuming and its success varies due to influence from animal sex and age, diet duration, fat content and fat source. Nevertheless, diet-induced models of DN have proven useful in studies of prediabetes and obesity-associated neuropathies [467].

1.4.2.4. Considerations when selecting a model

DN progression is undeniably multifactorial and a complete model has not yet been developed for either of the diabetes types. Therefore, when selecting a model, it is critical to do so with the disease aspect being investigated in mind. Further to that, model choice must also take into consideration factors such as duration and type of stimulus exposure, measurement tools, phenotype assessment techniques, and in the case of animals – their sex, age, diabetes type being induced, diet and diet duration, and degree of exposure to diabetes-inducing compounds and methods. For example, it has been well-documented, that male and female mice show significantly different DN phenotypes even when the same diabetes-induction approach is used [447]. Female mice are more often used as genetically NOD models of diabetes, because they develop T1D symptoms much earlier [449], while male rodents are more suitable models for nutritionally-induced diabetes. Female rodents have also been reported to show a much more muted level of painful DN than males, presumed to be due to influence by specific female steroid hormones modulating the release of peptidergic neurotransmitters during pain

signalling [469,470]. In addition, currently, an imbalance exists in the field of DN research. T1DN and its painful aspects are extensively researched (mainly in the STZ model) while clinical studies are mostly focused on T2DN cases due to their greater global prevalence. Although both diabetes forms lead to DN, it is vital that the model is of the diabetes type the findings will be translated to. Ensuring such synchronisation might contribute to closing the gap between animal and clinical studies.

The elaborate study of *in vitro* and *in vivo* models of DN would not have been possible without the development of powerful techniques and tools. To investigate the neuronal excitability of DRG neurons from *db/db* mice, I employed a functional assay previously developed in our laboratory which combines Ca^{2+} imaging and the VGSC activator veratridine. In the final section of this chapter, I will briefly outline the characteristics of the different components of this assay and discuss its experimental applications to date.

1.5. Veratridine-based calcium imaging assay for neuronal excitability assessment

The major goal in neuroscience research is the understanding of the complex patterns of neuronal activity and the physiological and molecular events underlying different neurodegenerative conditions. To address this, one must be able to record the activity and analyse the changes in excitability of nerve cells. Electrophysiology and Ca^{2+} imaging are the two most popular approaches implemented to that purpose. Electrophysiological techniques such as voltage clamp, current clamp and patch-clamping are the 'gold standard' in neurophysiology research, providing direct report of a neuron's electrical properties with high temporal precision [471]. However, electrophysiological approaches are often invasive, usually requiring the insertion of electrodes in the cell, and limited to assessing only a small subset of neurons (most often – a single cell at a time), thus unable to monitor the neuronal dynamics in a heterogeneous population such as DRG neurons.

For the simultaneous assessment of a great number of neurons in a diverse population, a medium-to-high throughput approach is more appropriate such as Ca^{2+} imaging. Furthermore, the research for new treatments against pain has been re-targeted from the CNS towards the nociceptors in the PNS and, more specifically, the ion channels they expressed that regulate pain signalling. The development of novel, more efficient and safer analgesics creates a need to be able to single out and specifically target nociceptors with minimal or no impact on non-nociceptive cells. To that end, a suitable assay is needed that is not only high throughput but

also able to discriminate nociceptors in a heterogeneous DRG neuronal population. Calcium imaging is a technique with the potential to answer these requirements.

1.5.1. Calcium imaging technique

In the neuron, Ca^{2+} ions play a central role in various processes across all parts of the nerve cell. A charge carrier and an important intracellular messenger, Ca^{2+} is involved in the regulation of neuronal development, apoptosis, neurotransmitter release and membrane excitation. At rest, the intracellular Ca^{2+} ion concentration in the neuron fluctuates between 50 and 100 nM, however they can rise 100-fold during electrical activity. Therefore, the direct measurement of intracellular baseline Ca^{2+} levels as well as Ca^{2+} spikes is highly informative of a neuron's activity and excitability [472]. By harnessing this neuronal property, Ca^{2+} imaging can directly visualise the Ca^{2+} status of hundreds of individual neurons simultaneously. Compared to electrophysiological methods, it does so in a less invasive way and can be used to track the activity of neurons over time. Calcium imaging has been used widely by neuroscientists to record activity in neuronal populations [473]. The imaging of Ca^{2+} ions depends highly on the use of appropriate Ca^{2+} sensor indicators. Calcium indicators allow for the real-time monitoring and recording of cellular Ca^{2+} signals *in vitro* as well as *in vivo,* discussed next.

1.5.1.1. Calcium indicators

To monitor neuronal activity based on intracellular Ca^{2+} levels, a Ca^{2+}-binding indicator is required that would enable the optical measurement of neuronal Ca^{2+} concentrations. Such indicators have been in development since the 1960s [474,475], but it was Tsien et al. that revolutionised Ca^{2+} imaging by introducing a series of highly sensitive fluorescent Ca^{2+} sensors. These Ca^{2+} indicators alter their spectral properties upon binding free Ca^{2+} ions. Broadly speaking, they can be classified into two main categories: chemical Ca^{2+} sensors for *in vitro* imaging [476] and genetically encoded Ca^{2+} indicators (GECIs) for *in vivo* imaging [477]. A summary of the structure and general properties of each class is presented in the diagrams of Figure 1.9.

- *Chemical calcium indicators*

The most successful example of an *in vitro* chemical Ca^{2+} indicator is the widely used high-affinity dye Fura-2. It is the result of the hybridisation of a highly Ca^{2+}-selective chelator with a fluorescent chromophore (or fluorophore) [478] (Figure 1.9). Its ester form, Fura-2AM, is its membrane-permeable derivative that freely crosses the cell membrane. Once inside the cell, cellular esterases cleave the ester bond thus "locking" the dye inside [479]. Fura-2 binds Ca^{2+} ions with high affinity (Kd ~ 145 nM), and has a relatively wide Ca^{2+} sensitivity, ranging between ~100 nM and ~100 μM [480]. The primary advantage of Fura-2 is that it is a ratiometric dye. The binding of free intracellular Ca^{2+} induces conformational changes in Fura-2, shifting its excitation wavelength from 380 nm (free Fura-2) to 340 nm (Ca^{2+}-bound Fura-2). Its emission

Figure 1.9. Structure and properties of chemical and genetically-encoded calcium indicators. Chemical calcium indicators, such as Fura-2, are made of a Ca2+-chelating site and a fluorophore. Binding of Ca2+ (red circle) changes the fluorescence emitted. In contrast, genetically-encoded calcium indicators (GECIs) are made of three main components: an enhanced green fluorescent protein as a fluorophore, calmodulin, which binds Ca2+ and M13, which binds Ca2+-bound calmodulin. The interaction between Ca2+-bound calmodulin and M13 leads to conformational intrmolecular changes resulting in an increased emitted fluorescence of 515 nm. Source: Grienberger & Konnerth (2012)

wavelength remains stable at ~ 500 nm at either excitation wavelength. The higher the intracellular Ca^{2+} concentration is, the stronger the excitation at 340 nm and the weaker the

excitation at 380 nm is. Thus, a quantitative measure of intracellular Ca^{2+} concentrations can be estimated, independently of the intracellular dye concentrations, by calculating the ratio of the emissions at the two excitation wavelengths (340/380). The ratiometric property of Fura-2 makes it a highly popular choice due to eliminating issues with variables such as variable dye concentration, unequal cell thickness, dye leaking and photobleaching [481].

- *Genetically encoded calcium indicators*

After the establishment of chemical Ca^{2+} sensors, protein-based genetically encoded Ca^{2+} indicators (GECIs) were the next big breakthrough as they allowed for the imaging of neuronal activity *in vivo* [477]. Their expression can be targeted to specific subpopulations of cells and maintained stable over months, allowing for the repeated observation of neurons. Thus, GECIs are invaluable in the research of nervous system development, maintenance, learning and memory. However, they remain slightly inferior to chemical Ca^{2+} indicators in terms of their signal-to-noise ratios. The most widely used form of GECIs is the single-fluorophore GCaMP family. It is composed of three main parts: a circularly permutated enhanced green fluorescent protein (eGFP) with the Ca^{2+}-binding protein – calmodulin attached on one end and a calmodulin-binding protein M13 on the other. Upon binding free Ca^{2+}, calmodulin interacts with M13, which induces a conformational change in eGFP. This results in the increase of emitted fluorescence by the fluorophore [477,482] (Figure 1.9). Substantial progress has been made in recent years to develop improved GECIs classes with high-affinity sensors, improved dynamics range, kinetics and better signal-to-noise ratio for the reliable detection of neurons with low activity rates [483].

1.5.2. Activating neurons

To record neuronal activity *in vitro*, apart from an appropriate Ca^{2+} indicator, Ca^{2+} imaging requires neuronal stimulation. This can be by a shift in extracellular conditions (e.g. temperature), electrical stimulation or pharmacological agents. Extracellular solutions with high K^+ concentration are generally used to activate neurons by depolarising the membrane potential across the whole neuronal population thus opening multiple voltage-gated ion channels simultaneously in the process. Hence, the excitability information derived by such stimulation is limited to a 'yes or no' interpretation and is impossible to discriminate among neuronal subtypes [484].

1.5.2.1. The constellation pharmacology approach

Apart from activating all neurons in a population simultaneously, separate neuronal subsets can also be targeted by applying specific pharmacological agonists. This method of "profiling"

a mixed population of neurons was popularised by Olivera et al. under the term "constellation pharmacology" [485,486]. Specifically, they applied a variety of pharmacological agents (e.g. allyl isothiocyanate, capsaicin, histamine, ATP, menthol and acetylcholine), as well as agonists targeted at voltage-gated ion channels (e.g. TTX, tetraethylammonium – a VGKC blocker) to a heterogeneous DRG population of neurons and recorded individual responses by Ca^{2+} imaging. Based on the distinct sets of Ca^{2+} responses produced by each neuron they were able to identify and functionally characterize neuronal subtypes [485,487]. The constellation pharmacology approach can therefore be applied not only for the functional characterisation of neuronal subtypes but also in drug screens to determine the specific selectivity of a drug, as well as the degree of any side effects. These can be established by comparing the Ca^{2+} responses induced by markers in control versus drug-treated cells [488]; or by pre-establishing a Ca^{2+} response template to high K^+ solution in cells and comparing the changes the drug would induce to changes induced by ion channel blockers with pre-determined specificity [485,487,488]. Albeit highly informative, this cellular neuropharmacological approach is not without limitations. Due to its nature, this method demands the testing of multiple markers in succession in order to characterise the constellation of neuronal ion channels and receptors. The more detailed the characterisation is, the greater number of agents need to be applied, creating lengthy protocols, which cannot be supported due to limitations by other factors, e.g. the eventual photobleaching of the Ca^{2+} indicator used. The need for elaborate, pre-established Ca^{2+} response templates as well as the application of high K^+ solution as a universal neuronal activator during drug screens are further examples of drawbacks of this experimental method.

1.5.2.2. Veratridine

One pharmacological agent used in drug screens is veratridine (VTD) due to its ability to activate VGSCs. Veratridine is a lipid-soluble alkaloid toxin derived from the seeds of lilaceous plants. It was first purified in the 1950s and became commercially available as a pharmacological agent in the early 1970s. Initially used mainly as an insecticide, the interest in VTD's neurotoxic properties gradually grew sparking research into its mechanism of action on VGSCs [489–491].

Veratridine binds reversibly to the S6 VGSC segment – part of the pore domain of the channel. The toxin is selective for TTX-S VGSCs and preferably binds them in their open state. Notably, voltage-clamp experiments in neurons from rat DRG have shown that VTD can also bind TTX-R channels although the dissociation rate is much faster than TTX-S channels, leading to transient responses [492]. Once bound, VTD locks the channel in its open conformation and prevents inactivation by shifting the activation threshold of the channel to a more negative potential [493]. Thus, VTD leads to an increase in intracellular Na^+ levels, depolarising the membrane and activating other voltage-gated ion channels resulting in increase in intracellular Ca^{2+} concentrations and increased overall neuronal excitability (Figure 1.10). Of note, for VTD to exercise its strong nerve depolarisation effect, the external medium needs to include Na^+ ions in its composition, as demonstrated by early experiments on frog and single nerve fibres[489]. With its VGSC activator profile confirmed by further electrophysiology experiments[491,494–496], VTD is now extensively used as a pharmacological tool in drug screening protocols to activate sodium channels and test the efficacy of VGSC blockers [497].

Figure 1.10. Veratridine (VTD) action on voltage-gated sodium channels (VGSCs). VTD preferably binds TTX-S VGSCs such as Nav1.7. Upon binding, VTD "locks" the channel in its open conformation state, increasing the flux of sodium (Na^+) ions into the cell. This causes membrane depolarisation and triggers the opening of other voltage-gated channels, including voltage-gated calcium channels (VGCCs). Their activation allows for the influx of calcium (Ca^{2+}) ions into the cells, increasing the intracellular Ca^{2+} concentration ($i[Ca^{2+}]$) which can be detected and measured using Ca^{2+} indicators and Ca^{2+} imaging.

1.5.3 Veratridine-induced calcium response profiles as functional indicators of DRG neuronal subpopulations

In DRG, the TTX-S VGSCs available for VTD to bind to are Nav1.6 and Nav1.7. Of the two, Nav1.7 is the most predominantly expressed in all DRG, especially nociceptors, whereas Nav1.6, although expressed by all DRG neurons, is found mainly in large, non-nociceptive neurons [498]. Although VTD preferentially binds to TTX-S VGSCs, it does not have specificity to a particular isoform. Applying VTD to a mixed population of DRG neurons, therefore, would be expected to produce mixed responses based on the constellation of VGSCs (and other voltage-gated ion channels) expressed by each neuronal type.

This concept was elegantly demonstrated previously in our lab by Mohamed et al. [499], in Ca^{2+} imaging experiments with VTD on mouse DRG neurons. They showed that VTD elicited heterogeneous Ca^{2+} responses. Based on their decay rate and oscillation, the responses were classified into four distinct VTD-induced Ca^{2+} response profiles: rapid decay (RD), intermediate decay (ID), slow decay (SD) and oscillatory (OS) profile with the SD and OS profiles being the most frequently occurring (Figure 1.11 A, B). Additionally, three nociceptive agonists were also applied to the neurons after VTD:

- **α,β-methylene adenosine 5'-triphosphate (ATP)** to mark non-peptidergic, IB4+ nociceptors,
- **allyl isothiocyanate (AITC)** – marker of LTMRs and some peptidergic and non-peptidergic nociceptors
- **capsaicin (CAP)** to mark peptidergic nociceptors.

The VTD-response profiles correlated with responses to the nociceptive markers (Figure 1.11 C) revealing a strong association between the OS profile and nociceptors, whereas the SD profile correlated with non-nociceptors. These observations were further confirmed by size analysis of the imaged neurons, showing that virtually all OS, ID and RD neurons are small to medium diameter, while SD was the most prevalent profile in neurons of large diameter. An additional finding of this study was that 25-30% of the identified nociceptors did not respond to VTD and were therefore presumed to be the population of high-threshold "silent nociceptors", activated under inflammatory or nerve injury conditions [4] (Figure 1.11 C).

Figure 1.11. *Veratridine (VTD) applied to mouse DRG neurons produces four distinct VTD-response profiles that could be indicative of specific neuronal subpopulations. **A)** Example traces for each of the four distinct VTD-response profiles: Slow decay (SD, top left), rapid decay (RD, top right), intermediate decay (ID, bottom left) and oscillatory profile (OS, bottom right). **B)** Frequency of the occurrence of the VTD-response profiles in VTD(+) DRG neurons. Most frequent is the OS profile (48 ± 4%), followed by SD (24.4 ± 4%), RD (13.4 ± 4%) and ID (12.7 ± 2%). **C)** VTD-response profiles can be correlated to functional markers of nociceptors. DRG neurons were applied nociceptive agonists: α,β-methylene ATP, allyl isothiocyanate or capsaicin. Neurons responding to at least one of the three agonists applied are defined as nociceptors. nociceptors constitute 66% of all DRG neurons. Of them, 64% were of the OS, ID and RD VTD-response profiles. Non-nociceptors (neurons insensitive to any of the three nociceptive agonists) constituted 34% of all DRG neurons, 51% of which were represented by the SD VTD-response profile. Source: Mohammed et al. (2017)*

Thus, the study generated the first detailed characterisation of VTD responses in DRG neurons and their correlation with neuronal subtypes. What is more, it proposed the use of the VTD response profiles as broad functional markers of neuronal subpopulation that can discriminate nociceptors from non-nociceptors. Importantly, it presented the opportunity of developing the VTD-Ca^{2+} imaging assay into a platform suitable for drug screening as well as characterisation of neuropathology phenotypes [499]. The promising findings by Mohamed et al. opened the door to exploring the possibilities of this assay and laid the foundations of the project described in this thesis.

1.6. Aims

The VTD-Ca^{2+} imaging assay has shown promise as a suitable technique for neuronal characterisation. However, before it can be applied, the link between the VTD-response profiles and neuronal subpopulations needs to be strengthened further. Therefore, the first aim and its objectives of this study are

<u>Aim 1</u>: Validation of the VTD-Ca^{2+} imaging assay as a suitable drug screen platform (Chapter 3)

- Confirm the link between VTD-induced Ca^{2+} response profiles and neuronal subpopulations by:
    o using DRG from mice with genetically ablated nociceptors
    o using specific and non-specific VGSC blockers and analyse the VTD response profiles patterns in DRG neurons

Then, I endeavour to investigate the application of the assay as a method for characterising the neuropathological phenotype in a diabetic neuropathy model. The *db/db* mouse is one of the most popular models of type 2 diabetes, however, its diabetic neuropathy phenotype is still poorly characterised and the accumulated literature on the matter to date is highly controversial. Therefore, the second aim and objectives of the research presented here are:

<u>Aim 2</u>: Application of the VTD-Ca^{2+} imaging assay and nociceptive agonists to characterise changes in excitability in neuronal subpopulations under diabetic neuropathy conditions (Chapters 4 and 5).

- Characterise excitability changes in DRG neurons from healthy mice cultured *in vitro* under hyperglycaemic conditions using the VTD-Ca^{2+} imaging assay and nociceptive markers
- Characterise morphological and excitability changes in DRG neurons isolated from diabetic *db/db* mice using VTD-Ca^{2+} imaging assay and nociceptive markers. Investigate the distinct excitability changes in the two diabetic neuropathy phases in the *db/db* mouse:
    o Early diabetic neuropathy phase
    o Late diabetic neuropathy phase

CHAPTER 2

MATERIALS & METHODS

CHAPTER 2: MATERIALS & METHODS

2.1 Materials

2.1.1. Animals:

C57/BL6 adult male mice (Charles River, Margate, UK), obtained at 5-7 weeks old were used in the experiments with healthy mice outlined in this thesis. For experiments investigating effects of diabetes on DRG neurons, a total of 12 male diabetic C57BKS*db/db* mice (BKS.Cg-+Leprdb/+Leprdb/OlaHsd) were used and 12 lean littermate mice were used as non-diabetic controls (BKS.Cg-+Leprdb/-Leprdb/OlaHsd) (Envigo, The Netherlands). Animals were kept in a 12h light-dark cycle with free access to food and water. Mice were housed in groups of 3-4 and were allowed a week for acclimatisation upon arrival at the Biological Services Unit at the University of Sheffield in a temperature- (23 ± 2°C) and humidity- (40-70%) controlled holding room. During experimental periods, no animal was left alone in its unit for longer than 24h. All efforts were made to minimise animal suffering and reduce the number of mice used in the reported studies. All experiments with animals were conducted according to the UK Home Office Animals (Scientific Procedures) Act 1986.

2.1.2 Cell culture reagents

Name	Supplier	Catalogue No
Albumin Bovine fraction V (BSA)	Melford	A1302
Collagenase Type XI,100X (0.6 mg/mL)	Sigma	C9407
Dispase, 100X (1 mg/mL)	Sigma	D4693
Dulbecco's Modified Eagle's Medium/F12 with Glutamax medium (DMEM/F-12 + Glutamax)	Gibco™ life technologies	31331-028
Dulbecco's phosphate buffered saline (DPBS), without Ca2+ and Mg^{2+}, 0.0095 M (PO$_4$)	Lonza	BE17-512Q
FBS (fetal bovine serum) – EU approved origin, origin: South America	Gibco™ life technologies	10500-064

Penicillin/Streptomycin 100X	Sigma	P0781
Poly-L-orinithine (20μg/mL)	Sigma	P3655

2.1.3 Pharmacological compounds

Name	Supplier	Catalogue No	Stock concentration	Solvent
4,9-anhydrotetrodotoxin	Tocris	6159	300 μM	water
A-803467	Abcam	Ab120282	10 mM	DMSO
Allyl isothiocyanate (AITC)	Sigma	377430	100 μM	-
Capsaicin	Tocris	0462	10 mM	ethanol
Isoflurane (IsoFlo®)	Abbott	B506	100%	-
PF-04856264	Sigma	11916	10 mM	DMSO
PF-05089771	Sigma-Aldrich	PZ0311	20 mM	DMSO
Veratridine	Abcam	Ab120279	5 mM	ethanol
α, β-methylene ATP	Sigma	M6517	10 mM	water

2.1.4. Reagents, chemicals and solvents

Name	Supplier	Catalogie No
Calcium Chloride (anhydrous)	Melford	C1103
D-glucose (anhydrous)	Fisher Scientific	G/0500/53
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	276855
Ethanol, absolute (HPLC grade)	Fisher Scientific	E/0665DF/17
Fura-2, AM (20 X 50 μg unit)	Molecular Probes	F1221

HEPES	Sigma	H3375
Magnesium chloride (Anhydrous)	Melford	M0535
Potassium chloride	Melford	P0515
Sodium Chloride	Fisher Scientific	S/3160/65
Sodium hydroxide	Fisher Scientific	S/4920/53
Mannitol	Sigma-Aldrich	63559

2.1.5. Tools, equipment and labware

Name	Supplier	Catalogie No
Calcium imaging perfusion chamber	Warner instruments	RC-25F
Cellview™ cell culture dish, PS, 35/10MM (Glass bottom, one compartment)	Scientific Laboratory Supplies LTD	-
Cover glass ø16 mm (0.13-0.17 mm thick)	Scientific Laboratory Supplies LTD	MIC3310
Disposable Scalpels (Surgical steel blades)	Swann-Morton	0505
Dumont #5 Forceps	Fine Science Tools, FST	15018-10
Luer-Lok syringes (50 mL)	Becton Dickinson (BD)	300865
Minisart® Syringe Filters (0.45 µm, Sartorius)	Appleton woods	25926
Portex Tubing ,30m non-sterile polythene tubing, [0.86 mm ID, 1.27 mm OD]	Fisher Scientific	800/110/260

2.1.6. Solution and medium recipes

- DRG neurons digestion mix (stored at -20°)
  - Collagenase XI (0.6 mg/mll)
  - Dispase II (1 mg/ml)
  - DMEM/F-12 + Glutamax

- Standard Extracellular Ringer Solution (stored at room temperature)
  - 140 mM Sodium Chloride,
  - 4 mM Potassium Chloride,
  - 2 mM Calcium Chloride,
  - 1 mM Magnesium Chloride,
  - 10 mM HEPES,
  - 5mM D-glucose (added on the day of experiment)*
  - pH = 7.4 at 25 °C, PH is adjusted with Sodium Hydroxide

- High Potassium Ringer Solution (stored at room temperature)
  - 104 mM Sodium Chloride,
  - 40 mM Potassium Chloride,
  - 2 mM Calcium Chloride,
  - 1 mM Magnesium Chloride,
  - 10 mM HEPES,
  - 5 mM D-glucose (added on day of experiment)*
  - pH = 7.4 at 25 °C, PH is adjusted with Sodium Hydroxide

*stored at 5°C once D-glucose is added

2.2. Methods

2.2.1. Preparation of mouse dorsal root ganglia neuronal culture

2.2.1.1 Isolation of DRG

The mouse was anaesthetized using isoflurane in an anaesthetic machine, then moved to a surgical table and culled by cervical dislocation in accordance with Schedule 1 procedures of UK Animals (Scientific Procedures) Act 1986. The animal's spinal column was isolated by careful incisions along its length and unnecessary extra tissue (muscle and fat) surrounding it was gently shaved off using a scalpel. The spinal column was then cut in half longitudinally, 1-2 vertebrates

at a time, using a scalpel in order to be able to access the spinal cord. Halves of the spinal column were placed under a light microscope and spinal cord matter pushed away carefully using forceps in order to expose the sockets containing DRG underneath. Then DRGs were extracted one by one out of the spinal columns with forceps and placed into 35mm dish with PBS until end of isolation procedure. Once all DRGs were isolated, long extending axons were trimmed whilst still in PBS dish under the light microscope, using a scalpel. For experiments investigating mice with $Na_v1.8$-expressing neurons ablated, DRG from $Na_v1.8$Cre-DTA mice [99] and littermate control mice were stored in ice-cold Hibernate-A medium (Gibco) containing penicillin/streptomycin whilst being transported from University College London (approximately 5-hour journey), after which were dissociated as outlined.

2.2.1.2 DRG digestion

The PBS in the dish containing isolated and prepped DRG bodies was replaced with DRG digestion mix made up of 1 ml Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) with Glutamax medium containing Dispase (1mg/ml) and Collagenase Type XI (0.6 mg/ml). DRGs were then left in an incubator for 60 mins at 37°C and 5% CO_2 for digestion.

2.2.1.3 Dissociation of neurons and seeding

At the end of digestion time, cells were triturated 10 times to ensure tissue separation from neurons. Resulting cell suspension was then carefully layered on the surface of 1.5 ml 15% Bovine Serum Albumin (BSA) in DMEM/F12. Mixture was centrifuged for 10 minutes at 21° at 800 g (2000 rpm (rotor no. 4624/ Hettich Rotina 46R centrifuge)) set at minimum deceleration speed. The produced cell pellet was then gently washed with DMEM/F12 containing 10% Foetal Bovine Serum, 100 units/mL penicillin and 100 µg/mL streptomycin. Mixture was centrifuged once more at 200g (1000 rpm) for 3 minutes. Supernatant was removed and cells were resuspended in 60 – 100 µl of the above-described DRG culture medium and seeded on autoclaved and poly-L-ornithine- coated (20 µg/ml) 16mm glass coverslips with each seeding drop being 3-4 µl. Neurons were left in the incubator at 37°C / 5 % CO_2 for 1 hour to allow adhesion to coverslip surface. Finally, plated cells were carefully flooded with DRG culture medium and incubated for a minimum of 24h at 37 °C / 5 % CO_2 prior to experiments.

2.2.1.4. Adjustments to glucose concentration in culture for in vitro hyperglycaemia experiments

Mouse DRG neurons were cultured with standard DMEM/F12 (17.5 mM glucose) with 32.5 mM added glucose (total 50 mM) to induce hyperglycaemic, diabetic conditions; control cultures

were supplied with DMEM/F12 medium containing default amount of 17.5 mM glucose deemed optimal for neuronal survival [318] and were added 32.5 mM mannitol for osmotic control. Additional glucose administration to the culture was initiated after 1 day after culturing the neurons and maintained for up to 5 days with measurement taken on days 1, 2, 4 and 5 post - glucose addition.

2.2.2. Calcium imaging of DRG neurons

2.2.2.1. DRG cells loading with Fura-2AM

All loading of neurons with a calcium dye took place in the dark. Working concentration of 2 µM of the calcium dye Fura-2AM was made up by mixing 1 µl Fura-2AM stock with 1ml standard extracellular Ringer solution (see section 2.1.6. Solution and medium recipes). Using a P1000 pipette, solution was vigorously mixed for up to a minute to ensure complete solubilisation of the dye. Coverslips were carefully removed from culture plates with DRG medium and placed in a 35mm dish, up to three at a time, taking care not to overlap. They were washed once with standard extracellular Ringer solution before loaded with Fura-2AM solution and left to incubate at 37 °C / 5 % CO_2 for 30 minutes. After 30 minutes, Fura-2AM solution was removed and replaced with 1 ml standard extracellular Ringer solution and cells were left to incubate at 37 °C / 5 % CO_2 for another 15 minutes. Finally, cells were taken out and stored in an opaque container outside of incubator for another 15 minutes in order to allow for neurons to adjust to the room temperature at which calcium imaging experiments would take place.

2.2.2.2. Experimental set up

Recordings were performed at room temperature of 23 ± 2°C. Each Fura-2AM-loaded coverslip was mounted on a recording chamber (RC-25F, Warner instruments) and slowly and carefully added 1 ml standard extracellular Ringer solution to prevent dehydration of cells. Chamber was placed on the stage of an inverted Olympus, IMT-2 microscope fitted with a 40X oil-immersion objective (Olympus, 160/-, DPlanApo40UVPL). Due to technical issues this objective was replaced with a 40X dry objective. Coverslip surface was examined to locate a suitable field of view, comprising of optimal number of cells (20 – 45) and cell density. Simple PCI6 software was used for recording set up and image acquisition with background subtraction and ratiometric measurements (F350/380 nm). Regions of interest (ROI) were obtained for each cell of interest in the selected field, as well as an additional one for the background. Neurons were excited with alternating 350 nm and 380 nm light (Cairn Dual OptoLED system) at 1.6 s intervals. Image acquisition was carried out using a Hamamatsu C4742-95 camera. Solution perfusion was carried out using a perfusion system reliant on gravity at a flow rate of 3 ml/min.

2.2.2.3. Recording of calcium responses

Upon launching of the perfusion system, a laminar stable flow across the coverslip was confirmed before recording commenced. Prior to addition of drug solutions during recording, neurons were initially perfused with standard extracellular Ringer solution for at least 5 minutes to ensure a stable initial calcium trace baseline is established. A wash of minimum of 5 minutes with standard extracellular Ringer solution was also included in between drug solution perfusions. Finally, high potassium extracellular Ringer solution (KCl, 40 mM) (see 2.1.6. Solution and medium recipes, page 66) was perfused at the end of each recording as a universal neuronal depolarising means to confirm viable neurons. Neurons irresponsive to high potassium extracellular Ringer solution were excluded from analysis.

2.2.3. Data processing and statistical analysis of calcium imaging data

2.2.3.1. Analysis of recorded calcium imaging responses

Viable neurons identified by high potassium extracellular Ringer solution were selected for analysis. All recorded traces were analysed using GraphPad Prism 8 software, also used to generate all graphs in this thesis. Calcium signal baseline was established from 30 frames of signal prior to the addition of any drugs. A calcium response to a drug was identified as an increase in F350/380 ratio of 6 standard deviations or more above the mean of the baseline. Veratridine (VTD) responses were classified according to the four VTD-response profiles, or shapes, outlined previously by Mohammed et al. (2017) [499].

The fractional difference (ΔF/F0) in fluorescence was established prior to amplitude, response onset and area under the curve analysis, using the formula:

$$\frac{\Delta F}{F0} = \frac{F350/380 - Baseline}{Baseline}$$

Area under the curve was calculated using a built-in analysis function in GraphPad Prism 8 implementing the 'trapezoid' principle for area under the curve measurement. The measured data included a 10-minute stretch of the recording, beginning at VTD application, due to the diversity in shapes and decay of VTD responses. Amplitude and response onsets were analysed manually using GraphPad Prism 8 for each trace individually.

2.2.3.2. Neuronal cell size analysis

Neuronal cell diameter analysis was carried out using the ImageJ software (version 1.53a for Windows). Cell circumference was obtained by manual tracing of each cell's soma outline. Cell area was then calculated by the in-built software formula. The generated cell area was then used to calculate cell diameter following the formula:

$$Diameter\ (d) = \sqrt{(\frac{4\ xArea}{\pi})}$$

2.2.3.3. Statistical tests

The unit used in statistical analysis was the mouse (N). The mean percentage of responsive neurons from each culture an N produced was calculated. Upon accumulation of sufficient independent samples (independent Ns) for each experiment, means of the Ns of each experimental group were compared to each other. To test the frequency distribution of cell sizes for normal Gaussian distribution, a normality test was performed (D'Agostino-Pearson and Kolmogorov-Smirnov normality tests), which yielded normal Gaussian distribution in all cases. To calculate the significant difference between two groups, a Student's t-test was used. To test for significant difference in the mean values between three or more groups, one-way analysis of variance (ANOVA) with post Sidak's multiple compariso

as the mean ± standard error of the mean (SEM).

CHAPTER 3

VALIDATION OF THE DISTINCT VERATRIDINE CALCIUM RESPONSE PROFILES AS FUNCTIONAL MARKERS FOR NOCICEPTORS AND NON-NOCICEPTORS

CHAPTER 3: VALIDATION OF THE DISTINCT VERATRIDINE CALCIUM RESPONSE PROFILES AS FUNCTIONAL MARKERS FOR NOCICEPTORS AND NON-NOCICEPTORS

The research presented in this chapter has been published under the title:

"An unbiased and efficient assessment of excitability of sensory neurons for analgesic drug discovery"

Authors: Zainab A. Mohammed, Katerina K. Kaloyanova and Mohammed A. Nassar

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Authors' contribution:

I co-designed, carried out and analysed all experiments presented here, excluding experiments with knock-out mice (Figure 3A and C). Contributed to the preparation of figures. Contributed to manuscript editing and revisions.

Zainab Mohammed co-designed and carried out experiments presented in Figure 3A and C; and co-designed and contributed to carrying out experiments presented in Figures 4 and 5. Also, contributed towards manuscript editing and revisions.

Mohamed Nassar conceived, designed and supervised all experiments and prepared the manuscript.

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An unbiased and efficient assessment of excitability of sensory neurons for analgesic drug discovery

Zainab A. Mohammed, Katerina Kaloyanova, Mohammed A. Nassar*

Abstract

Alleviating chronic pain is challenging, due to lack of drugs that effectively inhibit nociceptors without off-target effects on motor or central neurons. Dorsal root ganglia (DRG) contain nociceptive and non-nociceptive neurons. Drug screening on cultured DRG neurons, rather than cell lines, allows for the identification of drugs most potent on nociceptors with no effects on non-nociceptors (as a proxy for unwanted side effects on central nervous system and motor neurons). However, screening using DRG neurons is currently a low-throughput process, and there is a need for assays to speed this process for analgesic drug discovery. We previously showed that veratridine elicits distinct response profiles in sensory neurons. Here, we show evidence that a veratridine-based calcium assay allows for an unbiased and efficient assessment of a drug effect on nociceptors (targeted neurons) and non-nociceptors (nontargeted neurons). We confirmed the link between the oscillatory profile and nociceptors, and the slow-decay profile and non-nociceptors using 3 transgenic mouse lines of known pain phenotypes. We used the assay to show that blockers for $Na_v1.7$ and $Na_v1.8$ channels, which are validated targets for analgesics, affect non-nociceptors at concentrations needed to effectively inhibit nociceptors. However, a combination of low doses of both blockers had an additive effect on nociceptors without a significant effect on non-nociceptors, indicating that the assay can also be used to screen for combinations of existing or novel drugs for the greatest selective inhibition of nociceptors.

Keywords: Calcium imaging, Veratridine, Voltage-gated channel blockers, Nav1.7 Knockout, Nociceptors, Mouse DRG, Capsicin, Drug screening

1. Introduction

Chronic pain has significant negative impacts at personal, social, and economic levels, affecting millions of people worldwide.[10,19] Several drug classes are used to treat chronic pain, but their effectiveness is limited by either lack of potency or adverse effects. Therefore, the treatment of severe or chronic pain is a challenging clinical need.

The search for new analgesics focuses on targets in pain neurons in the dorsal root ganglia (DRG). Dorsal root ganglia contain a heterogeneous population of neurons that can be classified according to expression of sensory markers into 11 subtypes,[23] but they can be broadly classified as nociceptors and non-nociceptors (proprioceptors and touch neurons). Drug screens use cell lines expressing the nociceptive target of interest. Although this platform allows for high-throughput screening, it provides no information on drugs' effects on non-nociceptors. This information would weigh the potency on nociceptors against adverse effect on non-nociceptors

before costly and laborious in vivo testing. However, screens on DRG neurons are currently not practical because they are low throughput, and there is not a simple protocol to distinguish between drugs' effects on nociceptors vs non-nociceptors.

Voltage-gated sodium channels (VGSCs) are critical for the excitability in DRG neurons. We discovered that the VGSC opener, veratridine, produces distinct calcium responses in cultured sensory neurons.[16] We found that most nociceptors show an oscillatory response (OS), whereas most non-nociceptors show a slowly decaying response (SD), **Figure 1**. Here, we provide evidence that the OS and SD veratridine-response profiles can be used as readouts in an assay to evaluate the effect of drugs on nociceptors and non-nociceptors. We first showed that the OS population is drastically reduced in a mouse model where most nociceptors are ablated. We then determined the pattern of veratridine responses in a $Na_v1.7$ knockout mouse, a pain-free model. Subsequently, we used this pattern as a reference criterion against which 2 VGSC blockers, against the $Na_v1.7$ and $Na_v1.8$ channels, were evaluated. These channels are validated targets for analgesic drug development with several promising compounds identified in recent years,[6,25] but lack of in vivo potency and selectivity on nociceptors has contributed to failure in clinical trials.[9] The 2 VGSC blockers were unable to match the $Na_v1.7$ knockout pattern when applied separately but showed an additive effect when applied together. This suggests that our assay can also be used to screen for combinations of drugs that act on different targets in nociceptors for effective pain relief.

2. Methods

2.1. Dorsal root ganglia culture

C57BL6 mice were sacrificed according to Schedule 1 of the Animal (Scientific procedure) Act 1986. Dorsal root ganglia from

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Figure 1. Assessments of the excitability of nociceptors and non-nociceptors based on veratridine-response profiles. Dorsal root ganglia (DRG) contain a heterogeneous population of sensory neurons. Nociceptors are typically small in size, express the $Na_v1.8$ channel, and respond to nociceptive compounds (eg, ATP and capsaicin). Most nociceptors responded to veratridine with an oscillatory (OS), rapid decay (RD), or intermediate decay (ID) profiles. Non-nociceptors are typically large in size, do not express the $Na_v1.8$ channel, and do not respond to nociceptive compounds. Most non-nociceptors respond to veratridine with a slow-decay (SD) profile. The percentages of the OS and SD veratridine profiles plus the percentage of veratridine-irresponsive (VTD-) neurons can be used for an efficient assay of the excitability DRG neurons. Changes in the SD population reflect changes in non-nociceptors (blue, typically 15%-20% of all neurons). Changes in the OS population (red, typically 30%-40% of all neurons) reflect changes in nociceptors. Changes in the veratridine-irresponsive population (gray, typically 30%-40% of all neurons) can reflect sensitisation of high-threshold and normally "silent" neurons. The 2 minor profiles, ID and RD, are mostly nociceptors but can be excluded from an assay for simplicity as both account for less than 5% to 10% of all neurons.

all spinal levels were dissected and collected in phosphate-buffered saline at room temperature. Phosphate-buffered saline was then replaced with 1-mL dissociation solution containing Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) with Glutamax (Gibco, United Kingdom), 1× penicillin/streptomycin mix (Gibco), dispase (1 mg/mL, Sigma, United Kingdom), and collagenase Type XI (0.6 mg/mL, Sigma) and incubated at 37°C and 5% CO_2 for 90 minutes. Dorsal root ganglia were triturated with a P1000 pipette tip 10 times at 60 and 90 minutes. After the second trituration, the cell suspension was carefully layered on top of 15% bovine serum albumin (Melford, United Kingdom), dissolved in DMEM/F12 containing penicillin/streptomycin, and centrifuged at 800g for 10 minutes with the minimum deceleration speed. The cell pellet was then washed in a culture medium composed of DMEM/F12 containing 10% fetal bovine serum and penicillin/streptomycin (all from Gibco). Cells were pelleted again and then resuspended in 60-µL culture medium. A 3-µL "drop" was placed on the centre of a glass coverslip coated with D-polyornithine (20 µg/mL, Sigma). Coverslips were flooded with a 1 mL of culture medium after 15 to 30 minutes. Cells were imaged 24 to 48 hours after plating. Dorsal root ganglia from 1.8-DTA, $Na_v1.7$KO, and $Na_v1.8$KO were placed in ice-cold Hibernate-A medium (Gibco) containing penicillin/streptomycin while being transported from UCL (about 5 hours). Dorsal root ganglia were then dissociated as above.

2.2. Calcium imaging

All recordings were performed at room temperature (22-25°C). Dorsal root ganglia neurons were loaded with 2 µM Fura-2AM (ThermoFisher, United Kingdom) in standard Ringer solution (140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, and pH = 7.4 with NaOH) for 30 minutes at 37°C. Coverslips were then washed with ringer solution and left for 15 minutes at 37°C/5% CO_2 after which the Ringer was replaced. Coverslips were stored in a dark container at room temperature until imaged (within 2 hours). Cells were perfused at a flow rate of 3 mL/minute with Ringer solution for at least 5 minutes to establish a stable calcium baseline. Ringer with 40 mM KCl was perfused at

the end of recordings to identify viable neurons. Cells were imaged with a 40× objective and a Hamamatsu C4742-95 camera. Cells were excited with 350 and 380 nm for ratiometric measurement of intracellular calcium using Cairn Dual OptoLED system. *Simple PCI 6* software was used for data acquisition, background subtraction, and Fura-2AM ratiometric measurement (F350/380 nm).

All drugs were made in standard Ringer solution from stock solutions of the following concentrations: veratridine (5 mM in ethanol, Abcam ab120279), capsaicin (10 mM in ethanol, Tocris 0462), α, β-methylene ATP (10 mM in water, Sigma M6517), allyl isothiocyanate (AITC; 100 µM, Sigma 377430), 4,9-anhydrotetrodotoxin (300 uM in water, Tocris 6159), PF-04856264 (10 mM in DMSO, Sigma 11916), and A-803467 (10 mM in DMSO, Abcam Ab120282).

2.3. Data and statistical analysis

Neurons were included in the analysis if they respond to 40 mM KCl. On rare occasions, neurons responded to veratridine but not KCl (or the KCl response was not clear due to the calcium signal not returning to baseline after the application of last agonist). We defined a response as an increase in (F350/380) ratio of >6 SD above the baseline. Differences in fluorescence ($\Delta F/F_0$) were calculated according to the following formula: F350/380 ratio during agonist application (F) minus the average F350/380 ratio of the 2.5 minutes before agonist application (F_0). Mean values from each experiment (each N is a culture from one mouse) were compared with each other by one-way analysis of variance (ANOVA) with Sidaks' post-test. All statistical analysis and comparisons were performed by *GraphPad Prism* software (version 7.00 for Windows).

3. Results

3.1. The oscillatory response population is highly diminished in the $Na_v1.8$-DTA (nociceptor-ablated) model

To confirm the link between neurons with the OS profile and pain behaviour, we examined DRG from the $Na_v1.8$Cre-DTA (1.8-DTA) mouse.[1] The $Na_v1.8$ channel is expressed in 80% to 90% of

nociceptors.[5,21] In the 1.8-DTA mouse, Na$_v$1.8-expressing neurons are ablated (**Fig. 2A**) leading to the loss of most nociceptors and a profound loss of pain.[1] Therefore, we hypothesised that the veratridine-response profile that pertains to nociceptors (ie, OS) would be reduced in the 1.8-DTA DRG. First, we determined the percentage of remaining nociceptors in 1.8-DTA DRG using the 3 nociceptive agonists (capsaicin, αβ-methyleneadenosine triphosphate, and isothiocyanate) to identify all subtypes of nociceptors as described previously.[10] The percentage of neurons responding to any of the nociceptive agonists was drastically reduced from 75% in controls to just 8% in 1.8-DTA (**Fig. 2B**). This shows that most neurons in 1.8-DTA DRG are non-nociceptors with very few nociceptors left.

The ablation of most nociceptors caused opposing changes in the percentages of the veratridine OS and SD populations. The OS population decreased from 34% in controls to 7% (**Fig. 2C**). By contrast, the SD population increased from 17% in controls to 66%. This confirms that the OS population represents most nociceptors, whereas the SD population represents most non-nociceptors. Of note, the total number of veratridine-irresponsive neurons decreased

from 39% in control to 23% in 1.8-DTA (**Fig. 2C**). The veratridine-irresponsive population can be either nociceptors (ie, respond to the nociceptive agonists but not veratridine, yellow section in **Fig. 2D**) or non-nociceptors (ie, respond to neither the nociceptive agonists nor veratridine, orange section in **Fig. 2D**). The observed decrease in veratridine-irresponsive neurons was in fact due to the loss of almost all veratridine-irresponsive nociceptors, a population that accounted for 26% of all neurons in controls but only 0.8% in 1.8-DTA.

Results from the 1.8-DTA mouse link the reduction of the OS population in vitro to the previously described[1] pain deficits in vivo. Therefore, drugs can be screened based on their ability to reduce the OS population (an indication of potency on nociceptors) without an effect on the SD population (an indication of unwanted effects on non-nociceptors), **Figure 1**.

3.2. A reference veratridine-response pattern for a potent and safe analgesic

Next, we established the reference criterion by which drug potency and selectivity on nociceptors can be quickly ascertained
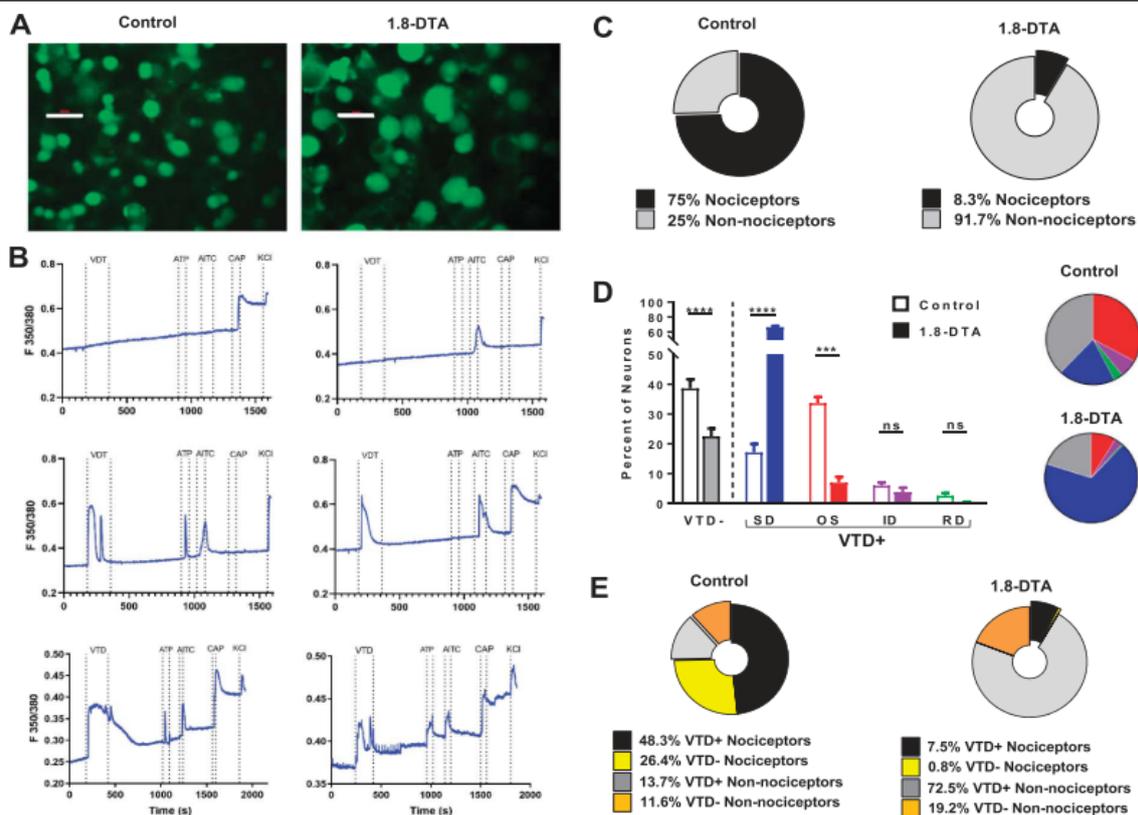
Figure 2. The OS population is reduced in mice lacking most nociceptors. (A) Representative images from control and 1.8-DTA cultures loaded with fura-2AM for imaging (contrast enhanced for both). Ablation of Na$_v$1.8-expressing neurons in 1.8-DTA mouse leaves behind mostly non-nociceptive large neurons. Scale bar is 50 μm. (B) Example traces from our imaging protocol. Dashed lines indicate the periods of agonist application in recordings typically 25 to 35 minutes long. The 4 agonists and KCL are applied in the same order for all coverslips. The first row shows examples of neurons irresponsive to veratridine but respond to capsaicin and AITC, the second row shows neurons responding to veratridine and 2 nociceptive agonists, whereas the third row shows neurons responding to all 4 agonists: veratridine (VTD), capsaicin (CAP), α, β-methylene ATP (ATP), and allyl isothiocyanate (AITC). (C) In control DRG, 75% of neurons respond to one or more of the 3 nociceptive compounds and are classified as nociceptors (top) while only 8.3% do so in the 1.8-DTA confirming the loss of 89% of nociceptors. (D) Ablation of Na$_v$1.8-expressing neurons decreases the percentage of veratridine-irresponsive neurons, decreases the percentage of OS neurons, and increases the percentage of SD neurons. VTD− CTR = 38.6 ± 3.1 vs VTD − DTA = 22.5 ± 2.6; SDCTR = 17.11 ± 2.8 vs SDDTA = 66.4 ± 1.4; OSCTR = 33.7 ± 2.1 vs OSDTA = 6.9 ± 1.9; IDCTR = 5.9 ± 1.1 vs IDDTA = 3.8 ± 1.5; RDCTR = 2.6 ± 0.9 vs RDDTA = 0.3 ± 0.2%. One-way ANOVA with Sidak's test. Pie charts represent mean percentages in the histogram. (E) In control DRG, veratridine-irresponsive neurons can be nociceptors (yellow section, 26.4% of all neurons) or non-nociceptors (orange section, 11.6% of all neurons). Veratridine-irresponsive or "silent" nociceptors are almost completely lost in 1.8-DTA (become 0.8% of all neurons). Overall, there are less veratridine-irresponsive neurons in 1.8-DTA. Data for C–E are from 940 neurons from 3 control mice and 360 neurons from 4 1.8-DTA. ANOVA, analysis of variance; DRG, dorsal root ganglia.

in our assay. We base this criterion on the change to the percentages of the OS and SD populations in the $Na_v1.7$ knockout mouse where pain behaviour is lost due to a decrease in the excitability of nociceptors (as opposed to their ablation as in 1.8-DTA). $Na_v1.7$ is a VGSC that is highly expressed in nociceptors and is a critical determinant of their excitability. Deletion of $Na_v1.7$ in DRG neurons leads to profound loss of pain without adverse motor or central nervous system (CNS) effects.[14,17] Therefore, the changes in the OS and SD populations in the $Na_v1.7$ knockout represents the effects a potent and safe analgesic would have. We used the advillin-1.7 KO mouse[14] ($Na_v1.7$KO thereafter), where $Na_v1.7$ is deleted in all DRG neurons. We hypothesized that $Na_v1.7$ deletion will cause a major decrease in veratridine-responsive neurons through a decrease in the OS but not the SD population. In the $Na_v1.7$KO, the percentage of veratridine-irresponsive neurons increased from 51% in controls (floxed $Na_v1.7$) to 80% (**Fig. 3A**). This decrease in the responsiveness to veratridine was due to the reduction of the OS population, which decreased from 28% to 7% (0.25 of control). Deletion of $Na_v1.7$ rendered 3 quarters of the OS population veratridine-irresponsive. Of note, there was no significant decrease in the SD population. Therefore, drugs can be screened by our assay based on the similarity of their effect on DRG neurons to $Na_v1.7$ deletion.

To validate the use of the pattern of veratridine responses in $Na_v1.7$KO as the criterion for a "good" analgesic, we compared it with the pattern of a would-be "bad" analgesic, a drug affecting non-nociceptors and CNS neurons. For this purpose, we used a $Na_v1.6$ channel blocker. Although conditional $Na_v1.6$ knockout suggests it

contributes to pain,[3] and it is not a potential analgesic target because of its role in CNS and motor neurons.[12,18] In DRG, $Na_v1.6$ contributes more to sodium current in large (non-nociceptive) than in small (nociceptive) neurons.[3] Therefore, we hypothesised that a $Na_v1.6$ blocker will reduce the OS and SD populations, but the effect on the SD population will be greater. Low concentrations of the tetrodotoxin metabolite, 4,9-anhydro-TTX (4,9TTX), preferentially blocks $Na_v1.6$ (IC_{50} 120 times lower than $Na_v1.7$).[20] As expected, 300 nM 4,9TTX greatly increased the percentage of veratridine-irresponsive neurons from 42% to 68% (**Fig. 3B**). Unlike $Na_v1.7$ deletion, 4,9TTX reduced both the SD and OS populations, but the reduction in the SD population (0.48 of control) was greater than the reduction in the OS population (0.62 of control). Therefore, drugs that reduce the SD population may act on non-nociceptive neurons with potential unwanted physiological effects.

To test whether our assay has the potential to detect increases in neuronal excitability (ie, as a readout for changes that lead to hyperexcitability, **Fig. 1**), we examined veratridine responses in the $Na_v1.8$ knockout mouse ($Na_v1.8$KO). $Na_v1.8$ is also an important determinant of the excitability of nociceptors. The global $Na_v1.8$KO mouse, however, showed a compensatory increase in $Na_v1.7$ expression.[2] This is the most likely reason why the $Na_v1.8$KO mouse does not show a major loss of pain. Because $Na_v1.8$ is expressed in nociceptors, then it is logical to assume that the compensatory increase in $Na_v1.7$ will occur in nociceptors. Therefore, we hypothesised that this compensatory increase in $Na_v1.7$ will primarily affect the OS population. In
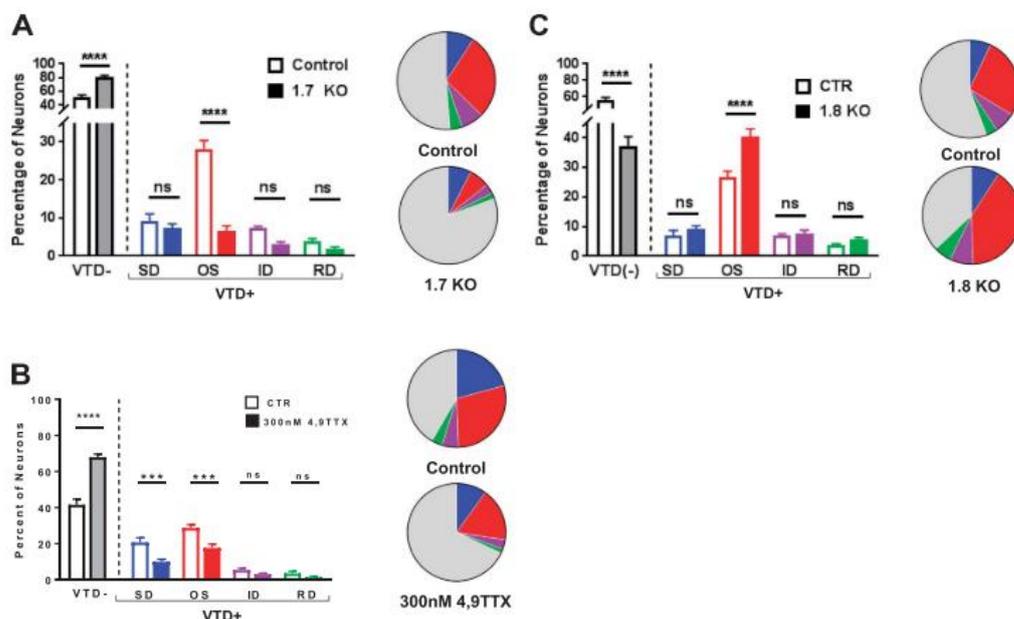
Figure 3. Reference veratridine-response patterns for "safe" and "unsafe" analgesic drugs. (A) Deletion of $Na_v1.7$ causes a major loss of pain without adverse CNS or motor effects. The veratridine-response pattern of the $Na_v1.7$KO represents that of a safe and potent analgesic. $Na_v1.7$ deletion leads to a decrease in responsiveness to veratridine (increase VTD− population) due to a decrease in the OS population but not the SD population. VTD− CTR = 51.0 ± 3.51 vs VTD− KO = 80.4 ± 2.8; SDCTR = 9.1 ± 1.9 vs SDKO = 7.5 ± 1; OSCTR = 28.2 ± 2.2 vs OSKO = 6.7 ± 1.3; IDCTR = 7.4 ± 0.4 vs IDKO = 3.2 ± 0.6; RDCTR = 4.0 ± 0.6 vs RDKO = 1.9 ± 0.5%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 1448 neurons from 6 floxed-control mice and 1630 neurons from 6 $Na_v1.7$KO. (B) 300 nM of the $Na_v1.6$ blocker 4,9TTX reduces responsiveness to veratridine (increases VTD−) through decreases in both the SD and OS populations. The decrease in SD is greater than that in OS. VTD− CTR = 41.6 ± 3.1 vs VTD− 4,9TTX = 68.0 ± 1.7; SDCTR = 20.68 ± 2.6 vs SD4,9TTX = 10.0 ± 1.5; OSCTR = 28.7 ± 1.8 vs OS4,9TTX = 17.6 ± 2.2; IDCTR = 5.4 ± 0.9 vs ID4,9TTX = 3.1 ± 0.5; RDCTR = 3.5 ± 1.1 vs RD4,9TTX = 1.4 ± 0.3%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 635 untreated and 927 treated neurons from 6 C57Bl6 mice. (C) The compensatory increase in $Na_v1.7$ channels in nociceptors of the $Na_v1.8$KO increases responsiveness to veratridine (decreases VTD−) due to an increase in the OS population. VTD− CTR = 55.4 ± 3 vs VTD− KO = 37.1 ± 3.2; SDCTR = 6.9 ± 1.7 vs SDKO = 9.1 ± 1.1, OSCTR = 26.7 ± 2.0 vs OSKO = 40.3 ± 2.7, IDCTR = 7.0 ± 0.5 vs IDKO = 7.65 ± 1.1, RDCTR = 3.6 ± 0.6 vs RDKO = 5.7 ± 0.5%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 1493 neurons from 5 littermate-control mice and 1028 neurons from 4 $Na_v1.8$KO. ANOVA, analysis of variance.

Na$_v$1.8KO, the percentage of veratridine-irresponsive neurons decreased from 55% in littermate controls to 37% in Na$_v$1.8KO (**Fig. 3B**). This increase in veratridine responsiveness came from the increase in the OS population from 27% in controls to 40%. The changes in the other populations were small and highly insignificant. In other words, the compensatory increase in Na$_v$1.7 expression caused previously silent neurons to respond to veratridine, and that these additional neurons had the OS profile, the profile of nociceptors. These results show that our assay is sensitive to increases in Na$_v$1.7 and thus can be used to detect pathological changes in Na$_v$1.7 function (eg, gain of function mutations or painful conditions).

Collectively, our results confirm that most nociceptors respond to veratridine with the OS profile, whereas most non-nociceptors respond to veratridine with the SD profile. Only the OS population is reduced when nociceptors are ablated (1.8-DTA) or lose a critical excitability determinant (Na$_v$1.7KO). Importantly, the veratridine-response pattern of the Na$_v$1.7KO can be used as the reference for potent and safe analgesic action, a reference pattern that potential analgesics can be evaluated against before-detailed in vitro characterisation or testing.

3.3. Evaluating the potency and selectivity of subtype-specific voltage-gated sodium channel blockers using the Na$_v$1.7KO as reference

Several blockers of Na$_v$1.7 and Na$_v$1.8 channels have been developed and are currently in clinical trials.[9,25] We used our assay to evaluate a candidate analgesic drug for each. PF-04856264 (PF-048) is from the arylsulfonamide class of selective Na$_v$1.7 blockers.[11] PF-048 reduced responses to veratridine at concentrations similar to or lower than those used in standard cell line–based FLIPR assays[4,11] and shows that our assay can identify nonpore blockers such as arylsulfonamides. PF-048 at 1 μM increased the percentage of veratridine-irresponsive neurons (**Fig. 4A**) from 46% to 58% through a decrease in the OS population, which decreased from 26% to 17% (ie, 0.65 of control). PF-048 at 5 μM (**Fig. 4B**) had a greater effect, increasing veratridine-irresponsive neurons from 44% to 72% through a larger decrease in the OS population from 28% to 12% (0.43 of control). However, 5 μM PF-048 decreased the SD population from 13% to 6%. Because a reduction in the SD population was not observed in Na$_v$1.7KO, this suggests that PF-048 will affect non-nociceptors at a dose that does not even reduce the OS population to the Na$_v$1.7KO level of 0.25 of control.

The Na$_v$1.8 blocker A-803467 (A-80) decreases the excitability of DRG neurons.[8] At 0.1 μM, it reduced the OS population from 25% to 12% (**Fig. 4C**) but without an overall increase in veratridine-irresponsive neurons (51% vs 55%). A-80 at 0.1 μM did not silence the OS population but rather prevented its multipeak oscillatory behaviour, converting them to the ID and RD profiles that respond to veratridine with a single peak (**Fig. 1**). A-80 at 0.3 μM increased veratridine-irresponsive neurons from 41% to 66% (**Fig. 4D**), through significant decreases in the OS population (from 28% to 12%) and the SD population (from 19% to 12%). These data suggest that A-80 is more potent in reducing the excitability of the OS population than PF-048, but it also affects the SD population at the higher concentration.

3.4. A combination of low concentrations of PF-04856264 and A-803467 have an additive effect on nociceptors

We suggest that our assay can be used not only to screen for novel drugs, but also to identify effective combinations of existing

ones. As a proof of concept, we used a combination of 1 μM PF-048 and 0.1 μM A-80 (**Fig. 5A**). When applied separately, neither reduced the SD population significantly, but their reduction of the OS population is considerably less than Na$_v$1.7 deletion. The combination reduced the OS population from 30% to 11%, greater than either alone. Importantly, the combination still did not significantly reduce the SD population. A direct comparison of the decreases in the OS and SD populations between the VGSC blockers used and Na$_v$1.7 deletion (**Fig. 5B**) clearly shows that the combination produced the closest effect on the OS population (reduction to 0.38 of control) to that observed in Na$_v$1.7KO (0.25 of control).

4. Discussion

Cell lines provide a high-throughput platform for analgesic drug discovery. However, their use leaves a knowledge gap that must be addressed on DRG neurons using lower throughput methods such as calcium imaging and patch clamping. The knowledge gap includes information on the effect of a drug on nociceptors as a whole (rather than just the molecular target of interest) and the concentrations needed for a potent effect on nociceptors with a minimal effect on non-nociceptors (as a proxy for unwanted side effects on CNS and motor neurons). We discovered that nociceptors and non-nociceptors respond to veratridine with distinct response profiles.[16] Here, we show that a veratridine-based assay allows for a simultaneous assessment of drugs' action on both populations and therefore provides an efficient and more informative assay for screening for analgesics on sensory neurons. First, we discuss the results from our findings and then discuss the key advantages of our assay.

We used the 1.8-DTA mouse that has well characterised pain deficits to demonstrate the link between the loss of the OS population and loss of pain.[1] In 1.8-DTA neurons, we recorded a loss of 89% of functionally defined nociceptors (**Fig. 2B**), which is in agreement with the expression of Na$_v$1.8 in 80% to 90% of nociceptors.[5,21] Our identification of nociceptors is based on responsiveness to one or more of 3 nociceptive agonists.[16] This definition will encompass 6 of the putative 11 molecular subtypes (the NP1-3, PEP1-2, and TH types) of sensory neurons as defined in one single-cell RNA-sequencing study.[23] By our functional definition, non-nociceptors will equate to the 5 neurofilament-positive subtypes (NF1-5).[23] Ablation of nociceptors leads to the loss of 78% of the OS population, confirming that most nociceptors respond to veratridine with the OS profile. In the Na$_v$1.7KO mouse, nociceptors are not ablated, but their excitability is greatly reduced to the extent that the pain loss in the Na$_v$1.7KO is very similar to that of the 1.8-DTA.[1,14] Not surprisingly, deletion of Na$_v$1.7 "silenced" 75% of the OS population (**Fig. 3A**), an almost identical percentage to that lost in 1.8-DTA.

Given that Na$_v$1.7 is expressed in all DRG neurons, why was there no significant reduction in the SD population in Na$_v$1.7KO? Veratridine primarily activates TTX-sensitive VGSCs, and indeed, we previously showed that TTX silences all OS and SD neurons.[16] However, although Na$_v$1.7 is the main TTX-sensitive channel in nociceptors, non-nociceptors rely on other TTX-sensitive subtypes, namely Na$_v$1.1, Na$_v$1.2, and Na$_v$1.6. In the absence of Na$_v$1.7, these subtypes allow veratridine to activate non-nociceptors. The predominance of other TTX-sensitive subtypes in non-nociceptors explains why deletion of Na$_v$1.7 in all DRG neurons has no effect on touch or proprioception.[14] This is also why 4,9TTX, which preferentially blocks Na$_v$1.6 channels, affected the SD population more than the OS population (**Fig. 3B**).
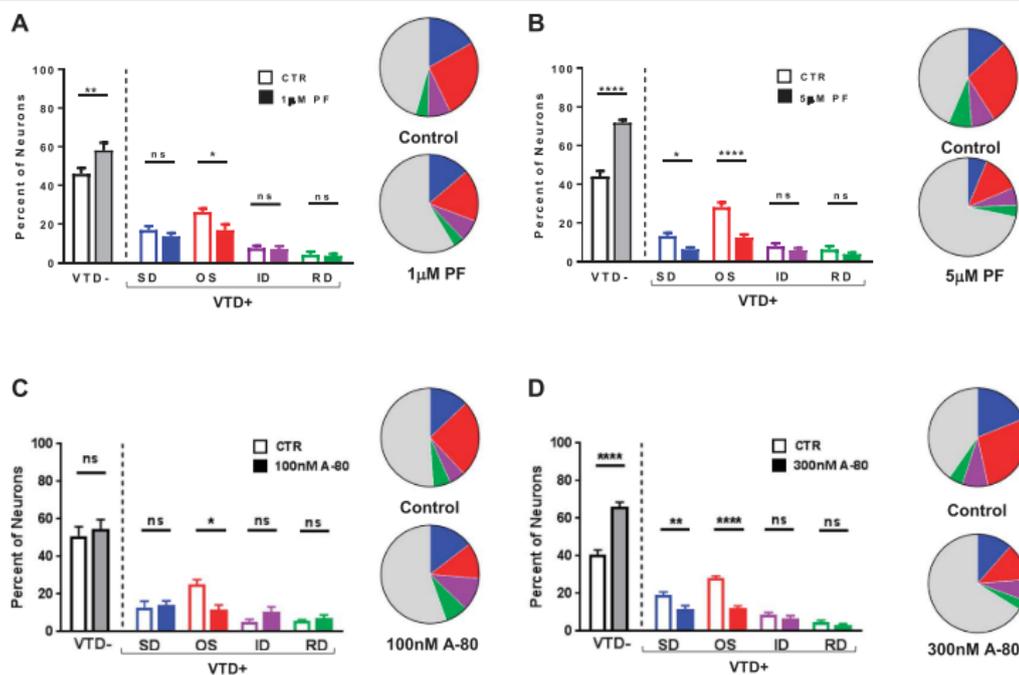
Figure 4. Evaluating the effect of PF-04856264 and A-803467 on nociceptors and non-nociceptors. (A) 1 μM PF-048 increases the percentage of veratridine-irresponsive neurons through a reduction of the OS but not the other 3 populations. VTD− CTR = 45.9 ± 3 vs VTD− 1 PF = 58.1 ± 4; SDCTR = 16.9 ± 2 vs SD1PF = 13.6 ± 1.7; OSCTR = 26.2 ± 1.7 vs OS1PF = 16.8 ± 3; IDCTR = 7.5 ± 1.3 vs ID1PF = 7.0 ± 1.4; RDCTR = 4.1 ± 1.6 vs RD1PF = 3.6 ± 0.9%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 813 untreated and 681 treated neurons from 7 C57Bl6 mice. (B) 5 μM PF-048 increases the percentage of veratridine-irresponsive neurons through a reduction of both the OS and SD populations. 5 μM PF-048 is equally potent on the SD and OS profiles reducing both to about 50% of control values. VTD− CTR = 44.1 ± 2.7 vs VTD− 5 PF = 71.8 ± 1.5; SDCTR = 13.2 ± 1.6 vs SD1PF = 6.3 ± 1.0; OSCTR = 28.1 ± 2.4 vs OS5PF = 12.4 ± 1.6; IDCTR = 7.9 ± 1.6 vs ID5PF = 5.9 ± 1.1; RDCTR = 6.2 ± 1.8 vs RDPF = 3.8 ± 0.7%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 605 untreated and 338 treated neurons from 5 C57Bl6 mice. (C) 100 nM A-80 does not change the percentage of veratridine-responsive neurons but reduces the OS population by about 50%. VTD− CTR = 51 ± 4.9 vs VTD− 100A80 = 54.8 ± 4.9; SDCTR = 12.9 ± 3.2 vs SD100A80 = 14.4 ± 2; OSCTR = 25.1 ± 2.6 vs OS100A80 = 11.61 ± 2.6; IDCTR = 5.2 ± 1.3 vs ID100A80 = 10.7 ± 2.5; RDCTR = 6.6 ± 0.8 vs RD100A80 = 7.3 ± 1.6%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 607 untreated and 594 treated neurons from 6 C57Bl6 mice. (D) 300 nM A-80 increases the percentage of veratridine-irresponsive neurons through a reduction of both the OS and SD populations. The reduction in the OS population is slightly greater than that in the SD profile. VTD− CTR = 40.8 ± 2.3 vs VTD− 300A80 = 66.2 ± 2.4; SDCTR = 19.1 ± 1.7 vs SD300A80 = 11.5 ± 2; OSCTR = 28.0 ± 1.3 vs OS300A80 = 12.2 ± 1.2; IDCTR = 8.5 ± 1.5 vs ID300A80 = 6.8 ± 1.5; RDCTR = 4.8 ± 0.9 vs RD300A80 = 3.3 ± 0.7%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 1197 untreated and 1294 treated neurons from 7 C57Bl6 mice. ANOVA, analysis of variance.

We propose to use veratridine responses of the Na$_v$1.7KO as the criterion VGSC blockers need to match for in vivo potency and safety. The pattern of veratridine responses we observed in vitro is due to the deletion of Na$_v$1.7 and are unlikely to be influenced by the reported increase in endogenous opioid agonism in the spinal cord.[15] This is because veratridine responses are recorded from disassociated neurons after 1 day in culture (that lacks opioid-releasing spinal cord cells) and under constant Ringer flow. Our assay detected a significant increase in the number of neurons responding to veratridine in Na$_v$1.8KO, and these "unsilenced" neurons have the OS profile, demonstrating for the first time the effect of the compensatory increase in Na$_v$1.7 expression.[2] All of the above strongly confirms the link between nociceptors' excitability and the OS population and suggests that a reduction in the OS but not the SD population correlates with a reduction in pain without motor/CNS effects.

Our assay has several advantages over cell line-based assays and patch clamping. First, a cell line-based assay examines the effect of a condition or a drug on one target at a time, whereas our assay evaluates the overall effect on DRG neurons, including non-nociceptors. For example, while A-80 was potent in reducing excitability, we noted clear cytotoxic effects at

concentrations >0.5 μM (not shown). The use of veratridine as a single "pan" activator of all sensory neurons has advantages over the commonly used potassium chloride (KCl). Dorsal root ganglion neurons respond to veratridine with distinct profiles that can be used to identify the main populations, whereas KCl produces a similar profile in all neurons. In addition, while 80% of Na$_v$1.7KO neurons did not respond to veratridine, they were all KCl-positive (Fig. 3A), strongly suggesting that KCl responsiveness is a poor predictor of the excitability of DRG in in vitro assays.

Second, our assay allows for changes in the population of high-threshold or "silent" sensory neurons that are observed in vivo,[13] to be monitored in vitro. This is possible due to the way veratridine activates sensory neurons. Veratridine acts on open VGSC at the resting membrane potential to prevent their inactivation. Neurons with low expression of threshold VGSC channels (eg, Na$_v$1.7 in DRG) or/and have a hyperpolarised membrane potential will have few open VGSC channels at resting potentials and will not respond to veratridine. We observed that between 30% and 50% of cultured DRG neurons do not respond to 3 minutes of 30 μM veratridine. Interestingly, these are similar to the percentages (38%-48%)
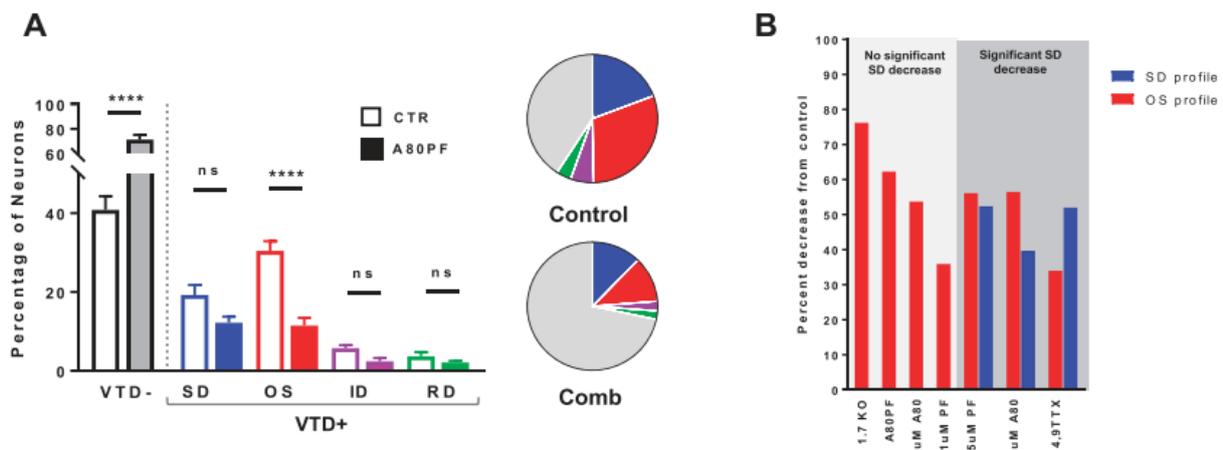
Figure 5. The additive effects of a combination of PF-04856264 and A-803467 on nociceptors. (A) A combination of 1 μM PF-048 and 100 nM A-80 increases the percentage of veratridine-irresponsive neurons through a decrease of the OS population only. VTD− CTR = 40.8 ± 3.5 vs VTD− A80PF = 71.5 ± 3.7; SDCTR = 19.2 ± 2.5 vs SDA80PF = 12.2 ± 1.5; OSCTR = 30.4 ± 2.4 vs OSA80PF = 11.5 ± 2; IDCTR = 5.7 ± 0.8 vs IDA80PF = 2.4 ± 0.8; RDCTR = 3.7 ± 0.4 vs RDA80PF = 2.1 ± 1.1%. One-way ANOVA with Sidak's test. Pie charts represent mean values in the histogram. Data from 980 untreated and 1114 treated neurons from 7 C57Bl6 mice. (B) Comparison of the changes in the OS and SD populations caused by VGSC blockers to those of the Na$_v$1.7KO. The higher doses of PF and A80 and 4,9TTX caused a significant reduction in the SD population. Notice that the combined action of the lower doses of the PF and A80 produced the closest reduction of the OS population to Na$_v$1.7 deletion. ANOVA, analysis of variance; VGSC, voltage-gated sodium channel.

of high-threshold "silent" DRG neurons that did not respond to 10- to 100-mA current injection in vivo but were unmasked by inflammation.[22] Because two-thirds of veratridine-irresponsive neurons responded to nociceptive markers (yellow segment in **Fig. 2D**), we speculate that the veratridine-irresponsive population includes the high-threshold silent nociceptors observed in vivo. However, we have no data to support this hypothesis at present.

Third, our assay allows the range of plasma concentrations for a potent action on nociceptors without an effect on non-nociceptors to be determined efficiently. Both PF-048 and A-80 reduced the OS population with A-80 being 10 times more potent than PF-048. However, at the higher concentration, both reduced the SD population, an effect not observed in either Na$_v$1.7 or Na$_v$1.8 KOs but obtained from the Na$_v$1.6 blocker 4,9TTX. The concentrations at which PF-048 and A-80 affected the SD population were not much higher than those at which they did not (5 fold for PF-048 and 3 fold for A-80). The reduction of the SD population by 5 μM PF-048 is larger than that by 0.3 μM A-80 (**Fig. 4**). This can be explained by the greater sequence homology between the VGSC subtypes expressed in non-nociceptors to Na$_v$1.7 than to Na$_v$1.8.

Finally, our assay can be used to screen for drug combinations to obtain the most potent effect on the OS but the least effect on the SD populations. This strategy may become a necessity considering that developing potent-selective Na$_v$1.7 blockers has been a challenging task[9]; although Na$_v$1.7 has been a validated target since 2004.[17] A combination strategy will be necessary when selective blockers against a single target do not produce the desired system effect. Because the pain phenotype seen in the mouse and human loss-of-function mutants[7,14,17] results from 100% loss of Na$_v$1.7 channels, it remains to be determined what level of pharmacological block of Na$_v$1.7 is needed in vivo for reduced pain and if selective Na$_v$1.7 blockers can achieve this level of inhibition. Furthermore, the pain phenotype seen in the mouse and human loss-of-

function mutants seems to involve changes in other systems (ie, opioid signalling in the spinal cord[15]). In these situations, a selective Na$_v$1.7 blocker may not be potent enough, and combination of drugs against different targets may produce the required potency on nociceptors. As a proof of concept, we demonstrate that combining low concentrations of PF-048 and A-80 produced the closest veratridine-response pattern to that of Na$_v$1.7KO, the reference genetic model (**Fig. 5B**). Efficient in vitro screening of drugs combinations against multiple targets is not possible using cell line–based expression systems. This is because a cell line has to be engineered to express multiple targets at levels similar to their native levels in DRG neurons.

Future work will focus on adapting the assay to high-throughput platforms by automating data analysis (including calculation of response onset, amplitude, and area under the curve) and making use of a nonwash protocol.[22] The potential of this assay to screen for drugs for other classes of ion channels (calcium and potassium) needs to be established.

For drug screening, the assay is most useful when the primary screening criterion is the decrease in the OS population where the screening window is large, a 75% drop in the 1.7KO and 62% in the A80PF drug combination (**Fig. 5**). Although a decrease in the total number of veratridine-responsive neurons can be used as the screening criterion, the screening window is narrower (A decrease of 38% in the 1.7KO). In all cases, the screening criterion should include a requirement for minimal effect on the SD population.

In summary, we described an assay to assess the excitability of nociceptors and non-nociceptors. The assay can be used in other applications (**Fig. 6**) in addition to drug development and screening. It can be used for in-depth, long-term characterisation of excitability changes in knockout mice and models of pain pathologies (diabetes, cancer, and aging). It can be used to optimise protocols to differentiate human stem cells into the different classes of sensory neurons.[24] Meanwhile, all this may have the added advantage of reducing the number of animals needed for in vitro and in vivo testing.

CHAPTER 3: VALIDATION OF THE DISTINCT VERATRIDINE CALCIUM RESPONSE PROFILES AS FUNCTIONAL
MARKERS FOR NOCICEPTORS AND NON-NOCICEPTORS

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Unbiased, medium-/high-throughput, and high content assessment of excitability of DRG neurons
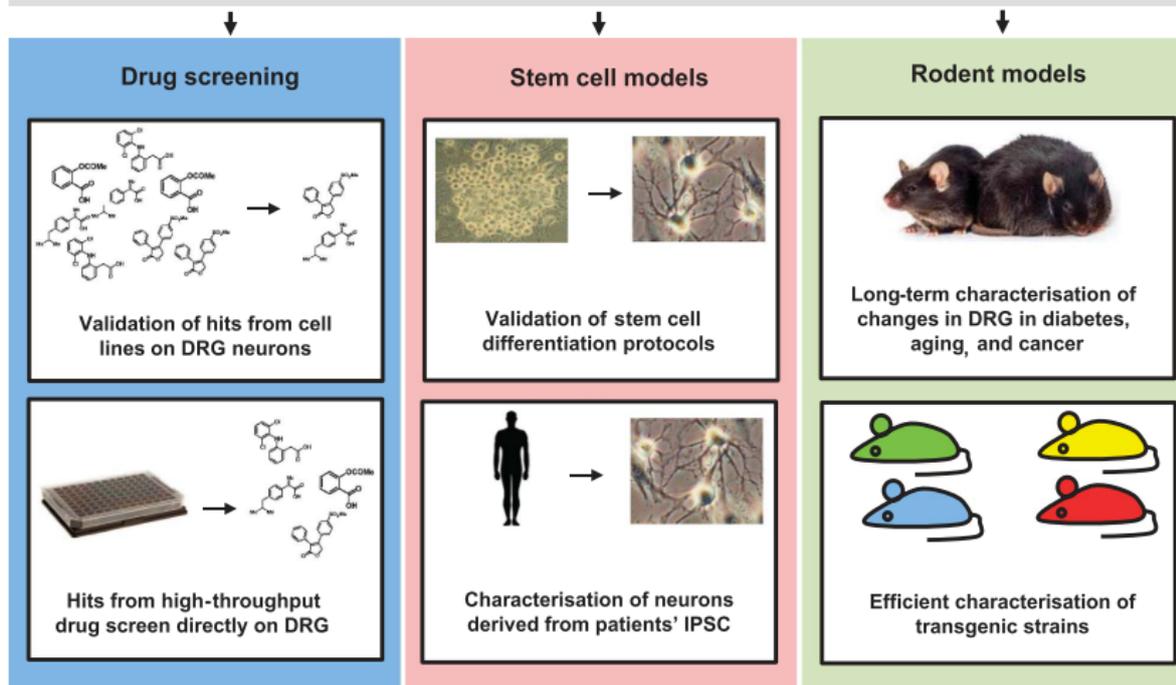
Figure 6. Applications of the veratridine-based calcium assay. The assay is a very efficient method to characterise changes in a heterogeneous population of neurons and therefore has several applications. The assay can be used to identify lead analgesic drugs either by validating hits from cell line–based screens on all types of DRG neurons or identification of hits by a direct screen on DRG neurons. The assay can be used to assess how stem cell–derived neurons compare to primary neurons of the same type more efficiently than by patch clamping. The assay can be used to compare neurons derived from patients' IPSC with known or unknown genetic mutations. The assay is suited to characterise pathologies that develop over time as in diabetes, aging, or cancer. Finally, the assay can be used to efficiently characterise changes in DRG from the large number of transgenic strains generated by phenotyping consortia. DRG, dorsal root ganglia.

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An unbiased and efficient assessment of excitability of sensory neurons for analgesic drug discovery

Zainab A. Mohammed, Katerina K. Kaloyanova, Mohammed A. Nassar*

Department of Biomedical Science, University of Sheffield, S10 2TN, UK

*Corresponding author m.nassar@sheffield.ac.uk

CHAPTER 4

ASSESSING EXCITABILITY CHANGES IN DRG NEURONS UNDER IN VITRO HYPERGLYCEMIC CONDITIONS

CHAPTER 4: ASSESSING EXCITABILITY CHANGES IN DRG NEURONS UNDER IN VITRO HYPERGLYCEMIC CONDITIONS

4.1. INTRODUCTION

The first step in answering this thesis's research question: "What are the excitability changes in DRG neurons during DN?", was to validate the use of the VTD-profiles as a functional marker for nociceptors and non-nociceptors in a heterogeneous neuronal population. In the previous chapter, VTD was shown to allow for the study of changes in VGSCs and place them in the context of specific neuronal subpopulations. Hence, the VTD-Ca^{2+} imaging assay was established as a suitable tool for the discrimination of neuronal subpopulations. As a result of its validation, several potential applications arose.

For the experiments in this and the next chapter, I apply the VTD-Ca^{2+} imaging assay together with selected nociceptive agonists to characterise diabetes-induced excitability changes in DRG neurons exposed to hyperglycaemic conditions. Determining the distinct excitability changes occurring in nociceptors and non-nociceptors during DN would provide valuable insights into the pathophysiological mechanisms driving DN in sensory neurons. To this end, in the current chapter, I used the *in vitro*-induced hyperglycaemia model.

In the literature, *in vitro*-induced hyperglycaemia has been mostly used to investigate the effects of acute exposure to high glucose on DRG neurons. Such studies have contributed vastly to DN research by demonstrating decreased cell viability [318,423,500,501], ROS rise [423,424,500], cell injury [423], increased Nav1.7 expression [351] and increased VGSC currents [424,502] in DRG when exposed to high glucose concentrations for up to 48h. However, to our knowledge, there are no studies exploring the effects of extended high glucose exposure time past the 48h mark on neuronal excitability neither are there studies sorting observed excitability changes to neuronal subtypes. Since VGSC currents and expression have been observed to increase in DRG neurons as early as 18h of acute high glucose exposure [351,424], we hypothesised that extending the exposure time will lead to further VGSC activity increase and neuronal excitability changes. Prolonged high glucose exposure of DRG neurons should more closely mimic the chronic hyperglycaemic conditions in humans and reflect physiological DN progression. Furthermore, apart from the immediate post-translational changes induced by hyperglycaemic conditions, transcriptional changes could also have the opportunity to take place. In time periods of 24h or less, such important transcriptional changes would be missed,

therefore, culturing DRG neurons in high glucose for an extended time period would allow for their detection.

4.2. AIMS

To assess the excitability changes in DRG neurons from C57BKS mice cultured in hyperglycaemic conditions for 1, 2, 4 and 5 days by analysing calcium responses induced by the VGSC agonist VTD and the nociceptive markers ATP, AITC and CAP.

4.3. METHOD

4.3.1. Glucose concentration and exposure time in *in vitro*-induced hyperglycaemia

Mouse DRG neurons were cultured with standard DMEM/F-12 (17.5 mM glucose) with 32.5 mM added glucose (total 50 mM) to induce hyperglycaemic, diabetic conditions. Control cultures were supplied with DMEM/F-12 medium containing its standard 17.5 mM glucose deemed optimal for neuronal survival [318] and added 32.5 mM mannitol as an osmolarity control to the high glucose medium. The hyperglycaemia-inducing glucose concentrations were selected on the basis of previous studies which have reported that *in vitro* glucose concentrations in the range from 30 to 45 mM lead to hyperglycaemic stress, oxidative stress, ROS elevation and cell injury in DRG neurons - all markers of DN (Table 4.1). Furthermore, 45 mM glucose treatment (1.8-fold above control) is similar to the ≥ 1.4-fold increase in blood glucose concentration in a person diagnosed as diabetic [503] and within the range of blood glucose concentration of diabetic mice [428–431,504] (e.g. *db/db* mice at 10 WoA have blood glucose concentration of ~ 32.7 ± 2.0 mM [504], which keeps increasing as diabetes progresses uncontrolled [428]). Therefore, a total concentration of 50 mM glucose for the present experiments is consistent with other diabetic animal models and human diabetes and should ensure a clear-cut hyperglycaemia manifestation.

Table 4.1. Studies that used varying concentrations and exposure times of glucose in in vitro DRG cultures and the main conclusions reached.

Team & Year	Type	Glucose concentration (mM)	Glucose exposure time (h)	Conclusions
Russell et al. 1999 [318]	Embryonic rat DRG	30 – 330 mM	24h- 48h	Observed concentration-dependent neuronal apoptosis and decrease in neurite outgrowth at glucose concentrations above 50 mM; optimal DRG survival and neurite growth require 25–30 mM basal glucose (optimal concentration).
Russell et al. 2002 [423]	Primary DRG neurons	45 mM	0- 24h	In vitro as in vivo, variations in administered glucose as small as 10 mM from basal value (25 mM) induce neuronal injury.
Singh et al. 2013 [349]	E4 Sprague-Dawley rat DRG	45 mM 60 mM	4h-24h	Short-term exposure of DRG neurons to high glucose concentrations enhance the VGSC activity and were attenuated via ROS-dependent mechanisms.
Leinninger et al. 2006 [501]	E15 Sprague-Dawley rat embryos	45 mM	3-6h	High glucose promotes convergence of Drp1-mediated mitochondrial fission with pro-apoptotic proteins, resulting in mitochondrial injury and apoptosis.
Vincent et al. 2005 [500]	E15 Sprague-Dawley rats	45 mM	0-24h	There were nearly identical levels of DRG neuron death at the endpoint with a 2h glucose exposure compared with prolonged hyperglycaemia for the whole 6 or 24h time course of the experiment. Programmed cell death starts as soon as 2h.

After isolation and culturing, neurons were left to adjust for 24 hours before adding glucose or mannitol as opposed to introducing hyperglycaemia during the first cell flooding. This was to ensure that both groups had an equal opportunity to acclimatise to being in culture before the introduction of the potentially injurious effects of high glucose. After the addition of high glucose, culture was maintained for up to 5 days. Calcium imaging experiments took place at days 1, 2, 4 and 5 in high glucose. Therefore, total days in culture are equal to [days after adding glucose] + 1, e.g., day 1 of experiments is equal to 1 day in high glucose conditions and 2 days in culture overall (Figure 4.1).

* Experimental day 1 = 24h in glucose/mannitol and 48h in culture

Figure 4.1. A schematic of the experimental protocol used in the current experiments. After 24h of post-dissociation (Day 1) acclimatisation, DRG neurons were added either glucose or mannitol (Day 2). Calcium imaging experiments took place In days 3, 4, 6 and 7 in culture which correspond to days 1, 2, 4 and 5 in high glucose conditions, respectively.

4.3.2. Calcium imaging

DRG neurons were loaded with Fura-2 and responses recorded to nociceptive agonists: capsaicin (CAP, 200 nM), allyl isothiocyanate (AITC, 100 µM) and α,β-methylene ATP (ATP, 1 µM) as well as VGSC potentiator veratridine (VTD, 30 µM). Standard calcium imaging protocol was followed as outlined in Chapter 2: Materials & Methods, section 2.2.2., page 68 .

4.4. RESULTS

4.4.1. The number of available neurons for imaging experiments declines with time in culture

When selecting neurons for Ca^{2+} imaging experiments, firstly regions of interest (ROI) are specified around each neuron from which fluorescence intensity will be recorded. These are selected based on the neuron's health (e.g., "shiny" membrane, absence of granulation, consistent round shape), quality and positioning relative to other neurons on the coverslip. After 48h in culture, neurite outgrowth and replication of non-neuronal cells begin [505]. This creates overcrowded networks of overlapping neuronal processes and non-neuronal cells that obstruct the neurons in culture from being easily identified and selected for Ca^{2+} imaging in a ROI. We compared the cell yield per mouse per time point (day 1, 2, 4 and 5) in order to determine whether the number of available neurons for imaging experiments is affected with time. A neuron was defined as "available" if it was easily distinguishable, not overlapping with other culture components and hence the fluorescence it would emit would have been unobstructed. The total number of neurons for each day decreased as time in culture increased (table 4.2). Indeed, when comparing the number of available neurons per mouse for each time point, there was a significant decrease in the quantity of available cells for each

consecutive day compared to day 1, for both the control and high glucose groups (figure 4.2). The number of cells imaged for each condition was 1,871 for CTRL and 1,888 for the 50 mM group. By day 5, there was a reduction of 4.2- and 4.7-fold for CTRL and 50 mM group, respectively, compared to day 1 (CTRL: from 191.6 ± 20 in d1 to 46 ± 3.8 neurons in d5; 50mM: from 194 ± 17.9 in d1 to 40.7 ± 11 neurons in d5, p < 0.001). There was no significant difference in the number of neurons available between the two conditions for each day. These results demonstrated a consistent decrease in the number of neurons available for Ca^{2+} imaging experiments the longer the culture was maintained.

4.4.2. Time in culture, but not *in vitro*-induced hyperglycaemia, affects responses to nociceptive agonists

Diabetes has been shown to sensitise nociceptors in DRG neuronal populations from animal models of DN as well as in *in vitro*-induced high glucose conditions [506–509]. Therefore, three

Table 4.2. The total number of neurons decreases the longer the culture is maintained. The total number of neurons was recorded for each day of culture (1, 2, 4 and 5) and compared between control (CTRL) and high glucose-treated (50 mM) neurons. The number of mice (n) is shown in columns next to the cell count.

Figure 4.2. The availability of neurons for Ca2+ *imaging declines the longer the culture is maintained. Each bar represents the average number of neurons available per mouse for the respective timepoint, derived by dividing total cells of each time point by the number of mice (n) they were derived from in Table 4.2. D1, CTRL = 191.6 ± 20.1; 50mM = 194 ± 17.8; D2, CTRL = 128 ± 8.1, 50mM = 132.4 ± 13.1; D4, CTRL = 67.5 ± 28, 50mM = 67 ± 28; D5, CTRL = 46 ± 3.8, 50mM = 40.1 ± 11.1; * = significance compared to D1 CTRL, *p<0.05, **p<0.01, ***p<0.001; & = significance compared to D1 50mM, &p<0.05, &&p<0.01, &&&p<0.001. One-way ANOVA with multiple comparisons*

well-established nociceptive agonists were applied: α,β-methylene ATP (ATP), allyl isothiocyanate (AITC) and capsaicin (CAP), to see whether *in vitro* hyperglycaemic conditions would lead to the sensitisation of corresponding nociceptive receptor channels and the nociceptive subpopulations they mark. Agonists were applied consecutively as opposed to

added together. In the control group of neurons, responses to AITC in days 4 and 5 were significantly lower than day 1 by 2.5- and 2-fold, respectively (decrease from D1 65 ± 3% to D4 25.6 ± 7.7% and D5 32.3 ± 1.9%). The percentage of AITC(+) neurons between conditions within each day were not significantly different. Thus, the percentage of AITC(+) control neurons decreased significantly with time in culture (Figure 4.3 B). In the high glucose group, all days of measurements (2, 4 and 5) showed significantly lower response rates to AITC than day 1 by 1.4-, 2.2- and 2.7-fold, respectively (decrease from D1 64.8 ± 1.9% to D2 48.1 ± 4.1%, D4 30.2 ± 0.7% and D5 24 ± 5.2%) (Figure 4.3 B). The percentage of ATP(+) (Figure 4.3 A) and CAP(+) (Figure 4.3 C) neurons was comparable between conditions as well as overtime within each condition. Collectively, these results indicate steadily decreasing response rates to AITC in cultured DRG neurons with time, irrespective of extracellular glucose levels.

__Figure 4.3.__ Responses of DRG neurons under control and hyperglycaemic conditions to nociceptive agonists.
*__A)__ no significant difference in the responses to ATP between conditions as well as overtime within each condition. CTRL: D1=24.1±2.9%, D =22.4±4.8%, D4 = 11.1±1.7%, D5 = 15.65±9.7%; 50mM, D1 = 20±2.7%, D2 = 16.5±2%, D4=13.8±1%, D5=17.4±5%; __B)__ The percentage of AITC(+) neurons was significantly decreasing with each day spend in culture compared to day 1 (D1) in both the control and hyperglycaemic cultures. CTRL: D1 = 65 ± 3%, D2 = 55.1 ± 3.2%, D4 = 25.6 ± 7.7%, D5 = 32.2 ± 1.9%; 50mM, D1 = 64.8 ± 1.9%, D2 = 48.1 ± 4.1%, D4 = 30.2 ± 0.7%, D5 = 24 ± 5.2%; __C)__ no significant difference in the responses to ATP between conditions as well as overtime within each condition. CTRL: D1 = 23.9 ± 4.4%, D2 = 34.1 ± 5.1%, D4 = 38.8 ± 7.5%, D5 = 37.9 ± 3.1%; 50mM, D1 = 23 ± 3.2%, D2 = 31.1 ± 4.3%, D4 = 43 ± 13.5%, D5 = 32 ± 2%. * = significance compared to D1 CTRL, *p<0.05, **p<0.01, ***p<0.001; & = significance compared to D1 50mM, &p<0.05, &&p<0.01, &&&p<0.001. Where significance is not denoted, the difference is not significant. One-way ANOVA with multiple comparisons. Mean±SEM. CTRL=control (open bars); 50mM = hyperglycaemic culture (blue bars); D# = day#. ATP=α,β-methylene ATP, AITC=allyl isothiocyanate, CAP=capsaicin. On the right handside of each, intracellular calcium signal trace following stimulation with the respective agonist, averaged from all responsive neurons (24) from one experiment. Black – average trace from one experiment; grey – showing the SEM for each second of the recorded trace; black arrows indicate the time point of respective drug application.*

A ATP

B AITC

C CAP
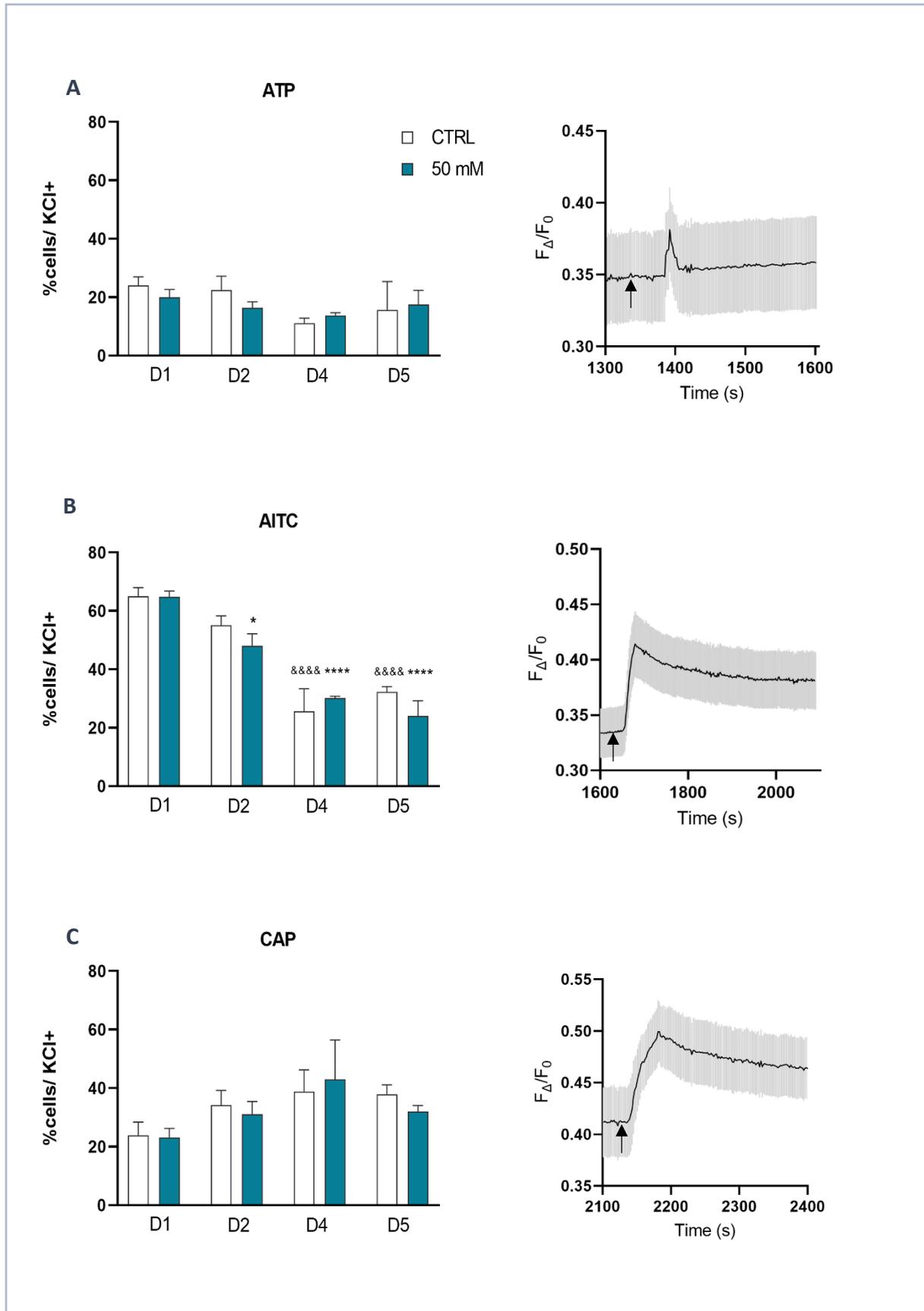
4.4.3. Time in culture leads to subtle changes in VGSC activity, affected by *in vitro* induced hyperglycaemia

High glucose concentrations have been reported to cause excitability changes in DRG neurons contributing to (P)DN [349]. We hypothesised that these changes are likely to involve modifications in VGSC activity and thus, will be detected using the VTD-based Ca^{2+} imaging assay. First, we looked at the percentage of VTD-irresponsive neurons – the percentage of VTD(-) neurons remained similar from day 1 to day 5 in culture in both the control and hyperglycaemic cultures (Figure 4.4 A). The percentage of VTD(-) neurons was also comparable between the CTRL and hyperglycaemic groups for each day, with non-significant differences. This suggests that neither hyperglycaemic conditions in this model nor time spent in culture influence overall VGSC-induced neuronal excitability.

Next, we classified the VTD(+) neurons into the four VTD-response profiles, in order to detect excitability changes within neuronal subpopulations. VTD-response profile rates overtime showed no significant differences between days within the CTRL and 50mM groups as well as between the CTRL and 50mM groups for each day (Figure 4.4 B,C,D,E).

*__Figure 4.4.__ **Responses to VTD by DRG neurons cultured for 1 to 5 days in high glucose or standard conditions.** Neither time spent in culture not high glucose levels influenced the responses to VTD in cultured DRG neurons.*
__A)__ Percentage of VTD(-) neurons, CTRL: D1 = 27.5 ± 1.4%, D2 = 28 ± 2.1%, D4 = 26.9 ± 4%, D5 = 26.1 ± 6%; 50mM, D1 = 32.1 ± 1.9%, D2 = 22.7 ± 3.6%, D4 = 35.8 ± 10.5%, D5 = 30.1 ± 4.7%; __B)__ Percentage of VTD(+) neurons of the SD profile, CTRL: D1 = 20.1 ± 2.8%, D2 = 24.2 ± 3.5%, D4 = 28.5 ± 2.9%, D5 = 29.7 ± 6.6%; 50mM, D1 = 18.2 ± 2.7%, D2 = 24.8 ± 2.2%, D4 = 22.2 ± 9.4%, D5 = 29 ± 4.2%; __C)__ Percentage of VTD(+) neurons of the OS profile, CTRL: D1 = 38.6 ± 2.4%, D2 = 34.2 ± 2.8%, D4 = 40.1 ± 1.6%, D5 = 30.9 ± 5.3%; 50mM, D1 = 35.4 ± 2.3%, D2 = 39.9 ± 3.6%, D4 = 30.1 ± 2.8%, D5 = 29.9 ± 4.1%; __D)__ Percentage of VTD(+) neurons of the ID profile, CTRL: D1 = 7.4 ± 1.2%, D2 = 6.8 ± 1.4%, D4 = 4.7 ± 0.5%, D5 = 8.2 ± 3.2%; 50mM, D1 = 7.8 ± 1.1%, D2 = 5.8 ± 1%, D4 = 8.3 ± 2%, D5 = 9 ± 3.3%; __E)__ Percentage of VTD(+) neurons of the RD profile, CTRL: D1 = 3.7 ± 0.4%, D2 = 5.6 ± 1.4%, D4 = 0 ± 0%, D5 = 4.8 ± 1.1%; 50mM, D1 = 5 ± 1.1%, D2 = 5.6 ± 0.6%, D4 = 2.9 ± 0.3%, D5 = 1.2 ± 1.2%; Where significance is not denoted, the difference is not significant. One-way ANOVA with multiple comparisons. Mean ± SEM. CTRL = control (open bars); 50mM = hyperglycaemic culture (filled bars); __F)__ Intracellular calcium signal trace following stimulation with VTD shaping the responses SD (left) and OS (right), averaged from all SD- or OS-profiled neurons (12 and 19, respectively) from one experiment.; Black – average trace from one experiment; grey – showing the SEM for each second of the recorded trace; black arrows indicate the time point of respective drug application.

Further analysis was performed of the average amplitude, time to peak and area under the curve (AUC) of the VTD responses for each of the four profiles (Figure 4.5). Significantly smaller amplitudes by 1.7-fold were observed in the OS profile in day 5 compared to day 1 of the control (decrease from d1 0.47 ± 0.06 to d5 0.27 ± 0.06, p < 0.05) but not the high glucose population (Figure 4.5. D). Significant amplitude reduction by 2-fold was also evident in the RD profile between control day 1 and control day 5 neurons (decrease from d1 0.39 ± 0.03 t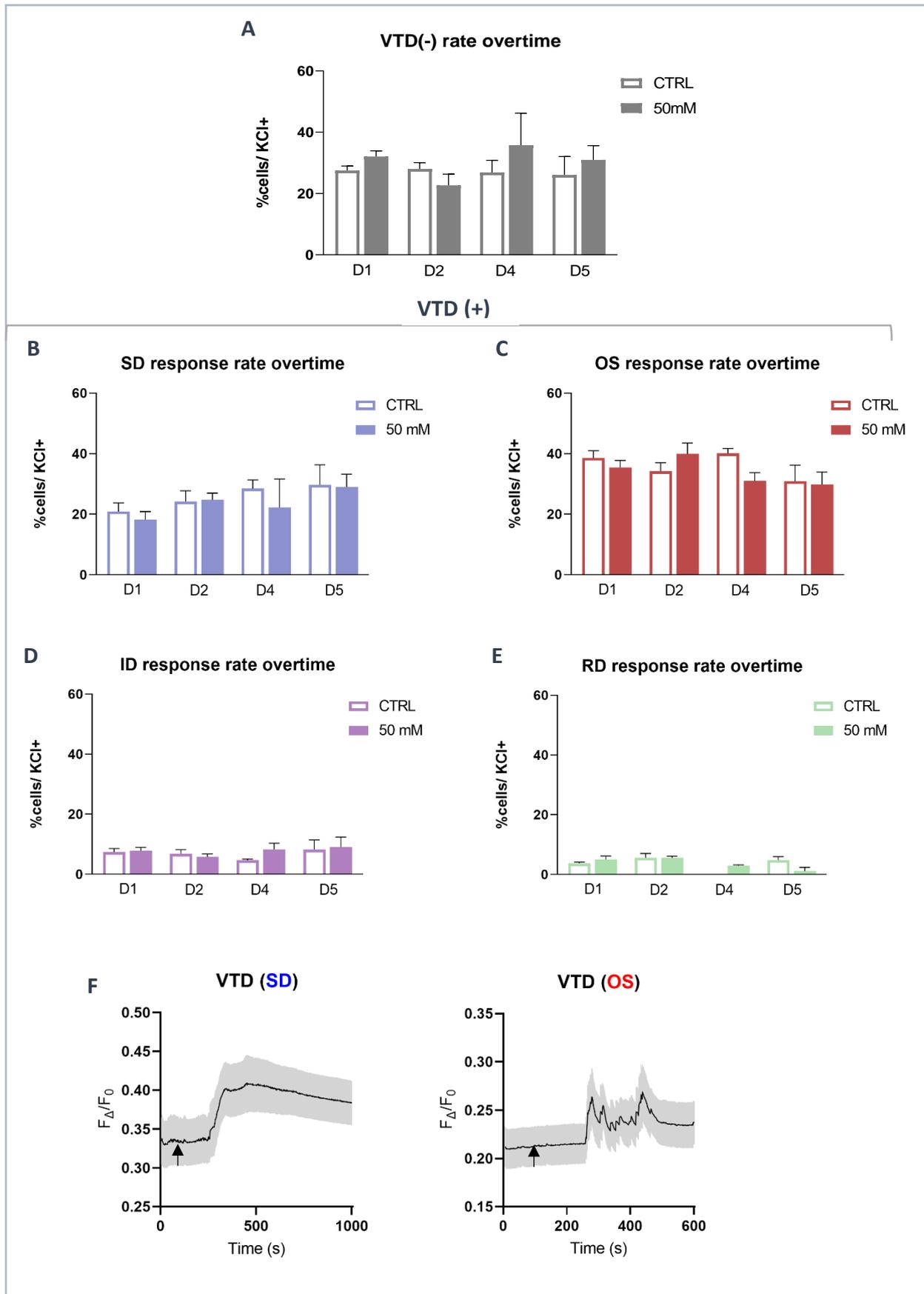o d5 0.2 ± 0.02, p < 0.05) (Figure 4.5. J). Similar amplitude trends were evident in the SD and ID profiles, albeit non-significant (Figure 4.5. A,G). These shifts indicate a possible time-in-culture-dependent effect on the Ca^{2+} signal amplitude in untreated cells. A significantly shorter onset to peak in the OS profile was also observed in day 4 high glucose neurons compared to same day control cells (d4 CTRL = 113.3 ± 29.3 s; d4 50mM = 42.2 ± 9.7 s, p < 0.05), although the sample size (n=2) is too small for concrete conclusions (Figure 4.5. E). These observations could point towards an effect increasing time in culture <u>exercises on VGSC activation-induced Ca^{2+} signal strength</u> in nociceptors that is attenuated by the presence of high glucose levels.

Taken together, these results indicate no effect of high glucose conditions on the response rates of cultured DRG neurons to nociceptive agonists and the VGSC agonist VTD. However, VTD-response amplitude changes with time in culture were noticed only in the untreated population of neurons, suggesting an effect high glucose conditions on signal amplitudes of nociceptors. Furthermore, time in culture might influence the response rates to some nociceptive agonists (AITC).

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*Figure 4.5. Analysis of the effect time in culture and hyperglycaemic conditions have on VTD-response parameters. The amplitudes of neurons responding to VTD with the OS and RD profile were significantly smaller on the 5th day in culture in control neurons compared to day 1. A) SD amplitude. CTRL: D1 = 0.39 ± 0.05%, D2 = 0.37 ± 0.03%, D4 = 0.29 ± 0.18%, D5 = 0.29 ± 0.05%; 50 mM: D1 = 0.44 ± 0.05%, D2 = 0.4 ± 0.04%, D4 = 0.34 ± 0.08%, D5 = 0.27 ± 0.04%; B) SD onset time to peak. CTRL: D1 = 100.5 ± 9.12%, D2 = 72 ± 13.42%, D4 = 84.8 ± 1%, D5 = 93.28 ± 20.36%; 50 mM: D1 = 94.54 ± 18.42%, D2 = 88.08 ± 18.78%, D4 = 74.5 ± 13.1%, D5 = 67 ± 17.23%; C) SD AUC. CTRL: D1 = 152.8 ± 35.29%, D2 = 147.4 ± 17.93%, D4 = 86 ± 65.4%, D5 = 108.8 ± 14.73%; 50 mM, D1 = 152.1 ± 19.79%, D2 = 149.2 ± 13.42%, D4 = 112.5 ± 23.55%, D5 = 92.38 ± 20.71%; D) OS amplitude. CTRL: D1 = 0.47 ± 0.05, D2 = 0.43 ± 0.02, D4 = 0.34 ± 0.07, D5 = 0.28 ± 0.06; 50mM, D1 = 0.42 ± 0.05, D2 = 0.41 ± 0.01, D4 = 0.35 ± 0.04%, D5 = 0.33 ± 0.04%; E) OS onset time to peak. CTRL: D1 = 74.78 ± 7.16%, D2 = 74.44 ± 6.28%, D4 = 113.3 ± 29.25%, D5 = 76.58 ± 8.07%; 50 mM: D1 = 74.96 ± 10.92%. D2 = 79.4 ± 11.8%, D4 = 42.15 ± 9.65%, D5 = 88.33 ± 5.42%; F) OS AUC. CTRL: D1 = 92 ± 15.94%, D2 = 98.64 ± 12.71%, D4 = 66.35 ± 33.85%, D5 = 63.88 ± 17.07%; 50 mM: D1 = 81.88 ± 16.03%, D2 = 155 ± 48.06%, D4 = 79.85 ± 15.45%, D5 = 81.53 ± 19.05%; G) ID amplitude. CTRL: D1 = 0.44 ± 0.04%, D2 = 0.49 ± 0.07%, D4 = 0.28 ± 0.04%, D5 = 0.36 ± 0.04%; 50 mM, CTRL: D1 = 0.45 ± 0.08%, D2 = 0.3 ± 0.03%, D4 = 0.34 ± 0.13%, D5 = 0.31 ± 0.03%; H) ID onset time to peak. CTRL: D1 = 110.7 ± 14.01%, D2 = 98.62 ± 15.14%, D4 = 141.2 ± 55.85%, D5 = 92.4 ± 12.81%; 50 mM: D1 = 112.7 ± 10.32%, D2 = 114.7 ± 24.73%, D4 = 116.1 ± 9.7%, D5 = 97.3 ± 14.24%; I) ID AUC. CTRL: D1 = 66.16 ± 11.99%, D2 = 81.58 ± 16.68%, D4 = 13.45 ± 0.95%, D5 = 45.65 ± 10.64%; 50 mM: D1 = 73.46 ± 11.53%, D2 = 61.76 ± 5.87%, D4 = 38.65 ± 10.65%, D5 = 48.1 ± 4.92%; J) RD amplitude. CTRL: D1 = 0.39 ± 0.03, D2 = 0.32 ± 0.05, D4 = no RD neurons recorded, D5 = 0.2 ± 0.02; 50mM, D1 = 0.47 ± 0.08, D2 = 0.32 ± 0.02, D4 = 0.19 ± 0.15, D5 = 0.19. K) RD onset time to peak. CTRL: D1 = 148.7 ± 8.49%, D2 = 157 ± 27.32%, D4 = no RD neurons recorded, D5 = 158.7 ± 38.49%; 50 mM: D1 = 147.4 ± 18.58%, D2 = 166.3 ± 13.52%, D4 = 180.8 ± 82.25%, D5 = 123.3 ± 0%; L) RD AUC. CTRL: D1 = 47.72 ± 8.51%, D2 = 45.98 ± 12.31%, D4 = no RD neurons recorded, D5 = 20.83 ± 4.73%; 50 mM: D1 = 46.3 ± 7.79%, D2 = 49.74 ± 8.64%, D4 = 22.55 ± 17.85%, D5 = 36.1 ± 0%. * = significance compared to D1 CTRL, *p<0.05. Where significance is not denoted, the difference is not significant. One-way ANOVA with multiple comparisons. Mean ± SEM. CTRL = control (open bars); 50mM = hyperglycaemic culture (filled bars); D# = day #.*
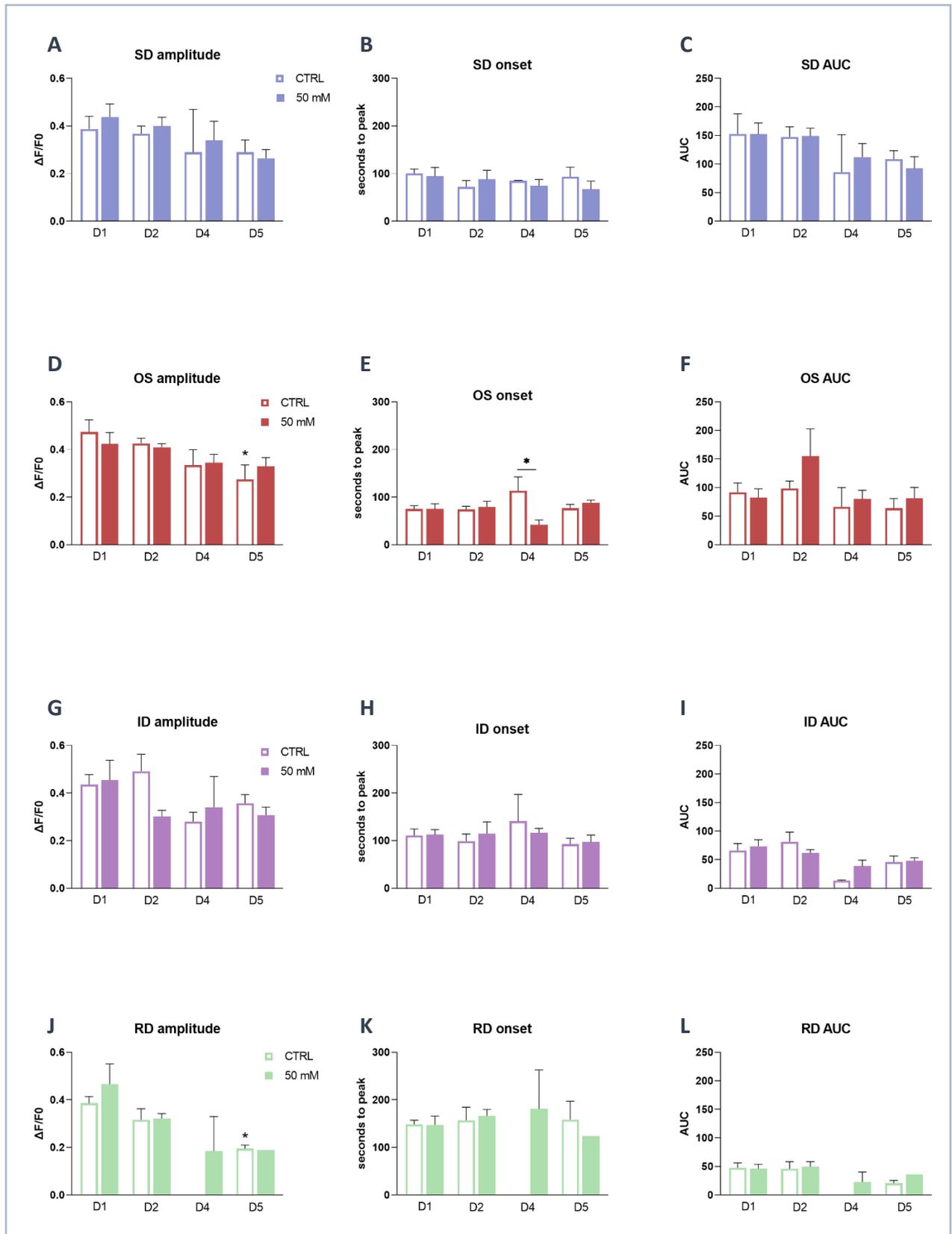
4.5. DISCUSSION

In this chapter, I outlined physiological experiments with DRG neurons under *in vitro* hyperglycaemic conditions. Cells cultured in a high glucose environment over 1, 2, 4 or 5 days showed no significant changes in their excitability, nociceptor sensitivity or VTD-induced Ca^{2+} response parameters compared to control. However, a decrease in the response rate to AITC was noted in both treated and untreated neurons as time in culture increased. Furthermore, we observed decreasing amplitude of the Ca^{2+} signal in response to VTD with time in culture in control but not in hyperglycaemic neurons, indicating possible extended effect of high glucose conditions on VGSC excitability.

In vitro hyperglycaemia has been routinely used to investigate changes in excitability of DRG, however predominantly only for short-term exposure. Previous research employing short-term *in vitro* hyperglycaemia has found that high glucose levels cause an increase in TRPV1 expression and currents in DRG neurons [506,510] as well as changes in VGSCs, including increased TTX-R current density [424,502] and increased $Na_v1.7$ expression [351]. Based on them, we expected that by inducing hyperglycaemia *in vitro* for short (1-2 days) and long-term (4-5 days) we will be able to detect changes in the VGSC and nociceptors excitability by Ca^{2+} imaging responses to different agonists. Here, *in vitro* induced hyperglycaemia did not produce the expected effects on DRG neurons excitability. However, there were differences in several values which were all associated with the time neurons had spent in culture. These findings can be summarised in the following:

- Decrease in the response rate to AITC in both high glucose-treated and control populations (Figure 4.3)
- Decrease in the VTD-induced Ca^{2+} response amplitudes of the OS and RD profiles in the control population, but not the high-glucose population of cells (Figure 4.5 D, J)

These observations are informative of possible changes in neuronal electrophysiology after maintaining neurons in culture for up to 6 days, with or without altered extracellular glucose conditions. On the other hand, these findings might be a product of the specifics of the particular protocol for culture conditions used (discussed in more detail in Chapter 6).

Neuronal dissociation and axotomy during the culturing process is injurious to neurons and has been well-documented to cause hyperexcitability in small DRG neurons via reducing AP threshold and increasing the AP duration and firing frequency [511–513]. Furthermore, the electrophysiological properties of acutely dissociated neurons are different from neurons cultured for 1+ days, with VGSC (such as Nav1.8) and VGCC expression and current density

decreasing with time [102,511,514]. The gradual decrease in neuronal excitability with time spent in culture could indicate neurons 'settling' and 'healing' after their excitability was increased immediately after dissociation due to its injurious nature [515]. Here, the decreases in VTD-response amplitude in nociceptors (OS) (Figure 4.5 D) were focused in the control population, whereas the high glucose group of neurons was not affected. The lack of significant decrease in nociceptive excitability in the hyperglycaemic population suggests that the high glucose environment could be preventing DRG neurons from bringing excitability down to normal physiological levels and thus "healing". Therefore, hyperglycaemia might render restoring normal neuronal physiology post-injury slower and more difficult. In an *in vivo* setting, wound healing has been well-documented to be delayed in diabetic rodents [516–518] and in diabetes patients [519] and so it is a possibility that a similar delay may occur also to neurons healing from damage during diabetes. The slower decrease of neuronal hyperexcitability post-dissociation might translate into prolonged pain sensation post-injury caused by diabetes. Currently, no clinical studies have explored this idea specifically, however DN patients are known to present with exaggerated pain responses to harmless or mild painful stimuli (allodynia and hyperalgesia, respectively) [234,356,520], which could support the concept of persistent and/or exaggerated wound-induced pain in diabetes patients. In any event, these results can serve as a caution for the interpretation of Ca^{2+} imaging data using ion channel agonists when recording responses from primary DRG neuron cultures over time.

Increased activity of nociceptive ion channels, such as TRPV1, TRPA1 and P_2X_3 has also been reported in DRG neurons during diabetes [58,343,521–523]. Here, short- and long-term exposure to high glucose conditions did not induce any significant changes in the response rates to the nociceptive agonists to these three receptors. Our results directly contradict two studies from 2018 reporting increased responses to CAP in DRG neurons cultured under high glucose conditions [506,510]. Lam et al. [506] report a significantly increased response rate to CAP in adult mouse DRG neurons cultured in high glucose conditions (25mM) glucose for 7 days. A possible reason for this discrepancy might be their choice of control and hyperglycaemic glucose concentrations of 5mM and 25mM, respectively. These are not comparable to the concentrations used in the experiments from this chapter, or the ones used in the majority of *in vitro* hyperglycaemia studies on DRG neurons [318,423,500]. An extracellular glucose concentration of 25mM can be considered within the optimal range for neuronal survival in culture, whereas a concentration of 5mM has been demonstrated to lead to neuronal apoptosis and cellular damage [423]. Therefore, the higher rates of CAP responses observed by Lam et al. are likely to represent the normal neuronal function, whereas the lower values seen in the control population are likely to be indicative of a hypoglycemic effect on CAP responses. Yet,

in direct contrast to the observations by Lam et al., others, employing the same experimental glucose conditions and exposure time as them, have observed a decrease in CAP-induced Ca^{2+} influx in mature DRG neurons [524]. The second study reporting increased CAP-evoked Ca^{2+} responses is by Bestall et al. (2018) who cultured DRG from naïve adult male rats in 50 mM glucose for 24 hours. Their study is the closest to ours in experimental design, yet we report conflicting results. A possible reason for the inconsistency could be due to a species difference (rat vs mouse) and/or the fact that relatively specific DRG were isolated (T1-L5), whereas, here, all DRG were isolated for experiments indiscriminately. Finally, another *in vitro* study using 50B11 immortalised rat DRG neuronal cell line reported an increase in responses to AITC when neurons were cultured in 66 mM glucose [525]. Albeit overlapping with observations *in vivo* [343,344], these *in vitro* results directly contradict ours, perhaps because of the different *in vitro* culture models utilised. An immortalised DRG neuronal cell line would not possess the complete arsenal of ion channel, receptors, exchangers and proteins contributing to shaping the net response to an agonist and therefore might not necessarily generate a complete picture of the final response. Therefore, the cell line may not be representative of the same populations of neurons with decreasing sensitivity to AITC. It is, thus, yet to be confirmed whether prolonged exposure to hyperglycaemic conditions *in vitro* is a suitable method for determining functional changes in nociceptive ion channels.

Neither short- nor long-term exposure to a hyperglycaemic extracellular environment affected the VGSC excitability of DRG neurons in my experiments. A literature search found very little on the question of prolonged exposure of DRG neurons to high levels of glucose *in vitro* (40-60 mM)) and the effect on VGSCs. A number of prior studies have noted an increase in the VGSC activity through increased levels of Nav1.7 expression [351] or increased density of TTX-R currents [349,502] of DRG neurons cultured in up to 60mM glucose for 18-24h. This corresponds to day 1 in my experiments, where no significant increase in VTD(+) cells and thus, in contrast to previous studies, no increase in overall VGSC activity was observed. There are a couple of possible explanations why these data differ. First, the studies reporting increase in VGSC activity derived their neurons from rats as opposed to the mouse neurons used here, which might account for potential interspecies differences in VGSC excitability during hyperglycaemia. However, to the best of our knowledge, there are currently no studies in literature comparing VGSC expression levels and activity between mice and rats and the VTD-Ca^{2+} imaging assay has yet to be validated in another rodent species. Second, only one study used DRG neurons from a mature rodent [502] like ours, however, only small (20-25 µm) L4-L5 DRG were selected for experiments. This, combined with the species difference (rat vs mouse here) and the very low control glucose conditions (5.5 mM) could contribute to the differences

in results reported by them and here. All other studies used embryonic DRG neuron cultures as opposed to the adult DRG neurons used here. Embryonic DRG neurons require supplementation with NGF to support neuronal survival in culture. NGF is known to alter electrophysiological properties of cultured DRG neurons [526–530]. It has also been widely implicated as a key driver of hyperalgesia during DN, with its expression levels significantly increased in DRG [456,531]. NGF is also demonstrated to sensitise TRPV1 and increase DRG responses to capsaicin [532,533]. Although the mechanisms of elevated NGF expression during diabetes are still unclear, it has been postulated that a hyperglycaemic environment is a trigger for increased NGF expression as a protective mechanism of tissues [456]. Therefore, the combination of supplemented NGF and high glucose to embryonic neurons in culture might produce a stronger hyperexcitability phenotype represented by increased VGSC activity compared to mature DRG neurons cultured in high glucose in the absence of NGF. Due to its role in DN hyperalgesia, addition of NGF to a hyperglycaemic culture could in fact be a sensible approach to recreating a physiological DN environment in a DRG culture. This and additional culture modifications to replicate DN more closely are discussed in more detail in Chapter 6 along with further limitations of the current experiment and future directions.

Taken together, the results from the *in vitro*-induced hyperglycaemia experiments are somewhat informative of events in the DRG culture when maintained for a prolonged time. Furthermore, our observation point to subtle changes hyperglycaemia might cause in VGSC excitability over extended exposure by maintaining neuronal excitability elevated after dissociation. Nevertheless, these experiments did not produce the expected, clear-cut overall increase in neuronal excitability after short- and long-term exposure to high glucose, seen in similar studies. The most likely reason for this outcome is the limited DN environment that we can be recreated in a dish which might not suffice to produce a phenotype strong enough to be detected by Ca^{2+} imaging with VTD and nociceptive agonists in our protocol. The multifactorial complexity of DN, especially T2DN, would require an individual extensive study of its own to extrapolate its components and optimise an in vitro T2DN model. Due to project time restrictions, it was decided to circumvent this lengthy process and opt for proceeding with investigating changes in excitability of DRG neurons derived from an in vivo model of diabetic neuropathy.

CHAPTER 5

ASSESSING EXCITABILITY CHANGES IN DRG NEURONS FROM DIABETIC DB/DB MICE

CHAPTER 5: ASSESSING THE EXCITABILITY CHANGES IN DRG NEURONS FROM DIABETIC *DB/DB* MICE

5.1. INTRODUCTION

The last chapter described experiments with DRG neurons under *in vitro*-induced high glucose conditions. Using an *in vitro* hyperglycaemia model to detect DRG excitability changes serves as a more ethical alternative to deriving neurons from diabetic animals. In addition, it reduces potential variability between controls and treated cells as both are isolated from the same animal as opposed to deriving diabetic cells from a diabetic animal and control cells from a healthy one. It also allows for the precise control of external glucose concentration, which varies in mouse models of diabetes and DN [419,534]. However, inducing hyperglycaemia *in vitro* represents only one, albeit main key drivers of DN pathogenesis– the high glucose levels. Furthermore, it is closer to reflecting T1DN rather than T2DN, as T2DN is driven by additional mechanisms such as aspects of the metabolic syndrome (insulin resistance, dyslipidaemia), together with hyperglycaemia. The many subcomponents and high complexity of T2DN pathogenesis renders it extremely difficult to model *in vitro* with high accuracy [419]. Therefore, to ensure all components of T2DN were in place, the second hyperglycaemia model I used was the T2D C57BL/Ks*db/db* mouse model.

The C57BL/Ks*db/db* (just *db/db* hereon) mouse is an excellent T2D model and, based on the established DN model requirements [448], is recognised as one of the most robust existing models of DN [453]. Its diabetic phenotype was first discovered in 1966 by Hummel et al. [535]. It wasn't until 1995, however, that its genetic mutation was identified [536,537]. The *db/db* mouse is a homozygous mutant knockout for the leptin receptor. Leptin is a hormone secreted by adipose cells and enterocytes in the small intestine after food intake. It controls appetite by binding to its receptor in the hypothalamus and inhibiting hunger and feeding behaviour (hence often referred to as the 'satiety hormone')[446,536]. The *db/db* mouse carries a deletion mutation in the gene encoding the leptin receptor, resulting in leptin signalling deficiency [536,538]. Eliminating leptin signalling leads to the mice exhibiting uncontrolled overeating behaviour (hyperphagia) simultaneous with decreased activity and thus energy expenditure. Blood insulin levels begin elevating at 10-14 days of age (DoA) followed by elevation in fasting blood glucose levels and dyslipidaemia (Figure 5.1). At 3-4 weeks of age (WoA), obesity and insulin resistance have developed and the phenotype culminates into T2D for the rest of the mouse's lifespan (Figure 5.2) [539]. The severity of the genetically induced disease causes continuous uncontrolled rise in blood glucose levels [428] and, at later stages, critical depletion of the insulin-

secreting pancreatic β-cells [540]. This, in combination with the secondary derangements brought about by obesity, it leads to death by 10 months of age [541].

	Db/db	Wild type
Age (weeks)	10	10
Body weight (g)	36.0 ± 1.2	25.2 ± 0.6
Food intake (g/day)	6.7 ± 0.4	3.9 ± 0.1
Water intake (g/day)	20.2 ± 2.4	9.8 ± 0.9
Fat mass (%)	58.9 ± 0.8	17.9 ± 1.0
Blood glucose (mM)	32.7 ± 2.0	7.9 ± 0.5
Plasma insulin (pg/μL)	4.6 ± 1.5	1.3 ± 0.2

Physical and molecular characteristics of db/db and wild-type mice. All characteristics apart from age were significantly different between the db/db and wild-type mice (unpaired *t*-test)

Figure 5.1. An excerpt from a paper by Guest and Rahmoune (2018) characterising the diabetic phenotype of a 10-week-old db/db mouse and comparing parameters to a wild type mouse. Source: [504].

Around 8 WoA, *db/db* mice develop DN [431]. Robertson & Sima were the first to describe the electrophysiological and morphometric changes in the nerves of *db/db* mice and were the first to provide a detailed characterisation of the DN phenotype in this model [428,542]. Their research revealed the key observation that DN progression in the *db/db* mouse mimics closely the phases in human patients.

The early phase of DN in *db/db* mice develops at 8–12 WoA and is called 'metabolic' (MET). It was characterised by severe motor (MNCV) and milder sensory (SNCV) nerve conduction velocity impairments as well as mild axonal atrophy in both myelinated and unmyelinated fibres (Sima and Robertson, 1978; Norido *et al.*, 1984). The MET phase was later further defined behaviourally with the presence of mechanical allodynia, hyperalgesia [456,461,507,543–546] and heat hypersensitivity [547,548], albeit the literature is highly controversial (see figure 5.1, Metabolic phase). In addition, a MET phase characteristic is that functional and structural deficiencies are generally reversible with metabolic interventions (usually, through glycaemic control with insulin administration) [429].
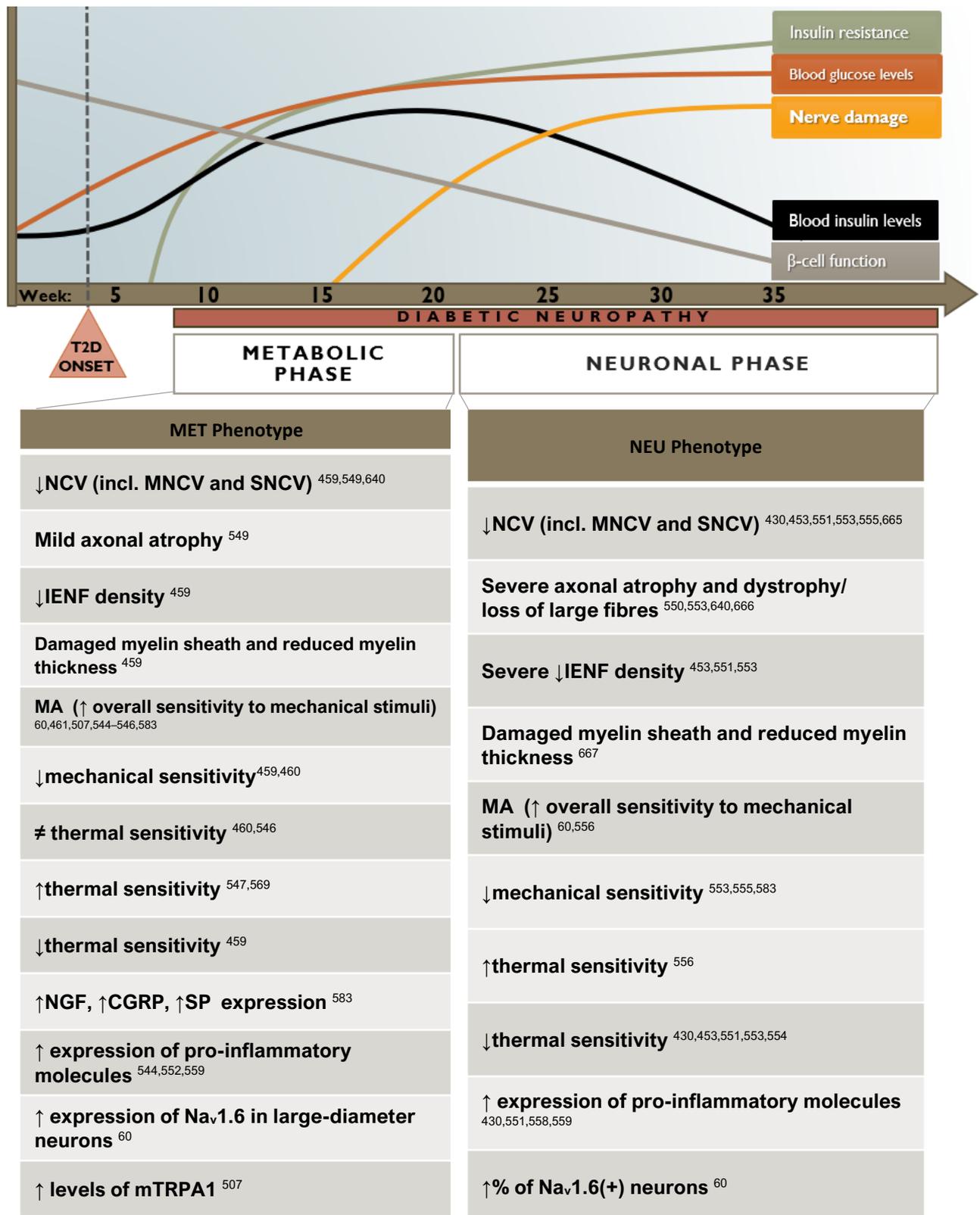
Figure 5.2. *A comprehensive summary of the available molecular, physiological, behavioural and transciptomic research on the db/db mouse model with observations categorised according to diabetic neuropathy phases observed by Sima and Robertson (1978). MA, mechanical allodynia; IENF, intraepidermal nerve fiber; NCV, nerve conduction velocity; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; ↑ denotes an increase, while ↓ denotes a decrease Figure adapted to the db/db mouse from*
https://www.sciencedirect.com/science/article/abs/pii/S0095454305701305?via%3Dihub

The late, 'neuronal' phase (NEU) in *db/db* mice develops from ~20 WoA and continues until the end of the mouse's life. It is characterised in the *db/db* mouse with an exacerbated MET phase phenotype of axonopathy and impaired MNCV and SNCV alongside with marked decrease of IENF density as well as loss of myelin sheathing [428,429,453,549–553]. Most studies agree on the presence of structural nerve damage but still report contradicting behavioural findings (see figure 5.1, Neuronal phase). For example, 20+ week old *db/db* mice have been reported to present with increased mechanical and thermal sensory thresholds and hypoalgesia by some [337,430,453,458,552–555], however others have observed increased sensitivity to mechanical stimuli and mechanical allodynia [544,556].

Additionally, several microarray and RNA-Seq analyses have shown an upregulation of immune and inflammatory molecules and pathways [430,552,557–559] in the peripheral nerves of *db/db* mice to begin as early as 8 WoA and continue throughout the rest of the DN progression contributing towards peripheral nerve fibre degeneration, as well as pain behaviour in the early stages of DN [456]. Finally, studies investigating the function, molecular mechanisms and expression levels of ion channels during DN in this mouse model are scarce. One study reported sustained increased expression levels of the large diameter neurons-associated VGSC $Na_v1.6$ in DRG of *db/db* mice from 8 WoA up until 20 WoA (end of experimental period) which was correlated to mechanical allodynia in these mice [60]. More recently, Wang et al. (2018) reported no increase in total protein expression of either TRPA1 or TRPV1. However, membrane-associated TRPA1, but not TRPV1, expression was increased and activity upregulated in *db/db* mice (6-7 WoA), further linked to impaired AMPK signalling and mechanical allodynia [507].

The abundance of behavioural, morphometric, and transcriptomic studies as well as the limited molecular research, has highlighted some key phenomena likely to underlie observed T2DN-associated behaviours in the *db/db* mouse, however there is no conclusive evidence on the pathophysiology of the pain during the progression of DN in T2D. Furthermore, the T1D STZ rodent is by far the most widely studied DN model, generating an imbalance in research between animal studies and clinical trials, which are largely targeting the T2D population of patients [241,418]. This demands more intensive research into the mechanisms driving T2DN. Finally, to the best of our knowledge, there are no physiological studies investigating the functional changes in *db/db* mouse DRG neurons during the MET and NEU phases of DN. Based on the above rationale, and on the established robustness of its DN phenotype, the T2D *db/db* mouse was selected as a model of DN for these experiments.

5.2. AIMS

To assess the excitability changes and changes in size distribution in specific subpopulations of DRG neurons from C57BKS*db/db* (*db/db*) mice, isolated at two different time points: 1) early (metabolic, MET) and 2) late (neuronal, NEU) stages of diabetic neuropathy by analysing the Ca^{2+} signals generated in response to the VGSC agonist VTD and nociceptive agonists ATP, AITC and CAP.

To compare excitability changes between the MET and NEU phases of DN in this mouse model in order to track the progression of diabetic neuropathy and its effect on DRG physiology.

5.3. METHODS

5.3.1. DRG neuron culture from *db/db* mice

DRG neurons were collected from *db/db* (BKS.Cg-+Leprdb/+Leprdb/OlaHsd) mice at 8 -12 weeks of age (WoA) (metabolic phase) and 22 - 28 WoA (neuronal phase) as well as from lean, DN phase-matched control mice (BKS.Cg-+Leprdb/-Leprdb/OlaHsd). Neurons were cultured in standard DMEM medium and left to incubate for at least 24 hours before experiments.

5.3.2. Calcium imaging

DRG neurons were loaded with Fura-2 and responses recorded to nociceptive agonists: capsaicin (CAP, 200 nM), allyl isothiocyanate (AITC, 100 µM) and α,β-methylene ATP (ATP, 1 µM) as well as VGSC potentiator veratridine (VTD, 30 µM). Standard calcium imaging protocol was followed as outlined in Chapter 2: Materials & Methods section 2.2.2., page 68.

5.4. RESULTS

5.4.1. The number of DRG neurons derived from *db/db* mice for each phase and condition

For the following experiments, 24 mice in total were sacrificed (6 MET CTRL, 6 NEU CTRL, 6 MET *db/db* and 6 NEU *db/db*). The total number of neurons imaged for each group is presented in table 5.1. The number of neurons used for these experiments from N5 and N6 in each phase and condition is generally smaller due to a diversion of some of the cells from these cultures to another set of experiments (not described in this thesis). Overall, the total numbers of neurons imaged for each phase of each condition were comparable.

Table 5.1 **Number of neurons for each mouse (N) of each phase (MET or NEU) for each condition (CTRL or db/db).** *Each cell with cell count represents one mouse's total cell yield. Twenty-four mice were used in total. MET = metabolic phase; NEU = Neuronal phase; CTRL = control (lean);*

phase & condition / Mouse	MET CTRL	NEU CTRL	MET *db/db*	NEU *db/db*
N1	496	425	521	409
N2	441	597	521	509
N3	267	326	610	384
N4	366	336	327	616
N5	270	463	338	374
N6	367	415	399	270
Total:	2473	2562	2716	2562

5.4.2. The overall cell size distribution is shifted towards smaller cells in DRG from *db/db* mice

The soma diameters of the DRG neurons from *db/db* and phase-matched lean control mice used for the experiments presented here were measured. Neurons from each condition group (*db/db* or lean) and phase (MET or NEU) were pooled for soma diameter analysis. Frequency distribution analysis of DRG sizes for each DN phase was performed and plotted as a histogram (Figure 5.3). In the MET phase, the median cell diameter was slightly smaller in *db/db* than in lean mice (lean = 21 µm, 2473 cells; *db/db* = 20.6 µm, 2716 cells) (Figure 5.3A). In the NEU phase, the mean cell diameter of DRG from *db/db* mice was still smaller than lean (lean = 21.4 µm, 2562 cells; *db/db* = 19.8 µm, 2562 cells), however here, the distribution of the diabetic DRG is shifted to the left (Figure 5.3B).

Figure 5.3. *Frequency distribution of neuronal diameter sizes from control (lean) and diabetic (db/db) mice for each phase of diabetic neuropathy: early, metabolic and late, neuronal.* **A)** *Metabolic, lean = 21 μm, 2473 cells; db/db = 20.6 μm, 2716 cells;* **B)** *Neuronal, lean = 21.4 μm, 2562 cells; db/db = 19.8 μm, 2562 cells. Dotted vertical lines represent median for their respective population. Plotted as number of cells observed in each 2 μm bin of diameter. Lean = grey bars, db/db = light blue bars; darker blue bars represent lean and db/db overlap.*

Neurons were classified according to their soma diameter into three categories: small- (<20 um), medium- (20-30 um) and large (>30 um), consistent with diameter range groupings commonly used in other studies [560,561] (Figure 5.4). During the MET phase, the percentage of large cells in *db/db* mice was significantly lower than that of lean cells by ~35% (lean = 13.1 ± 1.5%, *db/db* = 8.5 ± 0.7%; p<0.05, n = 6 each) (Figure 5.4 A,B). The vast majority of neurons in both lean and *db/db* mice were small and medium-sized, however, there was no significant difference in the percentage of small and medium-sized cells between lean and diabetic mice in the MET phase. In the NEU phase, 52% of *db/db* DRG neurons fell in the small-sized group, compared to 37.5% of lean neurons (n = 6 mice from each group, p < 0.05) (Figure 5.4 C,D). The increased number of small-diameter cells in diabetic mice was matched by the significantly fewer medium-sized cells compared to lean (38% vs 49%, respectively, n = 6 each, p < 0.05). There was no significant difference observed in the large-sized DRG group.

Collectively, the results from both DN phases point to a shift in the size distribution of diabetic cells towards smaller-diameter and away from medium and large-diameter neurons that starts during early and progresses through late DN stages.

Figure 5.4. Distribution of neuronal soma diameter in three size groups: small (< 20 μm), medium (20 - 30 μm) and large (> 30 μm). A) Percentage of small, medium and large neurons from lean and db/db mice derived during the metabolic phase. Lean: small = 41.2 ± 2.9%, medium = 45.7 ± 1.8%, large = 13.1 ± 1.5%; Db/db: small = 43.6 ± 2.8, medium = 48 ± 2.8%, large = 8.5 ± 0.7%; B) Pie chart representation of the mean percentage of small, medium and large neurons from lean and db/db mice of the metabolic phase; C) Percentage of small, medium and large neurons from lean and db/db mice derived during the neuronal phase. Lean: small = 37.5 ± 3.7%, medium = 48.9 ± 2.5%, large = 13.6 ± 1.4%; Db/db: small = 52 ± 4.8, medium = 38.1 ± 3.3%, large = 9.9 ± 1.6%; D) Pie chart representation of the mean percentage of small, medium and large neurons from lean and db/db mice of the neuronal phase. *p<0.05; Two-tailed paired Student's t-test, shown mean ± SEM.

5.4.3. Persistent increase in the sensitivity to capsaicin in small DRG neurons from *db/db* mice

We wanted to assess DRG neurons from *db/db* mice for excitability changes in the context of nociception. To this end, we applied ATP, AITC and CAP. Each cell can respond to only one of the agonists, to a combination of any two (ATP/CAP+, ATP/AITC+ or AITC/CAP+), to all three together (all(+)) or none of them (agonist (-), or non-nociceptors). To identify if any of these specific populations is affected, we dissected the responses to the three nociceptive agonists into all 8 possible response combinations. Diabetes caused an increase of 1.8-fold in the number of neurons responding to all three agonists simultaneously (all(+)) (from $3.3 \pm 0.7\%$ to $6.1 \pm 0.9\%$, $p < 0.05$) in the MET phase (Figure 5.5A). The increase in this subpopulation, together with the non-significant increases in the rest of the CAP(+) subpopulations (Figure 5.5A), lead to a 1.2-fold increase in the percentage of total CAP(+) DRG neurons in *db/db* mice (Figure 5.5C) (from $28.4 \pm 1.8\%$ to $34.2 \pm 1.7\%$, $p < 0.05$). The total percentages of agonist(-), ATP(+) and AITC(+) *db/db* cells remained comparable to control levels (Figure 5.5C). The results from this phase indicate an early on increase in the sensitivity of DRG neurons to capsaicin, mainly occurring in one subpopulation of neurons (all(+)).

Likewise, in the NEU phase, the percentage of all(+) cells remained significantly higher than lean controls (1.9-fold, from $1.4 \pm 0.2\%$ to $2.7 \pm 0.5\%$, $p < 0.05$) (Figure 5.5B). Here, the percentage of neurons responding to CAP-only as well as ATP and CAP together (ATP/CAP) was significantly higher in *db/db* mice by 1.5-fold (from $10.2 \pm 0.9\%$ to $15.3 \pm 1.1\%$, $p < 0.005$) and 4-fold (from $0.4 \pm 0.1\%$ to $1.6\% \pm 0.4\%$, $p < 0.05$), respectively (Figure 5.5B). These rises were at the expense of cells responsive to AITC-only, the percentage of which decreased by 1.3-fold (from $35.7 \pm 1.1\%$ to $24.8 \pm 1.5\%$ $p < 0.01$). The changes in response rates within each of these individual nociceptive subpopulations brought about a significant 1.3-fold increase in the total response rates to CAP (from $31.3 \pm 1.2\%$ to $40.7 \pm 2.5\%$ $p < 0.001$) and 1.1-fold decrease in total percentage of AITC(+) DRG neurons (from $57.1 \pm 0.6\%\%$ to $51.3 \pm 2.1\%$, $p < 0.05$) (Figure 5.5D). These findings point to an ever persisting and increasing sensitivity of DRG neurons to CAP in the NEU phase, which seems to have spread to affect more subpopulations of neurons, coupled with a decreased sensitivity to AITC.

To further characterise the additional CAP(+) neurons in each phase, the size distribution of CAP(+) DRG neurons from *db/db* and lean mice was analysed. In the MET phase, the medians of lean and *db/db* neuron sizes were 18.8 µm (n = 661 cells) and 19.6 µm (n = 928 cells), respectively (Figure 5.6 A). The additional CAP(+) neurons in the *db/db* mice fell in the 14-26 µm diameter range (figure 5.6 A).
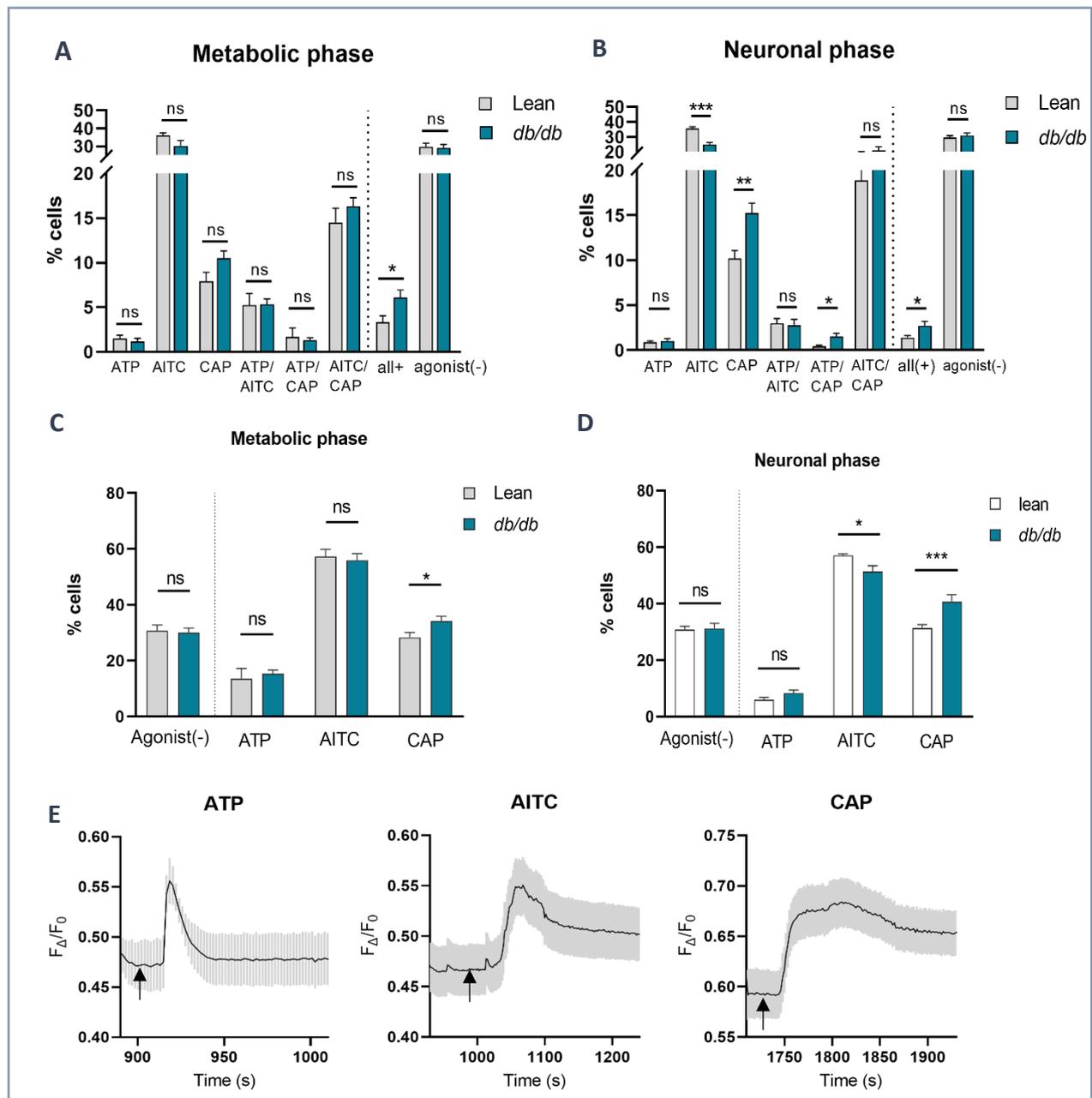
Figure 5.5. *The percentage of lean and db/db-derived neurons responsive to nociceptive agonists in each phase of diabetic neuropathy. A, B) Percentage of neurons responsive to each nociceptive agonist individually (ATP, AITC, CAP), in combination with another (ATP/AITC, ATP/CAP, AITC/CAP), all together (all(+)) or none (agonist(-)) during the metabolic and neuronal phases. A) Metabolic, lean: ATP = 1.5 ± 0.4%, AITC = 36.1 ± 1.4%, CAP = 7.9 ± 1%, ATP/AITC = 5.3 ± 1.3%, ATP/CAP = 1.7 ± 1%, AITC/CAP = 14.5 ± 1.6%, all(+) = 3.3 ± 0.7%, agonist(-) = 29.8 ± 1.9%; Metabolic, db/db: ATP =1.2 ± 0.4%, AITC = 30.3 ± 2.9%, CAP = 10.5 ± 0.8%, ATP/AITC = 5.4 ± 0.6%, ATP/CAP = 1.3 ± 0.3%, AITC/CAP = 16.4 ± 0.9%, all(+) = 6.1 ± 0.9%, agonist(-) = 29 ± 2%. B) Neuronal, lean: ATP = 0.9 ± 0.2%, AITC = 35.7 ± 1.1%, CAP = 10.2 ± 0.9%, ATP/AITC = 3 ± 0.5%, ATP/CAP = 0.4 ± 0.1%, AITC/CAP = 18.9 ± 1.3%, all(+) = 1.4 ± 0.2%, agonist(-) = 29.5 ± 1.3%; Neuronal, db/db: ATP = 1.1 ± 0.2%, AITC = 24.8 ± 1.5%, CAP = 15.3 ± 1.1%, ATP/AITC = 2.8 ± 0.6 %, ATP/CAP = 1.6 ± 0.3%, AITC/CAP = 21 ± 2.3%, all(+) = 2.7 ± 0.5%, agonist(-) = 30.8 ± 1.8%. Two-tailed paired Student's t-test. C, D) Total percentage of neurons which responded to each of the three agonists irrespective of the response's nature (e.g., CAP here is the total responses when CAP, ATP/CAP, AITC/CAP and all(+) from Figure 5.5A or C are added together). A) Metabolic, lean: Agonist(-) = 30.7 ± 2.1%, ATP = 13.6 ± 3.7%, AITC = 57.3 ± 2.7%, CAP = 28.4 ± 1.7%; Metabolic, db/db: Agonist(-) = 30.1 ± 1.5, ATP = 15.4 ± 1.3%, AITC = 56 ± 2.4%, CAP = 34.2 ± 1.7%; D) Neuronal, lean: Agonist(-) = 30.8 ± 1.2%, ATP = 6 ± 0.8%, AITC = 57.1 ± 0.6%, CAP = 31.3 ± 1.2%; Neuronal, db/db: Agonist(-) = 31.2 ± 1.8%, ATP = 8.4 ± 1%, AITC = 51.3 ± 2.1%, CAP = 40.7 ± 2.5%. Multiple comparisons following a One-way ANOVA. *p<0.05, **p<0.005, ***p<0.001. Shown mean ± SEM; E) Intracellular calcium signal trace following stimulation with the respective agonist, averaged from all responsive neurons (20) of one experiment. Black – average trace from one experiment; grey – showing the SEM for each second of the recorded trace; black arrows indicate the approximate time point of respective drug application.*

Figure 5.6. *Size distribution of CAP(+) neurons from control (lean) and diabetic (db/db) mice for each phase of diabetic neuropathy: early, metabolic and late, neuronal*. **A)** *Metabolic: lean = 18.8μm, 661 cells; db/db = 19.6 μm, 2716 cells;* **B)** *Neuronal: lean = 19.9 μm, 789 cells; db/db = 18.8 μm, 1053 cells. Dotted vertical lines represent median for their respective population. Plotted as number of cells observed in each 2 μm bin of diameter. Lean = grey bars, db/db = light blue bars; darker blue bars represent lean and db/db overlap.* **C)** *Percentage of small, medium and large CAP(+) neurons from lean and db/db mice derived during the metabolic phase. Lean: small = 15.7 ± 1.2%, medium = 9.8 ± 1.2%, large = 0.6 ± 0.1%; Db/db: small = 17.3 ± 1.5%, medium = 13.9 ± 1%, large = 0.5 ± 0.1%;* **D)** *Percentage of small, medium and large CAP(+) neurons from lean and db/db mice derived during the neuronal phase. Lean: small = 14.3 ± 1.1%, medium = 14.2 ± 2.6%, large = 0.8 ± 1.2%; Db/db: small = 22.9 ± 2.6%, medium = 13.9 ± 1.2%, large = 0.8 ± 0.2%; *p<0.05. ***p<0.001; Two-tailed paired Student's t-test, shown mean ± SEM.*

Sizes were also grouped into small medium and large. In the MET phase, there was a significant increase in the number of CAP(+) *db/db* neurons of medium-diameter (1.4-fold increase, control med 9.8 ± 1.2%; *db/db* med 13.9 ± 1%, p < 0.05) (Figure 5.6. C). There was no significant difference between *db/db* and control neurons of small- and large-diameter in this phase. This suggests that the increased sensitivity to CAP in *db/db* mice during the MET phase is contributed to by medium-diameter neurons but not the small or large cells. Of note, the increase in all(+) neurons in the *db/db* mice during the MET phase (Figure 5.5 A) was also contributed for by a population of medium-diameter neurons, where a significant 3.5-fold increase in the percentage of medium-sized all(+) neurons was noted (from 0.9 ± 0.3% to 3.1

± 0.4%, p < 0.001) (Figure 5.7 A), further confirming the all(+) population of neurons as the main contributor to the total increase in CAP sensitivity in MET phase *db/db* mice .

In the NEU phase, the medians of lean and *db/db* neuron sizes were 19.9 um (n = 1053 cells) and 18.8 μm (n = 789 cells), respectively, with the additional CAP(+) cells from *db/db* mice falling in the 8-20 μm diameter range (Figure 5.6 B). This translated to a significant 1.6-fold increase in the number of CAP(+) small-sized *db/db* neurons in the NEU phase (from 14.3 ± 1.1% to 22.9 ± 2.6%, p < 0.001) (Figure 5.6 D).

The drop in AITC(+) neurons in the NEU phase (Figure 5.5 B,D) could be correlated to a significant 1.8-fold decrease in the percentage of medium-diameter AITC(+) *db/db* neurons in the NEU phase (from 20.1 ± 1.9% to 11.5 ± 2.2%, p < 0.001), suggesting that the neurons losing their AITC sensitivity are of medium-diameter (Figure 5.7 B).

*Figure 5.7. Distribution of neurons responsive to specific all nociceptive agonists in the MET phase or AITC-only in the NEU phase in three size groups: small (< 20 μm), medium (20 - 30 μm) and large (> 30 μm). A) Percentage of small, medium and large neurons responsive to ATP, AITC and CAP simultaneously (all(+)) from lean and db/db mice derived during the metabolic phase. Lean: small = 2.1 ± 1.4%, medium = 0.9 ± 0.3%, large = 0%; Db/db: small = 2.6 ± 0.5%, medium = 3.1 ± 0.4%, large = 0.03 ± 0.03%; B) Percentage of small, medium and large neurons responsive only to AITC (AITC(+)) from lean and db/db mice derived during the neuronal phase. Lean: small = 11.5 ± 1.3%, medium = 20.9 ± 1.9%, large = 1.3 ± 0.5%; Db/db: small = 10.6 ± 1.4%, medium = 11.5 ± 2.2%, large = 0.9 ± 0.3%;. ***p<0.001; Two-tailed paired Student's t-test, shown mean ± SEM.*

Together, the changes in the size distribution of CAP(+) neurons during DN progression are indicating that the increasing sensitivity to CAP in *db/db* mice is dynamic, migrating from medium to smaller-diameter DRG neurons as neuropathy advances. The common medium-diameter size between the additional all(+) and CAP(+) *db/db* neurons of the MET phase strengthen the role of the all(+) subpopulation of neurons in the overall increased CAP sensitivity.

5.4.4. VGSC activity in small DRG nociceptors from *db/db* mice is increased during the course of diabetic neuropathy

Diabetic conditions have been shown to lead to an upregulation of VGSCs expression and activity, especially during early phases of neuropathy [58]. We hypothesised that the VGSC excitability will have also changed in the *db/db* mice DRG neurons. We used the VTD-Ca^{2+} imaging assay to assess the VGSC excitability in nociceptors (OS VTD-response profile) and non-nociceptors (SD VTD-response profile). In the MET phase, responses to VTD showed an increase in excitability (Figure 5.8 A): the number VTD-irresponsive (VTD(-)) neurons decreased by 1.14-fold (from 36.6 ± 0.7% to 31.5 ± 2.1%, p < 0.005). This decrease was matched by the increase in the percentage of VTD(+) cells of the OS profile by 1.16-fold (from 34.2 ± 1.1% to 39.6 ± 1.1%, p < 0.005). The response rates of the SD, ID and RD VTD-response profiles were not significantly different from lean mice (Figure 5.8 A).

Figure 5.8. *Responses to VTD by DRG neurons derived from lean or db/db mice during the metabolic or neuronal phase of diabetic neuropathy.* **A)** *Metabolic phase, lean: VTD(-) = 36.6 ± 0.7%, SD = 21 ± 1.1%, OS = 34.2 ± 1.1%, ID = 4.6 ± 0.3%, RD = 3.6 ± 0.4%; db/db, VTD(-) = 31.5 ± 2.1%, SD = 20.5 ± 1.4%, OS = 39.6 ± 1.1%, ID = 4.4 ± 0.5%, RD = 3.9 ± 0.2%;* **B)** *Neuronal phase, lean: VTD(-) = 39.9 ± 2.2%, SD = 22.4 ± 1.7%, OS = 30.8 ± 1.9%, ID = 4.1 ± 0.4%, RD = 2.6 ± 0.6%; db/db, VTD(-) = 36 ± 1.3%, SD = 21 ± 1.2%, OS = 34.3 ± 0.7%, ID = 5 ± 0.5%, RD = 3.4 ± 0.4%. One-way ANOVA with multiple comparisons. **p<0.005. Mean ± SEM. Open bars = lean (control); filled bars = db/db.* **C)** *Intracellular calcium signal trace following stimulation with VTD shaping the responses SD (left) and OS (right), averaged from all SD- or OS-profiled neurons (12 and 19, respectively) from one experiment.; Black – average trace from one experiment; grey – showing the SEM for each second of the recorded trace; black arrows indicate the approximate time point of respective drug application.*
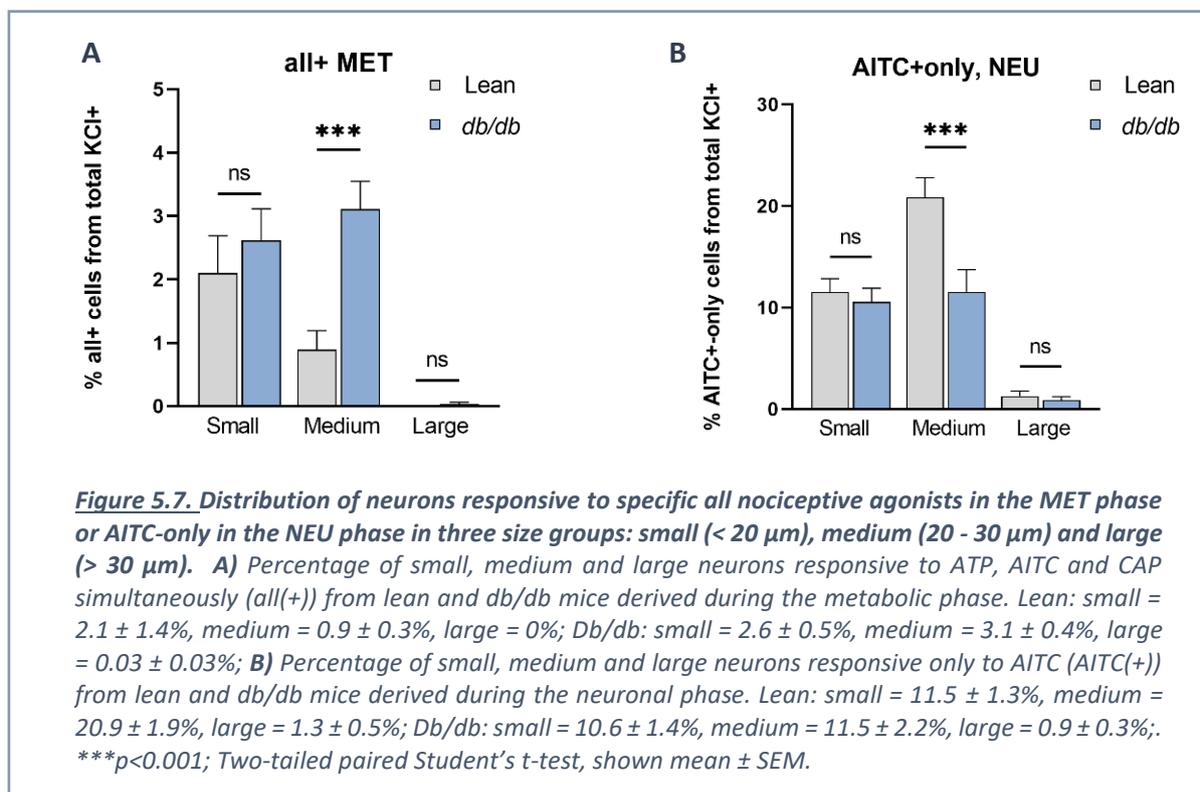
In contrast, in the NEU phase, there was no significant difference in the total response rate to VTD nor within any of the VTD-response profiles, however, albeit non-significant, a trend of increased OS responses in *db/db* neurons persisted (Figure 5.8 B). Of note, there were no significant differences in the amplitude, time to peak or AUC of calcium responses between *db/db* mice and controls (data not shown).

Analysis of the cell size distribution within the MET phase OS population (Figure 5.9 A,C) showed that there was no change in size distribution between lean and *db/db* neurons, but there were more OS *db/db* neurons in the 14-28 µm (small to medium-diameter) size range. This suggests that the additional OS neurons of the MET phase in *db/db* mice (Figure 5.8 A) are not focused in one neuronal size specifically but are likely to be mostly medium in diameter.

*Figure 5.9. Size distribution of neurons with the OS VTD-response profile from control (lean) and diabetic (db/db) mice for each phase of diabetic neuropathy: early, metabolic (MET) and late, neuronal (NEU). A) Metabolic: lean = 20µm, 825 cells; db/db = 20.1 µm, 1048 cells; B) Neuronal: lean = 20.1 µm, 793 cells; db/db = 19.3 µm, 894 cells. Dotted vertical lines represent median for their respective population. Plotted as number of cells observed in each 2 µm bin of diameter. Lean = grey bars, db/db = light red bars; darker red bars represent lean and db/db overlap. C) Percentage of small, medium and large OS neurons from lean and db/db mice derived during the metabolic phase. Lean: small = 15.8 ± 1.8%, medium = 13.9 ± 0.8%, large = 2.1 ± 0.3%; Db/db: small = 16.9 ± 2%, medium = 18.1 ± 1.3%, large = 1 ± 0.3%; D) Percentage of small, medium and large OS neurons from lean and db/db mice derived during the neuronal phase. Lean: small = 13.5 ± 1.7%, medium = 13.6 ± 1.2%, large = 1.7 ± 0.3%; Db/db: small = 18.3 ± 1.7%, medium = 12.5 ± 1.6%, large = 1.2 ± 0.3%; *p<0.05; Two-tailed paired Student's t-test, shown mean ± SEM.*

In the NEU phase, we saw an increase in the percentage of OS cells, however It was not significant (Figure 5.8 B). Therefore, we looked into the changes in the distribution and percentage of OS cells within the different sizes of *db/db* neurons to see whether the OS increase was focused within a particular neuronal subpopulation (Figure 5.9 B,D). There was a leftward shift in the distribution of *db/db* OS neurons, with more OS profiles in the 8-20 μm (small-diameter) diameter range (Figure 5.9 B). The additional OS neurons of the NEU phase in *db/db* mice were of small diameter – the percentage of small neurons in *db/db* mice was significantly higher than that of lean mice by 1.4-fold (lean = 13.5 ± 1.5%, *db/db* = 18.3 ± 1.7%, $p < 0.05$), while there was no significant difference between lean and *db/db* neurons in the small and large diameter groups (Figure 5.9 D). These insights suggest that the increase in OS neurons begins in the MET phase in small and, mostly, medium-diameter neurons and persists during the NEU phase, focusing into small-diameter neurons of *db/db* mice.

We wanted to find out whether the additional OS cells were also CAP(+). To do this, we performed isolated analysis of the OS population for the response rates to the ATP, AITC and CAP in the 8 possible combinations (Figure 5.10). In the MET phase, there was a significant increase of 2.2-fold in the percentage of all(+) OS neurons in *db/db* compared to lean mice (from 6.2 ± 1.3% to 13.4 ± 2.3%, $p < 0.05$) (Figure 5.10 A). This showed that the additional OS cells of the *db/db* mice in the MET phase were of the 'all(+)' population, identified to be also

Figure 5.10. The percentage of OS neurons from lean and db/db responsive to nociceptive agonists – alone or in combinations, in each phase of diabetic neuropathy. A) Responses of OS neurons to agonists during the metabolic phase, Lean: ATP = 2.4 ± 0.8%, AITC = 40.2 ± 3.2%, CAP = 4.7 ± 0.7%, ATP/AITC = 12.2 ± 2.9%, ATP/CAP = 3 ± 1%, AITC/CAP = 11.9 ± 1.3%, all(+) = 6.2 ± 1.3%, agonist(-) = 19.4 ± 2.4%; db/db: ATP = 2.2 ± 0.7%, AITC = 32.3 ± 2.9%, CAP = 5.7 ± 0.5%, ATP/AITC = 12.1 ± 1.8%, ATP/CAP = 2.5 ± 0.7, AITC/CAP = 11.6 ± 1.3%, all(+) = 13.4 ± 2.3%, agonist(-) = 20.3 ± 2%; *B)* Responses of OS neurons to agonists during the neuronal phase, Lean: ATP = 1.7 ± 0.6%, AITC = 48.4 ± 2.1%, CAP = 4.4 ± 2.5%, ATP/AITC = 7.7 ± 1.4, ATP/CAP = 0.5 ± 0.2%, AITC/CAP = 13.8 ± 1.2%, all(+) = 3.3 ± 0.5%, agonist(-) = 20.3 ± 2.7%; db/db: ATP = 2 ± 0.5%, AITC = 32.6 ± 2.7%, CAP = 11.2 ± 1.9%, ATP/CAP = 3 ± 0.7%, ATP/AITC = 7.2 ± 1.5%, AITC/CAP = 20.6 ± 1.8%, all(+) = 6 ± 1.5%, agonist(-) = 17.4 ± 2%. Two-tailed paired Student's t-test. *p<0.05, **p<0.005, ***p<0.001. Shown mean ± SEM

the main contributor to the overall increase in CAP sensitivity in that phase (Figure 5.5 A,C). The same analysis performed on the OS population of neurons in the NEU phase revealed that there was an increase in the percentage of OS neurons by responding to CAP alone by 2.5-fold (from 4.4 ± 2.5% to 11.2 ± 1.9%, p = 0.0559) or with another agonist (ATP/CAP, 5.9-fold, from 0.5 ± 0.2% to 3 ± 0.7%, p < 0.005; AITC/CAP, 1.5-fold, from 13.8 ± 1.2% to 20.6 ± 1.8%, p < 0.05) (Figure 5.10 B), identified to be amongst the main contributing populations to the overall CAP increase noted in the NEU phase in *db/db* neurons (Figure 5.5 B,D). These analyses suggest it is likely that the OS subpopulations of neurons affected by increased VGSC excitability could be the same subpopulations affected by increased sensitivity to CAP in each phase of DN.

Taken together, these results suggest that DN leads to an early-phase overall increase in VGSC excitability mainly in medium-diameter DRG neurons responsive to multiple nociceptive agonists. The concentration of this increase in the OS population confirms that these changes are occurring in nociceptors. As DN progresses into the NEU phase, the increased excitability of OS neurons shifts to affect small-diameter nociceptors. The subpopulations affected by increased VGSC activity overlap with those affected by increased CAP activity, suggesting that the same subpopulation of neurons could be affected during the course of DN.

5.5. DISCUSSION

The experiments described in this chapter aimed to examine the excitability changes occurring during the course of DN in DRG neurons isolated from the well-established T2DN *db/db* mouse model. A significant loss of large neurons was observed in the early, MET phase of DN, whereas there was a leftward shift in the size distribution in the late, NEU phase of DN. There were functional changes taking place in the DRG neurons of *db/db* mice during the MET and NEU stages of DN. DRG neurons from *db/db* mice showed increased sensitivity to CAP as well as increased VGSC excitability in nociceptors in both DN phases. The neurons with increased CAP sensitivity in the MET phase were mainly of medium diameter, expressing the receptors for ATP, AITC and CAP simultaneously (P_2X_3, TRPA1 and TRPV1, respectively), whereas those in the NEU phase were small in diameter, expressing TRPV1 alone or with another receptor. Parallel to the that, in the NEU phase there was also a significant decrease in the sensitivity to AITC, contributed by medium-diameter neurons. Collectively, these results point to distinct populations of neurons being affected by changes in excitability in each of the two DN phases: mostly medium-diameter nociceptors, expressing P_2X_3, TRPA1 and TRPV1 in the MET phase; and small-diameter TRPV1-expressing nociceptors as well as medium-diameter TRPA1-expressing neurons in the NEU phase. To our knowledge, we are the first to perform such

physiological functional and size characterisation affected DRG subpopulations during DN in the *db/db* mouse .

5.5.1. Cell size distribution in *db/db* mice DRG through the course of diabetic neuropathy

In the current study, we show that during the MET phase, there was a significant decrease in the number of large-diameter cells, where their percentage in *db/db* mice was 1.4-fold lower than lean. In comparison, during the NEU phase of DN, diabetic mice had a significantly increased percentage of small DRG neurons (<20 µm) by nearly 1.4-fold (Figure 5.3 B). At the same time, the percentage of medium-diameter (20–30 µm) DRG neurons was reduced as well as that of large-diameter neurons (>30 µm), albeit not significantly. These observations indicate a potential loss of medium-to-large-diameter neurons during late stages of DN, that could possibly have its beginnings in the MET phase.

Our observations are consistent with those made in the NEU phase by Shi et al. (2013) who found 33% neuronal loss in sections of lumbar 5 (L5) DRG of 32/33-week-old *db/db* mice but no change in 5/6-week-old mice. The lost neurons were of medium-to-large diameter (20–40 µm, according to their size groups). This loss is correlated with hypoalgesia and hyposensitivity demonstrated by behavioural experiments [555]. Here, we observe a total of 16% decrease in neurons *from db/db* mice of the same size range (~11% medium + ~5% large). The discrepancy in the percentages noted by us and Shi et al. could be attributed to the difference between the DRGs selected for analysis by them (L5 only) and us (all DRGs along the spinal column length). To our knowledge, theirs is the only study that investigated the changes in the distribution of DRG neuron sizes in the *db/db* mouse and in a T2DN model in general. As summarised in Figure 5.2, most studies on the *db/db* mouse model have focused on investigating the distal (axonal) as opposed to the more central (DRG) changes induced by DN. Therefore, as far as the same mouse model of DN is concerned, the size distribution results observed here agree to some extent with those observed *in vivo*.

The little information available on diabetes-induced morphological abnormalities at the level of DRG from other animal models of DN is controversial. In STZ rat models, a significant reduction in the perikaryal volume (size of the neuronal cell body) is noted in the DRG of STZ rats but only at 1 month of induced diabetes [562]. However, later, Zochodne et al. (2001) report no change in size distribution at 2 months but a significant reduction in the perikaryal volume after 12 months of induced diabetes [563], leading to a shift of the size distribution to the left, reminiscent of the one observed in the NEU phase here. In addition, apoptosis is confirmed to occur in DRG neurons of STZ rats at 1, 3 and 12 months of age, with large neurons being

particularly vulnerable [564]. On the other hand, several studies confirm that loss of DRG or reduced perikaryal volume is evident only in 12-13-month-old rats with chronic DN and not in the early stages [563,565–567]. Finally, in 10-month-old BB/Wor diabetic rats, a severe loss of small- but not large-diameter DRG has been noted [318], contradicting all previous and our own research results. Nevertheless, the vast majority of reports from the T1DN rat models outlined above are in overall agreement with the study in *db/db* mice and our results. It appears that most agree on some form of DRG neuronal loss to occur in late DN stages and predominantly in large-diameter neurons, similar to our observations. The general coherence amongst observations from T1DN and T2DN models suggests that the mechanism underlying DRG loss, at least in the late stages, is common and therefore likely to be driven by a shared mechanism underlying T1D and T2D such as hyperglycaemia. However, it remains unclear whether DRG loss preferably affects specific subpopulations and whether it is due to cell soma shrinkage[563,565,566], dying-back process [567] or apoptosis [318,564], as each has been reported to occur.

5.5.2. Diabetic neuropathy leads to increasing sensitivity to capsaicin in *db/db* mouse DRG neurons

Capsaicin is an agonist for the noxious heat-gated TRPV1 channel, a non-selective cation channel and a marker of peptidergic nociceptive neurons [132]. Physiologically, TRPV1 plays a key role in the generation of thermal hyperalgesia under inflammatory or tissue injury conditions [148,149,568]. During the MET phase of DN, our results showed an increase in the total responses to CAP (Figure 5.5 C) in medium-diameter (20-30 μm) neurons of *db/db* mice (Figure 5.6 A, B) and therefore, a likely sensitisation or increase in expression levels of its receptor TRPV1. We showed that this increase was focused within a specific neuronal subpopulation expressing P_2X_3 (ATP receptor), TRPA1 (AITC receptor) and TRPV1 simultaneously (the 'all(+)' subpopulation) (Figure 5.5 A).

In their comprehensive 2016 study, Li et al. combined transcriptomic, electrophysiological and morphological data from mouse DRG neurons and classified them into 16 molecularly distinct subtypes, each with particular set of proteins expressed [15]. Referencing the known expression ion channels of the identified all(+) subpopulation from our results with their categories revealed that it is likely the 'all(+)' subpopulation is of the C1 cluster – small (<30 μm according to their own size classification), mechano-heat-nociceptors, responding to noxious mechanical and thermal stimuli. Another cluster expressing P_2X_3, TRPA1 and TRPV1 together is the C10 cluster representing the large-diameter (36-42 um) mechanoreceptors [15]. However, this cluster was dismissed as a possibility since the diameter of the "all(+)" neurons from our

experiments did not exceed 29 µm. Behavioural studies in *db/db* mice confirm the presence of mechanical allodynia during the MET phase of DN (week 6-8) [456,507,547,569]. Some report mechanical allodynia to occur together with thermal hyperalgesia (TH) [547,548], consistent with the suggestion of an early stage sensitisation of mechano-heat-nociceptors. Pain of burning quality has also been reported to occur in DN patients, often in parallel to mechanical allodynia, prickling, itching, tingling and 'electric' pain sensation, mainly in the feet and hands [356,570–573]. These clinical observations agree with the early-phase behavioural data from *db/db* mice and the suggested increased overall TRPV1 activity we report in *db/db* DRG neurons.

Alternatively, referencing the all (+) subpopulation with the DRG subtypes defined by Zeisel et al. by using their online search tool for gene combinations (http://mousebrain.org/genesearch.html) revealed that the all(+) neurons are most likely a subpopulation of non-peptidergic neurons (particularly, the "PSNP6" group). In turn, Zeisel et al. reference their own classification with that of Usoskin's and the "PSNP6" group corresponds to their NP3 group of neurons. Usoskin et al. describe the NP3 cluster as non-peptidergic, unmyelinated neurons of small-to-medium diameter (~20 µm). Interestingly, they strongly suggest that the NP3 DRG neurons are most likely involved in inflammatory itch and pruritus (general itching), thus suggestive of an alternative or additional function of the all(+) population in our results. Chronic pruritus has been well-documented as a symptom in DN patients, reported by 3 – 49% of diabetics [574,575]. Albeit not investigated as a behaviour in the *db/db* mouse model of DN, increased scratching behaviour has been reported in STZ rats and linked to DN [576,577]. Itch-specific neurons have been identified to be unmyelinated C-fibre nociceptors expressing the Mas1-related G-protein-coupled receptor A3 (MrgprA3) [578] as well as P_2X_3, TRPA1 and TRPV1 together [14]. Albeit not the only itch-contributing neurons, they were defined as essential and sufficient for pruriception [578]. The mechanism underlying the pruritus phenotype in DN is still not clear, however, mounting evidence shows that pruriception is dependent on the recruitment of TRP channels [579]. Specifically, pruriception was shown to be alleviated and scratching behaviour reduced in mice after pharmacological blockade of TRPA1 and TRPV1 [580]. Furthermore, inflammatory mediators, known to be upregulated during DN, such as interleukin (IL-) 2, IL-4, IL- 13, IL-31 and NGF [256,552,557], have also been shown to sensitise TRPV1 and TRPA1 and contribute to itching and scratching [577,581]. This suggests that the increased activity of the all(+) subpopulation of *db/db* neurons in our results could be driven by their exposure to diabetes-driven inflammatory mediators in vivo and could translate to increase an increase in itch and scratching behaviour related to DN.

From the metabolic abnormalities in the early, MET phase arises progressive structural damage of the nerve fibres – the characteristic feature of the NEU phase [233,234]. This leads to

the well-established damage and reduction of IENF density as well as loss of myelin sheathing and slowing of NCV in animal models and patients [258]. In contrast to the positive symptoms (pain, prickling etc.) characteristic for the MET phase, the accompanying symptoms of the NEU phase are generally negative, i.e. decreased sensory function, numbness, impaired touch and vibration perception and in some patients, mechanical and thermal hypoalgesia [372,520]. It is now well-documented that pain is not just a defining feature of the MET phase and can develop at any stage of DN. In fact, the more severe the sensory deficits become, the higher the risk of developing neuropathic pain episodes is [237]. Moreover, one of the outcomes of IENF damage is bursts of spontaneous activity expressed as pain attacks [237,582]. Although the sensory deficit symptoms of DN have been well-established to originate from structural nerve damage, the mechanisms underlying the paradoxical pain during sensory deficits are still poorly understood.

Here, we report a further increase in total CAP responses, as DN progresses into the NEU phase in *db/db* mice (Figure 5.5 D). Compared to the MET phase, in the NEU phase, this total increase is contributed to mostly by small-diameter cells responding to CAP (Figure 5.6 C,D) and thus expressing TRPV1 (Figure 5.5 B). This points to the sensitisation to CAP spreading to envelop other subpopulations of DRG neurons, likely focusing on small, C-fibre nociceptors. Since the affected population in this phase is of small neurons expressing TRPV1, an increased activity in these cells could translate to increased thermal pain sensitivity *in vivo*.

Virtually all behavioural studies of *db/db* mice show increased mechanical and/or thermal pain thresholds in the NEU phase [430,453,553–557,583], thus contradicting the increase in TRPV1 activity observed here. However, it is worth noting that behavioural thermal sensitivity tests in animals are performed using assays such as the 'hot plate', 'Von Frey' and 'tail-flick' methods. These tests rely on the cutaneous perception of stimuli and the results are, therefore, affected by the integrity of the intraepidermal innervation. Thus, an increase in thermal and mechanical thresholds is perhaps more indicative of subcutaneous IENF damage rather than any central channel alterations. The conflict between *db/db* behavioural results of decreased thermal sensitivity and the increased TRPV1 activity we report could suggest that there is an ongoing central sensitisation, upstream of the subepidermal innervation, by stimuli affecting the DRG and not the terminal ends, causing pain of different types, degrees and frequency that might not be detected via conventional animal behavioural tests.

In humans with DN, thermal hyperalgesia has been reported in some patients with mild DN early in the disease's course [584], whereas the advanced phases of the disease are more characteristic with patients' reports of increased thermal perception thresholds [356,585].

Nevertheless, heat hyperalgesia is still experienced by some DN patients in the advanced DN stages [356]. Our results could provide a possible explanation for this by suggesting that increased TRPV1 sensitisation and/or expression in DRG is responsible for the increased responsiveness to CAP observed and thus might contribute to spontaneous heat pain. Consistent with our predictions, the most common combination reported in patients is numbness coupled with burning pain and prickling [238,356,586], shown by Baron et al. to occur together in 26% of tested DN patients, with the next most common sensory profile being episodic pain attacks (16%) [356]. The increased TRPV1 activity in DRG we report and the decreased sensitivity to mechanical and thermal stimuli reported by behavioural studies in *db/db* mice in the NEU phase confirm that functional changes can keep exacerbating in the soma of DRG neurons despite disconnection from the skin and impaired external inputs detection. Therefore, a continuous central sensitisation of TRPV1 in small C-fibre nociceptors could be a key player in the development of pain, specifically of burning quality, simultaneously with IENF damage and functional sensory deficits in DN patients.

What could lead to the ongoing central sensitisation of TRPV1 in DRG during DN? As mentioned, increased levels of inflammatory mediators have been reported in DRG neurons during early and late stages of DN and shown to sensitise TRPV1 by activating kinases such as PKC [142–144,256,552,557]. Protein kinase C activation by the overproduction of glucose metabolites (such as DAG) is also one of the main mechanisms underlying DN development (see Chapter 1, section 1.3.2.2., page 34). Thus, PKC overactivation by different pathways can lead to the hypersensitisation of TRPV1 in DRG [143,144]. The levels of one inflammatory mediator in particular – NGF, have been reported to be increased in the DRG of *db/db* mice during the MET phase, implied to contribute to the early phase painful phenotype, which could be as a result of NGF increasing TRPV1 sensitisation and expression levels [340]. Furthermore, insulin, which is abundant in the early phase of T2DN, has also been implied in increased TRPV1 activity by enhancing its translocation to the membrane and lowering its activation threshold, thus facilitating its activation by non-painful stimuli [291–293]. Therefore, ongoing central sensitisation of TRPV1 could be a potential source of the thermal hyperalgesia and/or pruritus during the early, MET phase and of the paradoxical spontaneous pain during sensory deficits in the late, NEU phase.

5.5.3. Diabetic neuropathy leads to a decrease in the sensitivity to AITC in the *db/db* mouse DRG neurons

Parallel to the increased sensitivity to CAP, in the NEU phase, we also observed a decrease in the percentage of total responses to AITC in *db/db* mice (Figure 5.5 D). This was caused by a

significant drop in the percentage of medium-diameter DRG neurons (Figure 5.7 B) responding to AITC only (Figure 5.5 B), (expressing TRPA1). The TRPA1 channel is activated by a variety of chemicals, cold temperatures and ROS [163,166,169]. Its role in painful DN pathogenesis has also been well-documented [184,185,587,588]. TRPA1 is activated and sensitised during DN by diabetes-generated endogenous compound, such as methylglyoxal [343,407,589] and conditions, such as oxidative stress [345]. This sensitisation of TRPA1 is linked to the DN phenotype of mechanical allodynia and hypersensitivity. In *db/db* mice, one group recently demonstrated that AMPK activity is impaired in DRG neurons causing an increase in membrane-associated TRPA1 and agonist-evoked TRPA1 currents and mechanical allodynia [507]. These findings contradict our observations of decrease in AITC response rate and hence possible TRPA1 desensitisation and/or decreased expression. However, their report on TRPA1 covers the MET phase of DN in the *db/db* mouse and not the NEU phase, where our observations take place. It is, thus, still inconclusive what changes occur in the activity of TRPA1 in the advanced DN stages in this mouse model.

The rest of the limited literature covering changes in TRPA1 activity during DN is largely focused on the STZ model of T1D and involves considerable controversy. For example, using mice with STZ-induced diabetes, Hiyama et al. demonstrated the involvement of TRPA1 in cold, but not mechanical, hypersensitivity in the early DN stage (2 weeks after diabetes induction). However, in the late stages (8 weeks after diabetes induction), mechanical hyposensitivity and loss of IENFs was shown to occur independently of TRPA1, as TRPA1-KO STZ mice developed a neuropathic phenotype comparable to WT STZ mice [590]. The involvement of TRPA1 in early-phase symptoms of mechanical hypersensitivity in STZ rats was further confirmed by others in the literature [588,591]. In contrast, increased expression levels and activity of TRPA1 in the STZ mice was reported at late stages (5 weeks after diabetes induction) and correlated to itch and hypoalgesia, as revealed by the attenuated neuropathic symptoms in TRPA1-KO mice and after pharmacological blockade of TRPA1 [592]. Further to that, all TRPA1-related findings derived from the STZ-induced diabetes rodents have to be interpreted with caution as Andersson et al. demonstrated a direct activation of TRPA1 by STZ *in vitro* and *in vivo*, where topical administration evoked TRPA1-dependent polymodal hyperalgesia and systemic administration produced acute sensory loss [466]. Thus, at this time the degree and exact mechanisms underlying the involvement of TRPA1 in DN pain and sensory deficits at the different disease phases is still largely uncertain.

With the literature on the subject being scarce, we can only speculate the possible events driving the decrease in AITC responses and therefore TRPA1 activity we see here. It is unlikely that TRPA1(+) neurons acquire TRPV1 and contribute to increased CAP responses, since the

reduction in AITC responses is noted in medium-diameter cells (Figure 5.7 B), whereas the increase in CAP is contributed for by small-diameter cells (Figure 5.6 D). Had there been a functional transformation of TRPA1(+) to acquire TRPV1 activity, we would have seen an increase in medium-diameter CAP(+) cells in the NEU phase as well as small. Therefore, the decrease in AITC responses we report in the NEU phase could be attributed to decreased TRPA1 activity due to desensitisation, downregulation of TRPA1 expression or an inhibitory mechanism triggered by DN, or, alternatively, due to loss of medium-diameter TRPA1(+) neurons. Further experiments in the *db/db* mouse model tracing TRPA1 expression and activity throughout the course of DN are required to confirm or refute these theories.

5.5.4. DRG neurons form *db/db* mice have increased VGSC activity in cells with the OS VTD-response profile

DN has been well documented to alter VGSC activity and expression in sensory neurons[350,424,593,594]. Here, we report an increase in VGSC excitability in *db/db* mice during DN (Figure 5.8). The increase in excitability was focused within the neuronal population of the OS VTD-response profile, previously confirmed to represent nociceptors [499,595]. The increase in OS neurons was evident during both phases of DN, however, in the MET phase it occurred in small and medium nociceptors (Figure 5.9 A,C), whereas in the NEU phase it was focused only within small-diameter nociceptors (Figure 5.9 B,D).

Is it possible to identify the VGSC contributing to the OS increase? DRG neurons express several different VGSCs: Nav1.3, Nav1.6 and Nav1.7 (TTX-S), and Nav1.8 and Nav1.9 (TTX-R). All have been demonstrated to be altered and to contribute to pain during nerve injury and neuropathies [31,360,593,596], including DN [351,352,424,597–600]. Veratridine preferably binds TTX-S channels in their open-state and prevents them from inactivating [492]. Due to the broad selective nature of VTD for TTX-S VGSC over TTX-R and not for any VGSC isoform in particular, it is difficult to correlate the increase in OS-response neurons seen to increased activity in any specific VGSC without further supporting experiments. As each neuron expresses a 'constellation' of ion channels, changed expression levels and/or activity of any of them can shape the net VTD response and lead to an increase in a particular VTD-response profile.

Only one paper investigated the expression levels and activity of a VGSC in the *db/db* mouse DRG during DN. In 2012, Ren et al. assessed the expression levels and activity of Nav1.6 in the *db/db* mouse during the progression of DN. Nav1.6 is a TTX-S VGSC, highly expressed in large-diameter DRG neurons, predominantly involved in transmitting tactile information [601] but also reported to contribute to painful DN in rodent models [58,593]. The research group reported significant and persistent (from 8 to 20 WoA) increase of Nav1.6 mRNA and protein in the DRG

of *db/db* mice. This upregulation was speculated to have been driven by the increased expression of neurotrophins and inflammatory mediators in the DRG during DN. It was also correlated with the demonstrated sustained mechanical allodynia in these mice, further implicating Nav1.6 involvement in painful DN [60]. These conclusions confirmed the increased Nav1.6 expression during DN noted by Craner et al. in the STZ rat [593]. We have shown that a low dose of a Nav1.6 blocker 4.9-TTX leads to a decrease in the percentage of OS DRG neurons in WT mice [595] see Chapter 3). It is then a possibility that the VGSC activity increase in OS neurons seen in the DRG of *db/db* mice here is as a result from increased expression levels of Nav1.6 in the DRG. However, it remains rather unlikely, given that Nav1.6 is predominantly expressed in large-diameter neurons and we only noted an increase in VGSC activity in small and medium neurons. Furthermore, the 4.9-TTX blocker led to a decrease in the percentage of SD neurons as well, therefore had there been an upregulation of Nav1.6 in the DRG of our diabetic mice, we would have expected it to be reflected by an increase in the SD profile as well as the OS. It can therefore be assumed that an upregulation of Nav1.6 is unlikely to have contributed to increase in the OS-response profile seen.

The rest of the TTX-S channels in DRG, Nav1.3 and Nav1.7 upon which VTD acts directly, have both been implicated in DN and pain [360]. Nav1.3 is another TTX-S channel shown to have upregulated expression in DRG neurons, triggered by inflammation and nerve damage [44,45,602,603]. It has also been implicated to have a role in nerve injury-related pain [47,105,604,605], however experiments with a Nav1.3 null mutant demonstrated that Nav1.3 is neither necessary nor sufficient for the development of such pain [606]. In STZ-rat models of painful DN with confirmed mechanical allodynia, Nav1.3 is reported to have lasting increased expression levels, usually accompanied by alteration in other VGSCs as well [352,593,607]. Given the conditional upregulation of Nav1.3, it is likely that the inflammatory environment in the *db/db* mouse has increased Nav1.3 expression levels in DRG neurons, which translated in an increased VGSC activity, as detected by the increase in the OS population.

In rats with painful DN, Nav1.7 has been shown to have robustly upregulated expression in small DRG neurons [58,350,594,608]. We have also previously suggested that increases in the OS VTD-response profile rates could be indicative of increase in the activity of Nav1.7 channel in DRG ([595], see Chapter 3). However, it is unlikely that the increase in OS VTD-profile seen in the DRG of *db/db* mice here is indicative of an increase in Nav1.7 activity or expression levels, as Nav1.7 is expressed in all DRG neurons (i.e. nociceptors and non-nociceptors). Had there been a significant increase in its expression or activity we would have expected to see this reflected by all VTD profiles.

The literature on the role of Nav1.8 in painful DN is controversial. Nav1.8 cooperates with Nav1.7 in DRG neurons, where Nav1.8 is reliant on Nav1.7's hyperpolarised activation to depolarise the membrane sufficiently for Nav1.8's activation [92,93]. Experiments with global Nav1.8KO mice show a compensatory increase in Nav1.7 expression [87]. Indeed, in diabetic animals downregulation of Nav1.8 is accompanied by an increase in Nav1.7 expression [593]. As well as downregulated [353], Nav1.8 is reported to be upregulated in diabetic rat DRGs too [594]. In addition, one study demonstrated that Nav1.8 expression is unchanged in diabetic STZ mice but methylglyoxal, a metabolite formed by excessive glucose, causes post-translational gating modifications of Nav1.8 which, together with causing slow inactivation of Nav1.7, contribute to the painful phenotype of the diabetic mice [342]. It could be the case that Nav1.8 activity or expression is upregulated in DRG of *db/db* mice and contributes to increase in OS, Alternatively, if Nav1.8 expression is downregulated during DN, Nav1.7 is expected to take over and sustain neuronal excitability and OS levels.

Nav1.9, a TTX-R VGSC, has also been demonstrated to be upregulated in diabetic DRG neurons from STZ rats with allodynia, where the increase of Nav1.9 mRNA and protein was observed in neurons of all sizes. The increase is greater in large-diameter neurons, whereas its expression in control DRG was primarily by small and medium-diameter neurons [593]. It is therefore unlikely that Nav1.9 solely contributed to an increase in the OS-VTD profile in small DRG neurons of *db/db*.

From all the information above, it is most likely that more than one VGSCs could have contributed to the increased OS responses we saw in *db/db* mice. Moreover, other classes of ion channels, such as VGKC and VGCC, shape the net Ca^{2+} signal response to VTD and so, any expression or functional changes in them can alter the VTD-profiles produced and contribute to the increase seen in OS profile. For example, gabapentin, a widely-prescribed VGCC blocker is effective at alleviating painful symptoms in DN patients [374,609]. Thus, functional and expression levels in VGCC and other channels should be investigated by further functional and biochemical assays.

5.5.5. Diabetic neuropathy leads to excitability changes in specific neuronal subpopulations

A summary of the known properties of the neuronal subpopulations affected and the changes observed is presented in Table 5.2 and Figure 5.11. In the MET phase of *db/db* mice, the net effect was an increase in CAP-responsive cells (Figure 5.5 C) and increase in VGSC excitability (Figure 5.8). The additional CAP(+) neurons were mostly medium diameter nociceptors (Figure 5.6 A,C) expressing TRPV1, TRPA1 and P_2X_3 together (i.e., of the "all(+)" population)

(Figure 5.5 A). In the same phase, the increased VGSC activity was due to increase in the percentage of OS neurons which were also small- to medium diameter nociceptors (Figure 5.9 A,C) of the all(+) subpopulation (Figure 5.10 A). Thus, there is a considerable overlap between the properties of the subpopulations affected by increased CAP and VGSC sensitivity in the MET phase in *db/db* mice.

In the NEU phase, the net effect was an increased number of CAP-responsive cells (Figure 5.5D). Here, the additional CAP(+) cells were focused within a subpopulation of small-diameter nociceptors (Figure 5.6 B,D) expressing TRPV1 (Figure 5.5 B). Also, the increased VGSC activity was attributed to additional OS-profile cells also of small-diameter (Figure 5.9 B,D) and also expressing TRPV1 (Figure 5.10 B). Therefore, the subpopulations affected during the NEU phase in *db/db* mice also overlap in their properties.

The features of the increased VGSC excitability subpopulations largely match those of the cell subpopulations identified with hypersensitisation to CAP in each phase. This could indicate a possible correlation between the increased TRPV1 sensitivity and VGSC excitability. I.e., the same neuronal subpopulations are affected by these two phenomena in DN in the *db/db* mice: predominantly medium-diameter nociceptors expressing P_2X_3, TRPA1 and TRPV1 together in the MET phase; and small-diameter nociceptors expressing TRPV1 in the NEU phase. In addition, the drop in AITC-responsive neurons in *db/db* mice during the NEU phase was attributed to a subpopulation of medium-diameter neurons of the OS profile expressing TRPA1. Since their parameters differ from those of the neuronal subpopulation affected by CAP(+) increase, it is likely that this is a separate effect on a different neuronal subpopulation.

Table 5.2. Summary table of the identified properties of the DRG neuronal subpopulations affected in db/db mice in the metabolic (MET) and neuronal (NEU) phases of diabetic neuropathy. Net effect for each phase shown in the bottom row. Green background denotes increase, whereas red background denotes a decrease observed. VTD = veratridine; VGSC = voltage-gated sodium channel; OS = oscillatory VTD-response profile.

Phase	MET	NEU		
Expression of noc. receptors	P_2X_3+TRPA1+TRPV1 (all(+))	TRPV1 (alone or in combination)	TRPA1	Population of neurons affected
Size (soma diameter)	small, mostly medium	small	medium	
VTD-response profile	OS	OS	OS	
Net effect	↑ CAP responses ↑ VGSC activity	↑ CAP responses	↓ AITC responses	
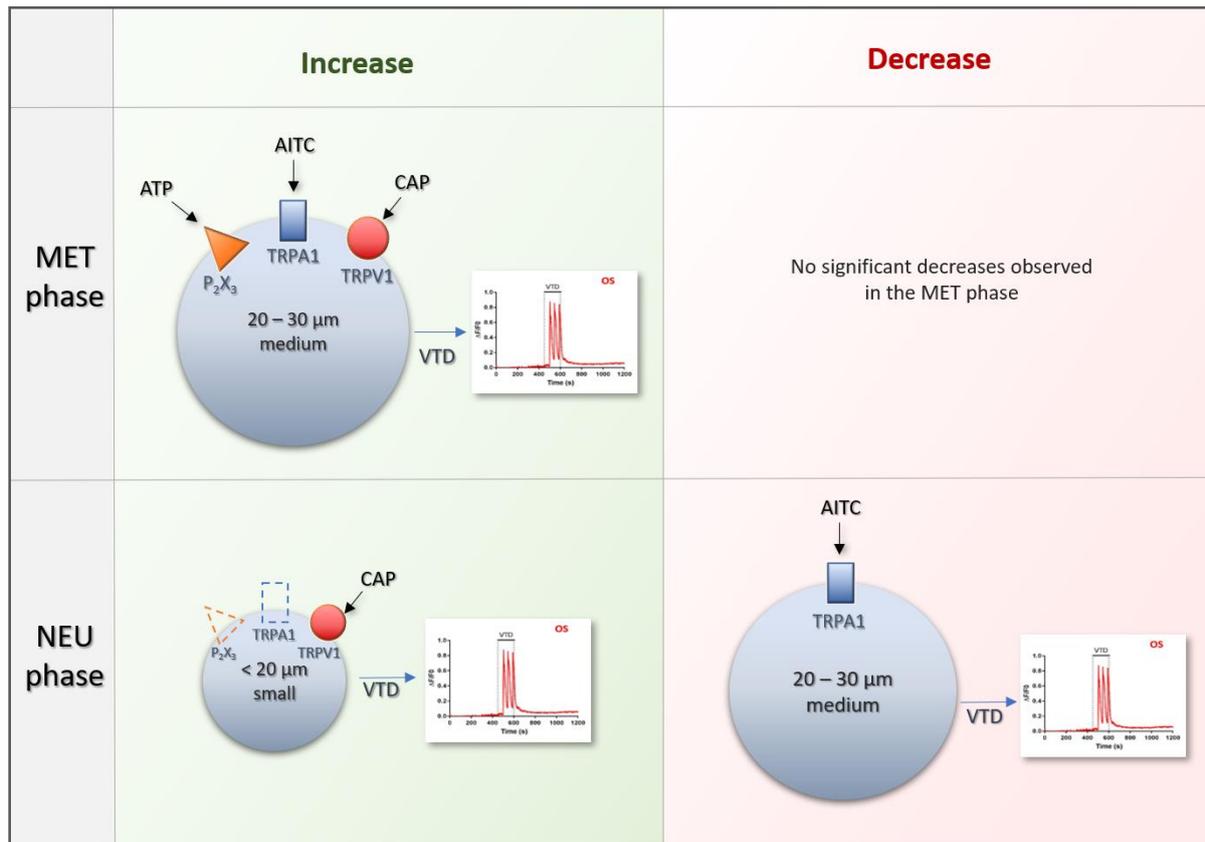
Figure 5.11. Graphic summary of the affected subpopulations of DRG neurons and their known properties in db/db mice in the metabolic (MET) and neuronal (NEU) phases of diabetic neuropathy. *Dotted outline of receptor channels mean that the neuron might express both, one or none of them together with TRPV1. Green background denotes increase, whereas red background denotes a decrease observed.*

Further experiments including tracing ion channel expression levels throughout the course of DN in the *db/db* mouse are needed in parallel to VTD-Ca^{2+} imaging to further characterise the identity of the specific subpopulations raising DRG neuronal excitability and potentially shaping a painful phenotype. The next and final chapter discusses further limitations and recommendations for future experiments to build on the results presented in this thesis.

CHAPTER 6

LIMITATIONS & FUTURE DIRECTIONS

CHAPTER 6: LIMITATIONS & FUTURE DIRECTIONS

Diabetic neuropathy is a debilitating condition significantly affecting the quality of patients' life, especially if accompanied by painful symptoms. Despite decades of animal and clinical research, the multifactorial mechanisms driving the positive and negative sensations caused by DN are still poorly understood. There are no disease-modifying or reversing drugs approved and DN continues to represent a therapeutic challenge [230]. The only successful strategy remains diabetes prevention via glycaemic control through diet and lifestyle management. To date, several treatments have been approved for the symptomatic relief of painful DN, however first- and second-line drugs target pain pathways in the CNS and produce modest analgesia at the cost of serious, potentially fatal side effects [376].

In recent years, research into novel analgesic agents for the treatment of pain has turned to targeting the PNS to avoid serious CNS adverse effects whilst improving drug efficacy. Several promising targets have been identified, the most popular being PNS-expressed ion channels implicated in pain for example VGSCs $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ [33,610]. Novel ion channel blockers or activators drugs are in development and ongoing pre-clinical and clinical studies [393,394,399–401] (also see Table 1.5). However, a common issue is the poor translation of drug efficacy from the animal model to the DN patient with most novel pharmacotherapies failing clinical trials. For example the failed translation of aldose reductase- and PKC-inhibitors efficacy from the STZ rat model of DN to DN patients [247,249]. To design better targeted drugs with minimal off-target effects, a better understanding of the mechanisms driving DN pathology and its modelling is required as well as a simple, efficient, medium-to-high throughput assay able to distinguish nociceptors from non-nociceptors.

Therefore, the aim of the current study was to aid this process by assessing the neuronal excitability changes occurring during DN in DRG neurons *in vitro* and from a diabetic mouse model. We expected that neuronal excitability shifts will be present at different degrees in the different subpopulations of neurons within a mixed DRG population. Therefore, first, the VTD-Ca^{2+} imaging assay was validated for its ability to discriminate nociceptors from a heterogeneous neuronal population and for its use as a potential drug screening tool, as outlined in Chapter 3. The next two chapters described following experiments exploring the application of the VTD-Ca^{2+} imaging assay in combination with other nociceptive agonists to characterise excitability changes during DN in DRG neurons from healthy mice cultured under *in vitro* hyperglycaemic conditions (Chapter 4) or DRG neurons derived from the diabetic *db/db* mouse model of DN (Chapter 5).

In this chapter, discussion will encompass advantages and future perspectives for the VTD-Ca^{2+} imaging assay regarding its optimisation and application. I will discuss the main limitations of our study with focus on the *in vitro* DN experiments and how realistic it is to replicate DN in a dish as well as using DN mouse models for assessing DRG excitability changes. Finally, the key findings and implications of the experiments will be briefly discussed with recommendations for future research.

6.1. Veratridine and Ca^{2+} imaging as a tool with multiple applications

This study began with the aim of validating the previously generated results by our lab for the use of VTD-induced Ca^{2+} responses as broad markers for DRG neuronal subpopulations. A detailed characterisation of the VTD-responses was carried out previously by our lab [499]. Here, using DTA mice with genetically ablated nociceptors, I demonstrated a strong link between the OS VTD-response profile and nociceptors, whilst non-nociceptors were associated with the SD profile (Chapter 3, Figure 2). Thus, the use of the VTD with Ca^{2+} imaging as a tool for assessing neuronal excitability in specific neuronal subpopulations was confirmed and the "VTD- Ca^{2+} imaging assay" was established.

6.1.2. Advantages

The VTD-Ca^{2+} imaging assay's main advantage is that it allows for the efficient screening of a heterogeneous population of neurons. Thus, the VTD response profile pattern generated integrates all responses present in the population, which would give a more realistic idea of what a drug's effect would be in a physiological environment. This contrasts with patch-clamping-based *in vitro* assays, where the investigated effect might not be detected as a result of the low-throughput screening efficiency. The ability to cover a heterogeneous population of neurons with a single assay screen is also advantageous over cell-line-based screening methods. Often the first choice in novel drug characterisations, heterologous expression systems are used to express the target protein against which drugs are screened. However, the absence of the full set of ion channels, exchangers and receptors can hide off-target effects of the compound investigated. The VTD-Ca^{2+} imaging assay is able to detect specific excitability changes in distinct neuronal subpopulations in each screen. Therefore, the development of the assay as a medium-to-high throughput screening tool in primary sensory neurons will aid in the complete characterisation of a drug's efficacy and safety profile, as it would appear in a heterogeneous population *in vivo*.

Apart from its functionality, another advantage of our system is the wealth of data generated with minimal resources and in a relatively short amount of time. For example, in our experiments with *db/db* mice, over 6 weeks of imaging experiments, a total of 10 313 neurons were imaged from 24 mice, giving an imaging efficiency of approx. 430 neurons per mouse. Such recording capacity ensured each animal made a maximum contribution to the study with minimal waste of cells. Moreover, the large numbers of neurons for each mouse contributed to the validity of the observed phenotype.

Our screening productivity is comparable to standard Ca^{2+}-imaging of DRG neurons. However, here, we employ VTD as a main neuronal exciting agent instead of a high-potassium solution (KCl), most commonly used to excite cells in Ca^{2+} imaging protocols. High-potassium solutions directly depolarise the membranes of all neurons in a DRG population, thus activating them simultaneously [484]. Here, due to VTD's action on VGSCs, the responses generated are shaped by the constellation of ion channels, receptors and exchangers expressed by each neuron. Therefore, neuronal responses are more nuanced and thus more informative compared to the binary "yes" or "no" information provided by responses to KCl.

Imaging Ca^{2+} responses to VTD and agonists in neurons provided us with basic information of the response rates to the drugs administered. Moreover, each recording also yielded a vast amount of data available for analysis, including measuring the amplitude of Ca^{2+} responses, time to peak and AUC. Each recording also provided an image of the neuronal population allowing for the estimation of neuronal diameter and subsequent analysis of cell size distribution. Such wealth of data allows for the in-depth multifactorial characterisation of the responses from each neuronal population imaged and hence the building of a more complete picture of the neuronal phenotype under investigation.

6.1.3. Limitations & Recommendations

Nevertheless, a major limitation of the VTD-Ca^{2+} imaging assay is the data analysis component. The diversity and abundance of information generated demands comprehensive analysis across several softwares. At present, this analysis is manual, with each neuronal trace of responses analysed individually. The process is laborious and time-consuming and manual data handling can be subject to conscious or unconscious bias.

Finally, AUC measurements are performed automatically by a software according to a method outlined in Chapter 2: Materials & Methods, section 2.2.3.1, page 70. Although facilitating the analysis, the application of the same estimation formula on each trace does not produce a completely realistic picture of the AUC. The formula computes the AUC of a response to a

drug based on a pre-specified baseline, which is the average value of the signal during the 30 seconds prior to adding the drug. Assuming a perfectly steady and horizontal baseline, areas under the curve calculated will be near 100% accurate for all traces. However, occasionally, even the slightest fluorescent dye bleaching during an experiment can cause a small deviation of the resting calcium baseline and transiently or steadily shift it. If this shift is within the 30 seconds specified for baseline, they are incorporated into the baseline calculations and lead to gain or loss of estimated AUC depending on whether the trace has been shifted upwards or downwards, respectively. Albeit infrequent, such cases occur and can "pollute" the estimated overall AUC value averaged for the whole neuronal population.

Therefore, perhaps the optimisation that this assay would benefit from the most, is automation of the data analysis process. Automating the calculation and tabulation of parameters will significantly reduce human error and the time taken. Furthermore, it would allow for the integration of a more complex formula for the AUC estimation which would account for potential deviations in the baseline of the Ca^{2+} signal.

To develop a full picture of the usefulness of VTD-response profiles, the assay needs to be further developed. For example, at present, the assay has only ever been applied to mouse DRG neurons. Further characterisation using rat and human DRG (hDRG) would elucidate differences in DRG excitability between species. Transcriptomic analysis of rodent and hDRG have demonstrated different levels of expression of some pain-related ion channels. Chang et al. showed that Nav1.7 had higher expression levels in hDRG than mouse DRG, whereas mouse DRG had higher expression of Nav1.8 [611]. Therefore, the VTD-response patterns produced by hDRG might be significantly different from that of rodent DRG, highlighting potential interspecies functional discrepancies. This could provide insights into why certain analgesics have shown efficacy in rodents but not in humans. Adapting the VTD-Ca^{2+} imaging assay to examine responses in hDRG would, therefore, benefit drug screening programmes.

The VTD-Ca^{2+} imaging assay can also be advanced by conducting more experiments with genetically modified animals (e.g., KO models of certain VGSCs, or knock-in for GCAMP Ca^{2+} sensors to allow *in vivo* imaging) as well as ion channel-blocking or activating drugs of various specificities. This would allow for the in-depth interrogation of each VTD-response profile and the contributing ion-channel(s) that shape it. This is of particular importance for clarifying the identity of the ID and RD profiles, which, although underrepresented, have a very distinct VTD-response shape of a single, transient peak.

Finally, experiments using DRG exposed to an inflammatory or injury-like *in vitro* environment (e.g. inflammatory soup-induced [612]) would aid in the characterisation of the population of

"silent nociceptors", believed to be "unsilenced" under such conditions [3,4]. It is hypothesised these neurons express predominantly TTX-R channels [499], but it is yet to be determined what VTD-profile they will produce once activated.

6.1.4. Applications

The confirmation of the VTD-Ca^{2+} imaging assay as a tool able to discriminate excitability changes between nociceptors and non-nociceptors has opened many doors for its potential uses. Its application as a drug screening assay was tapped into during the experiments with pharmacological channel blockers presented in Chapter 3. They demonstrated that the VTD responses produced by a certain drug can be informative of its specificity to nociceptors and degree of side effects on non-nociceptors. Moreover, we demonstrated that the assay can be used for investigating the effect of combining lower doses of 2 (or more) drugs to improve efficacy and minimise off-target effects. Combination therapies have been undergoing clinical trials and, in many cases, have proven more successful than individual ones and placebo [613–618], however the majority are still targeting the CNS. It would be interesting to see whether such approach can be applied to novel molecules targeting PNS-expressed ion channels. This can facilitate the analgesic drug development process, whereby failed ion channel-modulating agents can be resurrected into combination therapies instead of developing novel ones. This makes the VTD-Ca^{2+} imaging assay a suitable platform for pre-clinical drug tests on primary cultures of heterogeneous neurons. Furthermore, by using a primary culture of human DRG (hDRG) neurons, this could help bridge the translational gap between pre-clinical and clinical research. Thus, VTD-Ca^{2+} imaging can be used to assess the efficacy and safety of channel blockers and activators downstream of the VGSC activation step in the process of AP generation and propagation. However, a limitation to bear in mind is that, as a drug screening tool, the VTD-Ca^{2+} imaging assay would not be able to provide information on the efficacy of drugs inhibiting initial transducing events, e.g., blockers of TRP channels. This is because signal transduction is a step prior to VGSC activation, where VTD exercises its action.

The VTD-Ca^{2+} imaging assay can also be applied for the characterisations of DRG excitability phenotype in the context of different neuropathological conditions. In Chapter 5, I described and discussed experiments with DRG from the *db/db* mouse model of DN. In a DN setting, the assay was able to detect excitability changes in nociceptors expressed as increase in the percentage of neurons with the OS profile (Figures 5.8, 5.9, 5.10). The assay can be applied in other DRG-sensitisation models, such as paclitaxel-induced peripheral neuropathy [619] or inflammatory conditions [612]. It would also be interesting to compare VTD-response profiles produced by a T1DN rodent model, such as the STZ-induced model of DN, to those generated

by the *db/db* mouse. A comparison of this nature can elucidate DN-driving mechanisms in the different induced diabetes animal models and provide insights into the discrepancies seen in neuropathic complications between the two diabetes types.

Finally, the VTD-Ca^{2+} imaging assay can contribute for the validation of stem cell differentiation protocols [620]. Research efforts have been focused into the development of functional DRG cultures derived from iPSC for the study of peripheral neuropathies [621]. How well such novel cultures compare to natural human DRG could be determined by comparing the VTD-response profiles both will produce. Thus, one can estimate whether the expression of the constellation of ion channels in the membrane of stem cell-derived neurons is comparable to the physiological, natural DRG neuron.

6.2. Modelling diabetic neuropathy in a dish – a realistic goal or an unfeasible challenge?

It is common practice when investigating the pathology of a disease to replicate its aspects on cultured cells *in vitro* in order to study the mechanisms in a controlled and isolated environment. *In vitro* high glucose conditions have been used as a way of modelling DN extensively and have largely contributed to our knowledge of the mechanisms underlying diabetic neuropathy and pain [318,420–425]. In the experiments outlined in the present study, *in vitro*-induced hyperglycaemia did not produce the expected increase in neuronal excitability as increased VGSC activity [351,424,502] or increase in responses to nociceptive agonists implied elsewhere [506,510,525], as the response rates remained comparable to control (Figures 4.3, 4.4). It is difficult to explain this outcome, but it might be related to differences between the age and type of the DRG used, differences in glucose concentration and exposure time used by me and others, as well as other variables in the culture conditions.

Our *in vitro* results contradict virtually all previous studies using *in vitro* high glucose conditions, which report increase in CAP-evoked currents or VGSC activity or expression[351,424,456,502,506,510,531,622]. As discussed in detail in chapter 4, the possible reasons for these discrepancies are the extensive use of embryonic or neonatal DRGs by others [351,424,502,506] as opposed to adult DRG used here; the use of rat DRG [349,351,502,510] or immortalised rat DRG cell line [622] rather than mouse DRG used here; and the selection of specific DRG for culturing and experiments, e.g. lumbar DRGs [502,506,510] or only those of 20-25 μm [502] as contrasted with the isolation of DRG from the full spinal column length here, which might account for a potential "dilution" of a high glucose-driven excitability changes.

With our *in vitro* experimental design differing substantially in one or more aspects form others, we can only speculate how the conditions we introduced could be amended to achieve a high glucose-driven increase in DRG excitability. One thing to consider can be the degree and duration of hyperglycaemia exposure itself. Albeit well-supported by studies investigating its acute effects, the extracellular glucose concentration of 50mM used here might not have been potent enough to produce an effect on neuronal excitability represented by changes in VGSC activity detected in the VTD-response profile patterns generated. Also, in the present experiments, glucose concentration was increased to hyperglycaemic levels acutely after 24h and glucose in the medium was not replenished daily to maintain high concentration. DRG neurons have higher energy requirements and metabolic rates compared to other cells and tissues [318,623]. Their energy demands are likely to be even higher than normal for the first 24h following dissociation for culture as the neuron's metabolic rate is altered to support axonal regeneration [624]. Hence, cultured neurons might metabolise the available sugar abundance and re-adapt to control glucose levels in the 24 hours before experiments begin. Although, to our knowledge, this does not seem to be a practice in similar studies, daily replenishing of glucose in the medium might be an avenue worth exploring for Ca^{2+} imaging experiments with neurons cultured for more than 24h.

Another possible explanation for the absence of a hyperexcitability phenotype in our experiments is the lack of the full spectrum of DN aspects driving neurodegeneration. Historically, DN's pathology was largely believed to be "glucocentric". This is true to a large extent, as high blood glucose levels are toxic for the vasa nervorum, the neurons themselves and the Schwann cells supporting them. However, it is now known that glucose-independent mechanisms work in synergy with hyperglycaemia-driven processes to produce DN and pain. The major pathways were introduced in detail in Chapter 1: Introduction, section 1.3.3. One of the main glucose-independent triggers of neuronal damage in a DN setting is dysregulated insulin signalling. Insulin is an important neurotrophic factor for neuronal growth and is often added to neuronal culture media for trophic support [623]. Poor insulin availability, as in T1D and late stages of T2D, as well as insulin overabundance, like in early T2D, both contribute to neuronal health damage, independently of hyperglycaemic conditions [286,296–298,303]. For the experiments outlined in Chapter 4, the DRG cultures were not supplied with any insulin. Thus, the *in vitro* DN conditions we designed were closer to replicating those in T1DN and late T2DN, where neurons are in an environment with excess glucose and little insulin. Supplementation of the DRG culture with excess insulin together with high glucose levels might have brought culture conditions closer to those in early T2DN and might have produced detectable neuronal excitability changes. However, adding insulin to the culture at any concentration would still

introduce its trophic support effects to the cells, which may play down or negate hyperglycaemia-induced neuronal damage. In fact, it is possible that the large quantity of available insulin, at least in the initial stages of T2D, is responsible for maintaining neuronal health and, to an extent counteract the neurodegenerative effects of hyperglycaemia during T2D. This might explain the less aggressive symptomatic expression of T2DN compared to T1DN in the early stages of diabetes [233,252,253]. Insulin's production cessation due to β-cell destruction in T2DN advanced stages might then be responsible for the rapid progression of DN symptoms in the later stages of T2DN. This idea is reinforced in studies by Huang et al. who cultured mature sensory neurons with high insulin and normal glucose levels and reported increased hexokinase activity and ATP synthesis amongst other events. Hexokinase drives the first committed step of glycolysis, hence its upregulated activity indicates augmented glucose metabolism and energy generation [625]. In an *in vitro* hyperglycaemic setting, this could ameliorate the effects of high glucose on the neuronal health and thus mask a neuropathic phenotype. In another study, the group demonstrated that maintaining STZ-rat-derived DRG neurons in high glucose and supplementing them with insulin led to improved mitochondrial function. Furthermore, administering low dose insulin injection to STZ rats improved their sensory phenotype but without affecting hyperglycaemia in the rodent [626]. Their results call for caution when considering supplying *in vitro* cultures with insulin. Despite the growing evidence of its role in the DN pathogenesis, due to the hormone's nature it would be challenging to tease out its beneficial from neurodegenerative effects *in vitro*. Nevertheless, neuronal IR is a well-evidenced feature of T2DN [627] and future research into modelling DN *in vitro* should explore its adequate implementation in a culture model.

NGF is another trophic factor, considered an essential component of neonatal DRG culture protocols [628–630]. NGF expression is also upregulated in animal models of injury [631,632] and inflammation [340], including DN [456,531,567,633]. In animal models of both T1DN [531,633,634] and T2DN (*db/db* mice [456]), increased levels of NGF are detected and correlated to increased pain sensitivity. Although the mechanisms behind its increased expression are unclear, it is speculated that NGF levels increase during DN as a neuroprotective response to the metabolic stressors accompanying the disease [456]. From all of the above, neurotrophic factors such as NGF and insulin might be key drivers in the pathogenesis of DN and hence valuable components to include in an *in vitro* model, however should undergo careful consideration before being implemented and resulting outcomes should be interpreted with their trophic effects in mind.

Other components of DN pathology that have been directly introduced in *in vitro* DN models include pro-oxidants for replicating oxidative stress (Purves et al 2001), methylglyoxal for

direct glucose toxicity modelling [432] and oxidised LDLs to directly model hyperlipidaemia [434]. Although elucidative of the mechanism via which each might contribute to DN in isolation, no studies have yet modelled an *in vitro* DN environment incorporating several components together. Such an approach might pose difficulties, since an environment with a number of variables is less susceptible to tight control and manipulation. Nevertheless, identifying the optimal number and types of components able to be successfully and sustainably introduced in an *in vitro* neuronal environment might allow for a modelling of DN in a dish closer to that in humans.

Finally, a limitation of extending culture time to 6 days is the reduced number of neurons available for imaging with time. Twenty-four hours after plating, neurons start extending neurites and by day 3 in culture a dense axonal network is present [505]. In addition, proliferation of non-neuronal cells such as fibroblasts, satellite glia and SCs begins. This can obstruct neurons from being easily identified and selected for imaging experiments. The reduced availability of neurons as time in culture progresses inevitably leads to fewer cells imaged per day due to the limited number of coverslips and animals (Table 4.2, Figure 4.2). The reduced number of available neurons and hence ROIs of sufficiently good clarity for recording can be an important source of data variability. Unfortunately, to our knowledge, controlling neurite outgrowth without aggressive treatments that might introduce a phenotype is unfeasible. Future experiments might benefit from exploring compounds preventing minimising the amount of non-neuronal cells proliferation in culture, such antimitotic agents, e.g. 5-fluoro-2'-deoxyuridine [635,636] although caution should be taken when interpreting data from cultures containing additional elements. Alternatively, the restricted number of available neurons after 2 or more days *in vitro* can be compensated for by increasing the total sample size for the time point to ensure statistically valid quantity of data.

6.3. Studying excitability of DRG neurons derived from diabetic animals

The second big aim of the present study was to assess for excitability changes present in DRG neurons derived from diabetic *db/db* mice. This was also an opportunity to explore the application of the VTD-Ca^{2+} imaging assay as a tool for characterising the excitability phenotype of DN in sensory neurons. The VTD-Ca^{2+} imaging assay detected changes in VGSC excitability expressed as an increase in the OS profile in both phases of DN in *db/db* mice (Figures 5.8, 5.9, 5.10). Nociceptive agonists revealed an increase in the sensitivity to CAP which persisted and increased into the late, NEU phase of DN (Figures 5.5, 5.6, 5.7). We speculated it is likely that the functional changes observed start in a subpopulation of small-

to-medium nociceptors and later affect small, TRPV1-expressing nociceptors as DN progresses (Table 5.2, Figure 5.11). These results implicate specific neuronal subpopulations affected at early and late stages of DN and could serve as a foundation for further research into them and the molecular mechanisms driving excitability changes. Their further characterisation will, in turn, aid the identification of specific targets for the development of pain-alleviating or DN-modifying therapies. In addition, this is the first study to investigate physiological excitability changes in the *db/db* mouse in both phases of DN.

The *db/db* mouse is perhaps the most widely used model of T2D due to the collection of advantages they bring over other diabetic models (Table 6.1). The robust development of diabetes and subsequent complications follow closely the natural progression in human patients, making them also one of the most preferred models of DN [453]. Perhaps the main advantage of this model is that DRG neurons are chronically exposed to neurodegenerative DN conditions that go beyond hyperglycaemia, including oxidative stress, hyperinsulinemia and IR, inflammation and dyslipidaemia. We and others [431,637] have demonstrated that isolating and culturing DRG from *db/db* mice in standard medium and conditions produces a DN phenotype, hence allowing for their studying without supplementing the culture with additional elements complicating data interpretation.

Table 6.1. List of some advantages of the T2D db/db mouse model of diabetic neuropathy.

Advantages	References
Parallels human T2D and pathogenesis to a very high degree: • **Persistent hyperglycaemia** • **Obesity** • **Dyslipidaemia: persistent high blood cholesterol and triglyceride levels** • **Insulin resistance** • **Inflammation** **Mimics human DN progression qualitatively and temporally:** • **Hyperalgesia and allodynia 8-12 weeks** • **Thermal and mechanical hypoalgesia after 12 weeks*** • **Decreased NCV** • **Loss of large myelinated fibres** • **Axonal atrophy and dystrophy** • **Loss, shrinkage and breakdown of myelin**	456,458,541,549,666,671
T2D phenotype validated with anti-diabetic drugs	672–674
Shown to exhibit a pre-diabetic state	675
Develop severe hyperglycaemia and display advanced stages of the disease without the need of any external diet or pharmacological interventions that could disturb the natural phenotype	418,453,676

Table 6.2. List of some disadvantages of the T2D db/db mouse model of diabetic neuropathy.

Disadvantages	References
Short lifespan (30 – 40 WoA)	668
Leptin signalling impairments are much less severe in human patients	669
Leptin-based mouse models are often infertile, and generating significant numbers is time consuming.	447
Impairment of leptin signalling might have indirect consequences on other biochemical pathways	669,670
DN phenotype depends on strain used (C57BKS vs C57BL/6)	453,459,671

The *db/db* mouse does not come without some disadvantages, outlined in table 6.2. Perhaps the main disadvantage, and a limitation of these experiments, is that the *db/db* mouse strain, like every genetically modified disease model, presents with lower genetic differences thus producing lower variability in DN onset and/or progression. This is in contrast to humans, where different DN patients present with different degrees and timings of functional and structural changes [638]. Despite that, however, a similar trend is observed in both the *db/db* animal model and DN patients, whereby the early phase of DN is regulated by complex metabolic drivers producing contradicting behavioural reports in the *db/db* mouse (see table 4.2) and varying degrees of sensitivity changes in patients [639]. On the other hand, the sensitivity loss and structural changes during the late DN phase is well documented in both, animal models [252,453,640] and humans [230,234,639]. This perhaps implicates the *db/db* mouse as a more suitable model for investigating the late, structural effects on sensory neurons during DN. However, studies reporting positive functional and behavioural changes in the animal model during early phase DN could still benefit a subpopulation of patients experiencing similar events.

DN is characteristic with its stocking-and-glove distribution, affecting the toes and feet first, followed closely by the fingers and hands [641]. Each dorsal horn afferent nerve is dedicated to innervating a specific area of skin or organ, i.e., its dermatome. The nerves innervating the areas affected first by DN are lumbar spinal nerves 4 and 5 (L4 and L5) for the toes and feet, and cervical spinal nerves 6, 7 and 8 as well as sacral spinal nerve 1 (C6, C7, C8 and S1, respectively) for the fingers and hands. Often, studies exploring functional and structural changes in the nerves during DN would isolate only DRG from L4/L5, noting functional and structural alterations [254,337,544–546,637,642,643]. In the present study, DRG from the whole length of the spinal column were isolated for studying DN. Assuming that the DN phenotype is concentrated largely in the lumbar DRG, then dissection of all DRG indiscriminately could lead to "dilution" of the neuropathic phenotype and milder excitability changes being observed as opposed to isolating specific DRGs. Future studies can explore this concept by preparing DRG cultures from specific spinal column segments of nerves evidenced to be highly affected in DN, such as the lumbar nerves. This would provide a focused investigation of DN effects on specific organs and body areas and should be interpreted as part of the bigger picture of the DN pathogenesis. It would be interesting to see a meta-analysis of studies using all DRGs as opposed to specific ones from diabetic animals and whether the results obtained via these two different approached differ substantially.

One potential source of weakness in this study, which could have affected the neuronal excitability phenotype, is the decision to culture dissociated DRG from *db/db* mice in a

standard environment. That is, isolated DRG were not maintained in high glucose conditions, neither was insulin, NGF or other DN-characteristic elements added to the medium. Upon being faced with this choice when designing the experiments, we addressed relevant literature and established that virtually all studies using DRG from animal models of DN did not alter the culture medium to reflect DN conditions. Hence, the decision was made to undertake the same approach with the present experiments and observe the phenotype produced. Had it been the case that no significant differences were noticed between control and diabetic animal neurons, then experimental design would have been modified to introduce a modified DRG medium reflecting DN conditions. Since excitability changes were observed early in the course of the experimental period, it was agreed to proceed with the current method. However, it would be interesting to see whether maintaining neurons in a high glucose/NGF/insulin medium prior to experiments would produce a starker phenotype to the one observed here.

The scope of this study was limited to Ca^{2+} imaging experiments and some analysis of cell size and distribution. These were isolated experiments, unsupported by our own behavioural, structural, or other data. Further experiments are needed to fully understand the mechanisms underlying the DN-induced changes in neuronal excitability observed here. Is the increase in excitability in nociceptors due to altered expression and/or activity levels of a particular VGSC? Is the increase in sensitivity to CAP due to increased TRPV1 expression or simply enhanced activation? Immunohistochemistry experiments tracing changes in expression levels of ion channels during the MET and NEU phases of DN implicated would contribute to answering these questions. Are neurons from diabetic mice shrinking or apoptotic? Quantitative analysis of neurons' apoptotic tendencies throughout DN progression (e.g., using nuclei-labelling techniques) can provide insights. Further experiments would add to the physiological data provided by this research and contribute to a complete study on the DN phenotype progression in the *db/db* mouse sensory neurons.

6.4. CONCLUSION

The journey of this project started with the establishment of the VTD-Ca^{2+} imaging assay as able to discriminate between nociceptors and non-nociceptors via the VTD-response profiles generated by DRG neurons. It was then taken forward towards different applications including as a screening platform for ion channel blockers, individually or in combination, assessing their efficiency and safety. The application of the VTD-Ca^{2+} imaging assay as a tool for characterising neuropathological conditions was explored in the context of diabetic neuropathy *in vitro* as well as *in vivo*. By employing VTD-Ca^{2+} imaging as well as nociceptive agonists, we demonstrated that diabetic neuropathy produces excitability changes in distinct

subpopulation of DRG neurons from T2D *db/db* mice in the early, MET and late, NEU phase of the disease's course. This is the first detailed physiological characterisation of the diabetic neuropathy-driven excitability changes at the different phases in the T2D *db/db* mouse model. Identifying distinct neuronal subpopulations affected during DN could aid in their better targeting by novel pharmacological interventions developed. Finally, the VTD-Ca^{2+} imaging assay can have multiple applications, including as a screening platform and disease and iPSCs characterisation tool. In the future, this work can take several directions, for example focusing on the improvement of the VTD-Ca^{2+} imaging assay and its analysis process; and/or further characterisation of the *db/db* mouse model of DN and the identity of the neurons most affected during the different stages. Overall, this research hopes to eventually contribute to elucidating the complex mechanisms underlying diabetic neuropathy and aid in bridging the gap between pre-clinical and clinical research for treating this currently untreatable disease.

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