UNIVERSITY OF NOTTINGHAM

Alphavirus Infection and Nociception: Differential Effects on Primary Mouse Dorsal Root Ganglia

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Thesis submitted to the University of Nottingham for the degree of Master of Research

October 2021

ABSTRACT

Alphaviruses such as Chikungunya Virus (CHIKV) and O'Nyong-Nyong Virus (ONNV) present an increasing problem in the world, with an increased vector efficacy allowing infection in previously uninfected areas such as southern Europe and a total infection count of over 5 million in the last 20 years. Most infected patients experience acute pain and a significant proportion go on to develop chronic rheumatoid-like arthritis whose mechanisms remain unclear. One potential target and source of mechanistic insight are the sensory dorsal root ganglia (DRG) that detect peripheral events and propagate nociceptive action potentials for recognition as pain in the CNS. This study aimed to determine if alphaviruses can infect DRGs and if so, which neuronal subpopulations are preferentially infected.

DRGs were excised from C57BL/6J mice (6-8wo) and cultured with growth media and 4uM aphidicolin on coverslips for 24h. ONNV-coverslips were infected with an aliquot of the virus ($6.25x10^5$ $TCID_{50}$/ml) which had mCherry tagged to nsp3. Mock infection coverslips (CTRL) were not infected and both groups were incubated for 24h, 48h or 72h. Two immunohistochemistry experiments were performed on the DRG cultures with the following primary antibody target combinations: myelinated neurons ($NF200^+$) and peptidergic neurons ($CGRP^+$), as well as non-peptidergic neurons ($IB4^+$) and neurons ($NeuN^+$). Coverslips were imaged using a fluorescent microscope with cell counts analysed using ImageJ and GraphPad Prism.

No significant differences were found between CTRL and ONNV DRGs when considering total cell count. A statistically significant decrease in the mean relative percentage of $CGRP^-$ $NF200^-$ cells was found between CTRL and ONNV DRGs at 48h, (-11.86%, p = 0.0332), and 72h (-17.63%, p = 0.0024), as well as $IB4^-NeuN^-$ cells at 72h (-21.31%, p = 0.0220). This occurred in parallel with an increase in ONNV infection in these populations. The numbers and percentages of peptidergic neurons were significantly increased in ONNV infected coverslips compared to CTRL at 72h.

This study has shown that ONNV can infect DRG neurons which may explain the manifestation of pain. Using mCherry labelled ONNV it was shown that ONNV preferentially infects myelinated peptidergic neurons and non-neuronal cells between 24h and 72h post infection, resulting in a significant increase in the relative proportion of peptidergic neurons, and a decrease in non-neuronal cells. Further research is required to categorise the mechanisms underlying this infection, including the mechanism of axon transport, DRG responses to infection, and viral products released after cell death.

ACKNOWLEDGEMENTS

Many thanks to Professor Victoria Chapman and Dr Gareth Hathway for their expert and kind supervision, Paul Milns for culture infection and coverslip preparation, Dr. Stephen G Woodhams for his instruction, assistance, and patience in my immunohistochemical endeavours and all members of the Pain Centre versus Arthritis for their support.

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INTRODUCTION

ALPHAVIRUSES

Alphaviruses, such as Chikungunya (CHIKV) and O'nyong-nyong (ONNV) contain single-strand RNA genomes and are part of the Group IV *Togaviridae* family of the *Alphavirus* genus from sub-Saharan Africa. Both CHIKV and ONNV, discovered in Tanzania and Uganda respectively, (Robinson, 1955; Johnson, 1988), infect humans via sylvatic transmission (Althouse *et al*., 2018, Thiberville *et al.*, 2013), using the *Aedes Spp.* (all) and *Anopheles Spp.* (ONNV-only) mosquito vectors (Franz *et al*., 2015). The common vector *Aedes albopictus* present in 20 European Union countries, (Medlock *et al*., 2012), and adapted to better infect hosts at lower temperatures (Zouache *et al.*, 2014), these viruses are becoming a greater threat to public health in previously incompatible regions (Amraoui & Failloux, 2016). CHIKV alone has infected at least 5 million people in the last 20 years (World Health Organisation, 2017). These outbreaks have occurred all over the world, spreading from Kenya (Kariuki Njenga *et al*., 2008) to Reunion Island and Mauritius (Pyndiah *et al*., 2012), Italy (Rezza *et al.*, 2007), France (Granddam *et al*., 2011; Delisle *et al*., 2014) and Brazil (WHO, 2019). Leta *et al,* (2018), recently estimated that 78% of all countries and territories around the world were suitable for alphavirus infection (figure 1).

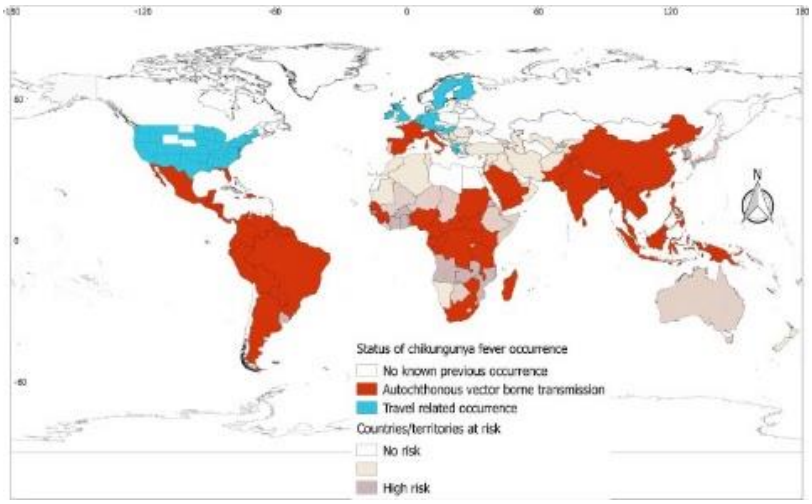

Figure 1: Global CHIKV fever occurrence and risk at the country level. Distribution extends to temperate regions including some European countries, source *Leta et al*,

Alphavirus infection is self-limiting with symptoms including fatigue, low-grade fevers, headaches, muscle aches and a maculopapular rash (Rezza *et al*., 2017). A hallmark feature of CHIKV and ONNV infection is Rheumatoid Arthritis-like polyarthralgia, (Miner *et al*., 2015), involving the wrists, ankles, elbows, and knees due to anti-viral immune response inhibition from reduced internal temperatures (Prow *et al*., 2017). In a prospective observational study of the outbreak in Brazil between 2014 and 2016, Brito Ferreira *et al*, (2020), evidenced that this polyarthralgia occurs in up to 85% of patients. Symptoms

typically subside after two weeks (Heath *et al*., 2018), however for a lot of patients polyarthralgia develops into a chronic state (van *Aalst et al*., 2017).

Both acute and chronic presentations result in diminished quality of life, by causing not only pain but significant socioeconomic stress due to work absences (Marimoutou *et al*., 2012). Uncovering alphavirus-induced pain pathophysiology is vital to not only increase patient quality of life, but also uncover novel mechanisms for potential therapeutic target development. To achieve this, we must first have a comprehensive understanding of the neurobiology of pain including the pain-sensing (nociceptive) mechanisms and pathways which will be discussed and evidenced in the following literature review.

THE PAIN EXPERIENCE

Pain is defined as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (Raja *et al*., 2020). Tissue damage and noxious stimuli are detected via nerve fibres via process of nociception. However, pain is the result of complex and dynamic neurophysiologic interactions that enable the conscious subjective experience of those events (Woolf & Ma, 2007). This experience is determined by a combination of biology, psychology, and social environment, and whilst pain is detected via nociceptive pathways and mechanisms, it is important to remember that sensory input does not directly predict perceived pain levels (Raja *et al*., 2020).

Pain can be categorised by the stimulus (stimuli) that provokes it; chemical, mechanical, or thermal; the pathologic cause, inflammatory or neuropathic or by the length of time it is experienced; either acute or chronic (Basbaum *et al*., 2009). Acute pain typically lasts no more than a few days to a week and has a defensive role to alert and protect the individual (Gupta *et al*., 2010). Chronic pain is pain that outlasts its underlying cause, is solely detrimental to the individual and persists or reoccurs for more than 3 months (Treede *et al*., 2019, Nicholas *et al*., 2019). Inflammatory pain arises from local surrounding tissue damage and peripheral events that initiate inflammation, presents both acutely and chronically, (Świeboda *et al.*, 2013), whereas neuropathic pain arises from direct nerve damage that causes nerve changes and also can present chronically (Jensen *et al*., 2011).

PERIPHERAL NOCICEPTION

Primary afferent sensory neurons in the Peripheral Nervous System (PNS) transduce external mechanical, thermal and chemical stimuli into action potentials at nerve terminals in target tissues, such as skin, muscle, and bone (Pace *et al.*, 2017). Briefly, this activation initiates a series of downstream events that open voltage-gated sodium ion channels (Na_v), resulting in the formation of an action potential (AP), (Julius & Basbaum, 2001). These APs travel afferently via the nociceptor cell bodies in the Dorsal Root Ganglia (DRG) and into the dorsal horn (Gemes *et al*., 2013). In the dorsal horn of the spinal cord these terminations synapse with second order neurons of the spinal cord within the Central Nervous System (CNS), (Julius & Basbaum, 2001). The second order neurons integrate inputs and convey

outputs to the brain for the processing of location, magnitude, and time (spatio-discriminatory) and emotive (affective) characteristics (Bushnell *et al.*, 2013). Finally, after processing, the brain can bi-directionally modulate dorsal horn excitability via descending systems which synapse with both first and second order neurons (Samineni *et al*., 2017).

Afferent sensory fibres are highly heterogenous and consist of three main types. Aβ fibres are the largest sensory fibre type, important for somatosensation and proprioception, but are non-nociceptive and thus will not be discussed extensively. Meanwhile, nociceptors can be classified into Aδ and C fibres (Basbaum *et al*., 2009). Thinly myelinated and fast conducting Aδ fibres relay first sharp and acute pain sensations (Andoh *et al*., 2018). Unmyelinated, slow conducting C-fibres relay second dull and chronic pain sensations (Zhang *et al.,* 2013). Both types can be categorised by AP conduction speed dictated by myelination level, stimuli modality dictated by the receptors expressed, and neurotransmitters used to pass APs across the synaptic cleft to second order neurons to enter the CNS.

Aδ fibres can be identified alongside their conduction velocity and cell size via positive heavy chain 200kD neurofilament (NF200) expression, an important cytoskeletal protein (Albisetti *et al*., 2017). They can be subcategorised into high and low threshold variants of mechanoreceptors, (Lenoir *et al.,* 2018), and capsaicin-insensitive polymodal A-mechano-heat (AMH) receptors; AMH-I (Slimani *et al*., 2018), and AMH-II (Armstrong & Herr, 2019).

C fibres can be divided into 2 major classes. The first subset innervates deeper dermal tissues as well as muscles and bone, use signalling neuropeptides Substance P (SP) and calcitonin gene-related peptides (CGRP), bind nerve growth factor (NGF), (Samanta *et al.,* 2018) and are termed peptidergic. The second subset innervates the epidermis, feature ATP-binding purinergic receptors (P2XR), bind glial-derived neurotrophic factor (GDNF), and in rodents can be labelled by the binding of the plant iso-lectin B4 (IB4; Basbaum *et al*., 2009; Pinto *et al*., 2019).

Most C fibres are polymodal and can be activated by many different stimuli including mechanical pressure, thermal fluctuations (noxious heat and cold), inflammatory mediators (interleukins, prostaglandins, bradykinin, histamine), markers of cellular destruction (H^+ and ATP), but also inhibited via the endocannabinoid system (Kaur *et al*., 2020) and G protein-gated inward-rectifying potassium channels (GIRK3), (Luján *et al.*, 2014), shown in figure 2. High and low threshold mechanoreceptors (C-HTMRs and C-LTMRs) that confer blunt pressure are the second largest population (Kuehn *et al.,* 2019). Finally, between 10% and 25% are silent adaptive thermoreceptors which only become sensitive to mechanical inputs when activated by noxious heat (Andoh *et al.,* 2018).

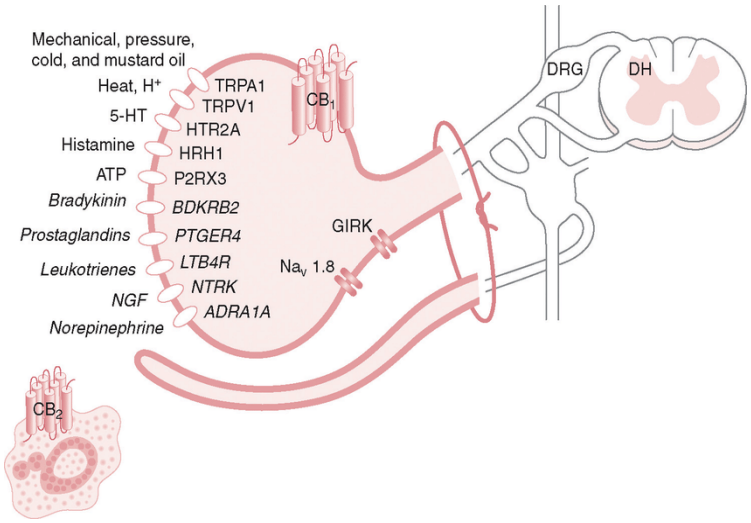

Figure 2: Schematic of common receptors present on the cell surface membranes of polymodal C-fibre terminals, source McPartland (2008). Abbreviations can be found in Appendix 1.

AP Initiation - Voltage-Gated Ion Channels

Ion channels present on the surface of nociceptors have key roles in the activation and propagated of action potentials, governed by the influx of sodium ions (Na^+) and efflux of potassium ions (K^+) along nerve axons (Lodish *et al.,* 2000).

The 9 subtypes of voltage-gated sodium channels (Nav1.1-1.9) activate to allow the entry of Na^+, increasing membrane potential and depolarising neurons forming an action potential (figure 3). Of these 9, Nav1.4 is absent from the PNS, being found in skeletal muscle (Trimmer *et al*., 1989), with Nav1.3 and Nav1.5 being expressed in low quantities in adult DRGs (Cummins *et al.,* 2007). Three subtypes have been extensively implicated in nociception, Nav1.7, 1.8 and 1.9, with these being recently evidenced to delineate silent from normal C-nociceptors (Jonas *et al*., 2020). Nav1.7 has slow closed activation and recovery, is found on 90% of C fibres and 40% of Aδ fibres and important in maintaining voltage thresholds (Djouhri *et al.,* 2003). As such the presence of loss of function (LOF) genetic mutations of the SCN9A gene that encodes Nav1.7 leads to congenital indifference to pain due to the lack of sodium entry and an ablation of C-fibres in cutaneous tissues (McDermott *et al*., 2019). Nav1.8 has an important role in the rising phase of activation and SCN10A mutations such as G1662S have been evidenced to impair fast activation, causing the development of compensatory mechanisms such as increased resurgent currents that result from the blockage of open channels during repolarisation (Bant & Raman, 2010). These resurgent currents and increased firing frequency contribute to increased excitability (Xiao *et al*., 2019). Luiz et al, (2019), used Nav1.8 negative cre-recombinase mouse models

and cold plates to provide evidence that the sensation of prolonged extreme cold (-5°C) is potentially mediated by Nav1.8, as the deficient mice did not exhibit any jumping behaviour indicative of stimuli-aversive behaviour. Nav1.9 has recently been shown to play an integral role in cold sensation through upregulation in cold-sensing neurons, in which the excitation switches to a subthreshold activity pattern, enabling AP amplification (Lolignier *et al.*, 2015).

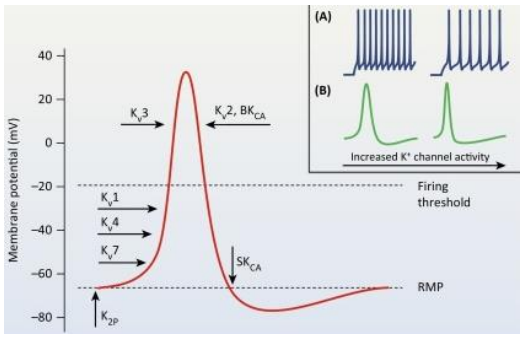

Figure 3: Depiction of sequential engagement of K^+ channels during an AP with typical effects of activation on AP waveforms, from Tsantoulas & McMahon, (2014).

Voltage-gated potassium channels (figure 3) regulate the frequency of action potentials by allowing K^+ efflux, causing neuronal repolarisation or even hyperpolarisation (Tsantoulas & McMahon, 2014). The stabilisation of neuronal resting membrane potentials is predominantly achieved by two pore K^+ channels (Gada & Plant, 2018). The low threshold rapidly inactivating transient A-type channels (IA), Kv1, Kv4 and Kv7, are involved in regulating basal membrane excitability (Manis, 2015). Kv7 channels can be inhibited by downstream inflammatory events, such as bradykinin binding to the bradykinin receptor, BK2, leading to the PLC activation cascade (Liu et al., 2010), PIP_2 depletion, (Zhang *et al.*, 2003), and Ca^{2+} release from the endoplasmic reticulum (Gamper *et al.,* 2005). High threshold Kv3 channels control AP duration whilst slower Kv2 channels regulate inter-AP duration to inhibit neuronal firing, but spinal nerve ligation causes downregulation, decreased inhibition and thus causing hyperexcitability (Tsantoulas *et al*., 2014). Finally, Ca^{2+}-activated K^+ channels are opened following repolarisation (BK) and hyperpolarisation (SK), (Tsantoulas & McMahon, 2014). In line with this, SK channels have been evidenced to reduce nociceptive DRG input to the spinal cord if sufficiently activated (Bahia *et al.,* 2005).

Synaptic Transmission – Voltage-gated Calcium Channels

Voltage-gated calcium channels (VGCC) play an integral role in nociception by enabling calcium influx into neurons (Castro-Junior *et al*., 2018). At DRG central terminals, this initiates the vesicle-mediated exocytotic release of excitatory neurotransmitters such as glutamate, CGRP and SP from the pre-synaptic bulb to the synaptic cleft of second-order neurons to continue the AP propagation into the CNS (Park & Luo, 2010).

Mechanical Sensation – PIEZO channels

Both Aδ and C-HTMRs and LTMR are involved in the detection and transduction of mechanical stimuli. The mechanisms of this were unknown, until a recent study that used optogenetic activation of the ion channel Channelrhodopsin-2 in $PIEZO2^+$ neurons to evidence painful behaviours such as paw licking in mice. This was further developed upon using von Frey filaments which when applied to PIEZO2 knockout mice, resulted in impaired cutaneous mechanosensitivity compared to wild type. Furthermore, using

electrophysiological recording of ex vivo saphenous skin-nerves, the authors found reduced Aδ and C-fibre activity in the first 3 seconds of vF application at the highest force used of 400mN. This implicated PIEZO2 channels as an important mediator of Aδ and C-fibre firing frequency and contributes to nociceptive mechanosensation (Murthy *et al.*, 2018).

TEMPERATURE SENSATION - TRANSIENT RECEPTOR POTENTIAL CHANNELS

Transient receptor potential (TRP) channels are non-specific cation transmembrane channels that can be activated by cytokine and chemokine binding as well as protons and subcategorised by additional sources of transducing stimuli (Szallasi *et al.*, 2007). TRP Vanilloids 1 to 4 (TRPV1-4) are sensitive to heat between 33°C and 52°C, TRPV1 is specifically sensitive above 43°C (Caterina *et al*., 1997). TRPV1 (figure 4) is activated by protein kinases, heat and protons resulting from cytokine binding and inflammatory responses, as well as capsaicin (Szallasi *et al.*, 2007). Capsaicin binding to an S4 and S5 transmembrane pocket opens an activation gate, increasing Ca^{2+} membrane permeability and initiating an action potential conferring pain (Hay *et al.*, 2017).

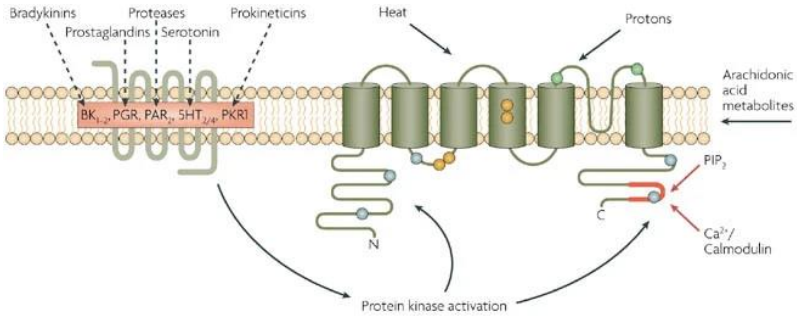

Figure 4: Schematic representation of a TRPV1 signalling within peripheral nociceptor terminals. Source Szallasi *et al*, (2007)

TRP melastatin 8 (TRPM8) can be activated by menthol as well as temperatures below 26°C and is involved in the detection of noxiously cold stimuli (Bautista *et al.*, 2007). TRPM8 has been shown to be coexpressed on $NF200^{+}$ neurons, unlike TRPV and TRPA channels (Kobayashi *et al*., 2005). Long TRP ankyrin protein 1 (TRPA1) is a polymodal receptor that can be sensitised by both hot and cold stimuli and formalin, alongside inflammatory mediators, and nitric oxide like other TRP channels (Miyamoto *et al*., 2009). TRPA1 is expressed mostly in non-peptidergic C-fibres (Barabas *et al*., 2012) and implicated in neuropathic pain mechanisms such as mechanical hypersensitivity and allodynia (Souza Monteiro de Araujo *et al*., 2020).

CHEMICAL SENSATION - ACID-SENSING ION CHANNELS

Acid-sensing ion channels (ASICs) are activated by low pH levels of pH4-6 (Alvarez de la Rosa *et al.,* 2002). This is in comparison to the activation of TPRV1 which is limited to pH6.0 (Alawi & Keeble, 2010). Cellular destruction releases large amounts of protons, lowering extracellular pH dramatically and this means that ASICs are among the first channels to be activated channels following peripheral tissue injury (Sun & Chen, 2015). Using a mouse model of peripheral nerve injury, Papalampropoulou-Tsiridou *et al*, (2020), found that different ASICs were expressed in different nociceptor subpopulations, with ASIC1 and ASIC3 expressed in peptidergic fibres, ASIC2a in non-peptidergic fibres and ASIC2b in both. Alongside these findings, ASIC1 and ASIC 3 in L4 and L5 peptidergic afferents respectively

were upregulated following peripheral nerve injury, conferring greater neuronal excitability (Papalampropoulou-Tsiridou *et al.*, 2020).

ASIC gene knockout studies performed on mice younger than 9 weeks old have shown that the absence of these channels, confirmed by the lack of corresponding RNA, are involved in thermal analgesia and formalin-induced pain behaviours. ASIC3 knockout mice displayed a small reduction in tail withdrawal latency compared to wild-type at 52°C, and ASIC1 and ASIC2 knockout mice showed increased spontaneous pain during the second phase of the formalin test (Staniland and McMahon, 2009), which is subsequently resolved by endogenous inhibitory mechanisms (Azhdari-Zarmehri *et al*., 2014).

ATP AND P2X RECEPTORS

The $P2X_3$ receptor is part of the ATP-gated cation channel P2X group of receptors (P2XR). These are expressed in about 40% of DRGs, 70% of those being monomodal thermoreceptors and 30% monomodal mechanoreceptors (Vulchanova *et al*., 2001). P2X receptors are colocalised with IB4, Mrgprd and GDNF and are thus present in non-peptidergic C fibres (Chen et al., 1995). Chen *et al*, (2020) used fluoro-gold retrograde trace labelling to indicate that P2X receptors modulate neuronal sensitivity in neuropathic pain states by changing expression in different subpopulations, evidenced by $P2X_3$ upregulation in medium Aδ fibres, and $P2X_2$ downregulation in non-peptidergic C-fibres.

THE ROLE OF NGF IN SENSORY NERVE FUNCTION

NGF is a neurotrophin and is important for postnatal neuronal development and for the survival and maintenance of adult neurone subpopulations. NGF^+ neurons can be identified by the prevalence of its low-affinity p75 receptor but more substantially by its high-affinity receptor, TrkA, constituting up to 40% of adult rat DRGs (Averill *et al*., 1995). NGF is released, alongside cytokines and chemokines, following acute peripheral tissue injury by activated mast cells, macrophages, and neutrophils as part of normally restorative

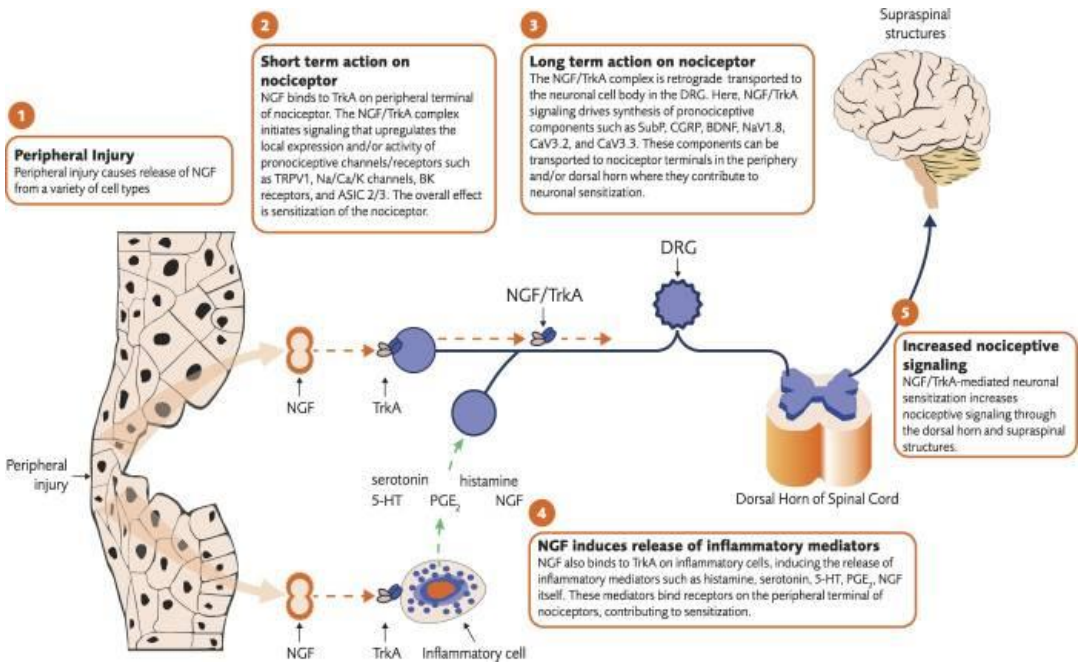

Figure 5: The pronociceptive actions of Nerve Growth Factor. Source Schmelz *et al,* (2019).

inflammatory processes (Thacker *et al*., 2007). NGF binding to TrkA causes the complex to be internalised by clathrin-mediated endocytosis (Uren & Turnley, 2014). Retrograde transportation to the DRG cell bodies occurs followed by a series of internal phosphorylation events that can increase the expression of a variety of receptors including TRPV1, ASICs 2 and 3, and the neurotransmitters CGRP and SP (Cohen *et al*., 2016). This binding can also lead to changes in gene expression, causing neurite outgrowth (Allen *et al.*, 2013). These events lead to $TrkA^+$ nociceptors being sensitised both peripherally via receptor upregulation causing reduced activation thresholds and neurite outgrowth causing larger receptor fields, and centrally via increasing the production of CGRP and SP, as well as voltage gated ion channels, summarised in figure 5 (Schmelz *et al.,* 2019).

The potency of NGF as an inflammatory mediator can be shown by the recent studies conducted by Schrenk-Siemens *et al*, (2019). The experiments conducted in this paper evidenced that NGF-binding to the 50% of peptidergic neurons in deep tissue that express nicotinic acetylcholine receptor subunit alpha-3 (CHRNA3) elicited the awakening of silent thermoreceptors to detect mechanical events via the PIEZO2 ion channels. Masuoka *et al*, (2020), evidenced that during chronic inflammatory states, NGF increases group I metabotropic glutamate receptor-expressing nociceptors via phosphorylating A-kinase anchoring protein 5 and activating TRPV1 channels to increase thermal pain levels.

DRG Cell Bodies and Central Terminations

DRG neurons are surrounded by satellite glial cell (SGC) lipid sheaths that serve as supportive environmental regulators (Pannese, 2013). SGCs have wide-ranging roles including the production of neural growth factors and TNFα to modulate DRG excitability via K^+ channel regulation (Takeda *et al*., 2009). Normally, these cells are supportive of neurons, however in injured states, SGCs become reactive, indicated by increased glial fibrillary acidic protein (GFAP) expression, and increase pro-inflammatory responses which leads to heightened neuronal activity and facilitated noxious inputs (Kim *et al*., 2016, Liu *et al*., 2018).

After passing the cell body in the DRG, APs travel along short central terminals which synapse with a heterogeneous population of second order neurons in the dorsal horn of the spinal cord. These include non-nociceptive specific and nociceptive-specific neurons, interneurons that modulate afferent excitability, as well as wide dynamic range neurons that synapse with all fibre types and include interneurons and projection neurons which convey information to the brain. The APs are transferred between neurons through the release of neurotransmitters from DRGs which bind to various receptors on the post synaptic membrane of second order neurons (Basbaum *et al*., 2009).

SENSORY NEUROTRANSMITTERS

Glutamate

Glutamate is the main excitatory neurotransmitter of the nervous system, expressed by all DRG neurones and is essential in the passage of nociceptive APs from DRGs to second order neurons (fig 6, Liu *et al*., 2010). Glutamate is stored in synaptic vesicles by three vesicular glutamate transporter (VGluT) proteins 1, 2 and 3, of which VGluT2 is highly expressed in DRGs and the spinal cord (Brumovsky *et al*., 2007). When an action potential arrives at DRG presynaptic termini, voltage-gated Ca^{2+} channels open allowing influx of this ion which causes VGLuT2 fusion with the presynaptic membrane, releasing glutamate into the synaptic cleft. Glutamate can bind to two classes of receptors, the first of which are ionotropic (iGlur) fast activating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, (AMPA) receptors, and slow activating N-methyl-D-aspartate (NMDA) receptors (Larsson, 2009) as well as metabotropic receptors. After release, glutamate is very quickly reabsorbed back to the presynaptic terminal using transporter proteins such as EEAC1 (Rothstein *et al*., 1994). Peripheral inflammation has been shown to increase the responses of $AMPAR^+$ DRG neurones, subsequently causing pain (Galan *et al.,* 2004). Conversely, the reduction or block in AMPA receptors on second order neurons has been shown to reduce pain from nerve injury (Chen *et al.*, 2013). Applying the antagonist ketamine to spinal NMDA receptors results in reduced pain by blocking afferent signals to nociceptor-specific and wide dynamic range neurons (Neugebauer *et al.,* 1993).

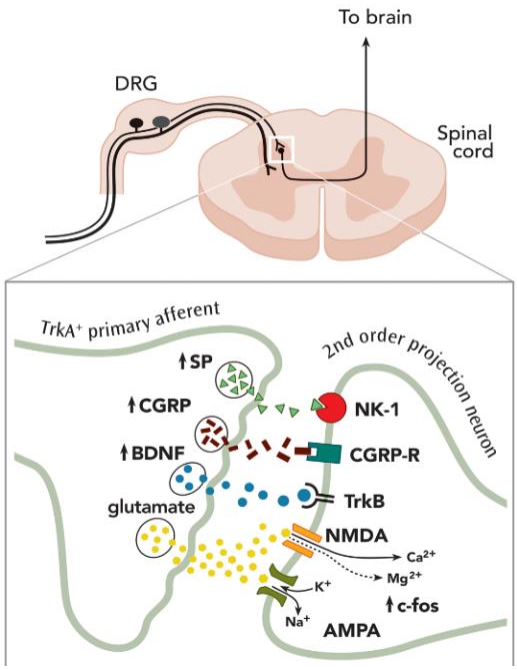

Figure 6: Neurotransmitters involved in peptidergic afferent signal transduction and their associated receptors in the post-synaptic cleft of second order projection neurons found in the Substantia Gelatinosa. Source Mantyh *et al,* (2011).

G-protein coupled metabotropic receptors (mGlur) constitute the other glutamate binding receptor type. 8 subtypes have been identified of which groups II and III are expressed on presynaptic membranes and group I are expressed on postsynaptic membranes (Pereira & Goudet, 2019). mGlur5, an important group 1 receptor, has been evidenced to inhibit A-type $K_v4.2$ channels in second order neurons, resulting in neuronal hyperexcitability and increased pain (Hu *et al.,* 2007). Conversely, due to the expression of group III receptors on presynaptic membranes, activation of these receptors leads to a reduction of glutamate received from PAFs to second order neurons, resulting in less pain signals and thus less pain experienced (Zhang *et al*., 2009).

Peptidergic Neurotransmission

Alpha Calcitonin gene-related peptide (αCGRP) is a regulatory product of the calcitonin gene, serving as an excitatory neurotransmitter for peptidergic neurons found within both the PNS and CNS (Lou & Gagel, 1998). CGRP is stored in large dense core vesicles (Kummer, 1992), diffusely released into the synaptic cleft via calcium-dependent exocytosis mediated by SNARE proteins (Meng *et al*., 2007). The peptides form glycoprotein complexes with one of three receptor-activity-modifying proteins (RAMP) and bind to calcitonin receptor-like receptors (CLRs) on the post-synaptic cleft. CLR-RAMP 1 binds to the high affinity CGRP receptor, which activates adenylyl cyclase, converting ATP into cAMP activating protein kinase A and resulting in a post-synaptic action potential (Iyengar *et al*., 2017). Substance P (SP) is a nociceptive neuropeptide that is expressed by 20% of DRGs and binds to Neurokinin (NK_1) receptors (Krug *et al*., 2019). SP can be released via axogenic reflex to recruit immune cells by upregulating cytokines and can be involved in inflammatory pathways in a positive feedback loop of constant peripheral inflammation (Mashaghi *et al.*, 2016). This axogenic reflex of substance P causes an increase the level of neurogenic inflammation in the skin (Holzer, 1998).

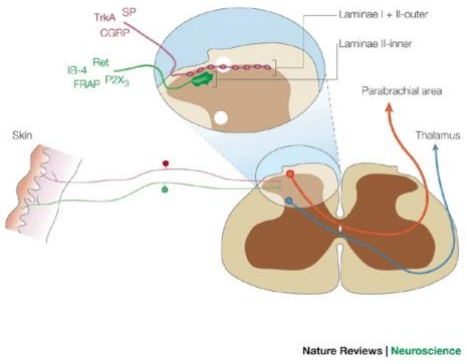

Figure 7: PAF terminations in the dorsal horn of the spinal cord. Source Mantyh & Hunt, (2001).

Central Mechanisms of Pain

All nociceptors synapse with second order neurons and travel up the spinal cord via the spinothalamic tract to the posterior thalamus and somatosensory cortices for the processing of spatial area, time, and intensity of the painful stimulus, termed the sensory-discriminative aspect of pain (Mantyh & Hunt, 2001). Within the spinothalamic tract, Aδ fibres terminate in dorsal horn Rexed laminas I and V, whilst peptidergic C-fibres terminate within laminae I and II outer, and non-peptidergic to II inner, shown in figure 7 (Serpell, 2005). C-fibres, consisting of mostly peptidergic fibres also synapse with second order neurons and ascend the spinoparabrachial pathway which innervates the periaqueductal grey (PAG) and parabrachial nuclei (PBN) , (Braz *et al*., 2005). The PBN further projects to the hypothalamus and amygdala (figure 8) to confer emotive, memory and behavioural responses, termed the affective aspect of pain (Bushnell *et al.*, 2013). Signals from these affective areas can inhibit dorsal horn excitability to modulate afferent pain signals through the PAG and rostral ventromedial medulla (RVM), (Mantyh & Hunt, 2001). These structures control many of the GABAergic inhibitory and glutamatergic excitatory interneurons which synapse with first and second order neurons to change excitability (Tobaldini *et al.,* 2018).

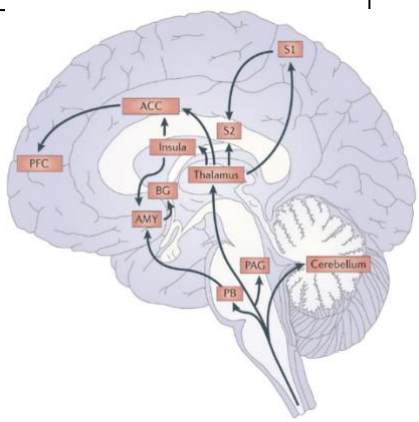

Figure 8: Illustration of nociceptive pathways within the brain, from Bushnell *et al*., 2013)

SENSITISATION

Previous research into pain mechanisms have characterised numerous sensory phenomena that develop from normal pain responses, including, sensitisation, hyperalgesia, and allodynia (Basbaum *et al*., 2009).

Sensitisation is the term given to describe the nociceptor adaptations that follow sufficient exposure to a stimulus either in duration or intensity (Slimani *et al.*, 2018). Specifically, action potential thresholds are decreased, and receptor fields are increased. Sensitisation results in the development of increased pain sensitivity, termed hyperalgesia, and can occur either peripherally via nociceptor changes alone or centrally in chronic conditions via alterations in central nociceptors or processing (Gold & Gebhart, 2010). Primary hyperalgesia is that which occurs within the injury area and can lead to both thermal and mechanical pain and is mediated by peripheral sensitisation (Slimani *et al*., 2018). Secondary hyperalgesia occurs in uninjured tissue surrounding the site via central sensitisation, which only increases perception of mechanical pain (Van den Broeke *et al.,* 2016). This central sensitisation can cause allodynia, pain elicited by normally innocuous mechanical stimuli (fig 9, Wilbrink *et al*., 2017).

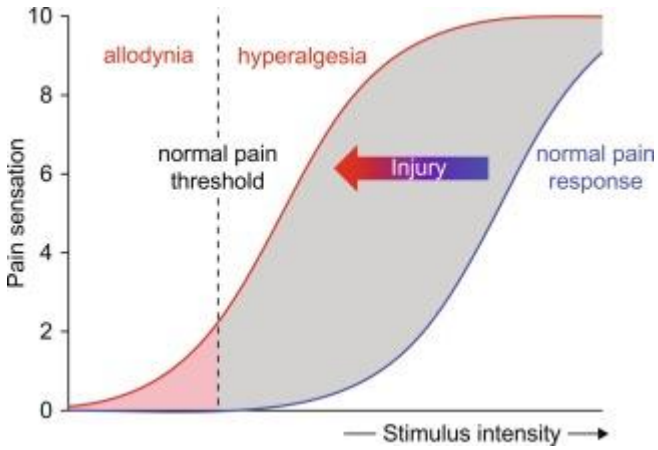

Figure 9: Illustration of how tissue injury causes peripheral sensitisation which leads to a leftward response shift resulting in an increased sensitivity to painful stimuli (hyperalgesia) and painful events arising from normally innocuous sensations (allodynia). Source Cervero *et al*, (1996).

ALPHAVIRUS CELLULAR AND MOLECULAR PROPERTIES

Now that we have a contextual understanding of nociceptive pathways and mechanisms, let us apply it to alphavirus infection. After mosquito feeding, E1 and E2 glycoproteins present on the capsid surface enable virus attachment and fusing with the plasma membranes of target cells, (Petitdemange *et al*., 2015), such as those in the epithelium (van Duijl-Richter et al., 2015). Alphaviruses enter through clathrin-mediated endocytosis, (Hoornweg *et al*., 2016), then release four non-structural proteins, (nsp 1 to 4), (Gorchakov *et al*., 2008), to hijack intracellular translation processes to replicate and repackage the positive-sense single strand RNA genome (Rougeron *et al*., 2015), releasing new particles (virions) into the extracellular space, as summarised in figure 10 (Rashad *et al*., 2013).

These virions enter the blood and infect cells of other tissues (muscle, bone) in a silent incubation phase (Maucourant *et al*., 2019). Infected cells can identify and halt viral RNA replication via viral pattern recognition receptors at key infection stages. The intermediary double strand RNA produced in viral replication can be detected by Toll-lke receptors (TLRs), cytosolic Retinoic Acid-Inducible Gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), (Goffic *et al*., 2006). Activation of TLRs, RIG-I and MDA5 cause downstream release of inflammatory molecules such as type 1 interferons (IFN), cytokines and chemokines (Olagnier *et al*., 2014).

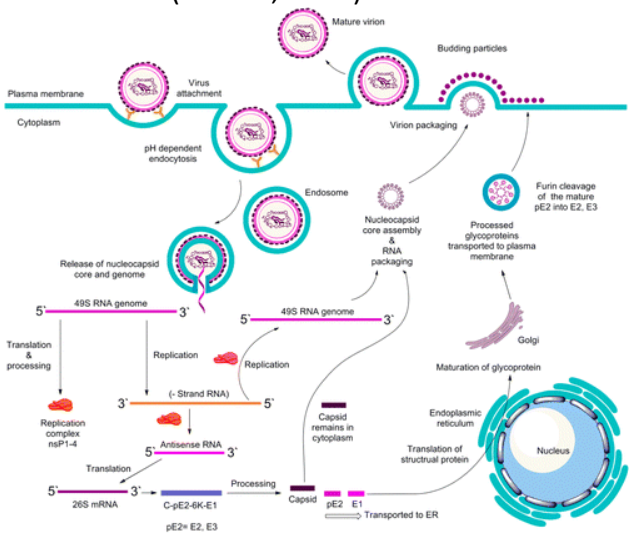

Figure 10: Schematic Representation of the CHIKV life cycle, from Rashad *et al*, (2013).

TLR3 has specifically been implicated as a major mechanism of Chikungunya virus replication. This was established by Her *et al,* (2014), who used a TLR3 knockout mouse model with CHIKV infection to evidence a 1 log increase in viremia 2 to 12 days post infection compared to wild type, alongside a sustained infection past day 18 which wild type had eliminated by day 12. This resulted in higher levels of CHIKV found in most tissues with reduced viral clearance being specifically due to impaired E2 glycoprotein recognition from TLR3. Knee joint immunohistochemistry further showed that symptomatic joint swelling was due to large scale myeloid cell infiltration into the tissues.

The release of these inflammatory molecules initiates the acute inflammatory phase, where immune system components such as natural killer (NK) cells and neutrophils (Costantini & Cassatella, 2010) infiltrate infected sites, destroy infected cells, and clean up cellular debris to halt viral replication and restore normal tissue function (Maucourant *et al*., 2019).

These inflammatory processes in turn produce more molecules, including ATP, protons, ions, and nitric oxide (NO) from direct cellular destruction, bradykinin release from infected cells (Rust *et al*., 2012) and prostaglandins via increased COX2 expression because of macrophage-mediated interleukin (IL) 8 and IL-1β release, (Verri et al., 2006). All of these molecules can directly sensitise nociceptors to cause pain by activating TRP channels, decreasing pH to activate ASICs and increasing extracellular ion concentrations to facilitate prolonged signalling from ion influx with voltage-gated ion channels (see the AP initiation, temperature sensation and chemical sensation sections).

USEFULNESS OF RODENT DRG NEURONS AS HUMAN DRG SURROGATES

In recent years, the study of human DRGs (hDRG) has become possible, highlighting that there are many differences between rodent model DRGs (mDRG) and human DRGs. Distinct

neuronal subpopulation differences have been identified, with cutaneous silent C-fibres in humans being around 25% of the total (Schmidt *et al.,* 1995), whereas in mice the proportions are below 10% (Wetzel *et al.,* 2006). Differences in subpopulations have also been identified, for example IB4 does not bind to human DRGs (Davidson *et al*., 2014). Rostock *et al*, (2018) showed that hDRGs have a larger population of TRPV1$^+$ peptidergic C-fibres (figure 11) when compared to rodent DRGs.

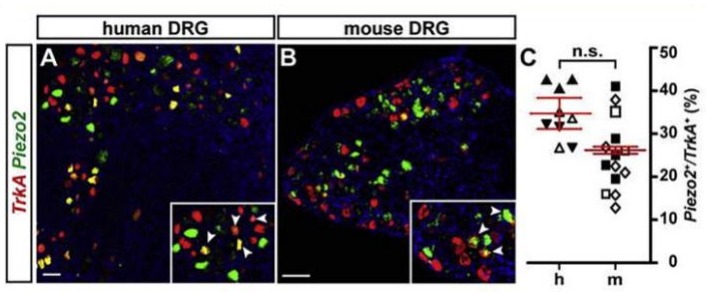

Figure 11: Humans have a higher percentage of PIEZO2$^+$/TrkA$^+$ neurons compared to mice DRGs. A and B show dual colour FISH probes detecting TrkA and PIEZO2 in red and green respectively. C shows comparisons between dual-labelled cells expressed as population percentages in humans (N=3) and mice (N=4) respectively. Source Rostock *et al*, (2018).

Recent advances in RNA transcriptomics have permitted more detailed comparisons. Shiers *et al*, (2020), used RNAscope in-situ hybridisation to elucidate that hDRGs have 25% more CGRP$^+$ fibres, 40% more TRPV1$^+$ fibres, 30% less TRPA1$^+$ fibres and near complete overlap between CGRP$^+$ (50.4%) and P2X3$^+$ (48.3%) neurons in comparison to mDRGs that have distinct populations.

However, these results rarely consider the impact of evolution in their assessment of differing expression, and many gene families are highly conserved between mice and humans with mouse models being faithful models of human DRGs (Ray *et al*., 2018). Given the inherent complexity of multiple interacting systems and the ethical considerations of in-vivo testing, in-vitro cultures are one experimental approach that yields isolated insight into nociceptive mechanisms with defined immunohistochemical methods (Price & Flores, 2007). As a result, this project used in-vitro primary mouse DRGs as a comparable model of human physiology.

Aim of Project

Given the important role of DRGs in peripheral nociception, the aim of this project was to investigate whether alphavirus infection preferentially infects nociceptive and non-nociceptive DRG subpopulations using immunofluorescence. To achieve this goal ONNV was used as a comparably safer alphavirus for infection due to being classed as a Containment Level 2 biological agent compared to CHIKV being CL3 (*The Approved List Of Biological Agents,* 2016).

The objective of this study was to use mCherry-labelled ONNV to identify which cell subpopulations were preferentially infected by ONNV in the acute stage and how neuronal

and glial populations changed over the course of three hour-post-infection (hpi) timepoints: 24hpi, 48hpi and 72hpi.

HYPOTHESIS

"Alphaviruses such as ONNV preferentially infect peptidergic nociceptors to cause pain"

METHODS

ANIMAL MODELS

C57BL/6J mice between the ages of 49 and 55 days and weight of 24g to 26g were purchased from Charles River UK, Ltd. These experiments were conducted in accordance with the Animal (Scientific Procedures) Act (1986). Mice were housed for a maximum of 14 days in a temperature and light controlled environment (21-23°C, 12h light/dark cycle) and provided with food and water *ad libitum*.

COVERSLIP PREPARATION

13mm Ø coverslips (VWR, 631-1578) were prepared by washing in 10% Decon 90 for 30 minutes, rinsed in ddH_2O 5x, immersed in HCl for 30 minutes, rinsed in ddH_2O 5x and autoclaved. Coverslips were incubated in 300ul of poly-DL-ornithine (2h, 37°C) before being washed with H_2O and dried. The coating agent, laminin was added (20ul of 20ug/ml, Sigma-L-2020) for incubation (2h, 37°C), with the coverslip being washed in ddH_2O and dried.

DRG PREPARATION

Animals were humanely killed using an approved schedule one method. Whole mice were then immersed in 70% IMS for 1 minute to reduce bacterial load and wet the fur before being transferred to a laminar flow hood with a dissecting microscope. The head, spine and dorsal muscular tissue were removed to expose the laminae. DRGs were removed using fine scissors and forceps and washed once in phosphate buffer solution free of calcium and magnesium (PBS 0.1M, pH7.4, Sigma D8537).

DRGs were disassociated by collagenase solution incubation (2h, 37°C, 5% CO_2). The collagenase solution consisted of 0.25% w/v collagenase (Sigma C5138) dissolved in Neurobasal media (NBM) containing 10% FCS, glutamine 2 mM, penicillin (200 units/ml) and streptomycin (200µg/ml). This was followed by three gravity PBS washes and incubation with 2ml trypsin stock (25g/L of porcine trypsin - 25,000-60,000 BAEE U/mL, Sigma T4549, diluted to 1:20) for 15 minutes at 37°C. 16% BSA 1ml aliquot (Sigma A7906) titration was . The cell suspension was layered on top of 3ml of BSA solution and centrifuged at 1200rpm for 10 minutes. The top layers were removed before adding 230ul of complete media to the cell pellet which is resuspended. 10uL of DRGs were then pipetted onto the centre of the

coated coverslips, placed into a 24 well plate (Corning incorporated, ref 3524) and incubated for 15 minutes at 37°C, 5% CO2, after which 390µl of complete media was added to the coverslip wells for incubation overnight.

The complete media consisted of NBM (Invitrogen 21103049) containing B27 supplement (Fisher Scientific, 17504044, final conc. 1:50 of stock), glutamine 2 mM, penicillin (200 units/ml) and streptomycin (200µg/ml), NGF (Sigma N6009, final conc. 50ng/ml), GDNF (Sigma G1401, 50ng/ml) and the mitotic inhibitor aphidicolin (final concentration 4mM). It is important to note that this concentration of NGF and GDNF could cause neuronal sensitisation. NGF is known to play an important role in the replication of Influenza, which can be blocked with TrkA inhibitors (Kumar *et al.,* 2011). Should this also be true of alphavirus replication mechanisms, then ONNV infectivity would be increased further by the addition of growth factors.

An aliquot of ONNV, with a fifty percent tissue culture infective dose concentration ($TCID_{50}$) of $6.25x10^5$ $TCID_{50}$/ml, was retrieved from the freezer (-80°C). This was added to NBM for final virus-media dilution factor of 1:80 and added to the ONNV-designated cell cultures after the removal of complete media. This dilution was decided upon after a series of 72h in-vitro infection experiments in which lower viral dilutions (1:20 and 1:40) killed too many cells and higher dilutions (1:160 and 1:320) did not infect many cells. 1:80 was chosen as it provided a significant level of infection with normal cell growth, but without skewing the infected population from excessive cell death at 72h. After incubation, DRGs were washed 3x with warm Hanks buffered saline solution (HBSS, pH7.4), to remove dead cells and fixed with 4% paraformaldehyde (PFA) for 30 minutes. Cells were washed for 3x in PBS before adding PBS containing 0.01% w/v sodium azide for storage in the fridge (4°C).

IMMUNOCYTOCHEMISTRY

Cultured DRG coverslips were transferred to 0.1M pH7.4 PBS-filled wells in a new 24 well plate (Corning incorporated, ref 3524). DRGs were incubated with 500uL of permeabilising and blocking solution consisting of 0.3% Triton X100, 10% NDS (Sigma, D9663) in PBS, pH7.4, for 1 hour at RT on an orbital shaker (OS, 100rpm). DRGs were then incubated with 2 primary antibody combinations (table 1). These consisted of 1; Sheep anti-CGRP (1:1000, Enzo Life Sciences, BML-CA1137-0100) and mouse anti-NF200 (1:1000, Sigma, N5389) or 2; IB4-AlexaFluor488 (1:500, Invitrogen, mp21410) and mouse anti-NeuN (1:1000, Merck Millipore, MAB377); in a solution containing 5% NDS in PBS overnight at 4°C on a rocker. Secondary-only control coverslips were incubated in 5% NDS in PBS. The following day, the DRGs were washed for 50 (5 x 10) minutes (RTOS). DRGs were incubated with AF-conjugated fluorescent secondary antibodies (1:500, table 2) in 5% NDS in PBS for 2 hours in the dark (RTOS), before being washed in PBS for 5 x 10 minutes. Coverslips were incubated with 150uL of Vecta TrueVIEW autofluorescence quenching media (Vector labs, SP-8400) for 3 minutes (RTOS) and counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:200) for 15 minutes (RTOS). Phosphate Buffer (0.1M, pH7.4) was used to wash for 2 x 10 minutes before coverslips were removed from wells, inverted, and placed atop a 10uL droplet of

VECTASHIELD Vibrance **(**Vector Labs, H-1700) mounting media on a labelled gelatinised slide. Slides were stored at 4°C until imaging.

PRIMARY ANTIBODY	COMPANY	CATALOGUE REFERENCE	DILUTION
SHEEP ANTI-CGRP	Enzo Life Sciences	BML-CA1137-0100	1:1000
MOUSE ANTI-NF200	Sigma	N5389	1:1000
IB4-AF488	Invitrogen	mp21410	1:500
MOUSE ANTI-NEUN	Merck Millipore	MAB377	1:1000

Table 1: Primary antibodies used in primary mouse DRG immunocytochemistry

SECONDARY ANTIBODY	COMPANY	CATALOGUE REFERENCE	DILUTION
DONKEY ANTI-SHEEP-488	Invitrogen	A21202	1:500
DONKEY ANTI-MOUSE-647	Invitrogen	A31571	1:500
DONKEY ANTI-MOUSE-647	Jackson Labs	715-605-150	1:500

Table 2: Alexa-conjugated secondary antibodies used in primary mouse DRG immunocytochemistry

MICROSCOPY

Images were captured using a CoolSNAP MYO 14-bit CCD camera mounted to a Zeiss Axiovert 200M fluorescent microscope controlled using Microscope Manager v1.4.21. Channels captured included white light/brightfield (BF) and the following filtered wavelengths; 405nm (DAPI), 488nm (FITC/AF488), 568nm (TRITC/mCherryONNV), 647nm (Cy5/AF647).

3 fields of view (FOV) per coverslip with 3 coverslips per condition were acquired except for 2 FOVs from a 72h CTRL coverslip from the CGRP and NF200 dataset and were excluded due to a lack of cells from excessive handling pressure, resulting in a total of 52 images and 6,050 cells. For the NeuN and IB4 experiment, 1 of the 24h CTRL coverslips and 2 of the 72h ONNV coverslips shattered while mounting, leading to a total of 45 images and 5, 306 cells. Each coverslip prep was a mix of 2 mice, thus the final n number for these comparisons was 2.

STATISTICAL ANALYSIS

All images were processed in Image J v2.52. Images of primary antibody combinations and secondary only antibody coverslips of the same condition and timepoint were visually compared. Manual dynamic ranges were set for each channel individually via look up table (LUTs) alteration, such that FITC (CGRP or IB4) and Cy5 (NF200 or NeuN) channels were negative in secondary-only images to comparatively eliminate autofluorescence. The TRITC (mcherryONNV) channel was set such that no expression was seen in the control condition.

Dynamic ranges were applied to each set of timepoints due to differing levels of intensity between sets.

Cell numbers were quantified manually via the multi-point tool. The area of each cell was calculated by drawing around each cell body in the brightfield channel using the freehand drawing tool and subsequently measuring the soma area for comparison. All measurements were saved using the ROI manager plugin.

It should be noted that the use of manual quantification does introduce the possibility of measurement bias. Attempts at automation through the generation of workflow pipelines in Cell Profiler v3.1.0 were made, however due to the counting being restricted to white pixel area values of single greyscale images, this provided no contextual interpretation of positive cells with overlapping markers of different intensities. To put this plainly, if a $CGRP^+$ axon passed over an $NF200^+$ soma, Cell Profiler would have incorrectly counted this as $CGRP^+NF200^+$. Additionally, due to the lack of ability to contextually integrate brightfield/DIC into the cell counts, same-intensity nuclei or cells situated next to each other were counted as one object. Automating the cell counts accurately would allow for an unbiased, much clearer understanding of the images at a much faster rate, but to this author's knowledge, no such software yet exists.

Cell counts were taken from 3 FOVs per coverslip of NF200, CGRP and IB4 neuronal subpopulations, with non-neuronal cells being determined by NeuN versus DAPI staining and ONNV infection determined by ONNV versus DAPI staining. These cell counts were converted into percentages of each subpopulation and averaged into a single data point per condition and timepoint. The respective results from each condition and timepoint were statistically analysed in GraphPad Prism (version 9.2) and tested for parametric normality with the Shapiro-Wilk test as the n was too small for D'Agostino and Pearson omnibus normality test. ONNV and control cell counts from the CGRP and NF200 dataset were compared using two-way ANOVA, with mixed effects models used for the IB4 and NeuN dataset due to the missing 72h ONNV coverslip values. ONNV infection levels were compared using One Way ANOVA tests. All multiple comparisons were followed by Šidák corrections to minimise type 1 errors. Cell counts, relative percentages and ONNV infection levels were presented as the mean of coverslip FOV averages with the standard error of the mean (±SEM).

RESULTS

The ability of ONNV to infect different sub-categories of neurons was investigated through two separate experiments, one using the peptidergic marker CGRP and heavy neurofilament marker NF200 (fig 12 and 13), and another using the non-peptidergic marker IB4 and neuronal nuclei marker NeuN (fig 14 and 15). These markers were chosen as they are established in the immunofluorescence literature, having strong accuracy and specificity of their respective subpopulation delineations, with CGRP labelling peptidergic C-fibres nociceptors, NF200 labelling myelinated neurons, a combination of CGRP and NF200

labelling nociceptive Aδ fibres, IB4 labelling non-peptidergic neurons in rodents and NeuN labelling neuronal nuclei to definitively identify the whole neuronal population from non-neuronal cells.

Comparing the individual subpopulations against each other alongside NeuN would be beneficial for reliable and exact quantification of population percentages and ONNV infection levels. However, due to limited supply of DRG tissue, time, and the fact that both NF200 and NeuN are raised in rabbit meant that this was not possible. Future experimentation investigating these differences in a more comprehensive approach would be advantageous.

Brightfield images (fig 12 and 16) are included to clearly illustrate the levels of infection (mCherry expression) without the multiple non-brightfield channels masking over this expression. The non-brightfield channel combinations (fig 13 and 17) are also included to show the relative expression levels of the different neuronal markers between conditions and across timepoints with statistical analysis on the following pages.

CGRP & NF200 BRIGHTFIELD IMAGES

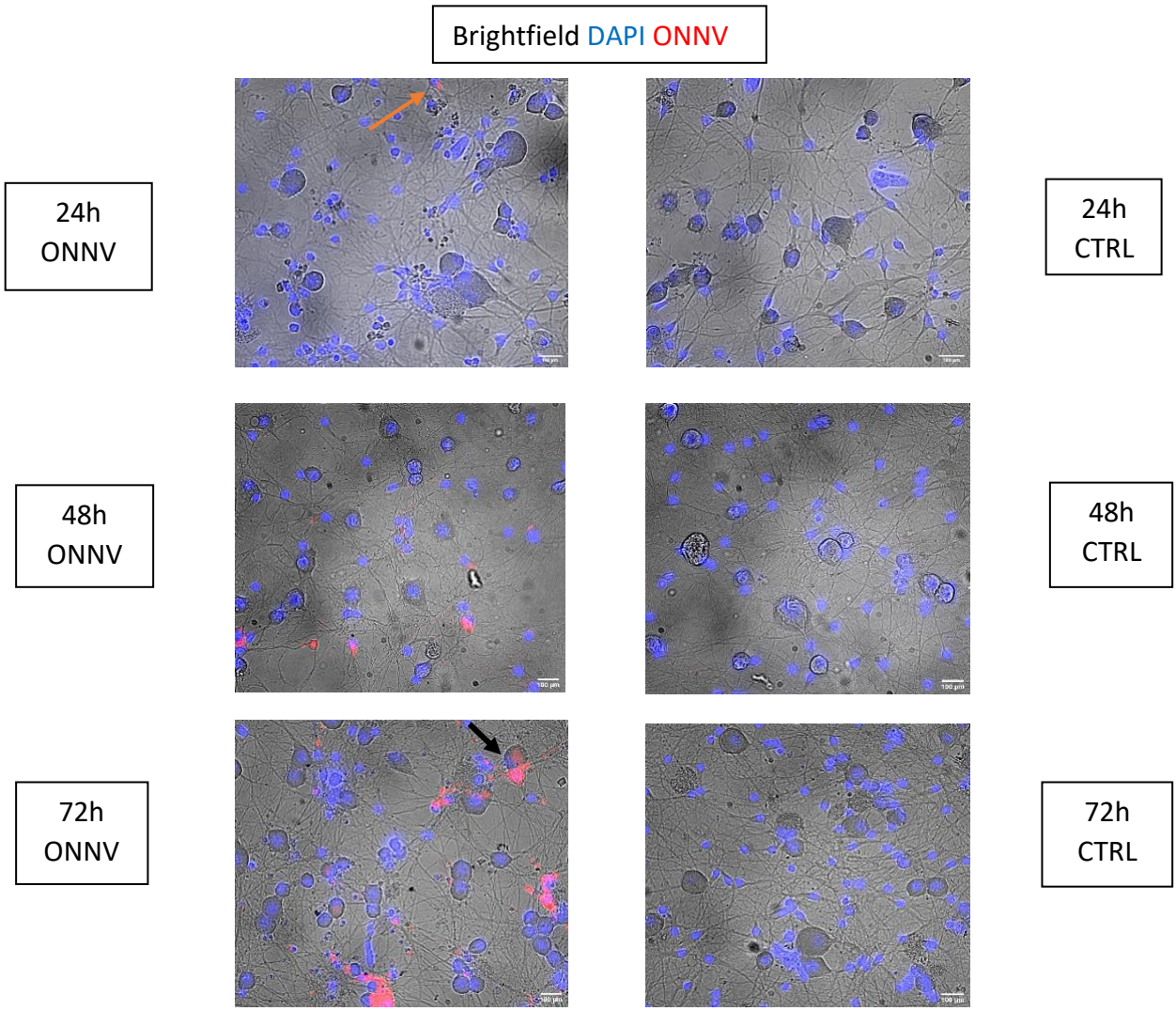

Figure 12. Representative 20x brightfield images of ONNV-infected and CTRL coverslips at 24h, 48h and 72h post infection (hpi). Labelling for the nuclear marker DAPI (blue) and mcherryONNV (red) from the CGRP and NF200 dual labelling experiment. Orange arrows show infected non-neuronal cells with black arrows indicating infected neurons with Scale bar: 100µm.

ONNV expression inside all cells was very low, and mainly concentrated within non-neuronal cells at the 24h timepoint, shown by the (figure 12). However, infection quickly increased at 48h and was evident in many cells at 72h, accompanied by a marked reduction in non-neuronal cells with no changes seen in the control group (CTRL).

CGRP & NF200 Fluorescent Channels

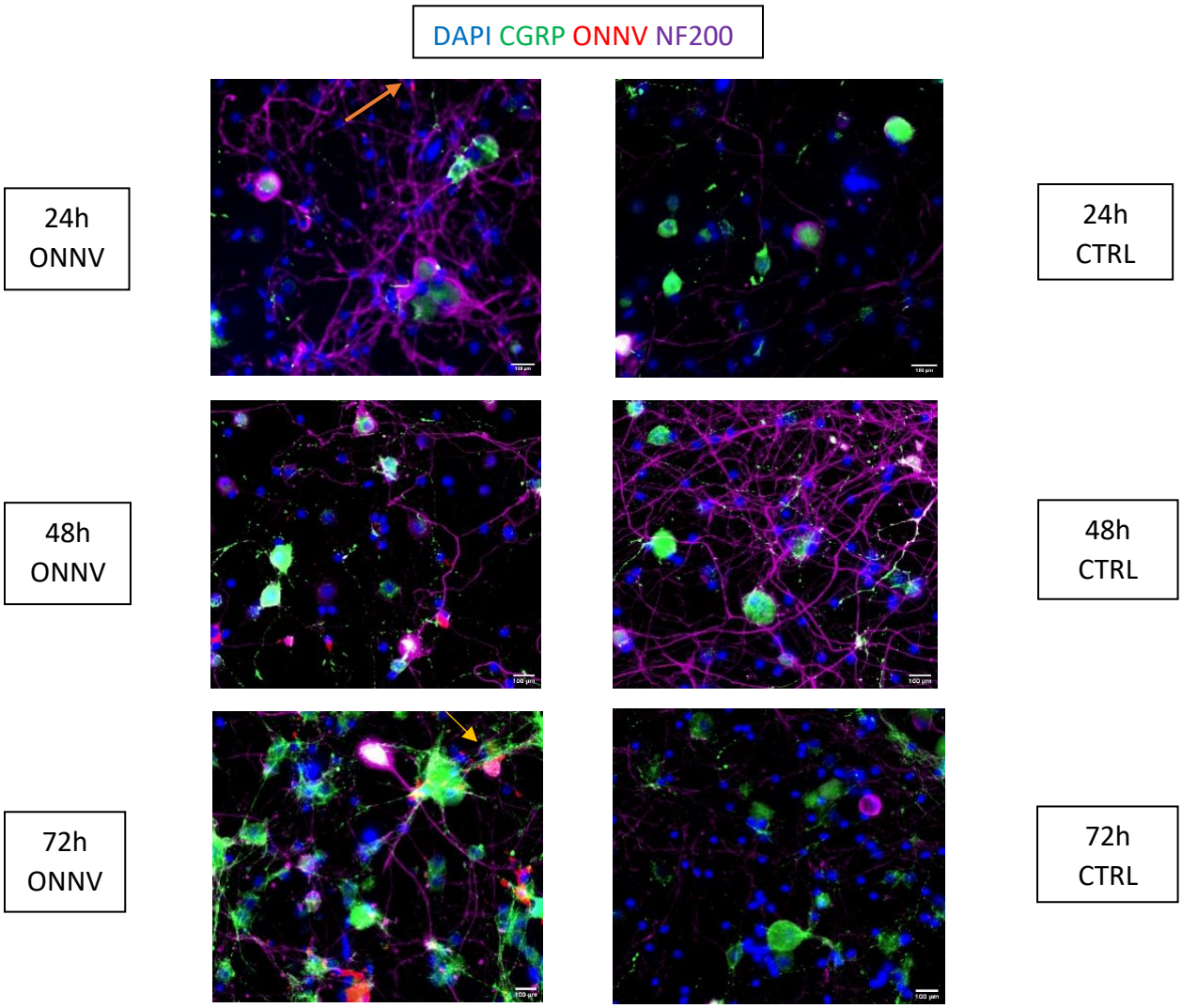

Figure 13. Representative 20x fluorescent images of ONNV-infected and CTRL coverslips at 24h, 48h and 72h post infection (hpi). Labelling for the nuclear marker DAPI (blue), peptidergic marker CGRP (green), mcherryONNV (red) and heavy neurofilament marker NF200 (magenta), from the CGRP and NF200 dual labelling experiment. Orange arrows show infected non-neuronal cells with yellow arrows indicating infected neurons with Scale bar: 100µm.

Compared to CTRL, there was an increase in the number of infected CGRP+ cells (green) and a marked reduction in the numbers of NF200+ neurons between 24h and 72h in the ONNV condition, with no change in the CTRL group.

ONNV INFECTION INCREASES CGRP$^+$ PEPTIDERGIC NEURONS

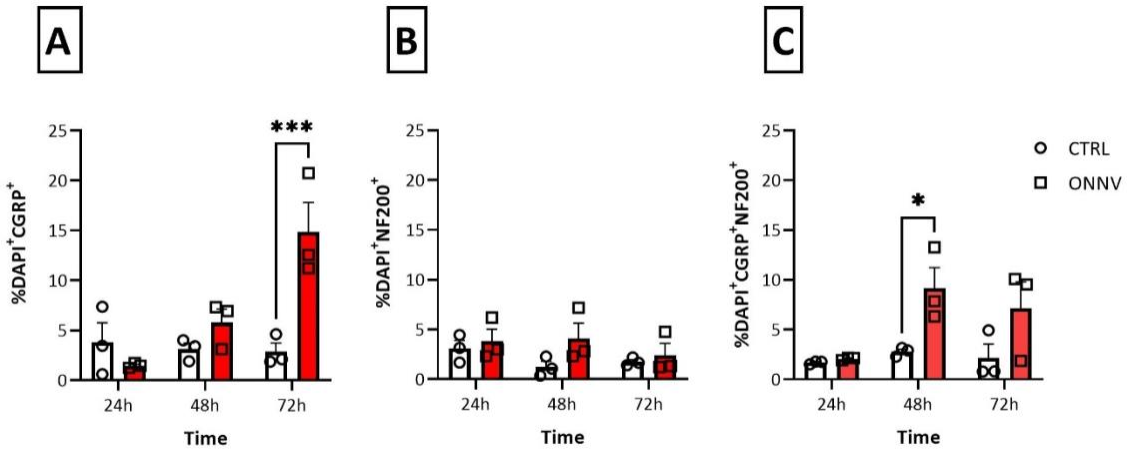

Figure 14. Analysis of peptidergic (CGRP$^+$), non-nociceptive (NF200$^+$) and myelinated nociceptive (CGRP$^+$NF200$^+$) DRG cell populations following ONNV infection at 24h, 48h, and 72h post infection (n= 3 per condition and timepoint). Percentage of total cells that are CGRP$^+$ (**A**), NF200$^+$ (**B**), and CGRP$^+$ NF200$^+$ (**C**). * = p<0.05, *** = p<0.001

There were significant increases in the percentage (11.97, p = 0.0006) of peptidergic (CGRP$^+$) neurons relative to total cell count in the ONNV-infected group (14.84±2.64) compared to the CTRL group (2.863±0.66) at 72h (fig 14A). Interestingly, the proportion of CGRP$^+$ cells at 24h was decreased in ONNV coverslips compared to CTRL, although this was not significant. No significant differences could be found between the percentage of CGRP$^-$ NF200$^+$ myelinated neurons at any timepoint although there was a decreasing trend between ONNV timepoints (fig 14B). The number of dual-labelled CGRP$^+$NF200$^+$ myelinated peptidergic neurones, counted as a discreet population, did increase between 24h (2.07±0.4) and 48h (9.14±1.51) in ONNV coverslips, with the 48h timepoint being significantly different (p = 0.0325) between CTRL (2.77±0.70) and ONNV conditions (9.14±1.51, fig 14C). This data suggests that the percentage of peptidergic C-fibres increases because of ONNV infection, with an unchanging level of myelinated CGRP$^-$ fibres, and a slight increase in the number of peptidergic Aδ nociceptors (CGRP$^+$NF200$^+$) from 24h to 48h, possibly as a result of non-neuronal cell death or specific division of these cells.

ONNV SIGNIFICANTLY INFECTS $CGRP^+NF200^+$ NEURONS

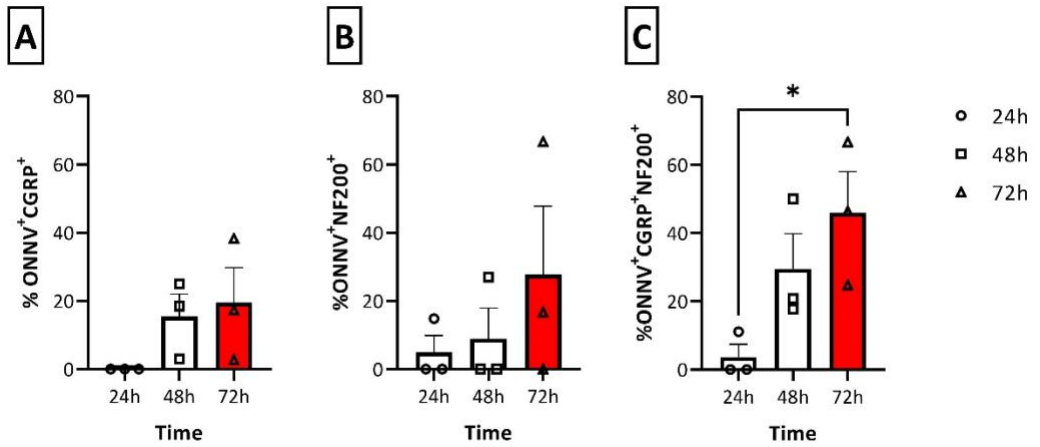

Figure 15. The percentage of peptidergic ($CGRP^+$), non-nociceptive ($NF200^+$) and myelinated nociceptive ($CGRP^+NF200^+$) DRG cell populations infected by ONNV at 24h, 48h, and 72h post infection (n= 3 per condition and timepoint). ONNV-infected $CGRP^+$ (**A**), $NF200^+$ (**B**), and $CGRP^+NF200^+$ (**C**) neurons. * = $p<0.05$

The percentage of peptidergic and myelinated neuronal subtypes that were infected with mcherryONNV were calculated to compare infection levels. At the 24h timepoint, no $CGRP^+NF200^-$ peptidergic C-fibres were infected by ONNV (fig 15A). If the mean values are taken into consideration, there is a clear increase from 24h to 72h (19.47± 7.73), however this did not reach significance ($p = 0.2666$). An increase in the infection of myelinated neurons ($NF200^+$) was also seen between 24h (4.94±2.38) and 72h (27.75±13.86, fig 15B) however this increase did not reach significance ($p = 0.5951$) due to large variability and small sample sizes. Significant differences ($p = 0.0439$) were found in the infection of myelinated peptidergic ($CGRP^+$ $NF200^+$) neurons (fig 15C) between 24h (3.70±3.49) and 72h (45.95±13.44). This data suggests that whilst ONNV can infect all three of these fibre types, the virus preferentially infects myelinated peptidergic neurons ($CGRP^+NF200^+$) during the acute phase of infection.

NeuN & IB4 Brightfield Images

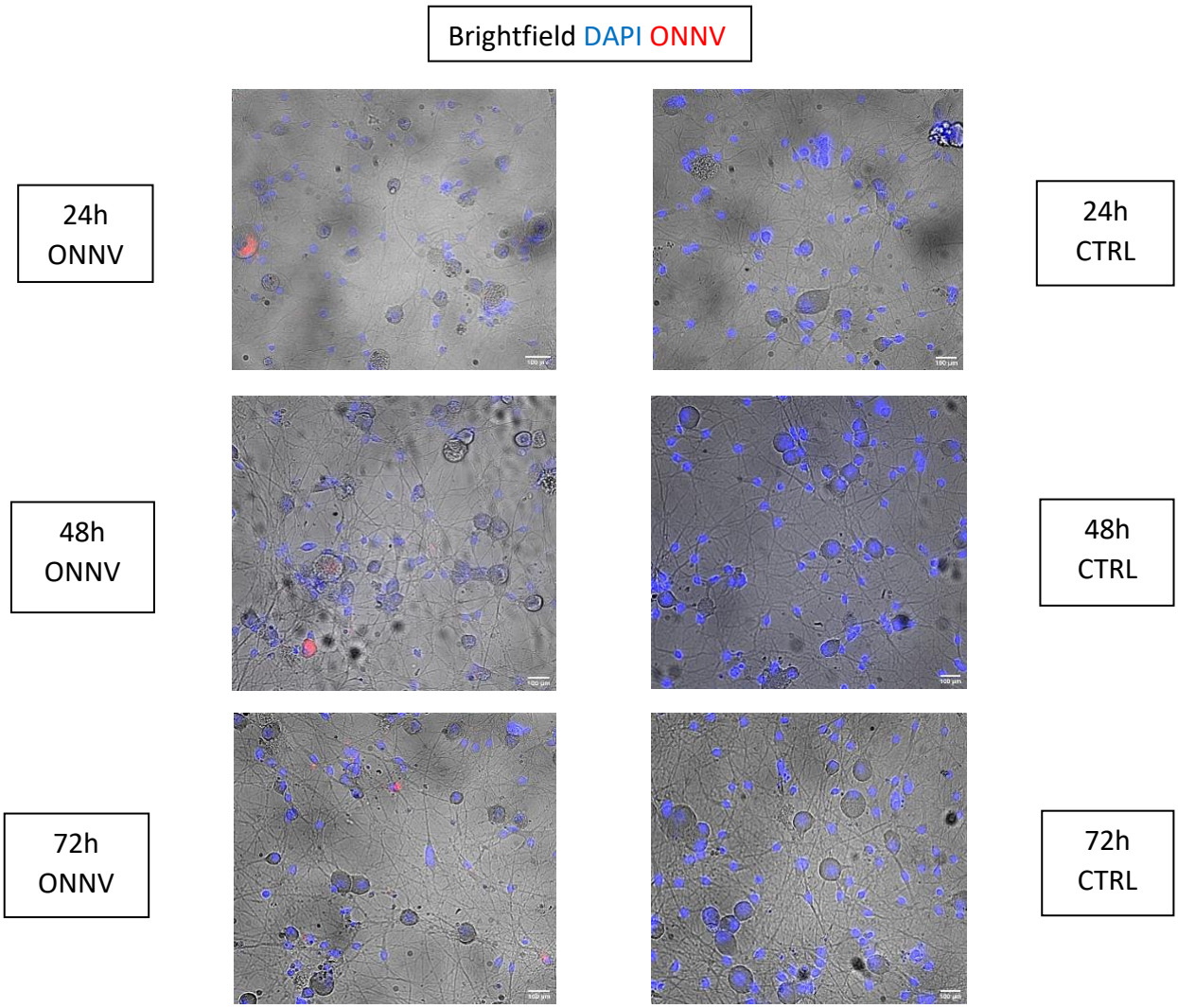

Figure 16. Representative 20x brightfield images of ONNV-infected and CTRL coverslips at 24h, 48h and 72h post infection (hpi). Labelling for the nuclear marker DAPI (blue) and mcherryONNV (red) from the IB4 and NeuN dual labelling experiment. Black arrows indicate infected neurons with orange arrows showing infected non-neuronal cells. Scale bar: 100µm.

In the NeuN and IB4 labelling experiment, there was no difference between the total number of cells between CTRL and ONNV conditions, however non-neuronal cells were significantly reduced in ONNV-infected FOVs, with no change in the mock infection (CTRL). ONNV expression increased from 24h to 72h particularly in non-neuronal cells, with marked reductions in the numbers of these cells compared to control coverslips.

NeuN & IB4 Fluorescent Channels

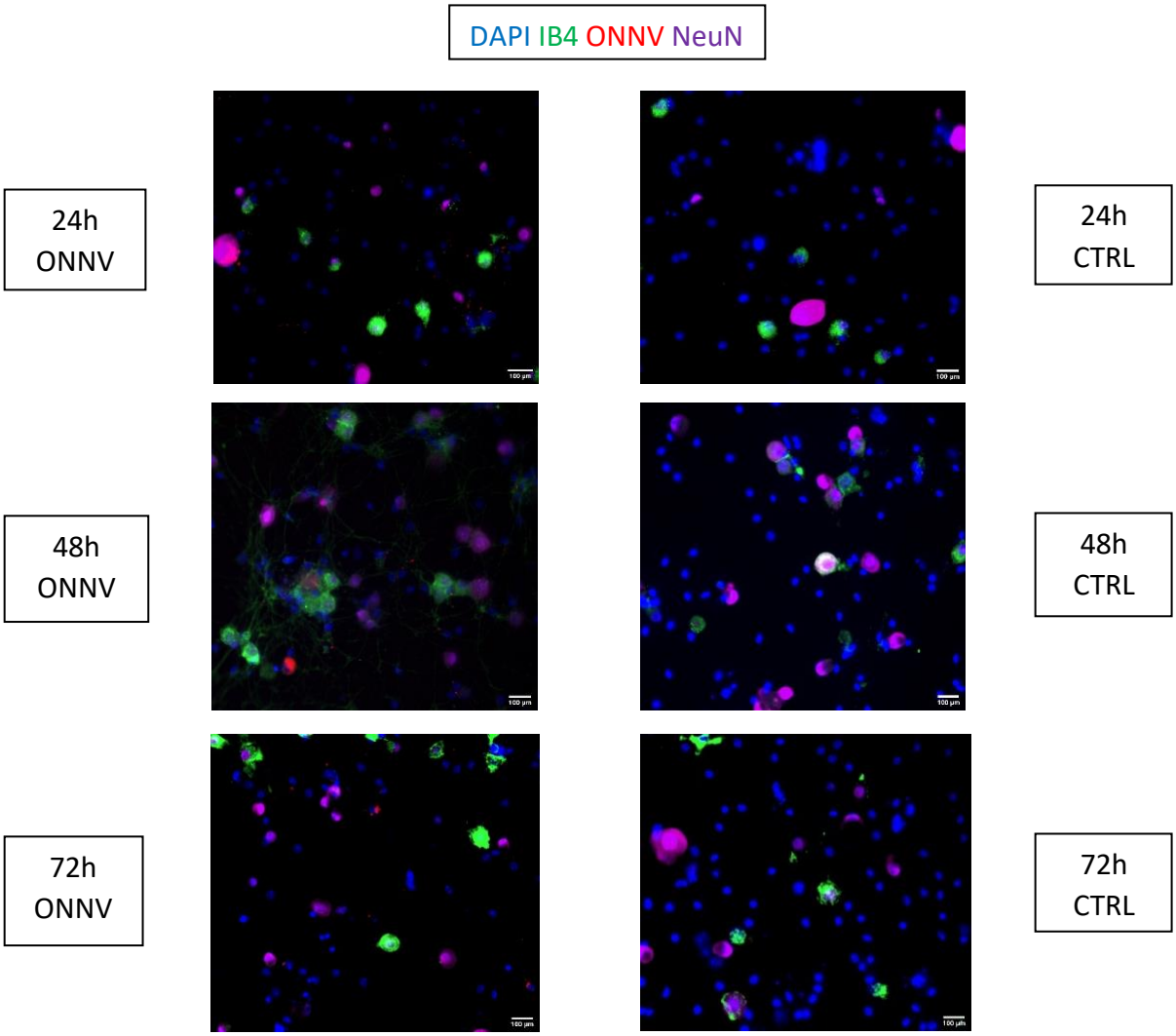

Figure 17. Representative 20x fluorescent images of ONNV-infected and CTRL coverslips at 24h, 48h and 72h. Labelling for the nuclear marker DAPI (blue), non-peptidergic marker IB4 (green), mcherryONNV (red) and neuronal nuclei marker NeuN (magenta), from the IB4 and NeuN dual labelling experiment. Blue arrows indicate infected neurons with orange arrows showing infected non-neuronal cells. Scale bar: 100µm.

During microscopy, no difference was detected in the numbers of either non-peptidergic neurons or neurons as a whole population between intra-condition timepoints. There were also remarkably more non-peptidergic neurons and fewer non-neuronal cells in the ONNV coverslip at the 72h timepoint compared to CTRL cultures.

ONNV INFECTION DOES NOT ALTER NON-PEPTIDERGIC NEURONS

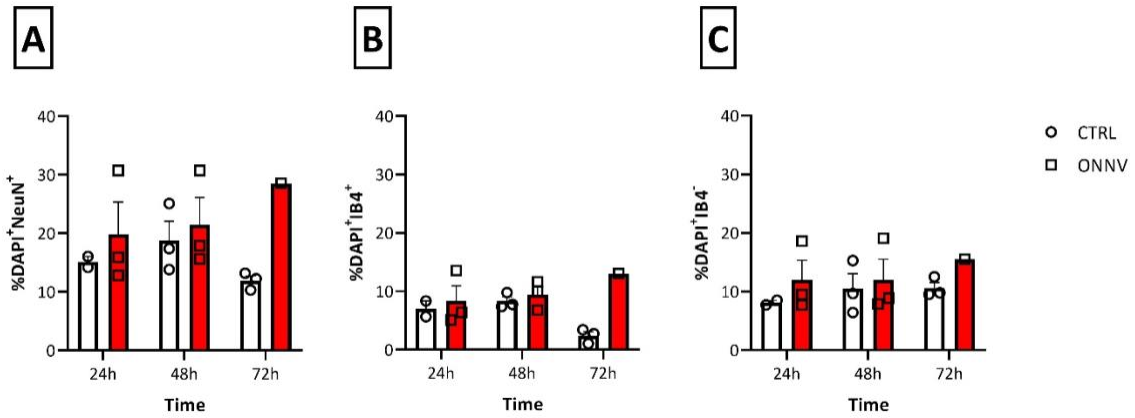

Figure 18. The percentage of (**A**) all neurones (NeuN$^+$), (**B**) non-peptidergic (IB4$^+$) and (**C**) peptidergic (non-IB4$^+$) DRG cell populations following ONNV or mock infection at 24h, 48h, and 72h post infection timepoint. N= 3 per condition and timepoint, excluding 24h CTRL (2) and 72h ONNV (1) due to lack of viable coverslips.

The percentage of neurons as a whole population (NeuN$^+$) and non-peptidergic (NeuN$^+$ IB4$^+$) neurons were calculated using an overlay of the DAPI channel. This analysis showed and that there were no significant differences in the percentages of total neurons as a whole population (NeuN$^+$, fig 18A) or non-peptidergic neurons (IB4$^+$, fig 18B) between ONNV and CTRL coverslips at any timepoint. Interestingly, there was also no statistically significant difference in the numbers or percentages of IB4$^-$ neurons (fig 18C) between ONNV and CTRL coverslips at any timepoint, suggesting that as a total population, peptidergic neuron infection does not increase over time. Perhaps this was due to the fields of view and limited 72h ONNV coverslip numbers. These results indicate that ONNV infection does not change the relative levels of neurons as a whole population, or non-peptidergic neurons specifically.

ONNV DOES NOT SIGNIFICANTLY INFECT NON-PEPTIDERGIC NEURONS

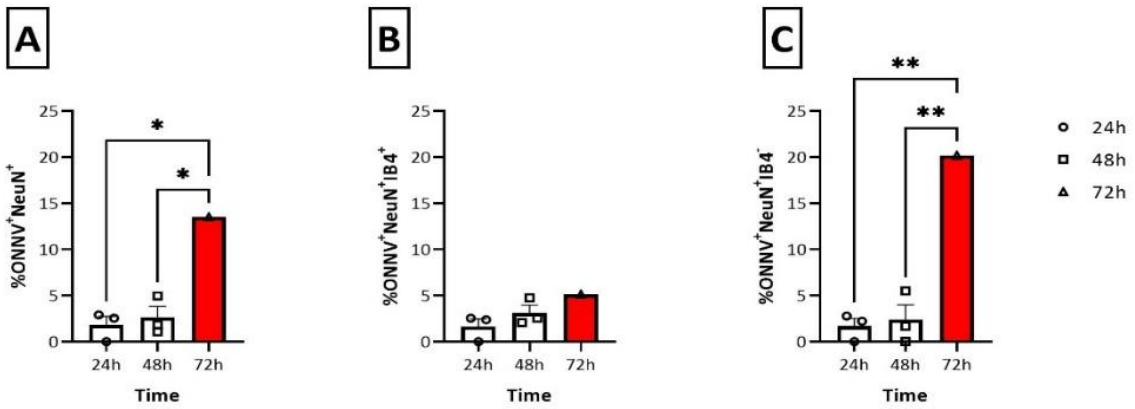

Figure 19. The percentage of NeuN$^+$ cells (**A**), IB4$^+$ neurons (**B**), and IB4$^-$ neurons (**C**), infected by ONNV at 24h, 48h, and 72h timepoints. N= 3 per condition and timepoint, excluding 24h CTRL (2) and 72h ONNV (1) due to lack of viable coverslips.* = p<0.05, ** = p<0.01

The infection of neurons as a whole population (NeuN$^+$) and non-peptidergic (NeuN$^+$ IB4$^+$) neurons were calculated using overlays of the nucleic DAPI channel and the TRITC channel showing mCherry-labelled ONNV nsp3. The infection of neurons as a whole population (fig 19A) increased significantly between 24h (1.82± 0.91) and 72h (13.54±5). In agreement with the insignificance of IB4$^+$ population changes, no significant difference in ONNV infection was seen in non-peptidergic neurons, however there was an increasing trend between timepoints and between 2.5% and 5% of IB4$^+$ neurons were infected (fig 19B). Furthermore, in agreement with the CGRP and NF200 labelling experiment, there was a significant increase ($p = 0.0319$) in the ONNV infection of IB4$^-$ neurons (fig 19C) between 24h (1.67± 1.05) and 72h (20.19) . This data indicates that ONNV minimally infects non-peptidergic neurons and most neuronal infection occurs in peptidergic neurones.

ONNV INFECTS AND REDUCES NON-NEURONAL CELLS SIGNIFICANTLY

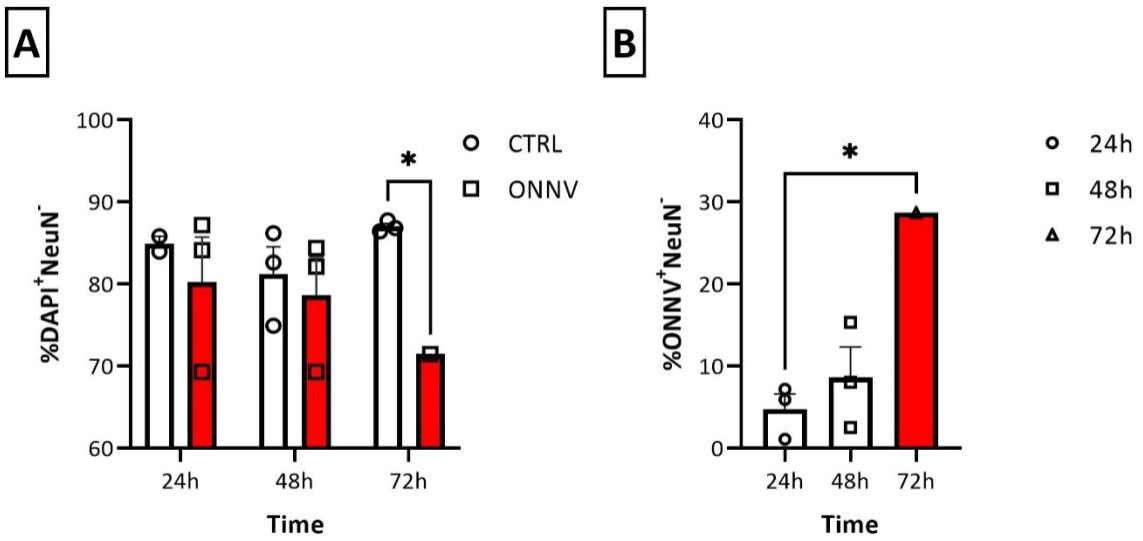

Figure 20. Non-neuronal ($NeuN^-$) cell counts expressed as a percentage of total $DAPI^+$ cell counts with SEM (**A**) and percentage of $NeuN^-$ cells infected by ONNV with SEM (**B**). Individual datapoints consist of coverslip FOV averages with non-neuronal cell counts being determined via non-labelled nuclei counting. N= 3 per condition and timepoint, excluding 24h CTRL (2) and 72h ONNV (1). * = $p<0.05$

To investigate whether non-neuronal cells were infected with the virus, a combination of neuronal brightfield and negative neuronal labelling was used during image analysis. There were significant reductions in non-neuronal populations ($NeuN^-$, fig 20A) between CTRL (118.67±14.58) and ONNV (75.33±15.57) at 72h ($p = 0.0220$). ONNV infection of non-neuronal cells ($NeuN^-$) also significantly increased, ($p= 0.0325$), between 24h (4.73±1.51) and 72h (28.68), shown in figure 20B. This indicates that ONNV significantly infects non neuronal cells and that this results in large reductions in cell numbers possibly via viral-induced apoptosis.

ONNV INFECTION DOES NOT SIGNIFICANTLY CHANGE TOTAL CELL COUNTS

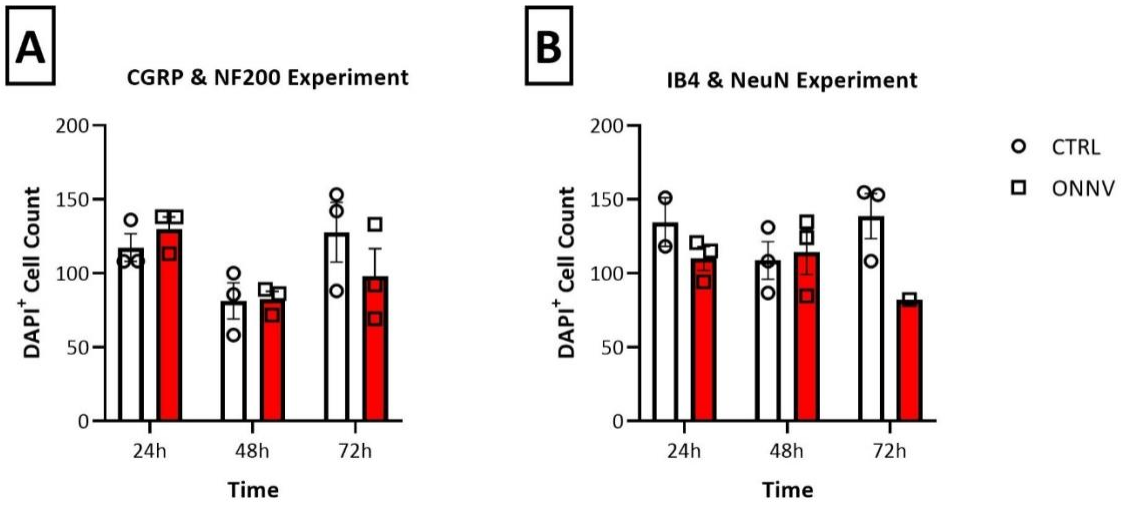

Figure 21. Total DAPI⁺ Cell Counts (**A**) CGRP & NF200 experiment with SEM, (**B**) IB4 & NeuN experiment with SEM. Individual datapoints consist of coverslip FOV averages, N= 3 per condition and timepoint, excluding 24h CTRL (2) and 72h ONNV (1) of the IB4 & NeuN experiment due to lack of viable coverslips.

The total cell counts were obtained via positive DAPI expression inside cell nuclei and compared between ONNV and CTRL coverslips. There was a clear trend for reduced mean numbers in ONNV-infected DRG cultures (98±11) compared to control (124±14) at 72h in the CGRP and NF200 dataset, however this did not reach significance with the small sample size (fig 21A). A clear trend was also found between control (139±15) and ONNV coverslips (82±14) in the NeuN and IB4 dataset (fig 21B), although due to a lack of replicate coverslips and statistical power, this difference was not statistically significant, and a Mann Whitney U-test could not be performed. Overall, whilst these experiments have yielded no statistically significant reduction in total cell numbers from ONNV infection, these trends indicate that ONNV may reduce total cell count, perhaps due to the large reduction in non-neuronal cells evidenced in figure 20.

CELL AREA ANALYSIS

The soma areas of positively labelled cells in the CTRL coverslips were acquired and compared to elucidate whether there were any differences between neuronal cell sizes. It should be noted that neurones that were positive for both CGRP and NF200 were counted as a distinct population, independent of other cell areas and values. Throughout all the timepoints in control cultures, no differences were found between the soma size of neuronal subpopulations, however myelinated neurons ($NF200^+$) and myelinated peptidergic neurons ($CGRP^+NF200^+$) were slightly larger than C-fibre positive markers (CGRP and IB4). This data reinforces the knowledge from the literature that DRGs are highly heterogeneous and cannot be differentiated by cell size alone.

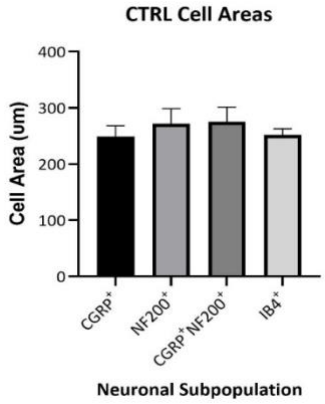

Figure 22. Area Analysis of neuronal subpopulations from CTRL coverslips across all timepoints.

SUMMARY

ONNV differentially infected the neuronal subpopulations from primary mice DRG cultures, most notably myelinated peptidergic neurons, as well as non-neuronal cells. These data further show that ONNV infection does not significantly increase between 24h and 72h in peptidergic and non-peptidergic C-fibres, nor myelinated neurons ($NF200^+$) that were $CGRP^-$. Non-neuronal infection was accompanied by a reduction in the percentage of non-neuronal cells. The percentage of myelinated peptidergic neurons significantly increased between at 48h, with the relative percentage of peptidergic neurons being significantly increased at 72h.

DISCUSSION

Alphavirus infection is known to involve a peripheral immune response (Maucourant *et al.*, 2019), with acute pain typically manifesting from the release of inflammatory mediators that sensitise nociceptors (Costantini & Cassatella, 2010). This acute pain is experienced by most CHIKV-infected patients with at least 16% progressing to chronic rheumatoid arthritis-like polyarthralgia (van *Aalst et al.*, 2017). Uncovering the mechanisms behind alphavirus-induced pain is thus of key importance in both immediately improving patient quality of life and eventual development of novel therapeutics. However, the question of whether DRGs could be infected by ONNV to cause pain remained elusive. This study has evidenced that alphaviruses such as ONNV can infect DRGs, and that infection increases in myelinated peptidergic neurons ($CGRP^+NF200^+$) between 24h and 48h post infection, as well as non-neuronal cells at 24h, 48h and 72h timepoints.

This infection induces significant increases in the relative percentage of peptidergic C-fibres (fig 15A) and myelinated peptidergic fibres (fig 15C) at the 48h timepoint and in addition to

non-neuronal cells (fig 21A) after 72h of in-vitro incubation. No significant changes could be seen between the percentages or infection levels of CGRP- myelinated neurons (fig 15B and 16B) nor non-peptidergic fibres (fig 18B and 19B). However, these peptidergic subpopulation changes do not contribute to a significant reduction in neurons as a whole population. Nevertheless, the reduction in non-neuronal cells could explain the reduction seen at the 72h timepoint.

ONNV-INDUCED SUBPOPULATION CHANGES

The PAF cell bodies are found in the DRG and synapse with second order neurons and interneurons in the dorsal horn to propagate action potentials for recognition, reaction, and experience in the brain (Basbaum *et al*., 2009). Neuropathic pain is caused by direct nerve injury resulting in neuronal hypersensitivity and sensitisation (Slimani *et al.,* 2018), and is another mechanism by which pain can be experienced in other alphaviruses such as the Semliki Forest Virus (Vernon & Griffin, 2005) and Sindbis virus (Burdeinick-Kerr *et al.,* 2009).

This study has evidenced that ONNV can infect all DRG cells and that ONNV may preferentially infect subpopulations of neurons as evidenced by increasing levels of viral nsp3 tagged by mCherry within neuronal cell bodies. Alphavirus nsp3 has a key role in facilitating viral RNA translation in cells (Rougeron *et al.*, 2015), and is an ideal target for the fluorescent tracking viral replication. The insertion of the red fluorescent mCherry protein into recombinant viruses is an effective method to study viral infection levels (Marzook *et al.*, 2014), which does not alter viral infectivity as shown by successful lethal infection (Nogales *et al*., 2015).

PEPTIDERGIC NEURONS ($CGRP^+$)

Alphaviruses enter target cells through clathrin-mediated endocytosis after incorporation via the fusion of E1 and E2 glycoproteins with the cell membrane (Fox *et al*., 2015). The virus then releases four non-structural proteins to replicate and package the single strand RNA into more virions which are released via exocytosis, (Rashad *et al*., 2013), or after cell death (Krejbich-Trotot *et al*., 2010).

The significant increase in the relative percentage of peptidergic C-fibres ($CGRP^+$) could have occurred because of the reduction in non-neuronal cells through infection potentially due to viral-mediated cell death assisted by nsp2 halting antiviral responses (Ahola and Merits, 2016). Intriguingly, ONNV infection did not significantly increase between 24h and 72h timepoints, however given the large population increase, perhaps infection increases at later timepoints in future research.

The terminals of peptidergic C-fibres are found in the deeper dermal layers (Pace *et al.*, 2017), with high expression in bone, being significantly activated in rodent models of osteoarthritis (Morgan *et al.*, 2019). Thus, it is possible that this increase in the proportional percentage of peptidergic cells due to non-neuronal cell death may contribute to the

development of both acute and chronic pain polyarthralgia seen as a hallmark of alphavirus infection (Miner *et al*., 2015). CGRP is also a potent vasodilator, (Brain *et al*., 1985), and plays a significant role in headaches, which are a common symptom of alphavirus infection (Rezza *et al*., 2017). $CGRP^+$ fibres have also been implicated in itch sensation (Rogoz *et al*., 2014), with the maculopapular rash seen in ONNV-infected patients (Rezza *et al*., 2017) also supports a role of this subpopulation of sensory afferents.

We know that peptidergic C-fibres are heavily involved in the development and maintenance of both acute and chronic pain states through the release of nerve growth factors such as NGF, which although not labelled in this study, overlap almost entirely (Shiers *et al*., 2020).

NGF has been implicated in the replication of influenza, and if this is the mechanism by which alphaviruses replicate, then increased levels of NGF release as a result of ONNV infection and replication within peptidergic fibres would thus cause neuronal sensitisation and the development of acute pain. We also know that the release of peptidergic neurotransmitters such as CGRP to second order neurons in the dorsal horn facilitates the transmission of pain signals to the CNS for the experience of pain to be realised. Thus, the increased population of myelinated $CGRP^+$ neurons seen at 48h, and peptidergic C-fibres at 72h, may start to explain the acute pain experienced by this infection.

Future experiments could use an in-vivo longitudinal cohort of infected and mock mouse models to elucidate these findings, potentially uncovering significant reduction in $NF200^+$ (fig 15B) and $CGRP^+NF200^+$ (fig 15C) populations that are shown as a trend in this study.

Additionally, infection levels of other tissues such as skin epithelia for initial infection mechanisms, surrounding muscle and bone in the knee for polyarthralgia monitoring and behavioural analysis with paw withdrawal thresholds and weight bearing asymmetry could be conducted for an all-encompassing analysis of infection that is not possible from human clinical data.

MYELINATED NEURONES ($NF200^+$)

The percentage of myelinated ($NF200^+CGRP^-$) neurons was not significantly different between 24h and 72h and mCherryONNV infection also did not differ significantly between 24h and 72h. Due to a lack of additional labelling such as SP or IB4, it is not possible to categorically define this population as non-nociceptive, and the mean cell areas indicate that the majority are likely to be Aδ fibres involved in fast pain signal transmission. The insignificant differences between 24h and 72h and lack of increased infection in these acute timepoints implies that the pain that patients experience from ONNV infection is not a result of this myelinated population of neurons.

NON-PEPTIDERGIC NEURONS ($IB4^+$)

The percentage of mCherryONNV infected cells did not increase significantly in non-peptidergic (IB4+) C-fibres between 24h and 72h timepoints, implying that ONNV does not significantly infect these neuronal subpopulations. This further implies that the itching seen from rashes in patients may be mediated by peptidergic fibres, independent from $MrgprD^+$ non-peptidergic fibres (Dong & Dong, 2018).

NON-NEURONAL CELLS IN DRG

There were significant increases in the percentage of non-neuronal cells that were infected by ONNV perhaps due to cellular division of infected cells, as well as significant reductions in the relative numbers of non-neuronal cells between 24h and 72h timepoints. This indicates that that ONNV may use non-neuronal cells for replication, killing and leaving them to infect other cells, such as the myelinated or unmyelinated peptidergic neurons. The death of non-neuronal cells would release sensitising agents such as protons and algogens into the extracellular space that could bind to nociceptors to increase pain signals (Costantini & Cassatella, 2010) to the CNS contributing to acute pain. Additionally, these inflammatory molecules would activate remaining glia, leading to the facilitation of immune responses, and increasing the coupling of action potentials (Lemes *et al*., 2018) to further increase pain signals in a neuropathic pain state that could contribute to polyarthralgia experienced by patients.

LIMITATIONS AND IMPROVEMENTS

Protocol optimisation was a particular challenge for this project, specifically minimising autofluorescence. To address this, the permeabilization and blocking buffer steps were combined. The 1% BSA was replaced with 10% Normal Donkey Serum (NDS) in the blocking buffer and Triton X100 was increased from 0.1% to 0.3% to improve permeabilization, as this had proved effective in the labelling of glial cells (Wang *et al.*, 2019).

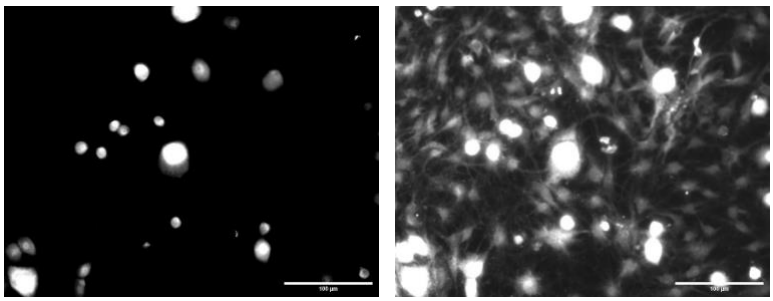

Figure 21: non-specific binding of GS ab49873 in CTRL 48h coverslips. (**A**)Mouse anti-NeuN (Merck Millipore, 1:100) labelling with 100µm scale bars. (**B**) Rabbit-anti Glutamine Synthetase (abcam, ab49873, 1:250).

The presence of large quantities of SGCs during culturing hampered the quantification of the infection of neurons, this was addressed by the inclusion of the mitotic inhibitor aphidicolin to reduce proliferation. Multiple attempts were made to selectively label glial cells, however labelling of neuronal somas by glutamine synthetase (GS, ab49873) at 20x on the Zeiss 200M (fig 21) and 60x by confocal microscopy (fig 22) prevented this. If time had been permitting, nestin could be used as an indicator of SGCs *in-vitro* as evidenced in Wang *et al*., (2019). Some impact of autofluorescence was still suspected at the latter stages of the study, which were remedied by increasing the number of PBS washes from 3x to 5x to improve antibody clearance, the addition of 0.3M glycine to the blocking buffer, adding Vecta TrueVIEW autofluorescence quenching media (kit from Vector labs, SP-8400), after post-secondary antibody PBS washes, and replacing fluoromount (Sigma-Aldrich, F4680) with VECTASHIELD Vibrance (Vector Labs, H-1700) mounting media.

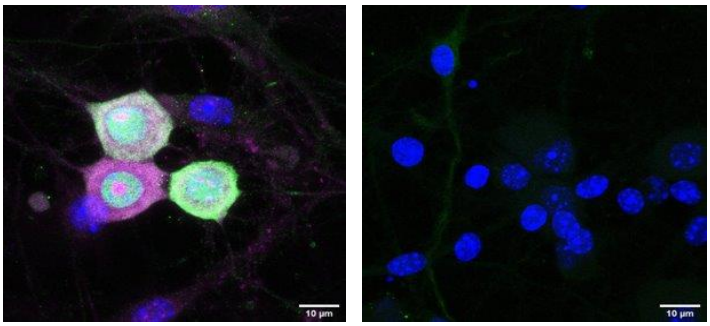

Figure 22: Intranuclear neuronal GS ab49873 labelling in CTRL 48h coverslips by confocal microscopy. (**A**) Primary antibody combination: NeuN (1:100) and GS (1:250). (**B**) Secondary-only labelling with 10µm scale bars.

All coverslips incubated for the 24h and 48h timepoints were from one animal, and thus despite multiple FOVs of multiple cultured coverslips, the N number for this study was very low (n=1). Additionally, due to an error with DRG preparation, the DRGs used for 72h timepoint coverslips were from a different animal than 24h and 48h, which may explain the stark differences in counts between these timepoints. A fragile batch of coverslips for the final IB4-and-NeuN-label experiments meant that considerable numbers of 72h ONNV-infected coverslips were lost at the beginning and end of the experiments. This removed the possibility of any further experimentation using the final DRG preps, in addition to necessitating a fixed effects (type 3) analysis for the IB4 and NeuN dual labelling experiment compared to a two-way ANOVA for the CGRP and NF200 dual labelling experiment. Additionally, this further resulted in the imaging of one 72h ONNV coverslip for the IB4 and NeuN experiment, invalidating any statistical differences between ONNV and CTRL at this timepoint.

It had been intended that an in-vivo ONNV infection animal study would be performed, but this was postponed due to the university closure at the end of March 2020 because of the SARS-Cov-2 pandemic. This would have provided significant insight into the effect of in-vivo infection and the potential quantification of axonal transport mechanisms. In response to this closure, the decision was made to postpone these experiments until the university reopened. However, the requirements for social distancing in the lab and the impact on time available resulted in the decision that in vivo studies would not be performed to allow focus on the in vitro study. There was a more general impact of the pandemic arising from

essential colleagues involved in my training and support being absent from work and my absence from the lab (both due to isolation requirements) which meant that my training was slowed and this resulted in less useable data being generated.

The results of this study suggest multiple avenues of research that could be further investigated. The significance of some of the findings is limited by the small number of the coverslips used for this study, future experiments involving larger datasets would elaborate upon these results and provide a more comprehensive view of ONNV infection in DRGs.

Future Work

This study only quantified non-neuronal cell counts and infection via brightfield morphological analysis. Future research with a successful glial antibody candidate would provide a categorical distinction between neurons, glia and other non-neuronal subpopulations such as immune cells.

Whilst this study evidenced significant increases in the infection of non-neuronal cells and $CGRP^{+}NF200^{+}$ neurons with significant increases in $CGRP^{+}$ neurons and reductions in non-neuronal cell counts, the exact mechanism of ONNV infection and following cell death remains elusive. Future experiments could use a marker of cell death such as cleaved caspase 3 to determine apoptotic events and immune markers for macrophage-mediated destruction (Kennedy *et al*., 2018).

A microfluidics chamber could also be used to establish exactly how ONNV infects sensory nerve fibres. Perhaps this could include a marker for the TrkA trafficking protein CD2AP, which has been evidenced to interact with CHIKV nsp3 (Mutso *et al*., 2018), to elucidate whether NGF is responsible for axonal translocation of virions to the DRG from the periphery.

Statement of Impact

Alphaviruses infections that cause acute and chronic pain are becoming more common, reducing patient quality of life. Understanding alphavirus pain mechanisms is vital to limiting pain for patients and providing the downstream possibility of therapeutic development.

Conclusion

In conclusion, this study has evidenced that alphaviruses such as ONNV can infect DRGs, preferentially infecting myelinated peptidergic fibres and non-neuronal cells that results in an increased peptidergic C-fibre population and reduced non-neuronal population. The infection of DRG populations could play an important role in the development of pain states alongside inflammatory pain in the acute phase of infection and provides a mechanism by which acute pain may transition to chronic pain through peripheral sensitisation. Additional

research is needed to define the more intricate mechanisms of viral replication in DRGs and fully comprehend the effect of alphavirus infection on the PNS and CNS.

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APPENDIX 1

ABBREVIATIONS

ACRONYM	FULL DESCRIPTION
CHIKV	Chikungunya Virus
AMH	A-Mechano-Heat Receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Action Potential
ASIC	Acid-sensing ion channels
ATP	Adenosine Triphosphate
AB	A-beta fibres
AΔ	A-delta fibres
BK	Big Potassium Channel
CAMP	Cyclic Adenosine Monophosphate
CGRP	Calcitonin gene-related peptide
CHRNA3	Nicotinic acetylcholine receptor subunit alpha-3 receptor
C-HTMR	C-fibre High Threshold Mechanoreceptors
CL	Containment Level
CLR	Calcitonin receptor-like receptors
C-LTMR	C-fibre Low Threshold Mechanoreceptors
CNS	Central Nervous System
DNA	Deoxyribose Nucleic Acid
DRG	Dorsal Root Ganglia
GABA	Gamma-aminobutyric acid.
GDNF	Glial-derived neurotrophic factor
GFAP	Glial Fibrillary Acidic Protein
GIRK3	G protein-gated inward-rectifying potassium channels
HDRG	Human Dorsal Root Ganglia

IA	inactivating transient A-type Potassium channels
IB4	Isolectin B4
IGLUR	Ionotropic Glutamate Receptor
KV	Voltage-gated Potassium Channel
LOF	Loss Of Function
MDRG	Mouse Dorsal Root Ganglia
MGLUR	Metabotropic Glutamate Receptor
NAV	Voltage-gated Sodium Channel
NF200	Heavy Chain Neurofilament 200
NGF	Nerve Growth Factor
NK1	Neurokinin Receptor
NMDA	N-methyl-D-aspartate Receptor
ONNV	O'Nyong-Nyong Virus
P2XR	Purinergic receptor type 2
PAF	Primary Afferent Fibre
PAG	Periaqueductal Grey
PBN	Parabrachial Nuclei
PLC	Phospholipase C
PNS	Peripheral Nervous System
RAMP	Receptor-activity-modifying proteins
RNA	Ribonucleic Acid
RVM	Rostral Ventromedial Medulla
SGC	Satellite Glial Cell
SK	Small-conductance Potassium Channel
SP	Substance P
TNFA	Tumour Necrosis Factor α
TRKA	Tropomyocin Kinase A
TRP	Transient receptor potential channels
TRPA1	Long transient receptor potential ankyrin protein 1
TRPM8	Transient receptor potential melastatin 8 channel
TRPV	Transient receptor potential vanilloid channels
VGCC	Voltage-Gated Calcium Channels
VGLUT	Vesicular Glutamate Transporter