

School of Life Sciences

New approaches to the study of nociception: identification and refinement of approaches for the use of *Drosophila melanogaster* in the drug discovery pipeline

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Submitted to the University of Nottingham for a Master of Research degree

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Abstract

Nociception is highly conserved between invertebrates and vertebrates. The bulk of basic biomedical research into pain and nociception relies on mammalian model systems with accompanying ethical, financial and time pressures. Thus, studies investigating the peripheral mechanisms of nociception in an invertebrate system where these concerns are reduced is highly desirable. *Drosophila melanogaster* utilise class IV dendritic arborization (C4da) neurons as polymodal nociceptors responsible for the detection of noxious mechanical, thermal, optical, and chemical stimuli. Transient receptor potential (TRP) channel mediated Ca^{2+} influx is crucial for the initiation of noxious signal transduction in both mammals and *Drosophila*, and the modulation of these responses by cAMP, following noxious stimulation, is common between both vertebrate and invertebrate sensory neurons. We selectively expressed a FRET (Foster resonance energy transfer)-based cAMP sensor and a genetically encoded Ca^{2+} indicator in the C4da nociceptive neurons. The spatiotemporal dynamics of signalling molecules underlying nociception have been monitored and quantified in *Drosophila* at single sensory neuron endings. We were able to induce a cAMP increase in the C4da nociceptive neurons with 50 uM Forskolin (FSK), an adenylyl cyclase activator (dynamic range ~6%). We report cinnamaldehyde (CA), a classic agonist of mammalian TRPA1, is able to activate the *Drosophila* TRPA1 homologues (dTRPA1 and/or Painless) expressed on these sensory neurons to induce a robust intracellular Ca^{2+} response (EC_{50} = 4.123 x 10^{-5} M). The CA-induced Ca^{2+} response observed is reproducible and can be pharmacologically inhibited by 20 uM AM0902. In mammalian sensory neurons there are temporal differences in the activation of distal and proximal terminals as well as the cell body. Within these cellular sub-compartments, it has been demonstrated that local processes, such as the regulation of expression key proteins involved in neuronal function take place. Importantly, we have demonstrated data can be obtained from different neuronal compartments of the C4da nociceptive neurons (distal dendrites, proximal dendrites, and soma). This model advocates the translational utility of *Drosophila*, demonstrating to the pain field that early proof of concept studies can be carried out in this non-mammalian system to reduce the numbers of successful candidate molecules being tested in rodents.

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1 Introduction

1.1 Nociception

The International Association for the Study of Pain defines nociception as "the neural process of encoding noxious stimuli", whereas pain constitutes "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (IASP, 2020). Nociception is an evolutionarily conserved process which first evolved more than 550 million years ago (Walters, 2018). Sherrington (1903) introduced the term nociception and later proposed nociceptors as being responsible for preferentially detecting noxious stimuli. Nociceptors are electrically silent, unless in the presence of imminent danger (Woolf and Ma, 2007). Noxious thermal, chemical, and mechanical stimuli activate high threshold ion channels expressed on primary afferent fibres to cause nociceptor membrane potential depolarisation. Sufficient depolarisation elicits an action potential at the peripheral nerve terminal that is propagated along the axon and then transmitted to the central nervous system (CNS) for processing (Smith and Lewin, 2009; Dubin and Patapoutian, 2010). This mechanism permits organisms to differentiate noxious and innocuous stimuli from each other and is critical for survival as a means to avoid tissue injury.

The fundamental physiology and molecular biology of nociception is highly conserved throughout the phylogenic tree from invertebrates to vertebrates. The fruit fly (*Drosophila melanogaster*) is therefore becoming an attractive model organism in which to investigate cellular processing in many neurological diseases. Publications utilising *Drosophila* to study the mechanisms of Alzheimer's Disease have doubled within a decade (46 publications in 2010 to 80 in 2020 out of a total of 1070 publications (1984 to 2021)). However, their use in pain research is distinctly less common than in other areas of neuroscience. Only 210 publications have used *Drosophila* to study nociception in the last 36 years. Cytokines, neuropeptides, and lipids, known to play pivotal roles in mammalian nociception, are conserved in *Drosophila* (Im and Galko, 2012; He *et al.*, 2022). Similarly, transient receptor potential (TRP) channels, the fundamental molecular sensors of noxious thermal stimuli across phyla, are expressed in *Drosophila* sensory neurons (Tracey *et al.*, 2003; Neely *et al.*, 2011).

This project has been devised to provide the experimental tools necessary for the identification and study of new analgesic drug targets through the optimisation of a novel invertebrate system that is highly amenable to genetic modification, specifically for the study of the peripheral mechanisms of pain. Currently there are no *Drosophila* models that have been validated for use in pain research and there is an urgent need for a fast and efficient method of identifying targets to accelerate the pipeline of analgesic drug discovery. The spatiotemporal dynamics of the signalling molecules underlying nociception can be monitored and quantified in *Drosophila* at single sensory neuron and within different neuronal components such as- dendritic arborization endings, axons and the cell body. This analysis will advance the mechanistic understanding of nociception. Our aim is for the model to produce reproducible responses to nociceptive stimuli, enabling the identification of potential nociceptive targets in *Drosophila* that have mammalian homologues. These targets can then be manipulated, either genetically or pharmacologically, to see how nociceptive responses are impacted, and to later introduce target manipulation into mammalian models. It is anticipated that this will provide a strong foundation for research teams worldwide to begin to replace rodent studies of nociceptive mechanisms, thereby complying with the 3Rs (replacement, reduction, and refinement) and providing major advances in scientific understanding beyond what is currently achievable using rodents.

1.2 Poor preclinical to clinical translation

Pain is a whole-body phenomenon, engaging the peripheral and central nervous systems and as such, in vivo experimental models are often used. The majority of pre-clinical pain research takes place in rodent models that are amenable to genetic modification, have well understood behavioural responses to pain and are relatively easy to use and manage. The global burden imposed by chronic pain, and diseases associated with pain, has been documented as the most important current and future cause of disability (Rice, Smith and Blyth, 2016). Breivik *et al.* (2006) conducted a survey to explore the prevalence, severity, treatment, and impact of chronic pain in Europe. One third of chronic pain sufferers were not being treated, 40% were identified as receiving inadequate pain relief from current analgesics and 14% had stopped taking pain medication due to the side effects. A recent survey, commissioned by BBC News, found that 28% of people in the East of England live with chronic pain, but the prevalence may be even higher in older age groups (Clegg and Hughes, 2022). Guidelines from the health regulator National institute of Health and Care Excellence

(NICE), for the assessment and management of all chronic pain (chronic primary pain, chronic secondary pain, or both), state that opioids should not be offered to manage chronic primary pain (NICE, 2021). Long term use of opioids in chronic pain have serious adverse effects such as the development of tolerance, addiction and opioid-induced hyperalgesia (Montgomery, 2020). Currently, nearly a quarter (24%) of individuals with chronic pain are currently taking opioid painkillers (Clegg and Hughes, 2022). Despite extensive research efforts, several decades have passed with almost no regulatory approvals of new clinically beneficial analgesic drugs for the treatment of chronic pain, even with the substantial clinical need. To develop treatment plans and prevention strategies we need to fill the significant gaps in our understanding of pain processing and its management.

Mogil (2019) reviewed the translatability of pain across species and considered the degree to which pain biology in rodents predicts pain biology in humans. Human pain biology is fundamentally different and more complex than rodent pain biology, with drugs that are developed in rats failing to show analgesic efficacy in human clinical trials. Despite this, there is strong evolutionary conservation among mammalian species. Comparative analysis of the mouse and human genome showed limited evidence for species differences in biology, with only 300 genes being unique to each species (Brent *et al.*, 2002). Sequencing of the rat genome found that almost all human genes, known to be associated with disease, have rat orthologues (Gibbs *et al.*, 2004). One possibility for poor translation in the pain field could be the non-predictivity of animal pain models, with criticisms surrounding the relevance of these models. For example, human clinical pain is largely spontaneous (non-evoked) in nature, whereas animal models largely measure withdrawal reflexes (Mogil, 2019). This lack of success has attributed to the failure of preclinical-to-clinical translation between pain studies in rodents and humans, nevertheless rodent use is still commonplace in research, exploring mechanisms of pain processing and target validation before moving to clinical trial (Mogil, 2009). Using the Medical Subject Headings (MeSH) terms ‘analgesic’ and ‘rodent’ Percie Du Sert and Rice (2014) identified an average of 1350 publications per year, in the last two decades, that used rodents in the area of pain research. To date, there are now over 49,000 publications in total.

1.3 Mammalian nociception

The detection and transmission of noxious chemical, mechanical or thermal stimuli is primarily governed by the expression and function of specific receptors and ion channels which regulate the excitability of primary afferent fibres (PAFs). Ion channels that have pivotal functional roles in the primary afferent pain pathway have emerged as attractive therapeutic targets, this includes: the voltage-gated sodium channel (VGSC) Nav1.7 and Nav1.8 isoforms, the N-type calcium channels and ligand gated ion channels such as acid-sensing ion channels (ASICs) and transient receptor protein (TRP) channels, many of which are dysregulated in chronic pain states (Waxman and Zamponi, 2014). Primary somatosensory neurons are pseudounipolar, the cell bodies located anatomically in the dorsal root ganglia (DRG), extending a peripheral axon to innervate the target tissue and a central axon that enters the dorsal horn (DH) of the spinal cord (Bell, 2018). PAFs are subdivided into different subclasses, based on their size, morphology, neurochemical phenotype, and modality of sensory information they encode. Three types of PAF convey sensory information to the DH of the spinal cord: Aβ, Aδ and C-fibres (Abraira and Ginty, 2013). Large diameter (6-12 μm) myelinated Aβ fibres are low frequency mechanoreceptors that convey non-noxious stimuli such as light touch, pressure, and vibration. Thinly myelinated Aδ fibres (6-12 μm in diameter) convey information about touch, temperature, and sharp localised pain. The small diameter (0.2-1.5 μm) unmyelinated C fibres, characterised by relatively high thresholds, carry information related to slow diffuse pain, noxious temperature, significant pressure, and itch. C fibres can be further categorised into two chemical subpopulations based on neuropeptide expression, peptidergic and nonpeptidergic. Peptidergic fibres express substance P (SP) and calcitonin gene-related peptide (CGRP), and the nonpeptidergic fibres are isolectin B4 positive (IB4+) cells that express the adenosine trisphosphate (ATP) purinergic P2X3 receptors (Fitzgerald, 2005).

Following membrane depolarisation, action potentials are initiated in the afferent pain fibres that synapse with second order neurons, either projection neurons or spinal interneurons, in spinal DH laminae I and II (Ozaki and Snider, 1997). The SP receptor Neurokinin 1 (NK1), present in peptidergic C fibres, is densely expressed in lamina I throughout the length of the spinal cord (Nakaya *et al.*, 1994). Todd *et al.*, (2002) reported that 80% of the lamina I projection neurons, the majority of which were activated by noxious stimuli, expressed NK1.

The excitability of the spinal DH, determined by the relationship between the sensory input and firing of the second order neuron, directly correlates with the pain an individual experiences. The heterogenous population of spinal interneurons, that relay and integrate noxious information to projection or motor neurons, precludes easy classification (Todd, 2010). Experimental approaches classifying spinal interneurons based on their firing pattern, in response to injected current, does not provide any information about the anatomy of the cell, the synaptic connections, or the area of the spinal cord they reside in. Equally, utilising a histological approach, which involves classifying based on somatodendritic morphology or neurochemistry, does not consider the electrophysiological properties of the interneuron. Therapeutically targeting the spinal inhibitory interneurons, to indirectly reduce the overexcitation thought to underlie maladaptive plasticity (e.g., neuropathic pain), could allow for efficacious, targeted treatment (Zholudeva *et al.*, 2021). Bezdudnaya, Lane and Marchenko (2020) reported that local intrathecal delivery of GABA and glycine antagonists, following cervical spinal cord injury, enhanced the efficacy of epidural stimulation for respiratory dysfunction. Restoring inhibition in an injured spinal cord, through the administration of an agonist of the neuron-specific K^+-Cl^- cotransporter (KCC2), also results in functional recovery (Chen *et al.*, 2018).

Thalamic third order neurons, within the spinothalamic tract, then transmit the sensory information to the primary somatosensory cortex for cognitive integration (Hodge and Apkarian, 1990). Ascending nociceptive fibres not only transmit pain signals to the higher centres in the brain, but also make connections to the brainstem and midbrain. Descending central controls within the brain can suppress or facilitate the transmission of signals related to pain. The periaqueductal grey (PAG) is a cytoarchitecturally and neurochemically heterogenous area of grey matter located in the midbrain. The PAG receives a very dense afferent projection from the spinal DH, with region-specific inputs. Parry *et al.* (2008) reported that second order neurons, activated by C fibres, project preferentially to the ventrolateral PAG (vlPAG). Whereas larger A fibres predominantly project to the dorsolateral/lateral PAG. Electrically stimulating the PAG of rats elicited stimulation, produced analgesia, and increased the threshold of spinal DH neurons (McMullan and Lumb, 2006). The rostral ventral medulla (RVM) mediates the effects of PAG stimulation, via a projection from the vlPAG to the nucleus raphe magnus, that is essential for descending pain control. The distinct ON and OFF cells within the RVM project to laminae I, II and V of the spinal DH, with stimulation of the RVM able to bidirectionally modulate spinal DH excitability (Walker, Fitzgerald and Hathway, 2015).

1.3.1 Mammalian peripheral sensitisation

Following injury, the release of proinflammatory mediators from macrophages, mast cells and damaged nerve terminals can stimulate PAFs directly. These mediators include bradykinin, cytokines (TNF-α, IL-1β), prostaglandins (PGE2), extracellular ATP, protons, and growth factors such as neurotrophins (nerve growth factor). Ion channels, either voltage-gated or ligand gated, are the primary targets of these mediators (Figure 1.1). The PAF itself can also release neuroactive compounds back into the interstitial space (SP and CGRP), increasing the blood flow and permeability of endothelial cells (Sann and Pierau, 1998; Basbaum *et al.*, 2009). Inflammation-mediated sensitisation of PAFs may include: the unmasking of silent nociceptors, increased evoked responses to stimuli during inflammation, decreased activation thresholds and the promotion of conditions, such as spontaneous pain, through changes in neuronal excitability (Kuner, 2010). Nociceptive sensitisation elicits behavioural changes such as an increased sensitivity to noxious stimuli (hyperalgesia) or a pain response to normally innocuous stimuli (allodynia).

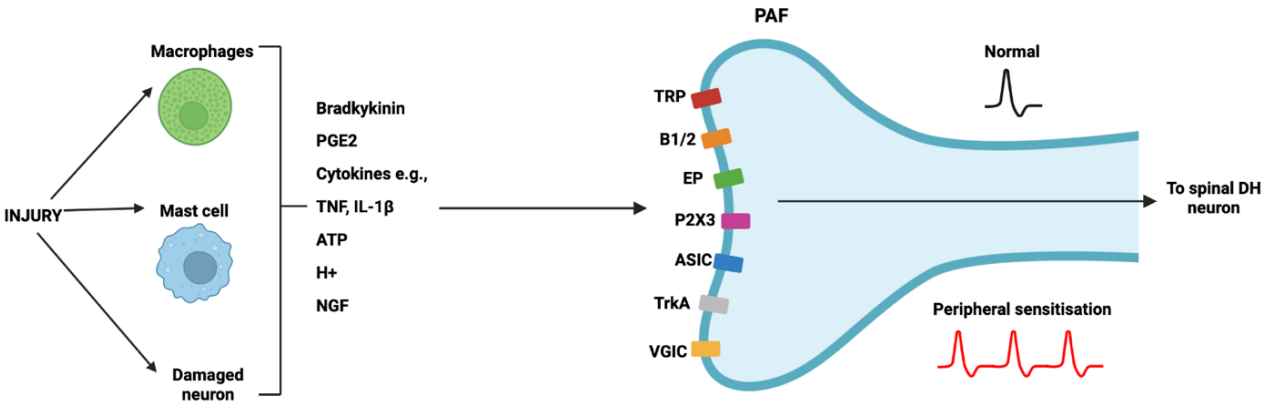


Figure 1.1: ***Mechanisms of peripheral sensitisation****. Tissue injury results in the release of numerous proinflammatory mediators from the damaged cells, nerve terminals and non-neuronal cells, such as macrophages and mast cells. These mediators then bind to their respective G-protein coupled receptors (GPCRs) or ion channels. This results in sensitisation of the PAFs, which synapse with second order neurons in the spinal DH. Abbreviations: NGF, nerve growth factor; TRP, transient receptor potential; ASIC, acid-sensing ion channels; TrkA, Tropomyosin receptor kinase A; VGIC, voltage gated ion channels. Image created myself using BioRender.com*

The cytokine tumour necrosis factor alpha (TNF-α) has a pivotal role in the development and maintenance of inflammatory and neuropathic pain (Cunha *et al.*, 1992; Woolf *et al.*, 1997). Woolf *et al.* (1997) reported that intraplantar injections of TNF-α in adult rats induced a dose-dependent increase in thermal and mechanical sensitivity. Ectopic discharge and a reduction in mechanical threshold was also observed in PAFs exposed to TNF-α (Sorkin *et al.*, 1997) . TNF-α primarily mediates its pro-inflammatory actions by acting directly on the ligand receptor TNF receptor 1 (TNFR1), expressed on PAFs. The p38 mitogen-activated protein (MAP) kinase has been shown to modulate tetrodotoxin (TTX)-resistant Na^+ channels in acute peripheral mechanical sensitisation, downstream of TNF-α via TNFR1, on cultured DRG neurons (Jin and Gereau IV, 2006). The production of TNF-α activates intracellular pathways, which regulate gene transcription events, leading to the production and release of other pro-inflammatory cytokines, such as interleukin-6 (IL-6) and interleukin-1β (IL-1β) (Issekutz, Meager and Issekutz, 1994; Lukacs *et al.*, 1995). The nuclear factor-κB (NF-κB) family of transcription factors are central mediators involved in the regulation of inflammatory processes. NF-κB translocates to the nucleus and binds to specific DNA sequences that promote the transcription of various pro-inflammatory genes including those encoding TNF-α, IL-6 and IL-1β (Palladino *et al.*, 2003; Hayden and Ghosh, 2008).

Prostaglandin E2 (PGE2), a metabolite of arachidonic acid in the cyclooxygenase (COX) pathway, is a potent inflammatory mediator that interacts at G protein-coupled PG receptors (EP). The four EP receptor subtypes (EP1-EP4) each activate distinct downstream signalling pathways. EP2 and EP4 are coupled with Gαs, which induces intracellular cyclic adenosine monophosphate (cAMP) production by stimulating adenylyl cyclase (AC) (Katsuyama *et al.*, 1995; Jang, Kim and Hwang, 2020). Activation of the EP3 receptor, that undergoes post-transcriptional RNA splicing to produce multiple isoforms, can either stimulate or inhibit cAMP production (Kunapuli *et al.*, 1994; Negishi, Sugimoto and Ichikawa, 1995). Although mammalian sensory neurons express multiple EP receptor subtypes, PGE2-induced sensitisation is mediated by the EP3C isoform and EP4 (Southall and Vasko, 2001). PGE2 is also implicated in prostaglandin-mediated central sensitisation, which involves sensitisation of the PAF to bradykinin. This enhances the response to glutamate receptor agonists in the DH second order neurons to produce pain hypersensitivity (Kohno *et al.*, 2008). Selective EP2 and EP4 receptor

antagonism reduced peripheral and central hyperalgesia in a preclinical mouse model of endometriosis, with the EP2 antagonist shown to be the most effective analgesic (Greaves *et al.*, 2017).

1.4 *Drosophila melanogaster*: a model organism

Drosophila melanogaster have been used extensively as a model for many human diseases, including cancer (Enomoto, Siow and Igaki, 2018), Alzheimer's disease (Tsuda and Lim, 2018), cardiac diseases (G Gregory Neely *et al.*, 2010), obesity (Warr *et al.*, 2018) and diabetes (Cronin *et al.*, 2009). Bier (2005) reviewed the genetic principles by which *Drosophila* can be used as an effective tool for analysing the function of human disease genes, referring to the organism as "the golden bug in the foreseeable future". The *Drosophila's* genome is 60% homologous to that of humans and 75% of human disease-causing genes have a functional homologue in *Drosophila*, demonstrating their translational utility (Fortini *et al.*, 2000; Reiter *et al.*, 2001). The use of *Drosophila* as a model organism presents many technical advantages over vertebrate models; they are inexpensive to culture in laboratory conditions, easy to genetically manipulate and have a rapid life cycle that takes approximately 10-12 days at room temperature (Figure 1.2). Female *Drosophila* are ready to mate less than 24 hours after eclosion (emergence from the egg) and can lay up to 100 eggs per day. This versatile model organism is also small and amenable to different quantitative physiological techniques.

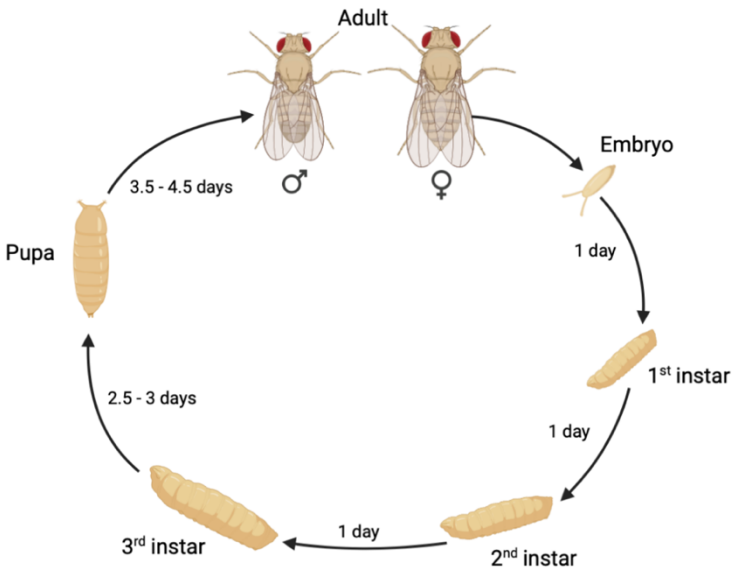


Figure 1.2: ***Drosophila life cycle.*** *The life cycle consists of four distinct stages: egg, larva (three instar stages), pupa and adult. Image created myself using BioRender.com*

1.5 Larval peripheral nervous system (PNS) of *Drosophila*

1.5.1 PNS organisation

The *Drosophila* PNS organises a stereotyped pattern of identified sensory neurons positioned along the body wall and, due to the abundant genetic tools available for genetic manipulation, provides an advantageous system to study. Anatomically, the body wall of the larva is segmented with three thoracic segments (T1-T3) and eight abdominal segments (A1-A8). In each abdominal half (hemi)-segment there are 30 body wall muscles, innervated by 36 neurons, that drive various locomotor behaviours (Figure 1.3) (Hoang and Chiba, 2001). *Drosophila* larvae have two main peripheral sensory neurons positioned below the barrier epidermis: type I, which innervate external sense organs and chordotonal organs, and type II. Type I neurons have ciliated monopolar dendrites, whereas type II neurons have multidendritic extensions and are structurally similar – type II are also known as multidendritic (md) or dendritic arborization (da) sensory neurons.

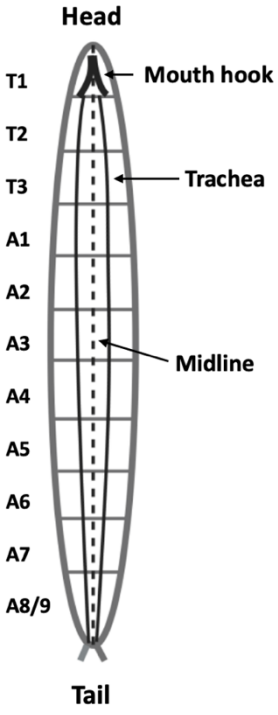


Figure 1.3: ***Schematic of a third instar larvae, positioned dorsal side up, depicting the segmentation of the body wall.*** *Third instar larvae have three thoracic segments (T1-T3) and eight abdominal segments (A1-A8).*
Image adapted from Maiellaro (2022) and created myself using Biorender.com

The da neurons of the peripheral sensory system in *Drosophila* larvae are placed into four distinct morphological classes (I-IV) based on the increasing complexity of their dendrites (Grueber, Jan and Jan, 2002). The dendritic domains of neurons belonging to the same class are non-overlapping, but there is extensive overlap among cells in different classes (Grueber, Jan and Jan, 2002). Shimono *et al.* (2009) further reported the persistence of the multidendritic da neurons in the adult *Drosophila* and the occurrence of dendritic remodelling following the third larval stage. Class I and Class II da neurons have simple branching patterns and have been proposed to function as proprioceptors and touch receptors, respectively (Hughes and Thomas, 2008; Tsubouchi, Caldwell and Tracey, 2008). Class III da neurons are low-threshold mechanoreceptors for sensing gentle touch that extend several long primary or secondary branches, with dendrites clearly distinguished by spiked protrusions (Yan *et al.*, 2013). The dendrites of five class III neurons provide 70% coverage of each abdominal hemisegment (Grueber, Jan and Jan, 2002).

The Class IV da (C4da) neurons -the cellular model of this study- are nociceptive, and are both sufficient and necessary for triggering the nociceptive escape behaviour evoked by the application of a noxious stimulus (thermal or mechanical) (Hwang *et al.*, 2007). This escape behaviour is highly stereotyped, characterised by a 360^{o} rolling response along the axis, followed by locomotion acceleration, is clearly distinct from normal locomotion used for other larval behaviours, such as foraging. Three C4da neurons in each hemisegment innervate and maintain full coverage of the epidermis (Figure 1.4), with the dendrites positioned between the epidermis and basal lamina extracellular matrix. The highly complex branching pattern of the C4da neurons distinguishes them from the three other da classes, with individual neurons generating as many as 900 terminal branches through more than six order branches (Sugimura *et al.*, 2003). The dendrite branches specifically express the *pickpocket* (*ppk*) gene that encodes a Degenerin/Epithelial Sodium Channel (DEG/ENaC) subunit. Basak, Sutradhar and Howard (2021) proposed a new function for the extensive branching of C4da neurons: to minimise the diffusion times of extracellular signals released from damaged adjacent cells, to enable neuronal activation. Despite the increase in *Drosophila* larva size, there is no addition of new C4da neurons through development. These polymodal larval pain-sensing neurons are functionally and morphologically analogous to vertebrate nociceptive neurons whose branched, naked nerve endings likewise innervate mammalian skin. Two members of the transient receptor potential (TRP) channel family, fundamental molecular sensors of noxious thermal and mechanical stimuli across phyla, are expressed on class

IV da neurons. TRPA1 is required for baseline thermal and mechanical nociception in *Drosophila* and Painless (Pain) mediates noxious heat and mechanical stimuli (Tracey *et al.*, 2003; Neely *et al.*, 2011). The expression of DmPiezo in the C4da neurons is also essential for larvae behavioural responses to noxious mechanical stimuli (Kim *et al.*, 2012), supporting the role of Piezo proteins in mechanosensory nociception.

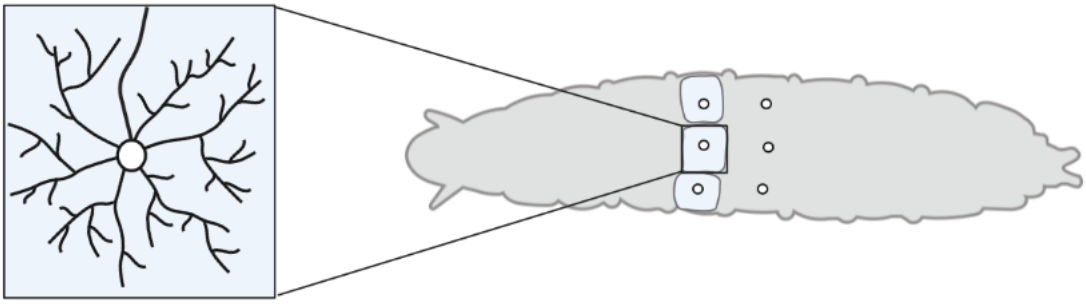


Figure 1.4: ***Schematic of the nociceptive C4da neurons with dendrites that allow the Drosophila larvae to detect noxious cues.*** *Arrangement of the C4da neurons and their dendritic territories in two hemisegments are schematised with the dendritic territories of the C4da neurons shown in the left segment and the cell body only in the right segment. Image created myself using BioRender.com*

Light avoidance behaviour is also mediated via the expression of the light sensor Gr28b on C4da neurons (Xiang *et al.*, 2010). Imambocus *et al.* (2022) recently showed noxious light avoidance in *Drosophila* larvae was gated by acute insulin-like peptide 7 (IIp7) release from neuromodulatory hub dorsal pair insulin-like peptide 7 (Dp7) neurons. Dp7 neurons were shown to integrate sensory input from multiple somatosensory neurons, including C4da neurons. Colocalisation of small molecule and neuropeptide transmitters, playing key functional roles in modulating neuronal activity, occurs in vertebrates and invertebrates (Girirajan, Campbell and Eichler, 2011; van den Pol, 2012). The release of neuropeptides, for example during opioid receptor signalling in situations of stress-induced analgesia, allows regulation of targets that are distant from the site of release (Fields, 2004).

Basak, Sutradhar and Howard (2021) discovered two distinct nociceptive pathways using focal laser stimulation to create highly localised physical damage to the C4da neurons and/or adjacent tissue. Calcium responses in dendrites, axons and the cell body were monitored by increases in GCaMP6f fluorescence, specifically expressed in C4da neurons. Focal laser stimulation induced intracellular Ca^{2+} increases in the axons, dendrites, and cell bodies when the laser made direct contact with the dendrites (the contact pathway). The peak magnitudes of Ca^{2+} responses increased with increasing stimulus intensity in the three different locations. A non-contact response, where stimulation adjacent to the dendrite was made, was observed primarily in the axons.

The vast majority of analgesic drug development targets the peripheral mechanisms of nociception, which are evolutionarily conserved, and thus studies could take place in invertebrates (Figure 1.5). Manev and Dimitrijevic (2004) proposed the use of *Drosophila* in neuropharmacological nociception research and developed methods to investigate the effects on heat avoidance following the injection of the $GABA_B$ receptor agonist 3-AMPA. Subcutaneous injection of the antinociceptive drug increased the threshold to noxious heat stimuli and paralleled what had been reported in mammalian studies (Thomas *et al.*, 1996). Invertebrate animals such as *Drosophila* are not protected under the Animals (Scientific Procedures) Act 1986. Therefore, the invertebrate model described is not only more ethically acceptable than the alternative *in vivo* mammalian animal models but is highly amenable to genetic modification. There is the possibility to improve target identification for new analgesic drugs through the discovery of novel evolutionarily conserved genes that may underlie pain in humans. An invertebrate model will provide a more robust phenotype for high-throughput screening, through random mutation or targeted RNA interference (RNAi) mediated knockdown, which will facilitate the identification of novel compounds and targets by streamlining the drug discovery process (Bell, McBride and Dockendorff, 2009).

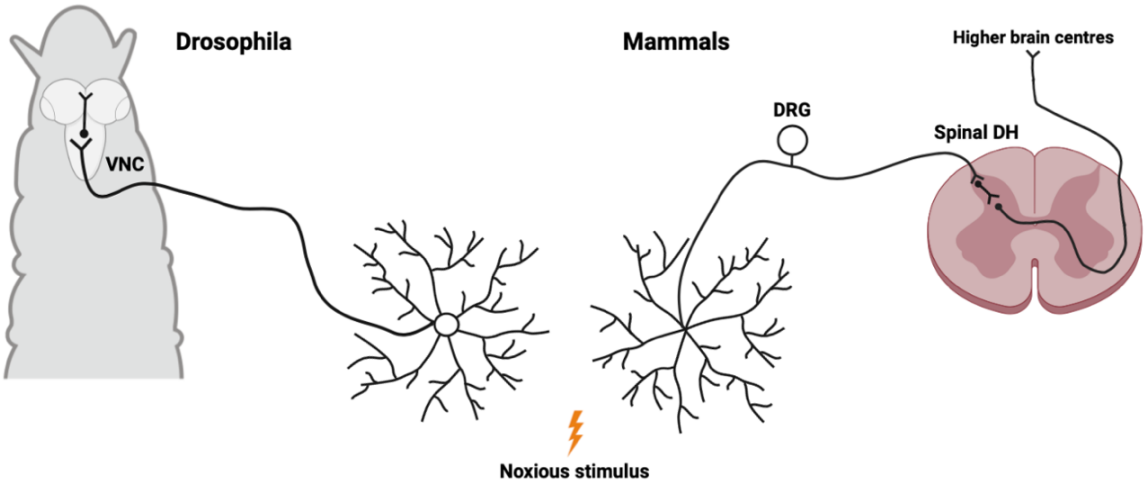


Figure 1.5: ***Comparative neuroanatomy of Drosophila and Mammalian somatosensory nervous system.*** *The polymodal C4da neurons in Drosophila are functionally and morphologically analogous to vertebrate nociceptive neurons (C fibres), whose free nerve endings innervate the skin. C4da neurons have cell bodies in the tissue which extend a central axon, synapsing with second order neurons in the ventral nerve cord (VNC). In mammals, pseudounipolar sensory neurons, with cell bodies in the dorsal root ganglia (DRG), extend a single axon into the spinal dorsal horn (DH) where synapses are made. Information is subsequently transmitted directly via ascending projection neurons, or indirectly via spinal interneurons, to the brain for conscious perception. Image created myself using BioRender.com*

1.5.2 Impact of *Drosophila* PNS development on nociception

Third instar larvae (Figure 1.2), the final stage of larval development, are primarily utilised in studies of nociception in *Drosophila*. Almeida-Carvalho *et al.* (2017) conducted behavioural assays, performed routinely on third instar larvae, on first instar larvae and found similar behavioural outputs to noxious mechanical stimuli between the two stages. The smaller size and lower speed of first instar larvae was also accounted for in parameters of nociceptive stimulation. Interestingly, Jaszczak *et al.* (2022) found noxious heat triggered increased rolling behaviour in the third instar larvae when compared to younger larvae. Previous studies had reported the absence of thermal nociceptive behaviour until final stage of larval development (Sulkowski, Kurosawa and Cox, 2011). Despite this, optogenetic nociception assays have shown C4da neurons are able to

function as nociceptors in newly hatched first instar larvae (Sulkowski, Kurosawa and Cox, 2011). Jaszczak *et al.* (2022) later determined C4da neurons displayed decreased Ca^{2+} activity, following stimulation with short wavelength UV light, during the larval development period. In third instar larvae, the steroid hormone ecdysone becomes localised to the nucleus of C4da neurons and the loss of nuclear ecdysone receptor (EcR) expression reduces noxious thermal sensitivity (McParland *et al.*, 2015). The authors proposed that a steroid-mediated shift, between first instar and third instar larvae, modulates C4da nociceptor activity by supressing the expression of the anoctamin family member *subdued* (encoding a TMEM16 channel) (Jaszczak *et al.*, 2022). It is possible that this regulation of sensory sensitivity is a development strategy as the larvae deviates from the food source, becoming more exposed to the external environment, in the third stage of larval development.

1.6 Genes and their involvement in nociceptive behaviour

1.6.1 Transient receptor potential (TRP) channels

Although there is a large cast of molecular mediators that contribute to nociception, TRP channels are the primary transducers of noxious stimulation. TRP channels are a family of six-membrane domain, non-selective cation channels with high Ca^{2+} permeability that have diverse roles in sensory signalling. TRP channels were first identified in mutant *Drosophila melanogaster*, whose photoreceptors failed to maintain a sustained response to a prolonged stimulus of light (Cosens and Manning, 1969). The *Drosophila* genome encodes 13 TRP channels (Figure 1.6), the majority of which are expressed and function in sensory neurons, and these are critical for sensory transduction in larval and adult *Drosophila* (Montell, 2021). For example, TRP and TRP-Like (TRPL) are required for multi-component phototransduction cascades (Montell *et al.*, 1985; Niemeyer *et al.*, 1996). The TRPN channel, NOMPC, contributes to mechanosensation in both adults and larvae (Walker, Willingham and Zuker, 2000; Tsubouchi, Caldwell and Tracey, 2012; Yan *et al.*, 2013). NOMPC, Nan and inactive (Iav) also function in auditory transduction (Kim *et al.*, 2003; Gong *et al.*, 2004; Cheng *et al.*, 2010).

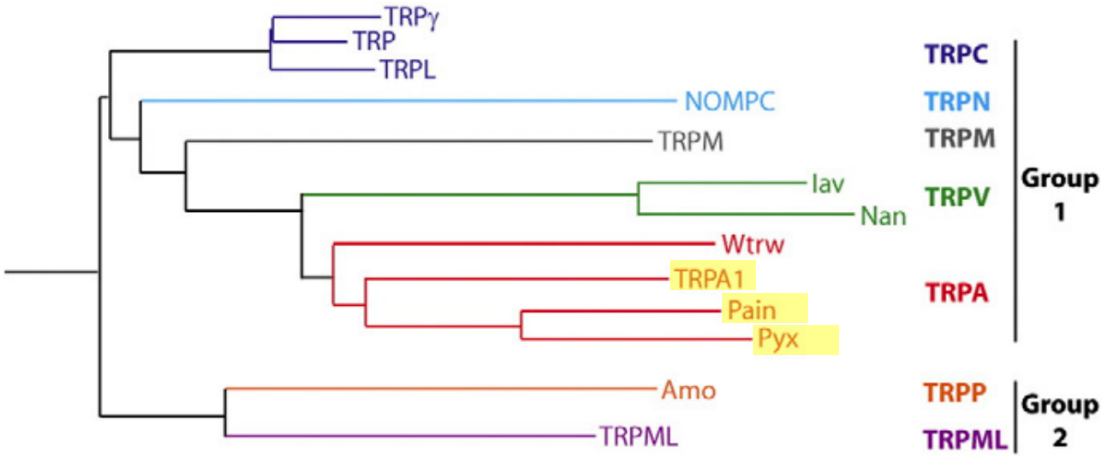


Figure 1.6: ***Phylogenetic tree of identified TRP Channels in Drosophila.*** *Drosophila TRP channels can be classified into seven related subfamilies: TRPC, TRPN, TRPM, TRPV, TRPA, TRPP and TRPML. The TRP channels highlighted: dTRPA1, Painless (Pain) and Pyrexia (Pyx), regulate nociception. Adapted from Fowler and Montell (2013).*

The mammalian TRP superfamily of ion channels include 28 members, which are subdivided into multiple subfamilies. Heat-sensitive TRPV1, cool-activated TRPM8, and cold-responsive TRPA1 have emerged as the primary transducers of noxious stimuli in mammals and constitute attractive targets for new generation analgesics. A nociceptive role of TRPM2 in the pathogenesis of inflammatory and neuropathic pain has also been proposed (Jang *et al.*, 2018). TRPA1 is the predominant sensor of chemical irritants that elicit acute and inflammatory pain (Bautista, Pellegrino and Tsunozaki, 2013). This channel is functionally expressed in a subset of mammalian DRG neurons that co-express the noxious heat-activated TRPV1 ion channel and the nerve growth factor receptor, TrkA (Kobayashi *et al.*, 2005; Anand, Otto and Anand, 2010). TRPA1 is directly activated by allyl isothiocyanate (AITC), cinnamaldehyde, and allicin, the pungent natural compounds present in mustard oil, cinnamon oil, and garlic extracts, respectively (Bandell *et al.*, 2004; Macpherson *et al.*, 2005; Bautista, Pellegrino and Tsunozaki, 2013). Bradykinin, an inflammatory peptide implicated in nociception, is also able to activate TRPA1 through its G protein-coupled receptor, bradykinin B2 (Bandell *et al.*, 2004).

Cinnamaldehyde (CA) is able to bind to mammalian TRPA1 with high specificity, known to be the most selective activator (Bandell *et al.*, 2004; Talavera *et al.*, 2020). TRPA1 activation, through topical administration

of CA, elicits significant spontaneous pain in humans (Prescott and Swain-Campbell, 2000; Namer *et al.*, 2005). In the area of application, there is also the development of CA-induced thermal and mechanical hyperalgesia (Namer *et al.*, 2005; Albin, Carstens and Carstens, 2008). This is comparable to the dose-dependent increase, in magnitude and duration, of thermal hyperalgesia and mechanical allodynia observed in rats following intraplantar injection of CA (Tsagareli *et al.*, 2010). Merrill *et al.* (2007) investigated the effects of repeated cutaneous CA application on spinal wide dynamic range (WDR) neuronal responses, to innocuous and noxious stimuli, in rats. Application of CA did not directly excite the dorsal horn WDR neurons and exhibited no effect on their responses to mechanical stimulation or skin cooling. However, the WDR neuronal responses to noxious heat were enhanced. This heat sensitisation by CA is also observed in rat trigeminal subnucleus caudalis neurons (Zanotto, Iodi Carstens and Carstens, 2008).

Venkatachalam, Luo and Montell (2014) reviewed TRP channel function and modes of action in the invertebrate model organisms *Caenorhabditis elegans* and *Drosophila melanogaster*. The evolutionary conservation between species supports the potential for *Drosophila* to be utilised to further study mammalian TRP channels. The heat activated TRPA1 channel in *Drosophila*, also referred to as dTRPA1, contributes to thermal nociception in both the larvae and adult (Neely *et al.*, 2011; Luo, Shen and Montell, 2017). This TRP channel has also been implicated in chemical nociception (Bandell *et al.*, 2004; Kang *et al.*, 2010). dTRPA1 is expressed as five isoforms (TRPA1-A, B, C, D, and E). Gu *et al.* (2019) modified the *Drosophila* TRPA1 locus and alternatively spliced exons 12 and 13, targeted using the genomic engineering framework isoEXPRESS, to generate knockin flies that only express a single TRPA1 protein isoform. The authors demonstrated alternative splicing of dTRPA1, a mechanism that increases the variability in protein products from a single gene, is required for polymodal nociception in *Drosophila*, alongside the co-expression of TRPA1-C and TRPA1-D. Zhong *et al.* (2012) had shown previously that *Drosophila* TRPA1 isoforms have functional specificity in thermal nociception, when examined in heterologous cells. Although the *in vitro* properties of TRPA1-C and TRPA1-D suggested these were polymodal transducers, there was stimulus specificity *in vivo* where heat- and UVC light-triggered nociception preferentially activated a distinct single TRPA1 isoform. TRPA1-D, expressed on C4da neurons, is preferentially activated by noxious heat, whereas UVC-nociception is mediated by TRPA1-C (Gu *et al.*, 2019). Gu *et al.* (2019) proposed these functional differences were the result of the two TRPA1 isoforms interacting with different protein or lipid partners.

Following the investigation of the distinct function of the TRPA1-D isoform in acute thermal nociception, Gu *et al.* (2022) explored the role of dTRPA1 isoforms in tissue injury-induced hypersensitivity. Despite sensitisation of the TRPA1-D isoform being hypothesised to underlie heat allodynia, it was TRPA1-C that mediated heat allodynia. Dillman *et al.* (2013) and Zhang *et al.* (2016) confirmed the high prevalence of alternative splicing within the mammalian nervous system, with alternative splice variants of the mouse and human TRPA1 gene shown to exhibit functional differences *in vitro* (Zhou *et al.*, 2013; Huang *et al.*, 2021). Further studies could explore the contribution of the identified splice variants to mammalian nociception and hypersensitivity *in vivo*.

Tracey *et al.* (2003) isolated the *painless* gene, encoding a non-selective cation channel in the TRPA family, and reported the requirement of Painless for the detection of both noxious heat and mechanical stimuli. Painless requires the presence of Ca^{2+} as a co-agonist for channel activation by noxious heat (Sokabe *et al.*, 2008). Although dTRPA1 has a higher amino acid sequence identity to the mammalian TRPA1 channel, TRPA1 remains the protein most comparable to Painless (FlyBase, 2022; He *et al.*, 2022). The polymodal functions of Painless are mediated by two distinct isoforms that vary in the number of N-terminal ankyrin repeats, necessary for their modality specific properties. Hwang, Stearns and Tracey (2012) demonstrated the importance of ankyrin repeats in thermal nociception, but not mechanical nociception. Painless is also required for chemical nociception in adult *Drosophila* (Al-Anzi, Tracey and Benzer, 2006; Kang *et al.*, 2010; Mandel *et al.*, 2018). Mandel *et al.* (2018) found that the behavioural aversion displayed in wild-type adult *Drosophila* to the active ingredient in wasabi, allyl isothiocyanate (AITC), was absent in *painless* mutants. Interestingly, both dTRPA1 and Painless co-express the neuropeptide diuretic hormone 31 (DH31) and Leucokinin (Johnson *et al.*, 2005; Mandel *et al.*, 2018). These are homologues of the mammalian transmitters CGRP and SP, respectively. This finding supports the conservation of the CGRP and SP signalling pathways that are implicated in nociception.

The third heat-activated TRPA channel, Pyrexia (Pyx) , provides tolerance in adult *Drosophila* under extreme heat conditions (Lee *et al.*, 2005). Pyx is ubiquitously expressed along the dendrites of multidendritic and non-multidendritic peripheral nervous system (PNS) neurons and is more permeable to K^+ than to Na^+. Upon exposure to 40^{o}C for 3 minutes, more than 60% of Pyx null mutant flies were paralysed, compared to the 9% of wild-type *Drosophila* paralysed under the same conditions (Lee *et al.*, 2005). This data further supports the

protective role of Pyx *in vivo.* Hamada *et al.* (2008) discovered that a set of previously uncharacterised cells located in the *Drosophila* brain, anterior cell (AC) neurons, contributed to thermal preference. AC neurons are activated, dependent on dTRPA1 activation, as the environmental temperature rises above optimum. Tang *et al.* (2013) later showed novel warm temperature integration at the level of AC neurons where an initial activation, via dTRPA1 expressed directly on AC neurons, is followed by a second activation due to the input of Pyx expressing neurons synapsing onto AC neurons. The Pyx TRP channel is also the first identified TRP channel to be involved in thermal circadian clock synchronisation, mediating low temperature cycles in *Drosophila* (Wolfgang *et al.*, 2013).

1.6.2 DEG/ENaC Family Channels

The acid-sensing ion channels (ASICs) are voltage-insensitive, proton-gated ion channels that belong to the epithelial sodium channel (ENaC)/Degenerin (DEG) superfamily (Waldmann *et al.*, 1997; Kellenberger and Schild, 2015). These cation channels are widely expressed in mammalian DRG neurons and the CNS (Wemmie *et al.*, 2003; da Silva Serra *et al.*, 2016). Activation of ASICs primarily triggers Na^+ influx, inducing membrane depolarisation (Waldmann *et al.*, 1997). The *Drosophila* gene *pickpocket1 (ppk1)* encodes an ion channel subunit of the DEG/ENaC family, highly expressed in C4da neurons and required for mechanical nociception. PPK1 is a critical pore-forming subunit, required for channel activation by acid (Boiko *et al.*, 2012). Zhong, Hwang and Tracey (2010) reported a reduction in nociceptive behaviour in *ppk* larvae mutants in response to harsh mechanical stimuli without causing deficits in thermal nociceptive behaviour. This supports the widespread and evolutionarily conserved mechanosensory function of DEG/ENaCs (Price *et al.*, 2001; Goodman *et al.*, 2002). Zhong, Hwang and Tracey (2010) argued that due to the function of *ppk1* being limited to mechanical nociception, this gene functions upstream of Painless which mediates both mechanical and thermal nociception.

Another member of the DEG/ENaC channel family, PPK26, is specifically expressed in C4da neurons and participates in mechanical nociception but not thermal nociception (Guo *et al.*, 2014). PPK and PPK26, both essential for mechanosensation, are independent in terms of their cell surface expression. PPK26 binds to PPK *in vivo*, forming a functional ion channel that regulates mechanical nociception in *Drosophila* larvae (Gorczyca

et al., 2014; Mauthner *et al.*, 2014). Jang *et al.* (2019) later reported a role for the PPK30 ion channel, a member of the *Drosophila* ppk family, in mechanosensation. Similar to previously identified members of the ppk family, PPK30 mutants displayed no deficits in noxious heat detection. Interestingly, PPK30 expressed in *Xenopus oocytes*, was also activated by acid.

1.6.3 Piezo Channels

Piezo channels are evolutionary conserved, mechanosensitive ion channels, that mediate touch and indirectly supress mechanical pain**.** In mammals, Piezo1 and Piezo2 have been identified as essential components of distinct mechanically activated cation channels (Coste *et al.*, 2010; Wang, La and Hamill, 2019; Zhang *et al.*, 2019). The single Piezo protein in *Drosophila*, DmPiezo, is a Ca^{2+} permeable non-selective cation channel, analogous to its mammalian homolog. DmPiezo is 24% identical to mammalian Piezo proteins, with sequence conservation throughout the length of the proteins (Coste *et al.*, 2012). DmPiezo expression in human embryonic kidney (HEK) cells induces mechanically activated currents (Coste *et al.*, 2012). In DmPiezo knockout larvae, behavioural responses to noxious mechanical stimuli were reduced and there was no observation of mechanically activated currents in C4da sensory neurons (Kim *et al.*, 2012). Kim *et al.*, (2012) hypothesised DmPiezo and PPK are involved in two parallel pathways within C4da neurons, supported by the complete abolishment of mechanical responses in combined knockdowns. Wang *et al.* (2020) further identified the expression of DmPiezo in mechanosensitive visceral neurons, required to monitor feeding to avoid overconsumption in *Drosophila*. These neurons are located in the pars intercerebralis (PI) of the brain, involved in homeostatic control. DmPiezo activation, following axon injury or during axon regeneration, inhibits axon regeneration in *Drosophila* sensory neurons (Song *et al.*, 2019). Song *et al.* (2019) proposed the mammalian homologue Piezo1 also functions as a regeneration inhibitor, with conditional knockout of Piezo1 accelerating regeneration. Piezo-like, a homologue of piezo gene families, is required for *Drosophila* larval locomotion. Piezo-like mutants displayed severe defects in crawling pattern and body gesture control, with human or mouse Piezo1 able to rescue this (Hu *et al.*, 2019). This supports the functional conservation between *Drosophila* and mammalian Piezo proteins.

1.7 Transmission of nociceptive information to the *Drosophila* central nervous system (CNS)

The ventral nerve cord (VNC) is the insect analogue of the vertebrate spinal cord, involved in the integration of sensory information and locomotor output that underlie *Drosophila* behaviours (Court *et al.*, 2020). Grueber *et al.* (2007) examined the morphology of da neuron axon projections in third instar larvae to determine whether different classes of da neurons develop differential connectivity to second order neurons in the VNC. The laminar arrangement of somatosensory neurons in Drosophila da neurons, displaying different morphological classes, have distinguished central projections and provide anatomical support for distinct functions among different da neurons. Evidence of somatotopic arrangements in the CNS were also displayed by class I and class IV da (C4da) neurons. Dorsal and ventral C4da neurons projected axon branches across the CNS midline. However, this did not occur in C4da neurons, positioned in the lateral regions of the abdominal body wall, and was rarely observed among other type II da neurons (Grueber *et al.*, 2007).

The presence of modality-specific layers of sensory axon terminals in the invertebrate VNC, comparable to the mammalian somatosensory system, was further supported by Tsubouchi *et al.* (2017) who systematically mapped the somatosensory neural circuitry of *Drosophila*. The C4da neurons project to the most ventral layer of the nerve cord, whereas mammalian C fibres innervate the superficial DH of the spinal cord (Grueber *et al.*, 2007). Peptidergic C fibres, marked by the expression of CGRP and SP, primarily terminate in lamina I and outer lamina II (Nakaya *et al.*, 1994). The isolectin B4 positive (IB4+) non-peptidergic subpopulation predominately terminate in inner lamina II (Bradbury, Burnstock and McMahon, 1998; Ribeiro-da-Silva and De Koninck, 2008). Tsubouchi *et al.* (2017) concluded that the terminals of functionally comparable somatosensory neurons within *Drosophila* are layered in an inverse dorsoventral order. Geoffroy Saint-Hilaire (1822) first proposed the inversion hypothesis: the ventral side of arthropods was homologous to the dorsal side of vertebrates. Functional studies later confirmed an inversion of the dorsoventral axis during animal evolution (Robertis and Sasai, 1996). The modality specific layered organisation of the somatosensory system therefore appears to be conserved between *Drosophila* and mammals.

Mapping of the VNC circuity using large-scale electron microscopic reconstruction, combined with thermogenetic activation of larval C4da neurons and vibration-induced activation of larval mechanosensory

chordotonal neurons, has identified a multilevel multimodal nociceptive circuit downstream of the primary sensory neurons (Ohyama *et al.*, 2015). First order Basin interneurons, located in the ventral sensory domain of the nerve cord, receive excitatory nociceptive and mechanosensory information from a local region of the body wall. Integration of information between distinct Basin cell types may converge again onto downstream neurons, increasing the probability of rolling escape behaviour. Basin cells impinge on command-like Goro interneurons in the nerve cord, through second order abdominal interneurons A05q and A23g, where further integration of sensory inputs occurs followed by projection to higher order centres, such as the brain (Ohyama *et al.*, 2015; Jovanic *et al.*, 2016). Burgos *et al.* (2018) later identified a population of somatosensory interneurons in the *Drosophila* VNC that function downstream of C4da neurons, termed Down-and-Back (DnB) neurons, that are activated by noxious heat and required for robust nociceptive rolling during nocifensive escape. DnB neurons receive inputs from multiple mechanosensory cell types and nociceptive C4da neurons, comparable to vertebrate spinal interneurons that receive direct excitatory input from nociceptors and Aβ mechanoreceptors (Duan *et al.*, 2014).

1.8 Ca^{2+} signalling as conserved components of nociception

Neely *et al.*, (2010) performed a *Drosophila* genome-wide neuronal-specific RNAi knockdown, to screen and identify the core conserved components of nociception. Many conserved genes between *Drosophila* and mammals, that are implicated in nociception, involved Ca^{2+} signalling components. The authors first identified dTRPA1 and its mammalian homologue, TRPA1. As previously discussed, dTRPA1 is a Ca^{2+} permeable ion channel that regulates thermal nociception in both larval and adult *Drosophila* (Neely *et al.*, 2011; Luo, Shen and Montell, 2017). Another Ca^{2+} gene identified through this conserved functional genomic screening strategy was *straightjacket (stj)*. The gene encodes a member of the $\alpha 2\delta$ subunit family, that function as accessory subunits of voltage gated calcium channels (VGCC) (Catterall, 2000). This $\alpha 2\delta$ subunit in has been shown to be critical for synaptic function (Dickman, Kurshan and Schwarz, 2008; Ly *et al.*, 2008; Kurshan, Oztan and Schwarz, 2009). In both larval and adult *Drosophila*, *stj* is required for thermal nociception. Mutant *stj* mutant adults failed to avoid noxious heat and larvae were unable to elicit thermal nociceptive escape responses. Mice mutant for the mammalian orthologue of *stj* , $\alpha 2\delta 3$, also exhibited deficits in acute thermal nociception (Neely *et al.*, 2010). Importantly, the authors found $\alpha 2\delta 3$ polymorphisms were significantly

associated with a reduced noxious heat threshold in both healthy volunteer and chronic pain cohorts. The analgesic actions of gabapentin and pregabalin have been shown to be mediated by $\alpha 2\delta 1$, closely related to $\alpha 2\delta 3$ (Field *et al.*, 2006). In addition to the important role of *stj* in acute nociception, Khuong *et al.*, (2019) later showed this $\alpha 2\delta$ subunit to be essential for the development of allodynia. Gabapentin treatment was also able to alleviate the nociceptive hypersensitivity observed in *Drosophila* with neuropathic injury.

The calcium-activated chloride channel Subdued, belonging to the anoctamin family, has also been implicated in larval thermal nociception (Montell, 2021). This is comparable to thermal nociception being mediated by Anoctamin 1 in mammalian DRG neurons (Cho *et al.*, 2012).

Expression of genetically encoded Ca^{2+} indicators, such as GCaMP, can be induced in the mouse genome using viral or gene knock-in approaches to allow the activity of populations of cells in the pain pathway, such as the dorsal root ganglia (DRG), spinal neurons or glia to be studied. Expression of a GCaMP indicator in a tissue-specific manner provides a powerful tool for understanding a neuronal subset's contribution to nociception and how this changes in neuropathic pain (St. John Smith, 2018). Recent two-photon imaging has enabled the visualisation of these fluorescent targets deep within tissue, with fluorescence intensity proportional to intracellular Ca^{2+} concentration (Anderson, Zheng and Dong, 2018). However, minimal research has been undertaken in the nerve terminals, where the noxious stimuli are first detected and processed (Vrontou *et al.*, 2013). There is a requirement for the development of a novel assay to answer important unresolved questions about the molecular mechanisms of peripheral nociception. Although in vivo measurement of intracellular $[Ca^{2+}]$ is an indirect method of neuronal excitability, with not every action potential generating a change in intracellular $[Ca^{2+}]$, this powerful tool has enabled significant advances in the mechanistic understanding of nociception and neuropathic pain (St. John Smith, 2018).

1.9 Cyclic AMP (cAMP), an important second messenger

1.9.1 Nociceptor signalling pathway component

Cyclic AMP (cAMP) is a universal secondary messenger, with its production initiated by the binding of an extracellular ligand to a G-protein coupled receptor (GPCR). Adenylyl cyclases are the class of enzymes

responsible for cAMP generation, synthesising cAMP from adenosine triphosphate (ATP). Phosphodiesterases (PDEs) are responsible for cyclic nucleotide degradation. The main effector proteins of cAMP are protein kinase A (PKA), guanine nucleotide exchanging proteins activated by cAMP (EPAC) and cyclic nucleotide gated ion channels (CNGCs) (Sassone-corsi, 2012). Activation of the cAMP-pathway has been strongly implicated in pain processing within the central nervous system. Evidence from *In vitro* models confirm increases in cAMP, through application of a membrane permeable cAMP analogue, induces significant spontaneous activity of DH neurons alongside enhancing the sensitivity of glutamate-gated ion channels (Cerne, Jiang and Randić, 1992; Cerne, Rusin and Randić, 1993). In inflammatory, non-inflammatory, and neuropathic pain models, activation of the cAMP pathway induces hyperalgesia and central sensitisation. Whereas inhibition of the cAMP pathway reduces hyperalgesia and central sensitisation (Sluka, KA; Karla, A; Moore, 2001; Sluka, 2002; Hoeger-Bement and Sluka, 2003). Thus, the cAMP pathways have been proposed as potential targets for analgesic drug development (Skyba *et al.*, 2004).

1.9.2 Epac-based cAMP sensor

The principle of fluorescence resonance energy transfer (FRET) relies on the non-radiative energy transfer from an excited donor fluorophore to a second acceptor fluorophore (Forster, 1946; Jares-Erijman and Jovin, 2003). For FRET to occur, several criteria must be fulfilled: (i) the interacting fluorophores must be in close physical proximity to one another (typically <10nm), (ii) the emission spectrum of the donor fluorophore and the absorbance spectrum of the acceptor protein must overlap adequately and (iii) there must be a favourable relative orientation between the two fluorophores. The use of FRET biosensors, to visualise the spatiotemporal dynamics of signalling pathways at the cellular and subcellular level, has been extensively reviewed (Calebiro and Maiellaro, 2014; Greenwald, Mehta and Zhang, 2018).

The FRET based biosensor employed in this project, Epac1-camps, harnesses FRET and the binding properties of EPAC (Figure 1.7). Previously, Shafer *et al.* (2008) established the use of the Epac1-camps sensor to visualise cAMP signalling mechanisms in central *Drosophila* neurons. Maiellaro *et al.*, (2016) later expressed Epac1-camps at the *Drosophila* neuromuscular junction and the authors were able to visualise local cAMP signalling

compartments within the motor neuron, demonstrating the capability of the sensor to monitor nanomolar changes in cAMP concentration.

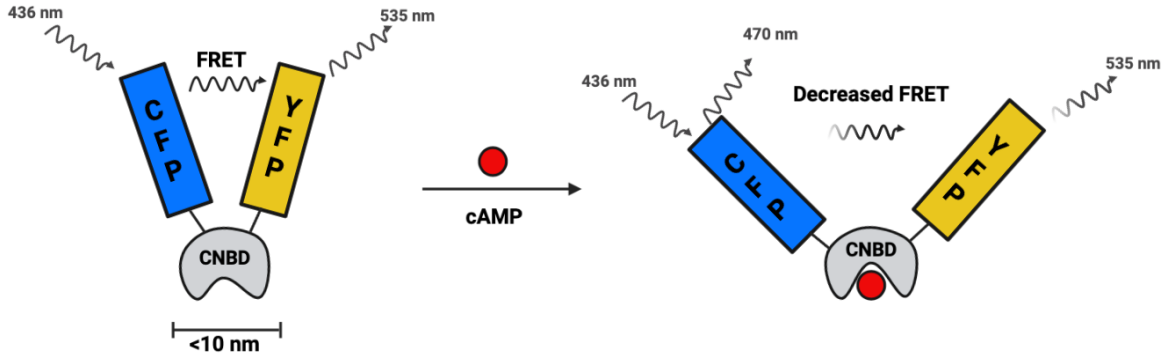


Figure 1.7: ***Principle of Epac-based cAMP sensor.*** *Epac1-camps reports the spatiotemporal dynamics of cAMP by exploiting the principle of FRET. In this case, the FRET pair is CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein). In the cAMP unbound state, the two fluorescent proteins are in close enough proximity to enable FRET to occur when CFP is excited. When cAMP binds to the cyclic nucleotide binding domain (CNBD), this induces a conformational change within the sensor that leads to an increased distance between CFP and YFP. An increase in cAMP is therefore detectable as a reduction in FRET efficiency. Image created myself using BioRender.com*

1.10 Nociceptive sensitisation of *Drosophila* sensory neurons following tissue damage

As previously discussed, both the fundamental physiology and underlying signalling mechanisms of nociception are conserved between vertebrates and invertebrates. It now remains to be determined whether nociceptive responses in *Drosophila* display sensitisation after injury, comparable to what is observed in vertebrates. *Drosophila* models of nociceptive hypersensitivity have been established to study bacterial-induced inflammation (Bachtel *et al.*, 2018), chemotherapy-induced peripheral neuropathy (Hamoudi *et al.*, 2018) and diabetes-induced mechanical sensitisation (Dabbara, Schultz and Im, 2021). Babcock, Landry and Galko (2009) developed a novel robust tissue injury model of nociceptive sensitisation in *Drosophila* larvae. Acute exposure to ultraviolet (UV) radiation, that elicits injury and apoptosis of epidermal cells, resulted in the development of thermal hyperalgesia in third instar larvae, which exhibited exaggerated responses to noxious thermal stimuli. UV-treated larvae also developed thermal allodynia, manifested as aversive withdrawal

behaviour to normally innocuous thermal stimuli. Cutaneous over-exposure to UV radiation in rats induces both dose-dependent thermal and mechanical hyperalgesia and allodynia, lasting several days (Davies, Siau and Bennett, 2005; Bishop *et al.*, 2007).

Babcock, Landry and Galko (2009)'s results demonstrated that UV-Induced nociceptive sensitisation is mediated by an evolutionary conserved pro-inflammatory cytokine. Apoptotic epidermal cells release a tumour necrosis factor (TNF) homologue, Eiger, which acts directly on the TNF receptor Wengen, expressed on the nociceptive C4da neurons. TNF-α has been shown to contribute to the hyperalgesic response to inflammation in mammals (Woolf *et al.*, 1997). Despite Eiger and Wengen both being required for the development of thermal allodynia; thermal hyperalgesia is not mediated by this genetic pathway (Babcock and Galko, 2009). The developmental morphogen Hedgehog (Hh), produced by C4da neurons following tissue damage, has been identified as a critical mediator for both allodynia and hyperalgesia, which acts in parallel to TNF signalling (Babcock *et al.*, 2011). Substance P-like tachykinin peptides, acting upstream of Hh signalling, are also required for damage-induced thermal nociceptive sensitisation (Im *et al.*, 2015). Downstream of Hh signalling, activation of the Bone Morphogenetic Protein (BMP) signalling pathway plays a crucial role in the formation of UV-induced sensitisation (McParland *et al.*, 2021). This highlights that the fundamental mechanisms and signalling pathways driving the transition from acute nociception to nociceptive hypersensitivity are conserved between invertebrates and vertebrates. Downstream of these signalling pathways, distinct TRP channels mediate allodynia and hyperalgesia. The TRP channel Painless, required for the development of allodynia, is the target of both Eiger/TNF signalling and Hh signalling. Hh signalling then mediates thermal hyperalgesia through dTRPA1 (Babcock *et al.*, 2011).

Bush *et al.* (2021) developed a *Drosophila* model of nociceptive hypersensitivity, induced by antiretroviral therapy (ART) in acquired immunodeficiency syndrome (AIDS) patients, to investigate the mechanisms that underlie the peripheral sensory neuropathy. Larvae exposed to the antiretroviral drug azidothymidine exhibited C4da neuron degeneration, quantified by an increase in fragmented terminal dendrites, compared to larvae raised in control conditions. The study demonstrated nucleoside reverse transcriptase inhibitors, the first class of antiretroviral drugs, affected the stability of peripheral sensory neurons. The fragmented C4da neuron phenotype observed modelled the peripheral sensory neuron degeneration described by Polydefkis *et*

al. (2002) in patients with HIV-associated sensory neuropathy. The authors initially proposed the Wallenda/di-leucine zipper kinase (DLK) pathway, known to be implicated in axon degeneration in cultured DRG and *Drosophila* neurons, to have a role in mediating the response to azidothymidine (Miller *et al.*, 2009; Bhattacharya *et al.*, 2012). However, the wnd/dlk pathway was determined to have no significant role in inducing the fragmented phenotype. This finding supports previous studies that had reported the degeneration and regeneration of peripheral dendrites was independent of this pathway (Stone *et al.*, 2014; Honjo and Tracey, 2018).

Studies investigating *Drosophila* nociceptive sensitisation have primarily focused on the larval stage of the life cycle. However, investigations of long-term sensitisation are limited due to the transient nature of the larval stage. Khuong *et al.* (2019) reported that the $\alpha 2\delta$ peripheral calcium channel subunit *straightjacket (stj)*, known to have an essential role in acute nociception, is required for nociceptive sensitivity in both larval and adult *Drosophila*. The authors proposed a role for *stj* in triggering GABAergic cell death, following tissue damage. In the absence of injury, GABAergic interneuron cell death in the *Drosophila* VNC is sufficient to trigger allodynia (Khuong, Wang, *et al.*, 2019). Loss of inhibition, manifested by a dysregulation of central GABA, is a conserved mechanism of neuropathic pain (Moore *et al.*, 2002; Henderson *et al.*, 2013; Inquimbert *et al.*, 2018). This further validates using *Drosophila* models for analgesic drug discovery.

1.11 Aims and hypothesis

Drosophila have been used for more than 100 years as an organism to answer many fundamental questions, across biology. The literature clearly demonstrates the great translational utility of the organism to accelerate the pipeline of drug discovery. We appreciate that pain is not the same concept in *Drosophila* as it is in mammals, but the fundamental physiology and molecular biology of nociception is highly conserved, including the signalling pathways that drive peripheral sensitisation. We anticipate that this novel invertebrate system will advance the knowledge of the peripheral mechanisms of nociception, allowing us to characterise the response to nociceptive stimuli. Our aim is to establish a series of experimental tools to enable the application of *Drosophil*a in pain research programmes.

2 Methodological Development

2.1 *Drosophila* stocks and genetics

The *Drosophila* were reared on cornmeal/agar media supplemented with yeast and kept in a 25^{o}C incubator (Parasonic, PHCbi, UK), with a controlled 12h light/12h dark cycle. A full list of *Drosophila* stocks is shown (see Table 2.1). Each genotype was maintained in separate polystyrene vials (Greiner Bio-One, Stonehouse, UK) with their food and plugged using reusable stoppers (Drosophilacentre, Germany), to prevent escape and allow air passage. Experimental stocks were transferred into a new vial every other day. Biosensors were expressed in specific cellular regions using the GAL4-UAS targeted expression system (Brand and Perrimon, 1993). To obtain selective expression of the Epac1-camps in the C4da sensory neurons, we crossed flies expressing GAL4, under the control of the pickpocket (ppk) promoter, with the reporter line carrying the gene of interest. The genetically encoded Ca^{2+} indicator GCaMP6m was also selectively expressed in the C4da neurons utilising the same system (Figure 2.1). To ensure consistency with the expected progeny genotype, crosses were performed using virgin females and males of the required genome. Acute CO_2 exposure was used to anaesthetise the flies, which were then transferred to a porous fly pad (Genesee Scientific, California, USA) to ensure optimal, consistent diffusion of CO_2. Virgin females were identified under a dissecting microscope (Zeiss, Germany) based on the presence of the meconium, a dark spot in the abdomen. Transgenic larvae 72-120h after egg laying were used for all imaging experiments.

Stock	Genotype	Chromosome	Description	Source
ppk-GAL4	w[*]; P{w[+mC]=ppk-GAL4.G}2	2		BDSC #32078
UAS-Epac1-camps	w[1118];+;P{UAS-epac1(camps) w[+]}/Sb	3	Red eyes	BestGene (California, USA) 11257-1-M-Ch3
UAS-GCaMP6m	w[1118];+; P {y[+t7.7]w[+mC]=20XUAS-IVS-GCaMP6m}VK00005	1, 2		BDSC #42748

Table 2.1: **A list of Drosophila stocks used throughout this project.** Abbreviation: BDSC, Bloomington Drosophila Stock Centre (Bloomington, USA).

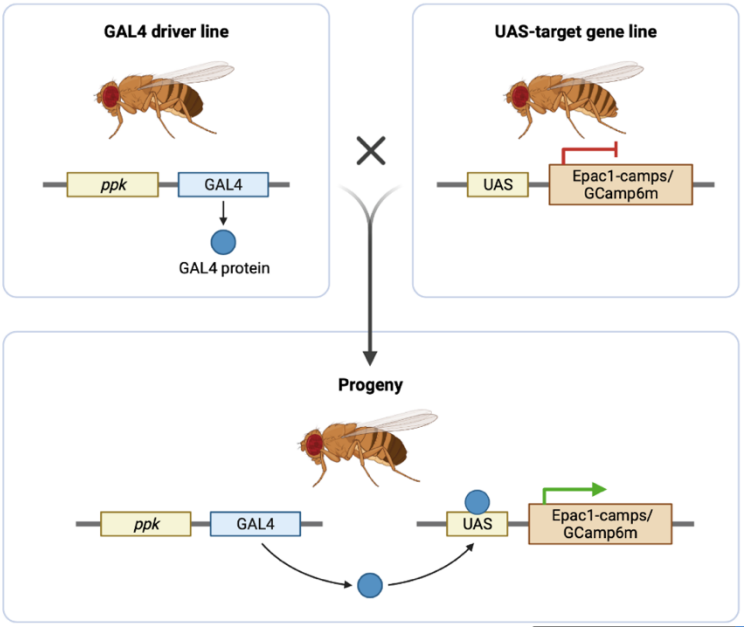


Figure 2.1: ***The principle of the GAL4-UAS expression system.*** *The driver line has GAL4 under the tissue specific promoter pickpocket (ppk), a gene exclusively expressed in C4da sensory neurons. The transgene of interest, either Epac1-camps or GCaMP6m, is placed downstream of a GAL4 upstream activating sequence (UAS). The tissue specific GAL4 lines are then genetically crossed with the UAS reporter lines. This allows for GAL4 to be produced in a tissue specific manner, that binds to the UAS region, to drive expression of the biosensor. Image created myself using BioRender.com*

2.2 Generalised methods

2.2.1 Larval preparation

Wandering male third instar larvae (Figure 1.2), sexed by the two lateral gonads, were dissected in ice-cold calcium free haemolymph-like HL3 saline solution (NaCl 70 mM, KCl 5 mM, $MgCl_2$ 5 mM, $NaHCO_3$ 10 mM, Trehalose 5 mM, Sucrose 115 mM, HEPES 10 mM, pH 7.2) (Stewart *et al.*, 1994). Initially there was pH fluctuation of the HL3 solution, which resulted in the increase in concentration of HEPES buffer from the original 5 mM to 10 mM. Dissections were performed using a stereomicroscope (Zeiss, Germany) with a total magnification range of 6.5x-50x. The larva was placed onto a Sylgard 184 pad (Sigma-Aldrich, Missouri, USA) and pinned dorsal side up, (this was identified by using the two tracheal tracts as visual reference points),

using stainless steel dissection pins (0.1 mm) (Figure 2.2). Pins were handled using straight forceps (tip dimensions 0.1 x 0.06 mm) and straight fine forceps (tip dimensions 0.05 x 0.02 mm), with incisions made using spring scissors (angled up blades). All dissecting tools and pins were purchased from Interfocus, Fine Science Tools (Cambridge, UK).

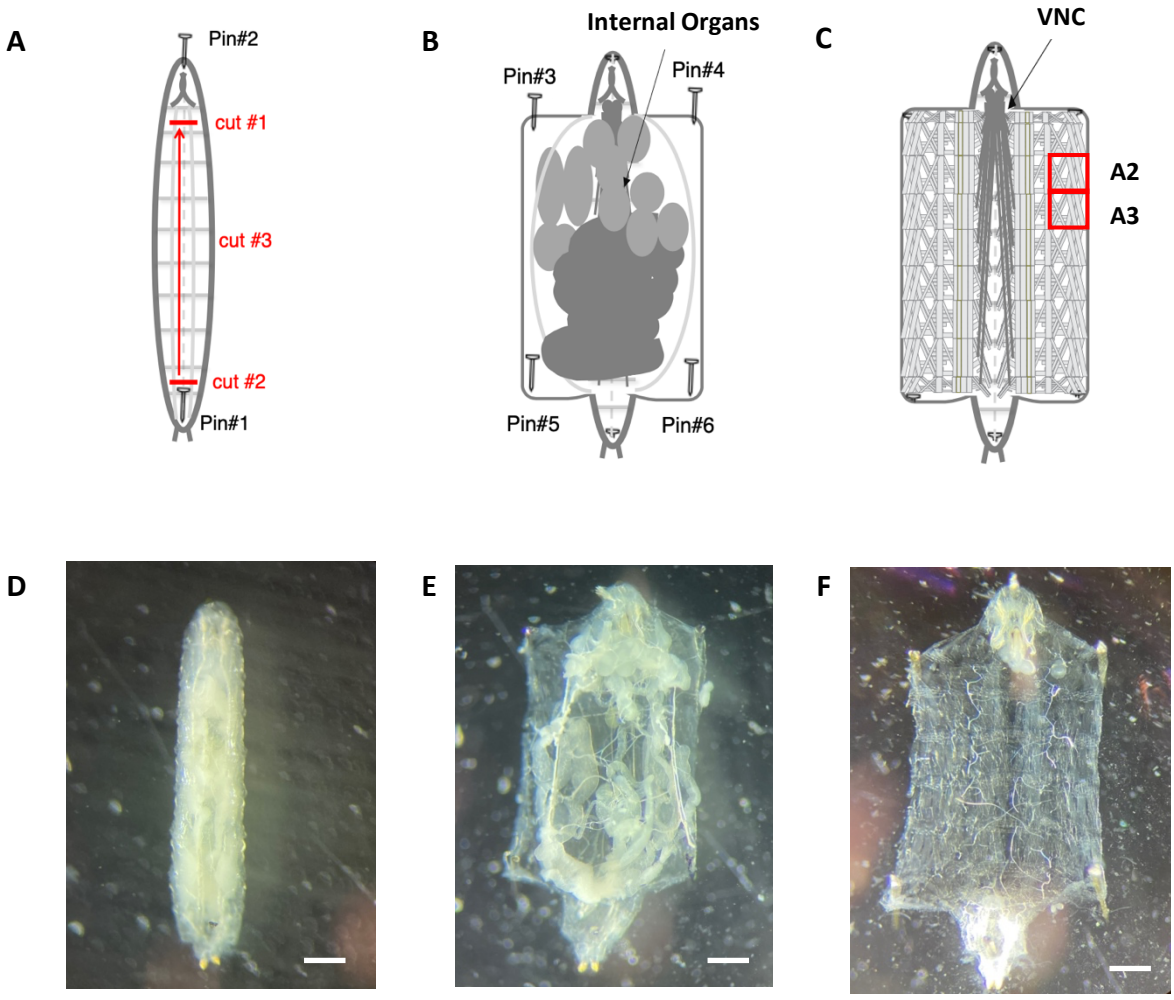


Figure 2.2: ***Third instar larva preparation.*** *(A, D) The head and tail of the larva were pinned on the Sylgard pad. Following this, one horizontal incision was made above the mouth hook and another at the tail (between the tracheae). A vertical cut was then made along the dorsal midline to join the two previous incisions. (B, E) The corners of the body wall were pinned down, ensuring that the larva was maximally stretched in all directions, using a further four pins. (C, F) The trachea and internal organs were removed. In this preparation (F), the ventral nerve cord (VNC) was also removed. Scale bars, 2 mm. (A-C) adapted from* Maiellaro (2022).

2.2.2 *In vivo* imaging set up

The Sylgard pad, with the dissected larva, was transferred to a diamond-shaped microscope chamber (Warner Instruments, Holliston, USA) and allowed to equilibrate in HL3 solution with 1 mM Ca^{2+} for 5 minutes at room temperature (RT). Preliminary experiments determined 2 ml as being an appropriate amount of HL3 solution in the imaging chamber, to cover the larva and bathe the objective. *In vivo* imaging experiments were performed on C4da neurons in abdominal (A) hemisegments A2 and A3 of male third instar larvae (Figure 2.2C). An upright epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a water-immersion objective (60x/1.0 numerical aperture) was used. CoolLED was used as the excitation source (pE-4000). Images were captured using a Prime 95B Scientific CMOS camera (Visitron Systems, Germany) with an OptoSplit II dual emission image splitter (Cairn research, Faversham, UK). The MetaFluor 5.0 software (Molecular Devices, California, USA) was utilised to monitor FRET and calcium fluorescence in real time. Agonists in HL3 solution were applied to the bath with a pipette. All pharmacological agents were purchased from Tocris Bioscience (Bristol, UK), unless stated otherwise. Experiments that required wash out of the reagents used a valve-controlled gravity perfusion system, calibrated to a flow rate of 1ml/min to avoid disruption to the larvae. The diamond-shaped chamber allowed for laminar flow and the excess solution was taken up by an aspirator. Only one C4da neuron was analysed per larva. Therefore, n indicates both the number of C4da neurons and larvae.

2.2.3 Data acquisition

Recorded experiments were analysed using the MetaFluor 5.0 software (Molecular Devices, California, USA). Regions of interest (ROI) were selected in different cellular components of the C4da nociceptive neuron such as the soma and dendritic arborization endings. Within this thesis, dendrites were subclassified as being either distal or proximal to the soma. All graphs were presented using Prism 6.0 software (GraphPad, La Jolla, USA).

2.3 *In vivo* imaging of cAMP dynamics in *Drosophila* nociceptive neurons

2.3.1 Experimental set up

FRET imaging was performed using the upright epifluorescent microscope (Section 2.2.2). The OptoSplit II dual emission image splitter was set to split the two FRET emission channels, to simultaneously acquire the donor (CFP) and acceptor (YFP) fluorescence. The filter cube (Cairn research, Faversham, UK), with a 505LP dichromic

mirror and emission filters for CFP (480/30) and YFP (535/40), was inserted into the OptoSplit II. CFP and YFP images were captured every 5 seconds upon excitation of CFP, occurring at 436 nm (4% LED power) for 20 ms. FRET was monitored as the ratio between CFP and YFP emissions (480 nm and 535 nm, respectively). Forskolin (FSK), an adenylyl cyclase activator, and 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, were used in these experiments to elevate the intracellular concentration of cAMP.

2.3.2 FRET specific data analysis

The FRET ratio, alongside the corresponding CFP and YFP emissions, were logged in Microsoft Excel (Washington, USA) for each ROI. A change in intracellular cAMP concentration, reported as a FRET signal, is characterised by antiparallel changes in intensity of the CFP and YFP emissions. Thus, FRET signals were discriminated from artefacts by inspecting the specific CFP and YFP wavelengths. The FRET dynamic range of the Epac1-camps sensor in C4da nociceptive neurons was calculated with the following equation:

$$FRET\ dynamic\ range\ \% = \left[\frac{R_{max} - R_{min}}{R_{min}}\right] * 100$$

2.3.3 Protocol optimisation

We were able to visualise the C4da nociceptive neurons, expressing the Epac1-camps sensor, at baseline by following the protocol described previously (Section 2.2). However, during preliminary experiments we found that the sensory neuron was moving out of the field of view as a consequence of muscle contraction, following the addition of FSK and/or IBMX. To minimize such effect, we cut all nerves emerging from the CNS during sample preparation, a standard procedure. The larval brain and ventral nerve cord (VNC) were then removed alongside the internal organs (Figure 2.2F). This enabled us to get stable recordings from the C4da nociceptive neurons, without movement. It is important to note that the primary focus of this project is the study of the peripheral mechanisms of nociception and therefore removal of the CNS will not limit the potential of this invertebrate model. However, no increases in intracellular cAMP were observed in the C4da nociceptive neurons following stimulation with FSK and IBMX (Figure 2.3). We produced replicates of this experiment which also proved to be unsuccessful.

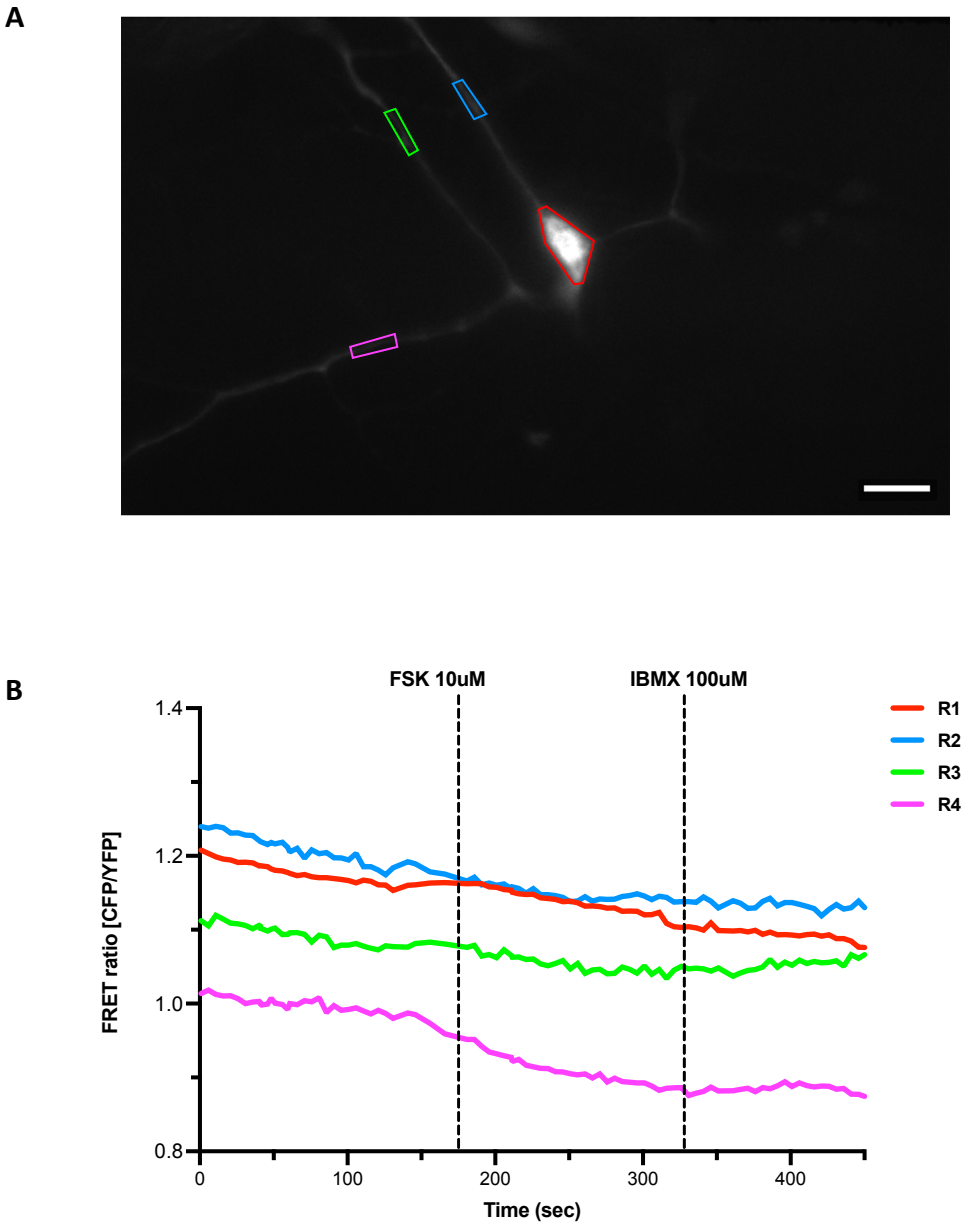


Figure 2.3: ***Stimulation of the transgenic larval preparations, with 10uM FSK and 100uM IBMX, produced no detectable increase in the intracellular cAMP concentration in C4da nociceptive neurons.*** *(A) Representative C4da neuron expressing the cAMP sensor, Epac1-camps. ROI are outlined in colour. Scale, 10um (B) Representative FRET trace obtained from the four ROI depicted in (A) upon the addition of 10uM FSK and 100uM IBMX.*

This issue was approached in a systematic manner, and we identified three possible explanations for the lack of experimental success at this stage: (i) the Epac1-camps sensor itself (e.g., limitations in the dynamic range), (ii) the set-up protocol for *in vivo* imaging (e.g., issues concerning the microscope) and (iii) the presence of the larval muscle tissue acting as a physical barrier.

2.3.3.1 Transfection of Human Embryonic Kidney (HEK) cells with Epac1-camps

To confirm function of the Epac1-camps sensor, we transfected the FRET sensor into Human Embryonic Kidney (HEK) 293 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Massachusetts, USA). Sterilised cover slips were coated with 1.5 ml of 50 µg/ml poly-D-lysine (Thermo Fisher Scientific, Massachusetts, USA) in a 6-well plate. Following incubation for 1 hour at RT, the poly-D-lysine was removed with aspiration, and the cover slips washed with de-ionised water. Prior to transfection, the cover slips were allowed to dry for at least 2 hours. HEK cells were plated, in 2 ml of growth media without antibiotics, at a concentration to ensure 70-90% confluence at the time of transfection. Epac1-camps DNA (6.67 ng/µl) and Lipofectamine 2000 (0.04% (v/v)) solutions were prepared in medium without serum, mixed gently, and incubated at RT for 5 minutes. The DNA solutions were added to the Lipofectamine 2000 solutions, mixed gently, and incubated at RT for 20 minutes. To each well, 250 µl of the DNA and Lipofectamine 2000 solution was added in a dropwise manner. After gentle rocking of the 6-well plate, the HEK cells were incubated at 37^{o}C for 18-24 hours before FRET imaging was performed (Section 2.3.1).

Prostaglandin E2 (PGE2) (Sigma-Aldrich, Missouri, USA), a potent inflammatory mediator, was initially added to the imaging chamber as an agonist. Providing we could optimise the FRET protocol, to visualise cAMP dynamics in the C4da nociceptive neurons, we then planned to investigate if these neurons were able to demonstrate PGE2-induced sensitisation. As described previously (Section 1.3.2), binding of PGE2 to its corresponding prostaglandin receptors EP2 and EP4 induces cAMP production (Jang, Kim and Hwang, 2020). In HEK cells transfected with the Epac1-camps sensor, the intracellular cAMP concentration was elevated in response to 100uM PGE2. This was reported as an increase in the FRET ratio (CFP emission/YFP emission) (Figure 2.4). Following this, 10uM FSK further increased cAMP production within these cells. Stimulation with 100uM IBMX induced no cAMP FRET signal, we expected that the Epac1-camps sensor was maximally activated. This data demonstrates Epac1-camps is able to serve as a highly sensitive cAMP indicator *in vitro*.

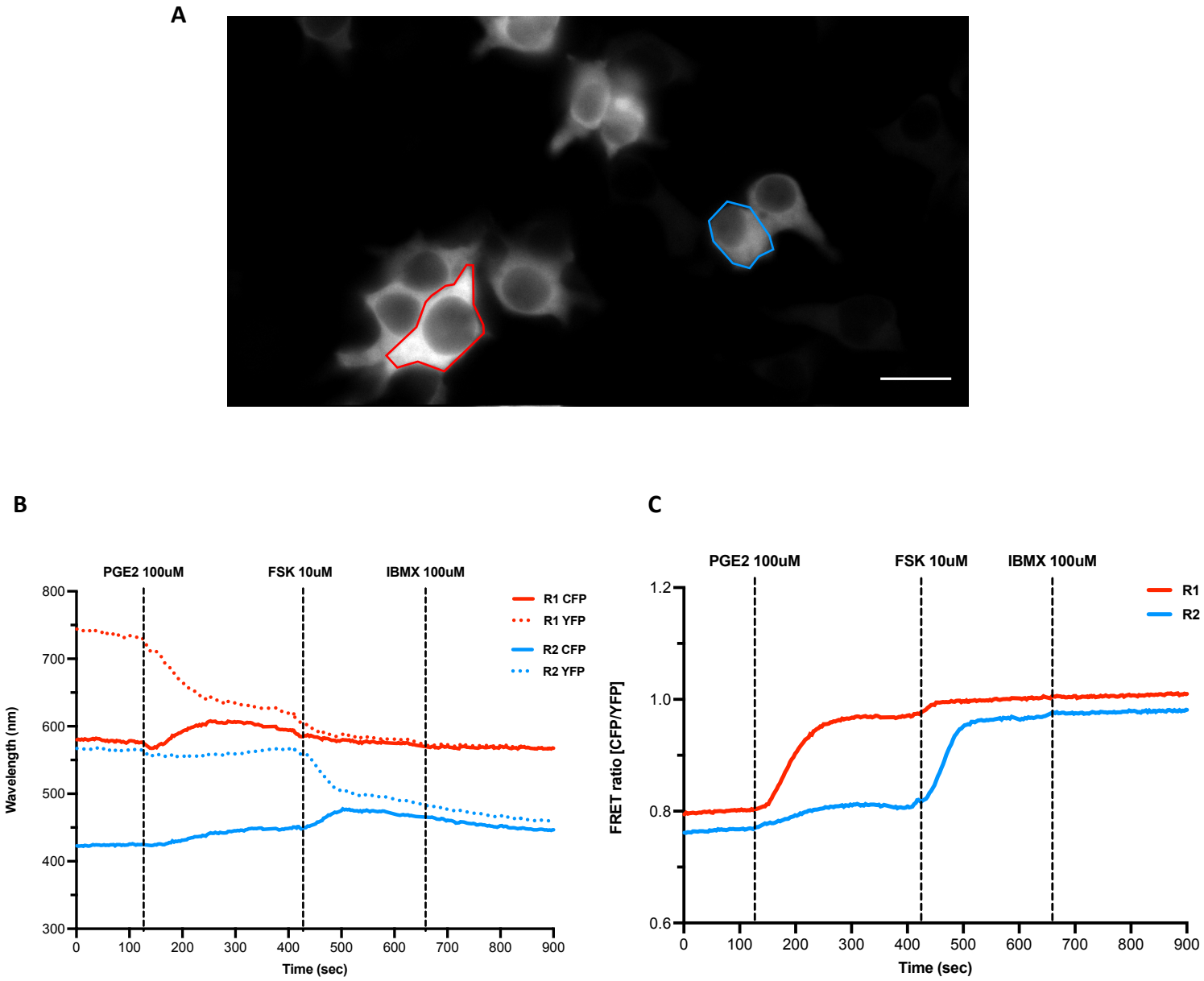


Figure 2.4: ***Robust cAMP responses can be induced in HEK cells transfected with Epac1-camps****. (A) Representative HEK cells, transfected with the FRET sensor, with ROI outlined in colour. For simplicity, only two representative ROI are depicted. Scale bar, 20um (B) Raw CFP and YFP emissions obtained from the ROI depicted in (A) upon the addition of 100uM PGE2, 10uM FSK and 100uM IBMX. An increase in cAMP is detectable as a reduction in FRET efficiency, characterised by antiparallel changes in intensity of the CFP and YFP emissions. (C) Corresponding FRET trace obtained from (B).*

2.3.3.2 Visualisation of cAMP dynamics in *Drosophila* motor neurons

We transgenically expressed Epac1-camps at the *Drosophila* NMJ, utilising the GAL4-UAS targeted expression system (Figure 2.1), to investigate the sensors function *in vivo*. We were able to monitor cAMP dynamics in glutamatergic type Ib motor neurons by targeting the expression of GAL4 to the tissue specific promoter *ok6* (ok6-GAL4). The G-protein coupled receptor (GPCR) agonist octopamine, comparable to vertebrate adrenaline and noradrenaline, was employed as a stimulant alongside FSK and IBMX. Stimulation of GPCRs for octopamine, expressed on the glutamatergic motor neurons, induces synaptic bouton outgrowth that is controlled by a cAMP and CREB-dependent positive-feedback mechanism (Koon *et al.*, 2011). In our experiments, octopamine triggered an increase in intracellular cAMP (Figure 2.4). These octopamine-induced cAMP signals were observed in different cellular compartments of the motor neuron- the axon, cell body, and boutons. Our results replicated a previous *in vivo* study that utilised the Epac1-camps sensor, to monitor the spatiotemporal dynamics of octopamine stimulated cAMP responses in these neurons (Maiellaro *et al.*, 2016). At the end of each experiment, we stimulated the larval preparations with FSK and IBMX to saturate the Epac1-camps sensor.

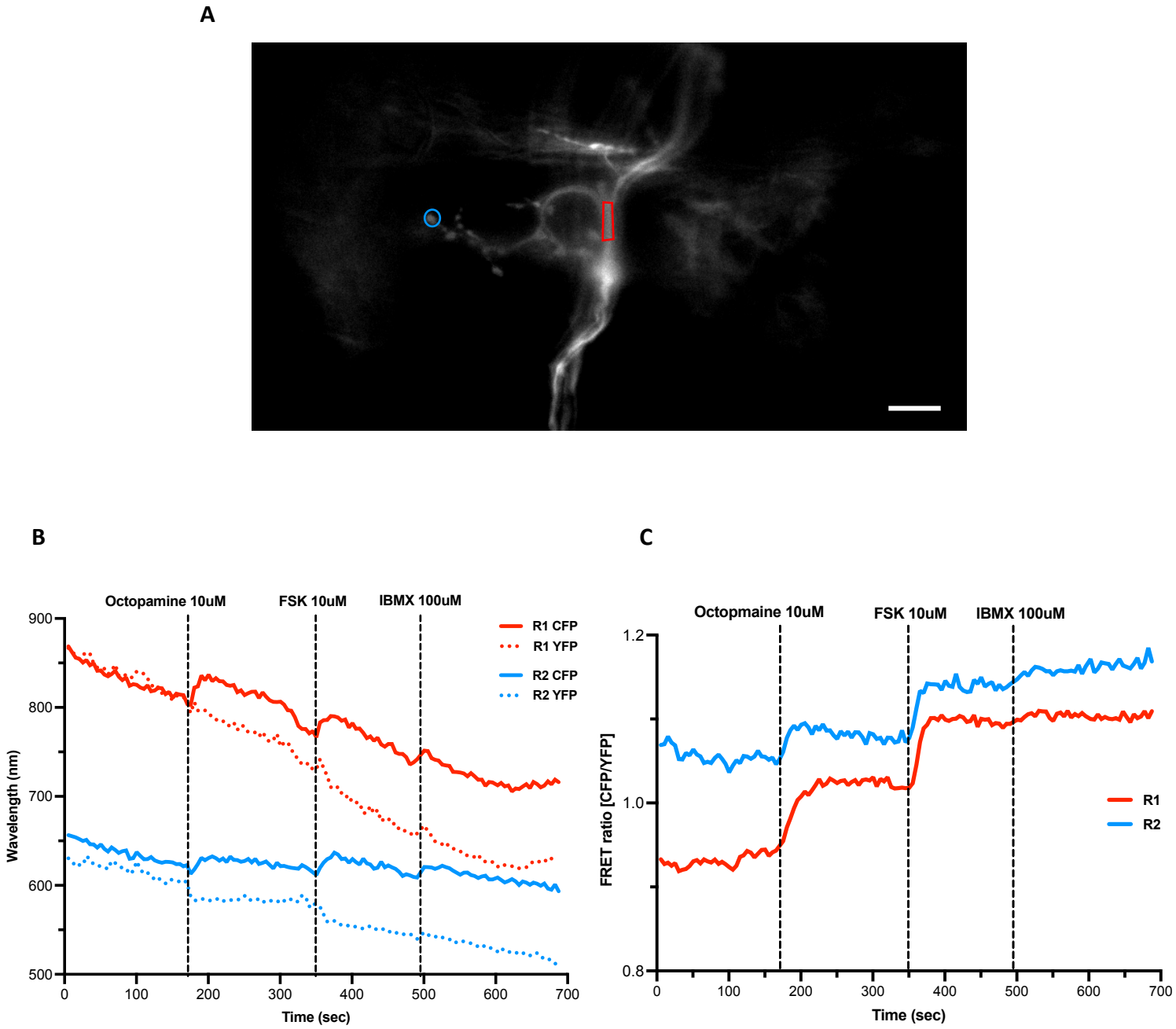


Figure 2.5: ***Stimulation of the transgenic larval preparations induced robust cAMP responses in Drosophila glutamatergic type Ib motor neurons.*** *(A) Representative* glutamatergic type Ib motor neuron *expressing the cAMP sensor, Epac1-camps. ROI are outlined in colour. For simplicity, only two representative ROI are depicted. Scale, 10um (B) Raw CFP and YFP emissions obtained from the ROI depicted in (A) upon the addition of 10uM octopamine, 10uM FSK and 100uM IBMX. (C) Corresponding FRET trace obtained from (B).*

Our data clearly show the Epac1-camps sensor is able to monitor changes in intracellular cAMP concentration both *in vitro* (Section 2.5.3.1) and *in vivo*. In addition, the success of experiments in HEK cells and *Drosophila* type Ib motor neurons confirmed the imaging protocol that had developed for this project was appropriate. Therefore, this eliminated concerns that the experiments in the C4da nociceptive neurons were unsuccessful due to an issue with the microscope. The FSK and IBMX-induced cAMP signals recorded also validated the function of the stock solutions that were used to stimulate cAMP in the sensory neurons.

2.3.3.3 Larval muscle tissue removal

Another possibility of our initial experiments being unsuccessful was the larval muscle tissue obscuring the cAMP FRET signals and/or acting as a diffusion barrier for the agonists applied. As previously discussed, the C4da sensory neurons are positioned between the basal surface of the epidermis and the epidermal extracellular matrix (ECM) (Figure 2.6).

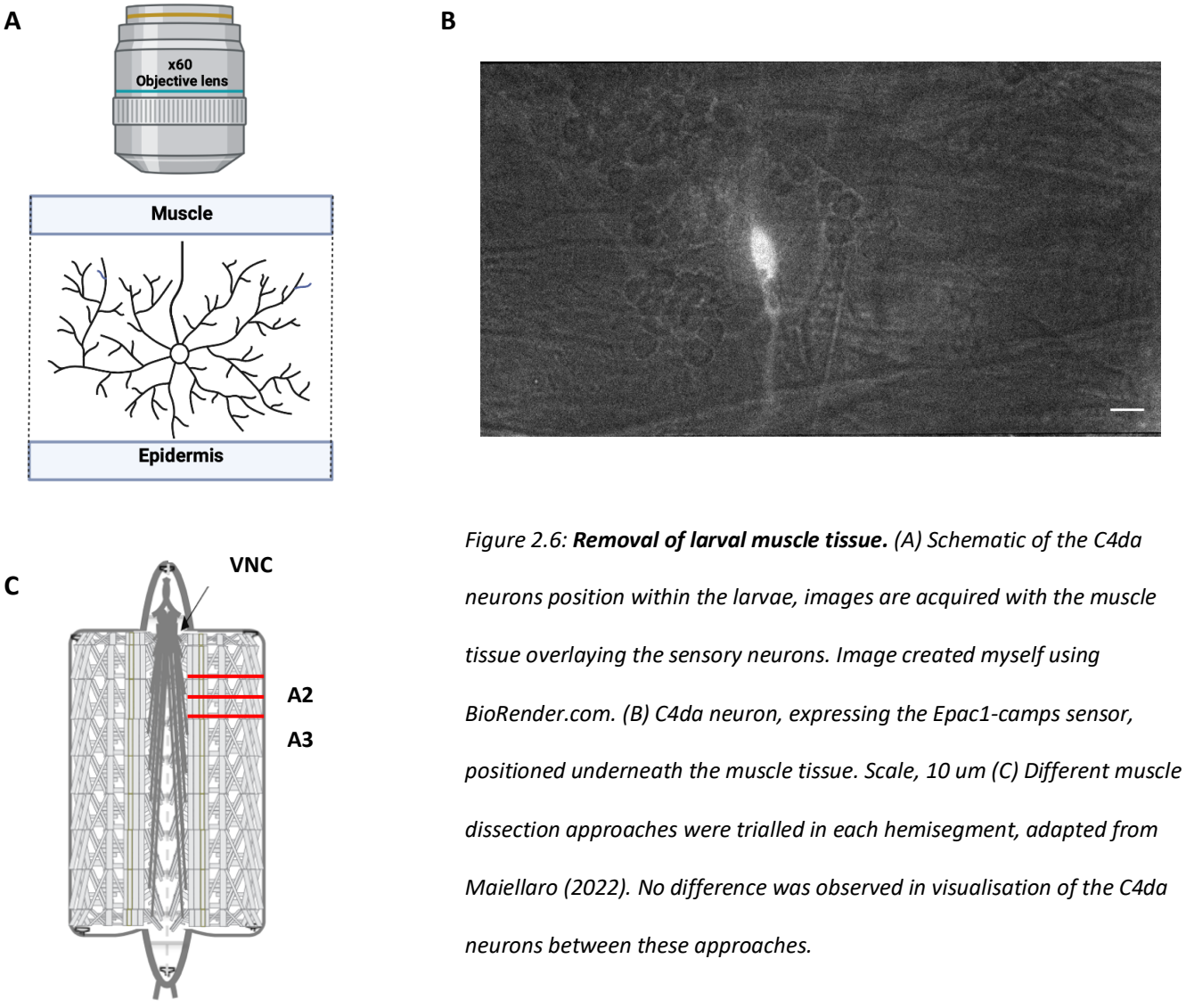


Figure 2.6: ***Removal of larval muscle tissue.*** *(A) Schematic of the C4da neurons position within the larvae, images are acquired with the muscle tissue overlaying the sensory neurons. Image created myself using BioRender.com. (B) C4da neuron, expressing the Epac1-camps sensor, positioned underneath the muscle tissue. Scale, 10 um (C) Different muscle dissection approaches were trialled in each hemisegment, adapted from Maiellaro (2022). No difference was observed in visualisation of the C4da neurons between these approaches.*

The larval muscle tissue, located anterior to the epidermal ECM, has been shown to greatly reduce visualisation of the C4da sensory neurons (Tenenbaum and Gavis, 2016). This group developed a protocol for the manual removal of *Drosophila* muscle tissue for immunofluorescence measurements in larval C4da neurons. This was a modification of previously described methods that had introduced muscle removal (Brent, Werner and McCabe, 2009; Rezaul Karim and Moore, 2011). Approaching from the dorsal midline, where the muscle tissue is absent, forceps were inserted between the muscle tissue and epidermal cell layer and pulled upwards to break the attachment. Following this, the larvae were dissected in HL3 solution containing 1.5 mM Ca^{2+} to aid the separation of the muscle tissue from the epidermis. cAMP FRET signals were successfully recorded from C4da neurons once the muscle tissue had been removed (Figure 2.7) which demonstrated that this step had optimised the protocol.

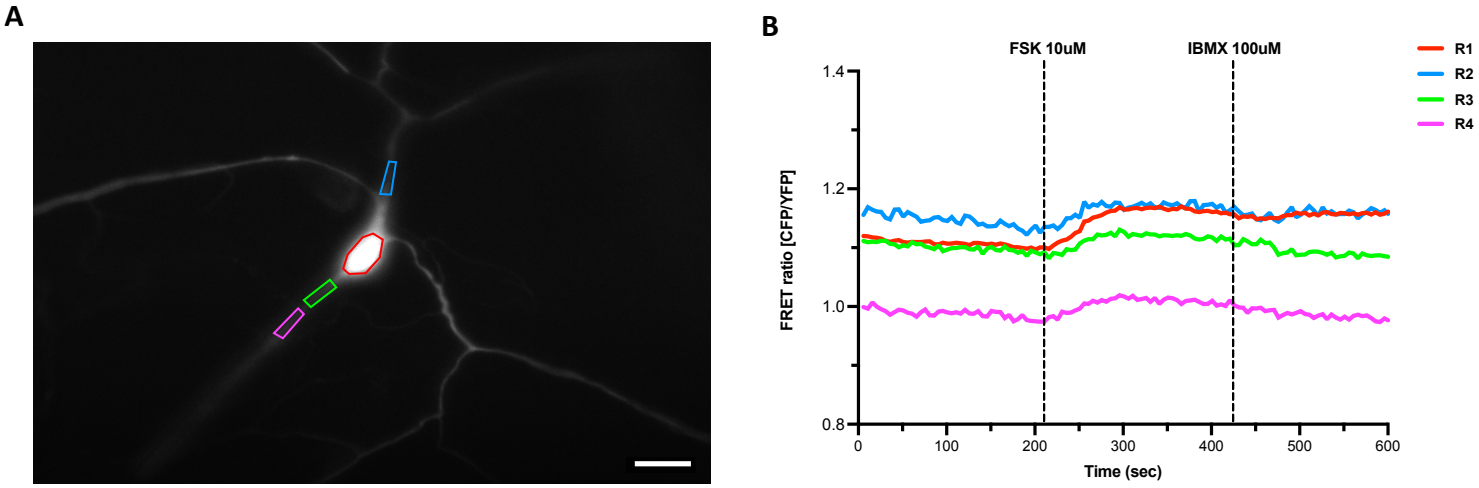


Figure 2.7: ***Removal of the larval muscle tissue resulted in cAMP FRET signals, upon stimulation with FSK.*** Scale, 10um. *(A) Representative C4da neuron expressing Epac1-camps following the manual removal of the muscle tissue. ROI are outlined in colour. Scale, 10 um (B) Representative FRET trace obtained from the four ROI depicted in (A) upon the addition of 10 uM FSK and 100 uM IBMX.*

2.3.3.4 Installation of new OptoSplit II Bypass image splitter

Following removal of the muscle tissue, increases in cAMP were induced by FSK and IBMX. Despite this, the cAMP FRET signals obtained in the C4da nociceptive neurons did not display a wide dynamic range. The Epac1-camps sensor was fully saturated by 10uM FSK, with 100uM IBMX showing no detectable increase in cAMP following this (Figure 2.7). The original beam splitter installed, that enables a single camera to record images simultaneously at two different emission wavelengths, was outdated. We proposed that the alignment was no longer sufficient to collect the FRET ratio in the sensory neurons, resulting in a loss of signal.

Following the arrival and installation of the OptoSplit II Bypass image splitter (Cairn research, Faversham, UK), we reduced the power of the LED exciting CFP from 50% to 4%. To obtain better characterisation of the Epac1-camps sensor dynamic range, the concentration of FSK was also increased from 10uM to 50uM. Following these changes to the protocol, robust cAMP FRET signals were consistently recorded following the addition of FSK (Figure 2.8). C4da neuron experiments with the larval muscle tissue intact, prior to the installation the new beam splitter, produced no detectable increases in intracellular cAMP in response to stimulation (Figure 2.3). Following the increase in FSK concentration and application of the new equipment, we observed removal of the muscle tissue was no longer necessary to acquire a cAMP FRET signal. When then attachment of the overlying muscle tissue was broken this changed the tension in the larval body wall, which in turn affected the image quality of the C4da neuron. Therefore, not only was the sample preparation stage simplified, but the morphology of the C4da nociceptive neurons were better preserved. The single lever bypass mode of the OptoSplit II Bypass image splitter also enabled single wavelength Ca^{2+} recordings to be conducted within the C4da neurons, in addition to the dual channel FRET experiments.

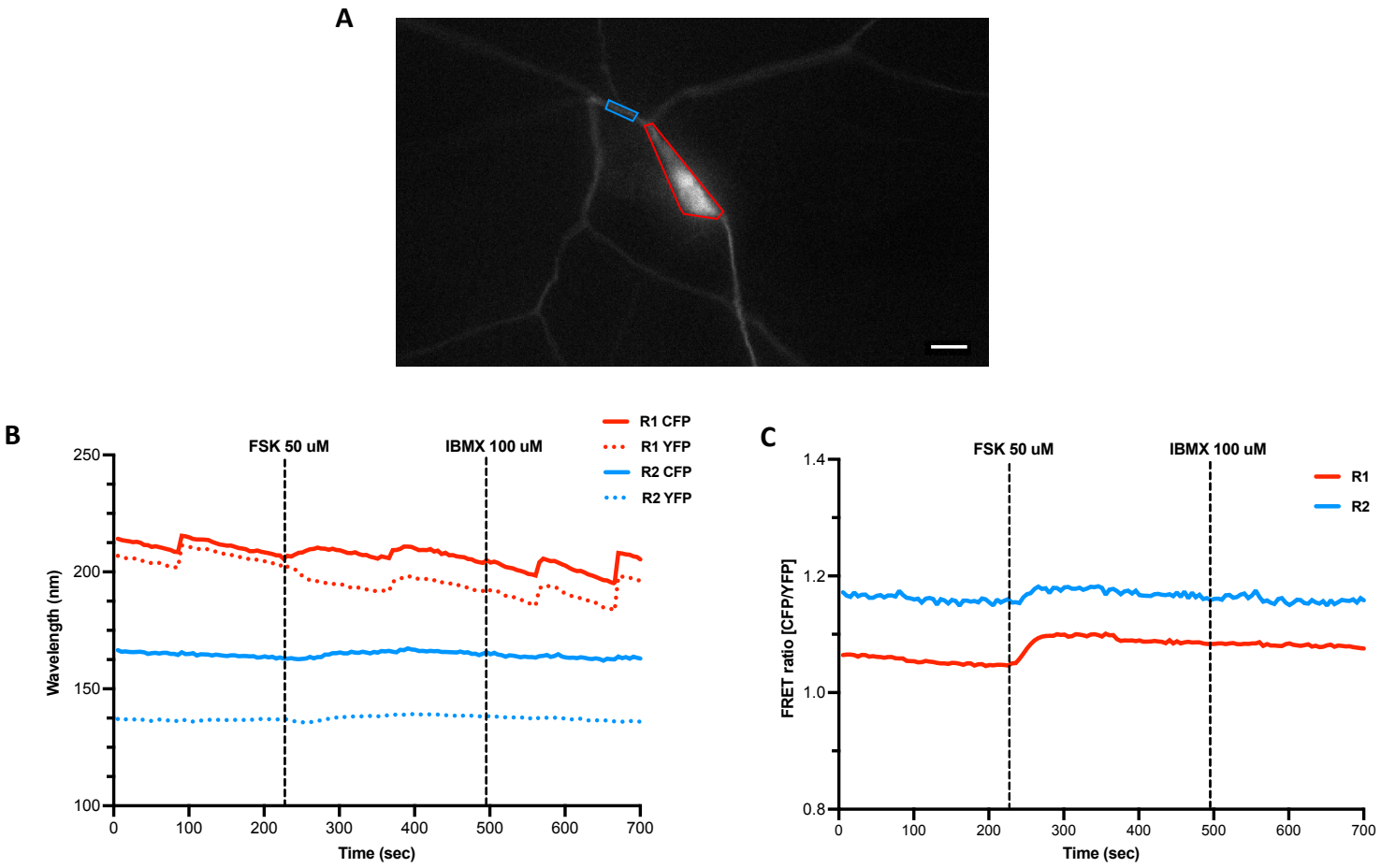


Figure 2.8: ***Following further optimisation of the protocol, an increase in the intracellular cAMP concentration can be induced in C4da nociceptive neurons without removal of the muscle tissue.*** *(A) Representative C4da neuron expressing Epac1-camps with* the larval muscle tissue intact*. ROI are outlined in colour. For simplicity, only two representative ROI are depicted. Scale, 10um (B) Raw CFP and YFP emissions obtained from the ROI depicted in (A) upon the addition of 50uM FSK and 100uM IBMX. (C) Corresponding FRET trace obtained from the ROI depicted in (A).*

2.4 *In vivo* Ca^{2+} imaging in *Drosophila* nociceptive neurons

2.4.1 Experimental set up

Ca^{2+} dynamics in the C4da nociceptive neurons were monitored using the previously described *in vivo* imaging protocol (Section 2.3). The OptoSplit II dual emission image splitter was configured into single channel (non-split) mode, to make use of the full input image. Images were captured every 500 ms upon excitation of GFP, occurring at 490 nm (50% LED power) for 50 ms. Cinnamaldehyde (CA) (Sigma-Aldrich, Missouri, USA), the selective TRPA1 agonist, was employed as a nociceptive stimulus in these experiments. Pharmacological manipulation experiments utilised the reversible TRPA1 channel blocker, AP18 and the TRPA1 antagonist, AM0902.

2.4.2 Ca^{2+} data analysis

Mean fluorescence intensity within each ROI was quantified and expressed as the ratio of fluorescence at time t divided by the mean intensity 0-20s before the addition of the agonist (F/F_0). Prism 6.0 software (GraphPad, La Jolla, USA) was used for statistical analysis and graphical presentation. Outliers were identified by Grubb's test and excluded from statistical analysis. Statistical comparisons between peak somal response values ($\Delta F/F_0$) in C4da nociceptive neurons were made using unpaired student's t tests. Statistical comparisons between defined cellular compartments of the sensory neurons were made using a repeated measures one-way ANOVA with Bonferroni's multiple comparisons test. A mixed-model ANOVA was performed when there was excluded data points. Individual data points were represented as mean ± standard error of mean (SEM). Differences were considered significant when $P < 0.05$.

2.4.3 Protocol optimisation

2.4.3.1 Supplementing the HL3 with L-glutamate

Preliminary experiments employed the Ca^{2+} ionophore ionomycin (Thermo Fisher Scientific, Massachusetts, USA) and cinnamaldehyde (CA) as stimulants. In all sample preparations the larval CNS was removed. However, immediately after the addition of the agonists to the imaging chamber, the overlying muscle tissue contracted and subsequently moved the C4da nociceptive neuron out of the field of view. We observed both compounds triggered an increase in the intracellular Ca^{2+} concentration, demonstrated by the increase in fluorescence intensity. Despite this, we were unable to accurately analyse these experiments as the larvae movement resulted in the sensory neuron no longer being visible when peak response was reached. One approach to this issue could have been the manual removal of the muscle tissue, as previously trialled for the in vivo FRET imaging (Section 2.3.3.3). However, the *Drosophila* neuromuscular junction (NMJ) synapse utilises glutamate as a primary neurotransmitter (Jan and Jan, 1976). The postsynaptic ionotropic glutamate receptors (GluRs) at larval NMJ synapses are homologous to AMPA-type GluRs in the mammalian brain (Menon *et al.*, 2012). Chen *et al.*, (2009) reported ambient extracellular glutamate reduces synaptic transmission at *Drosophila* larval NMJs, in a dose-dependent manner (EC_{50}: ~0.4mM). Extracellular glutamate initially triggers postsynaptic receptor activation, followed by desensitisation. Going forward, the HL3 saline solution was supplemented with 10 mM L-glutamate to desensitise the postsynaptic receptors. This suppressed muscle

contractions by attenuating synaptic transmission at the larval glutamatergic NMJs. Thus, we were able to obtain stable recordings following the application of agonists to the transgenic larval preparation (Section 2.4.3.1).

2.4.3.2 Heating of the CA to aid solubility

We trialled three different concentrations, diluting the from the neat chemical, for our initial CA experiments. CA concentrations greater than 1:100, in HL3 saline solution supplemented with L-glutamate, obscured the visibility of the C4da neuron when added to the imaging chamber. This is due to the organic compound being a yellow oily liquid at RT. To aid the solubility of CA in HL3, the solution was heated in a water bath at 90 ^{o}C for 5 minutes. Prior to amending the protocol, we observed that the compound was not fully dissolved in solution during experiments. The C4da nociceptive neurons produced varied responses to the same concentration of cinnamaldehyde (Figure 2.9). We expected this was attributable to the low solubility of the compound in aqueous solution.

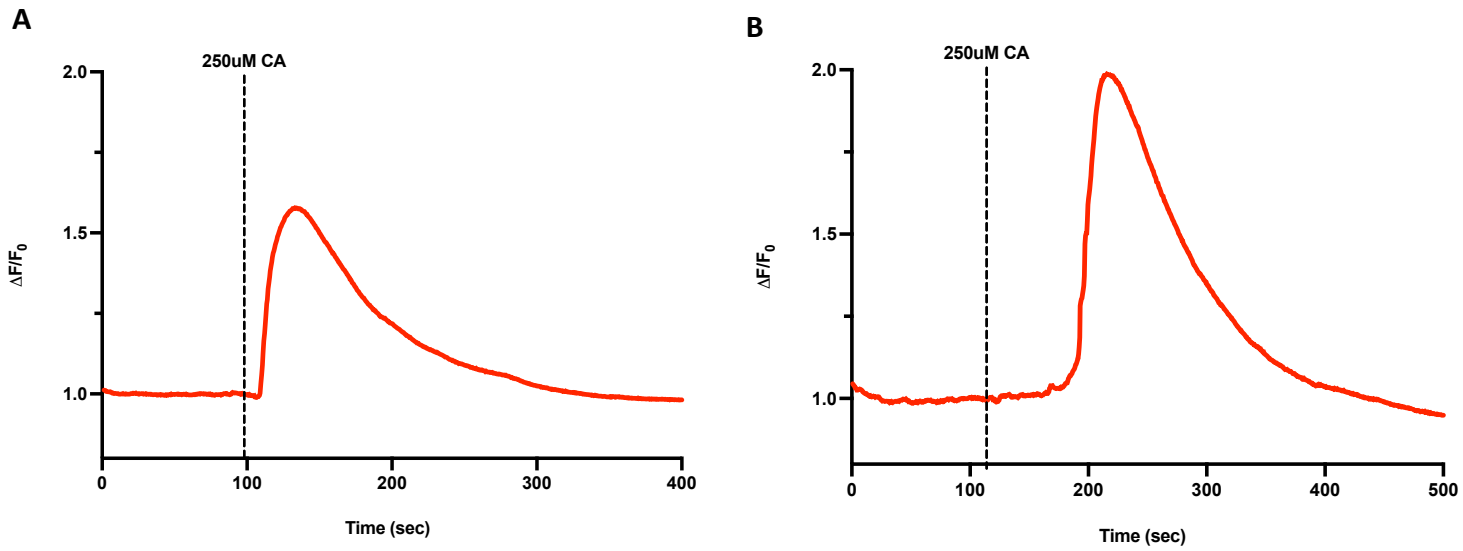


Figure 2.9: ***Stimulation of the transgenic larval preparations with a concentration of 250uM CA produced variable fold changes in Ca^{2+} concentration in the C4da soma.***

3 Results

3.1 Characterisation of the Epac1-camps sensor

C4da neurons function as primary nociceptors in *Drosophila* larvae, being sufficient and necessary for triggering the highly stereotyped avoidance behaviour evoked by noxious stimulation (Hwang *et al.*, 2007). We have shown that selective expression of Epac1-camps in C4da nociceptive neurons can be achieved through exploiting the GAL4-UAS expression system, using the well-described ppk promoter (Figure 2.1). First, we analysed the dynamic range of our sensor expressed in the sensory neurons (ppkepac). Dynamic range values were calculated as previously described (Section 2.3.2). R_{min} is the basal FRET ratio and R_{max} was obtained by saturating the sensor with 50uM FSK and 100uM IBMX (Nikolaev *et al*., 2004; Maiellaro *et al*., 2016). From nine experiments, the dynamic range values of our sensor in C4da neurons were around 6% (Figure 3.1), well below the dynamic range value reported in the literature (Nikolaev *et al.*, 2004; Maiellaro *et al.*, 2016). As control, to understand if this was a technical limitation more than a physiological one, we performed similar experiments in HEK cells and *Drosophila* type Ib motor neurons, expressing the cAMP sensor Epac1-camps. We calculated the dynamic range values in transfected HEK cells (25%) and *Drosophila* type Ib motor neurons (31%). Despite the reduced dynamic range, our data demonstrate we are able to monitor changes in intracellular cAMP concentration in these sensory neurons.

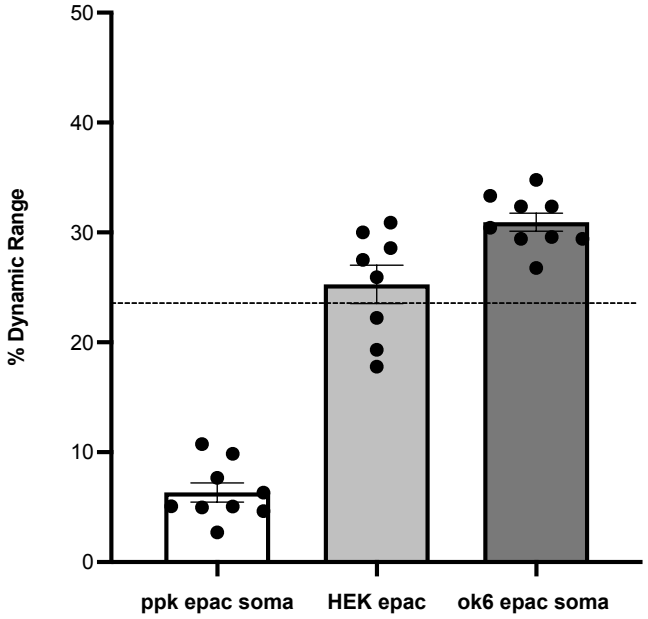


Figure 3.1: ***Dynamic range values of the Epac1-camps sensor.*** *Values obtained from C4da neurons (n=9), HEK cells (n=8) and type Ib motor neurons (n=9). Data are shown as a mean ± SEM. Individual data points are expressed as a percentage. The line shows the dynamic range value reported in the literature for this cAMP sensor (24%)* (Nikolaev *et al.*, 2004; Maiellaro *et al.*, 2016).

3.2 Effect of PGE2 on C4da nociceptive neurons

In mammals PGE2 is produced during inflammatory processes and is closely associated with pain. PGE2 interacting with EP receptors leads to G-protein mediated increased activity of adenylate cyclase and consequently increased cAMP production (Section 1.3.1). We therefore investigated whether analogous cAMP increases occur in *Drosophila* following PGE2 exposure. We performed seven experiments, in which the transgenic larvae expressing our cAMP biosensor were exposed to PGE2 followed by 50uM FSK and 100uM IBMX. We have previously shown the conditions in which we can control intracellular cAMP in C4da nociceptive neurons (Section 3.1). in all nine experiments stimulating with 50uM FSK and 100uM IBMX alone, a robust FRET cAMP signal was recorded, and the Epac1-camps sensor was fully saturated by 50uM FSK. At the beginning of each experimental day, to confirm function of the control solutions, we stimulated a transgenic larva with 50uM FSK and 100uM IBMX alone. Interestingly, the control response was inhibited in 71% of C4da nociceptive neurons pre-stimulated with PGE2. Thus, the data obtained was more difficult to interpret (Figure 3.2A).

Typically, FRET values are normalised to express the agonist response as a percentage of the sensor maximally activated at the end of each experiment. This eliminates differences in FRET that can be due to a difference in the expression level of the sensor. We are unable to calculate ΔFRET (% max), as a result of the small percentage of neurons that responded to the controls, therefore we cannot directly compare across experiments. Despite this, we can observe the effect of PGE2 on the FRET ratio (CFP emission/YFP emission). PGE2 alone does not produce a detectable change in the FRET ratio. In only two cases we could confirm there was no FRET cAMP response as these sensory neurons subsequently responded to FSK (Figure 3.2B). With the additional five experiments, we have no means of discriminating a lack of PGE2-induced cAMP FRET response or an issue in the detection of this.

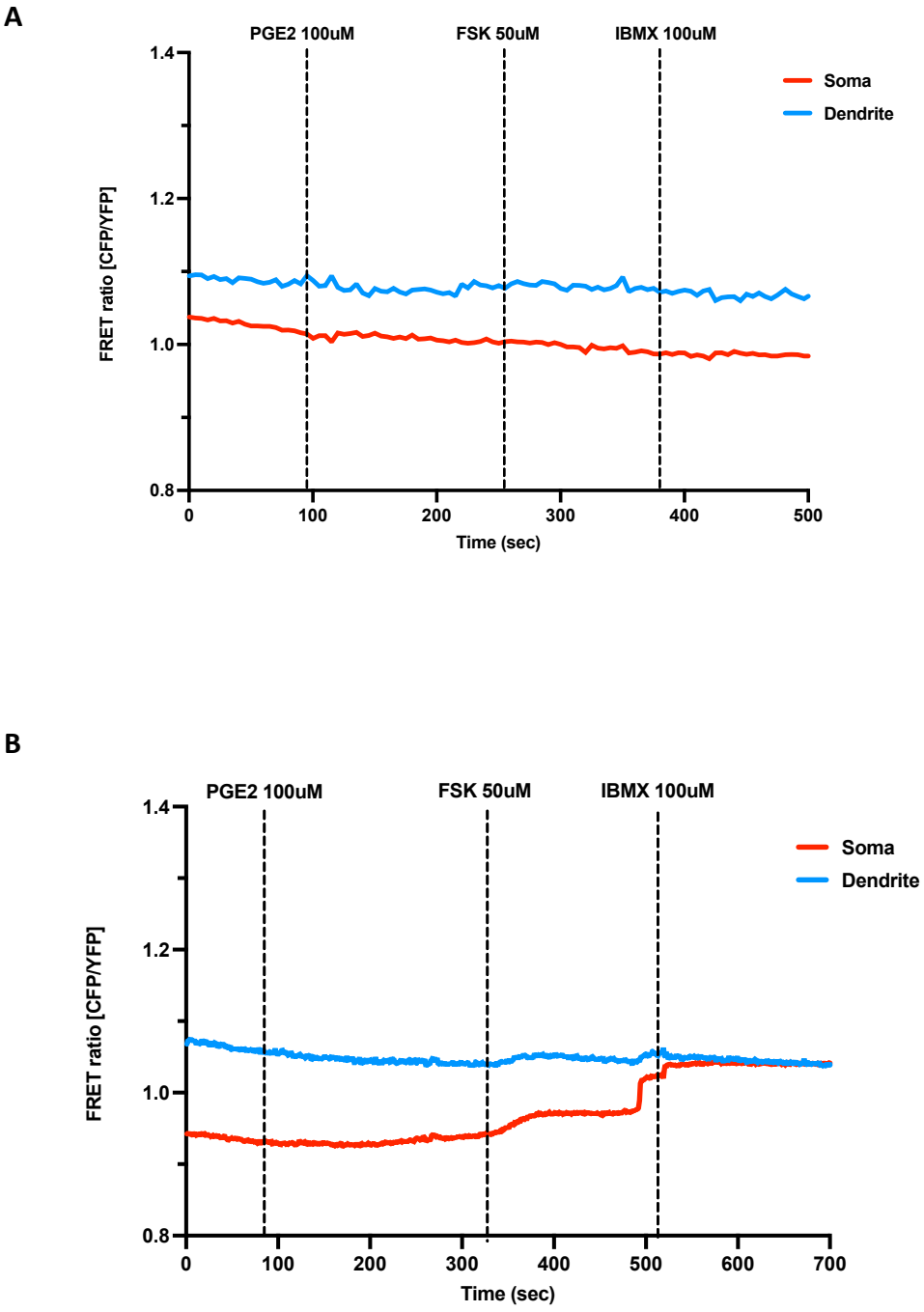


Figure 3.2: ***C4da nociceptive neurons pre-stimulated with PGE2.*** *(A) Representative trace of a C4da neuron that has been stimulated with 100uM PGE2, followed by 50uM FSK and 100uM IBMX. We were unable to get a control response to 50uM FSK, different to what had been observed previously. (B) Representative experiment, of a C4da neuron pre-stimulated with 100uM PGE2, that displayed a minor control response to 50uM FSK.*

3.3 Cinnamaldehyde (CA) induces an intracellular Ca^{2+} response in C4da nociceptors

Next, we selectively expressed the genetically encoded Ca^{2+} indicator GCaMP6m in the C4da nociceptive neurons (Figure 3.3A, C), utilising the GAL4-UAS expression system (Figure 2.1). To investigate whether a change in the cytosolic Ca^{2+} concentration ("a calcium response") could be induced in these sensory neurons, as an indicator of depolarisation of these neurons, the mammalian TRPA1 agonist cinnamaldehyde (CA) was employed as a nociceptive stimulus (Section 1.6.1). We performed a series of experiments in which the entire larva was exposed to CA, through direct application via the bathing solution. Regions of interest (ROI) were drawn on specific cellular compartments, either the soma or dendrites, and Ca^{2+} signals were followed. CA triggered a transient Ca^{2+} response, demonstrated by the increase in fluorescence (ΔF) (Figure 3.3A,C), that mimicked the kinetics of the GCaMP6m sensor (Chen *et al.*, 2013). The observed cellular response to CA was robust and reproducible (Figure 3.3B). Our results suggest that the mammalian TRPA1-specific agonist CA is also able to activate the *Drosophila* TRPA1 homologues (dTRPA1 and/or Painless), expressed on the C4da neurons, to stimulate a Ca^{2+} influx. This supports the evolutionary conservation of the peripheral mechanisms of nociception, thus demonstrating the great translational utility of this invertebrate model to accelerate the pipeline of analgesic drug discovery.

Next, we aimed to understand whether the CA-induced calcium responses observed in the soma were concentration dependent. Individual transgenic larval preparations were stimulated with one of six CA concentrations (5uM, 10uM, 25uM, 50uM, 100uM, 1mM). In the C4da soma, no detectable changes in Ca^{2+} concentration were induced by 5uM CA. Experiments stimulating with low concentrations (5uM and 10uM) were followed with 1 mM CA as a positive control. Maximum Ca^{2+} response was achieved with 1mM CA alone. Overall, the data presented show a CA dose-dependent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]$), with an $EC_{50} = 4.123 \times 10^{-5}$ M (Figure 3.3D). This data is in line with reported values calculated in mouse TRPA1-expressing Chinese Hamster Ovary (CHO) cells using ratiometric Ca^{2+} imaging (Bandell *et al.*, 2004).

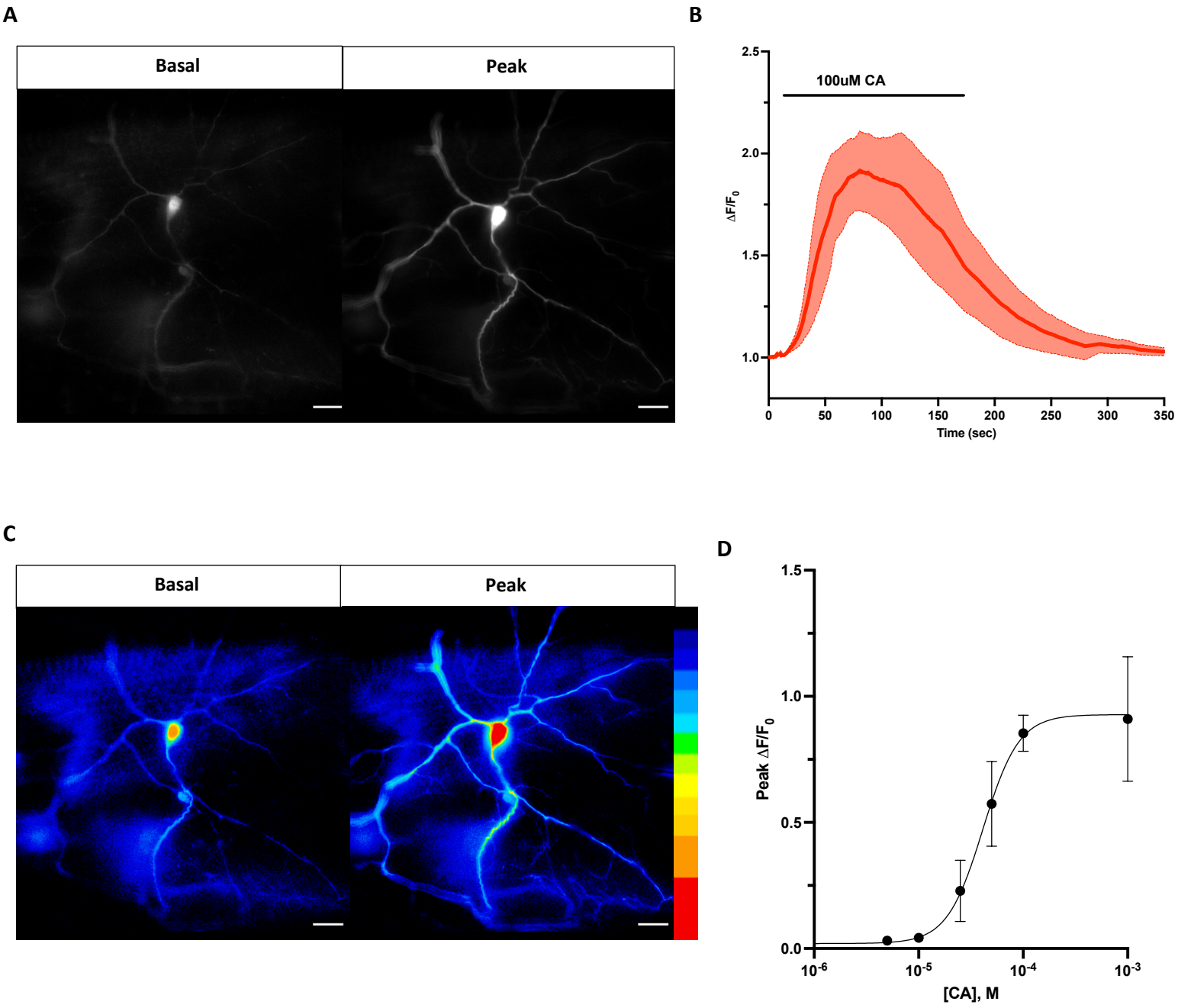


Figure 3.3: ***An intracellular Ca^{2+} response is induced in C4da neurons following CA application.*** *(A, C) Representative image of a C4da neuron, expressing the Ca^{2+} sensor GCaMP6m, at basal and peak fluorescence following application of 100uM CA. Monochromatic (A) and pseudo colour images (C) are shown, alongside the colour scale for fluorescent change. Scale bars, 20um (B) Average of individual Ca^{2+} responses obtained in C4da cell bodies stimulated by 100uM CA (n=5). Mean fluorescence intensity was quantified and expressed as the ratio of fluorescence at time t divided by the mean intensity for 0-20s before addition of CA (F/F_0). (D) Concentration-response curve obtained from traces like those shown in (B) following stimulation by generalised application of CA concentrations: 5uM (n=7), 10uM (n=6), 25uM (n=5), 50uM (n=8), 100uM (n=7) and 1mM (n=3). Data are shown as mean ± SEM.*

3.4 Pharmacological characterisation of the CA-induced Ca^{2+} response

It is important that we are able to establish whether *Drosophila* pharmacology is analogous to that seen in mammals for our aim to introduce invertebrate systems into the target identification and validation pipeline, for drug discovery in pain research programmes. Next, we pharmacologically manipulated the system and investigated how this impacted the nociceptive response to CA. AP18, a reversible TRPA1 antagonist, was initially selected as an appropriate antagonist for these experiments (IC_{50} values are 3.1 and 4.5 µM at human and mouse TRPA1 respectively) (Petrus *et al.*, 2007). AP18 has been highly used in the literature to pharmacologically inhibit CA-induced nociception, demonstrating biological activity both *ex vivo* and *in vivo* (Petrus *et al.*, 2007; Chen *et al.*, 2009; Taylor-Clark *et al.*, 2009).

Transgenic larval preparations specifically expressing GCaMP6m in the C4da nociceptive neurons were incubated with 20uM AP18 for 20 minutes. Larvae were initially stimulated with 50uM CA through direct application via the bathing solution. Following this, the TRPA1 antagonist AP18 was washed out of the imaging chamber using a perfusion system. During these experiments, there was continual perfusion of CA so that the concentration within the chamber remained at 50uM during and after washout of AP18. Our data showed no significant difference in peak soma Ca^{2+} response, following 50uM CA application, between larval preparations incubated with 20uM AP18 for 20 minutes and controls (Figure 3.4). We selected 50uM for these experiments due to this being a concentration close to the EC_{50} (Figure 3.4D). A percentage of C4da neurons could be said to exhibit partial antagonism of the CA-induced Ca^{2+} response by AP18 (n= 3). The nociceptive neurons displayed a second Ca^{2+} response, following washing of AP18 out of the chamber, in the continued presence of 50uM CA (Figure 3.4A). Previously, after an initial Ca^{2+} response to CA, a second stimulation of CA does not induce a Ca^{2+} response. This demonstrates the reversible property of this TRPA1 channel blocker, which has been shown by previous groups (Petrus *et al.*, 2007; Chen *et al.*, 2009). Whilst we cannot rule out the second CA-induced Ca^{2+} response is a wash-in delay, we think this is unlikely as this was only observed in preparations incubated with an antagonist, following perfusion of this antagonist out of the chamber. Overall, these results suggest that 20uM AP18 does not significantly block the nociceptive responses to 50uM CA.

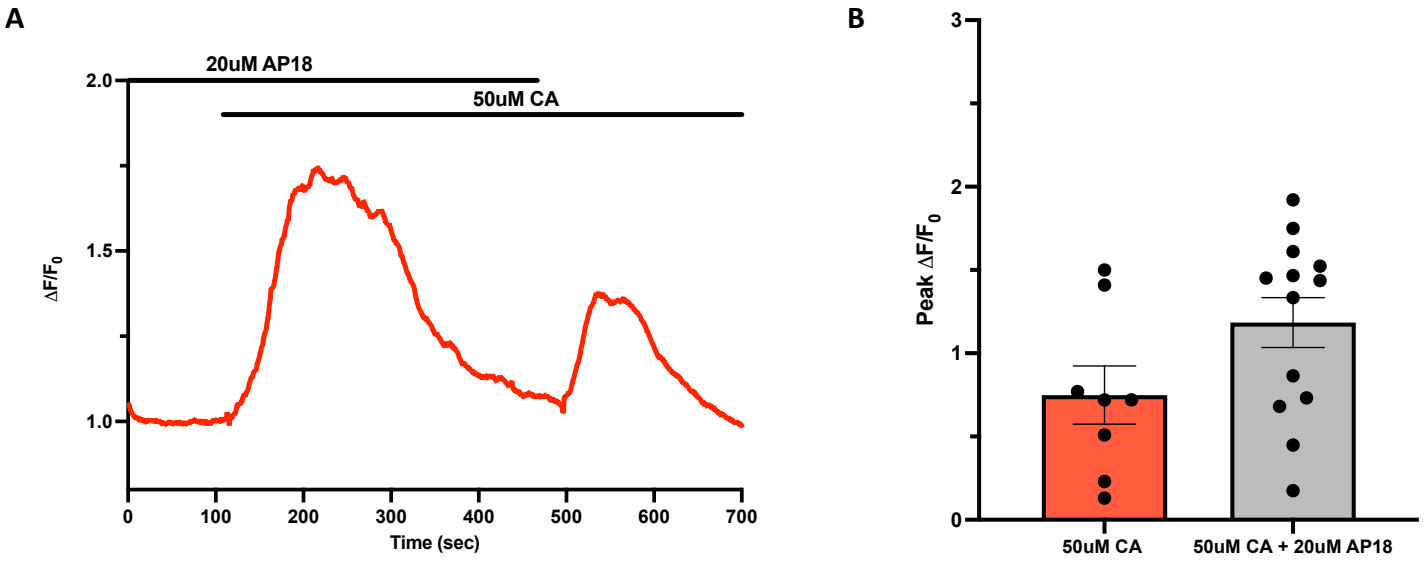


Figure 3.4: ***The CA-induced Ca^{2+} response in C4da neurons is not significantly blocked by the reversible TRPA1 channel blocker, AP18.*** *(A) Representative trace of a C4da neuron that has been incubated with 20uM AP18 and stimulated with 50uM CA, followed by a washout of the antagonist using a perfusion system. (C) Peak soma values of ΔF/F0 for C4da neurons stimulated with 50uM CA in the presence of AP18 (n=13) and without (n=8). Data are shown as mean ± SEM. Unpaired Student's t tests were performed.*

Following this, the selective and more potent TRPA1 antagonist AM0902 was employed (IC_{50}= 0.02 µM for human TRPA1). AM0902 has demonstrated a dose-dependent inhibition of allyl isothiocyanate-induced flinching in rats, which validates compounds in vivo target efficacy (Schenkel *et al.*, 2016). Similar to previous experiments, 50uM CA was selected as an appropriate nociceptive stimulus due to this concentration being close to the EC_{50} (Figure 3.3D). C4da nociceptive responses to 50uM CA are significantly blocked in the soma following incubation with 20uM AM0902, for 20 minutes prior to imaging ($t(14) = 3.915$, $p=0.0016$; Figure 3.5B). Following perfusion of AM0902 out of the imaging chamber, a second Ca^{2+} response was also observed (Figure 3.5A). Due to the variation within the control 50uM CA group, we also performed experiments to investigate the effect of 20uM AM0902 on the C4da response to 100uM CA. AM0902 was also able to significantly block the nociceptive response to 100uM CA ($t(12) = 6.962$, $p<0.0001$; Figure 3.5D). In the presence of AM0902, 100uM CA induced significant Ca^{2+} spiking activity within the C4da soma in all experiments. In summary, these data demonstrate that the nociceptive response to CA can be pharmacologically manipulated.

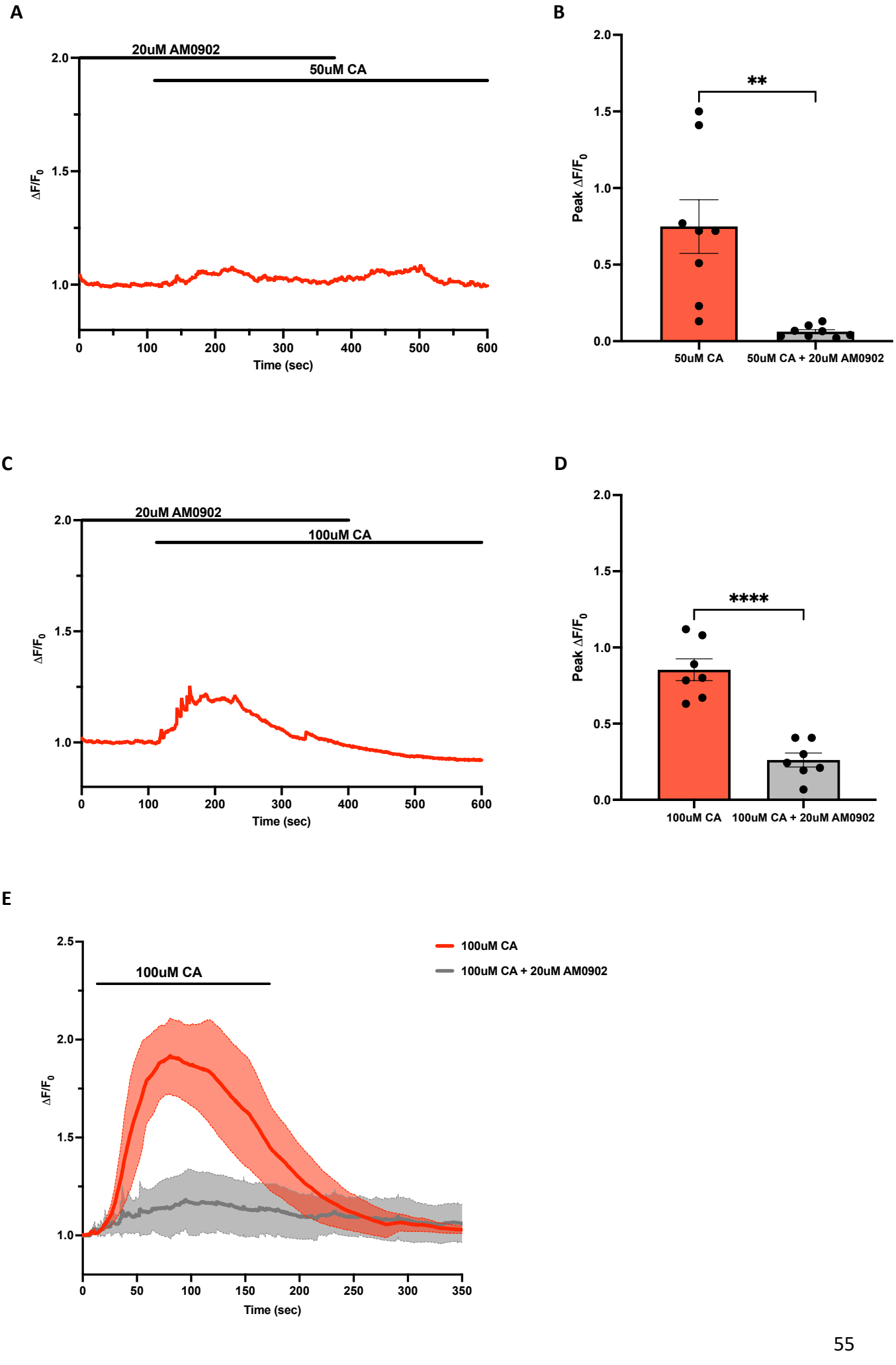
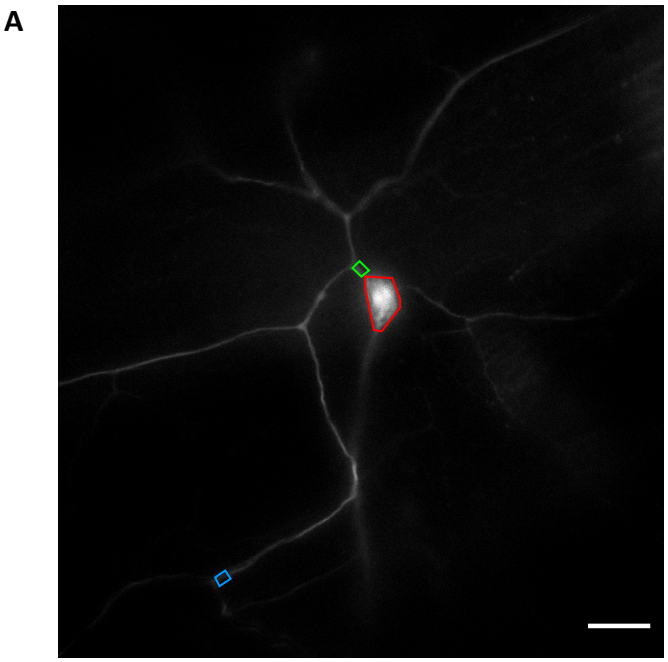

A
20uM AM0902
50uM CA
ΔF/F0
Time (sec)
B
Peak ΔF/F0
**
50uM CA
50uM CA + 20uM AM0902
C
20uM AM0902
100uM CA
ΔF/F0
Time (sec)
D
Peak ΔF/F0
****
100uM CA
100uM CA + 20uM AM0902
E
100uM CA
100uM CA
100uM CA + 20uM AM0902
ΔF/F0
Time (sec)

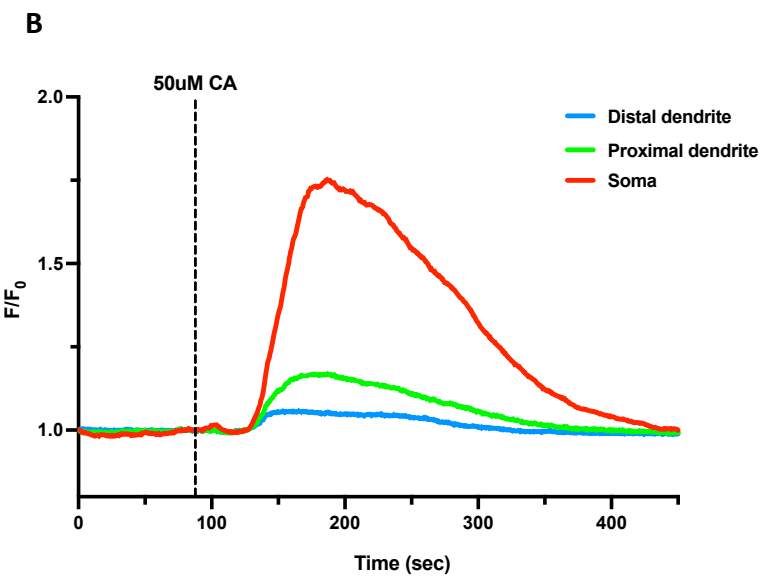
Figure 3.5: ***The CA-induced Ca^{2+} response in C4da neurons is blocked by the potent TRPA1 antagonist, AM0902****. (A, C) Representative traces of a C4da neurons that have been incubated with 20uM AM0902 and stimulated with 50uM CA (A) and 100uM (C), followed by a washout of AM0902 using a perfusion system. (B) Peak soma values of $\Delta F/F_0$ for C4da neurons stimulated with 50uM CA in the presence of AM0902 (n=8) and without (n=8). (F) Peak soma values of $\Delta F/F_0$ for C4da neurons stimulated with 100uM CA in the presence of AM0902 (n=7) and without (n=7). (E) An average of individual soma Ca^{2+} responses to 100uM CA in C4da neurons incubated with 20uM AM0902 (n=7) and controls (n=5). Data are shown as mean ± SEM. Unpaired Student's t tests were performed. **** p<0.0001, ** p<0.01 compared to control group.*

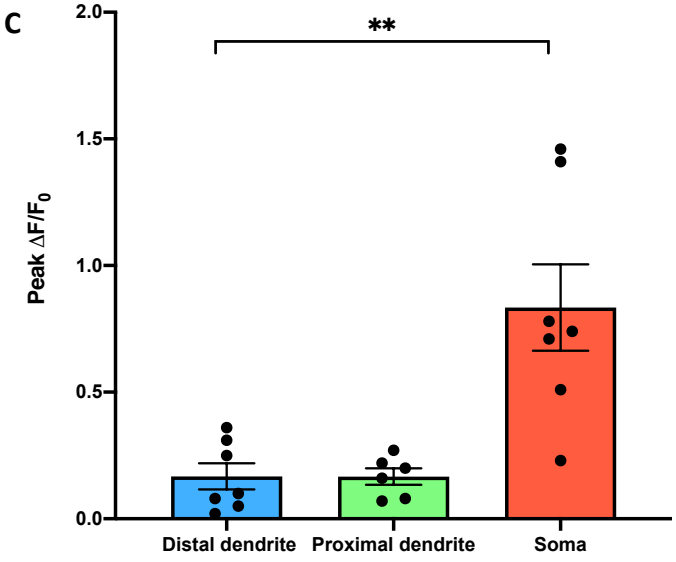
3.5 Spatiotemporal dynamics of the CA-induced Ca^{2+} response

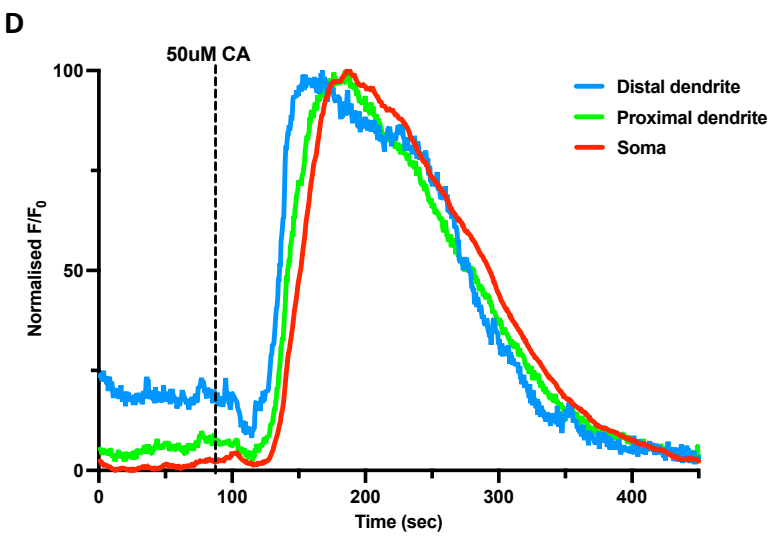
The data presented earlier show that with CA we are specifically activating the TRPA1 homologues in the C4da neurons (dTRPA1 and/or Painless). Now we have confirmed an appropriate agonist and the channel(s) activated by this, we aimed to understand the spatiotemporal dynamics of the nociceptive response. Nociceptive neurons in flies or mammals are large with extensive axons and dendrites which project some distance from the soma. Being able to measure local changes in Ca^{2+} dynamics in different cellular compartments may shed invaluable insight into encoding of stimuli in the periphery and integration of these signals in second order neurons. To this end, we analysed the calcium response in the sensory neuron cell body and dendrites proximal and distal to the cell body (for simplicity only 3 representative ROI are depicted in all the graphs). We examined the amplitude of the calcium response evoked by 50uM CA. The graph in Figure 3.6C shows that the amplitude of calcium increase evoked by 50uM CA was significantly different between the neuronal compartments ($F(1.032, 5.176) = 20.76$, $p=0.0054$). The observed cellular response is similar in the distal and proximal dendrites and higher in the cell body.

Next, we calculated the time constant (τ) of the Ca^{2+} response induced by 50uM CA in the defined cellular compartments of the nociceptive sensory neurons. A non-linear regression (one phase decay) was utilised to calculate the time constant (τ). Following 50uM CA stimulation, peak cytosolic Ca^{2+} concentration is reached within 5.701 (± 2.475) seconds in the distal dendrites. Within 10.83 (± 3.504) seconds, the proximal dendrites reach peak cytosolic Ca^{2+} concentration, followed by the soma at 30.60 (± 7.046) seconds (Figure 3.6E). These data demonstrate the peak amplitude of the Ca^{2+} response is higher in the C4da cell body, but the speed of accumulation is faster in the dendrites. Despite the distal dendrites reaching the peak Ca^{2+} response earlier, compared to the proximal dendrites, both compartments reach the same amplitude (Figure 3.6C).


A


B
50uM CA
Distal dendrite
Proximal dendrite
Soma
F/F0
2.0
1.5
1.0
0
100
200
300
400
Time (sec)


C
**
Peak ΔF/F0
2.0
1.5
1.0
0.5
0.0
Distal dendrite
Proximal dendrite
Soma


D
50uM CA
Distal dendrite
Proximal dendrite
Soma
Normalised F/F0
100
50
0
0
100
200
300
400
Time (sec)

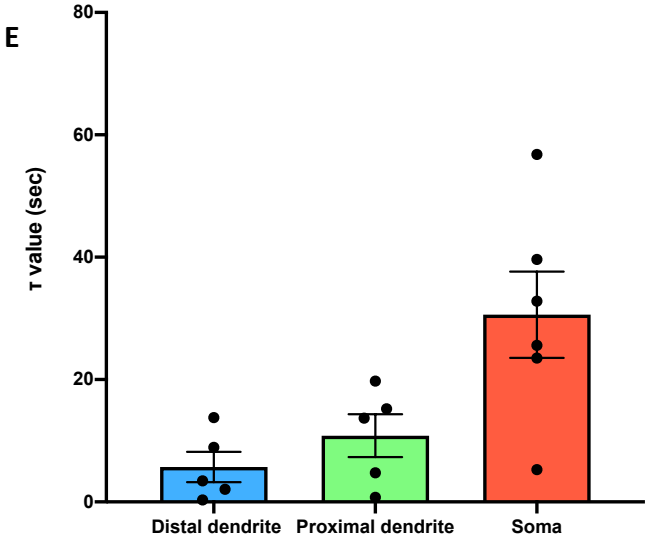

E
τ value (sec)
80
60
40
20
0
Distal dendrite
Proximal dendrite
Soma

Figure 3.6: ***Spatiotemporal dynamics of the intracellular Ca^{2+} response to 50uM CA.*** *(A) Representative image of a C4da neuron, expressing the Ca^{2+} sensor GCaMP6m, depicting examples of ROI selected on the dendrites distal (blue) and proximal (green) to the soma (red). Scale, 20um. (B) Representative Ca^{2+} trace obtained from the three ROI depicted in (A), upon the addition of 50uM. (C) Peak amplitude values of $\Delta F/F_0$ for C4da neurons stimulated with 50uM CA (n=6-7). A. (D) Identical Ca^{2+} trace shown in (B), where $\Delta F/F_0$ is normalised for the peak amplitude. The C4da distal dendrite initially reaches peak cytosolic Ca^{2+} concentration, followed by the proximal dendrite and finally the soma. (E) Time constant (τ) of calcium increase, in different cellular compartments, induced by stimulation with 50uM CA (n=5-6). Data are shown as mean ± SEM.*

Next, we aimed to investigate whether the spatiotemporal dynamics of the nociceptive response to 100uM CA was comparable to what we had observed at 50uM CA. Peak amplitudes, in response to stimulation with 100uM CA, are also significantly different within the defined cellular compartments ($F(1.694, 8.472) = 84.92$, $p<0.0001$) (Figure 3.7A). The C4da soma has a greater fold change in the intracellular Ca^{2+} concentration, compared to the distal dendrites ($p=0.0004$) and proximal dendrites ($p=0.0005$). However, we report no difference in the peak amplitude response between the distal and proximal dendrites. Likewise, both the distal and proximal dendrites of the C4da nociceptive neurons react before the soma (Figure 3.7). The distal dendrites reach peak $\Delta F/F_0$ within an average time constant of 8.841 (± 2.996) seconds. Following this, peak Ca^{2+} is reached within 13.62 (± 3.317) seconds in the proximal dendrites and 25.48 (± 2.278) seconds in the soma (Figure 3.7D). These results are consistent with the C4da nociceptive response to stimulation with 50uM CA.

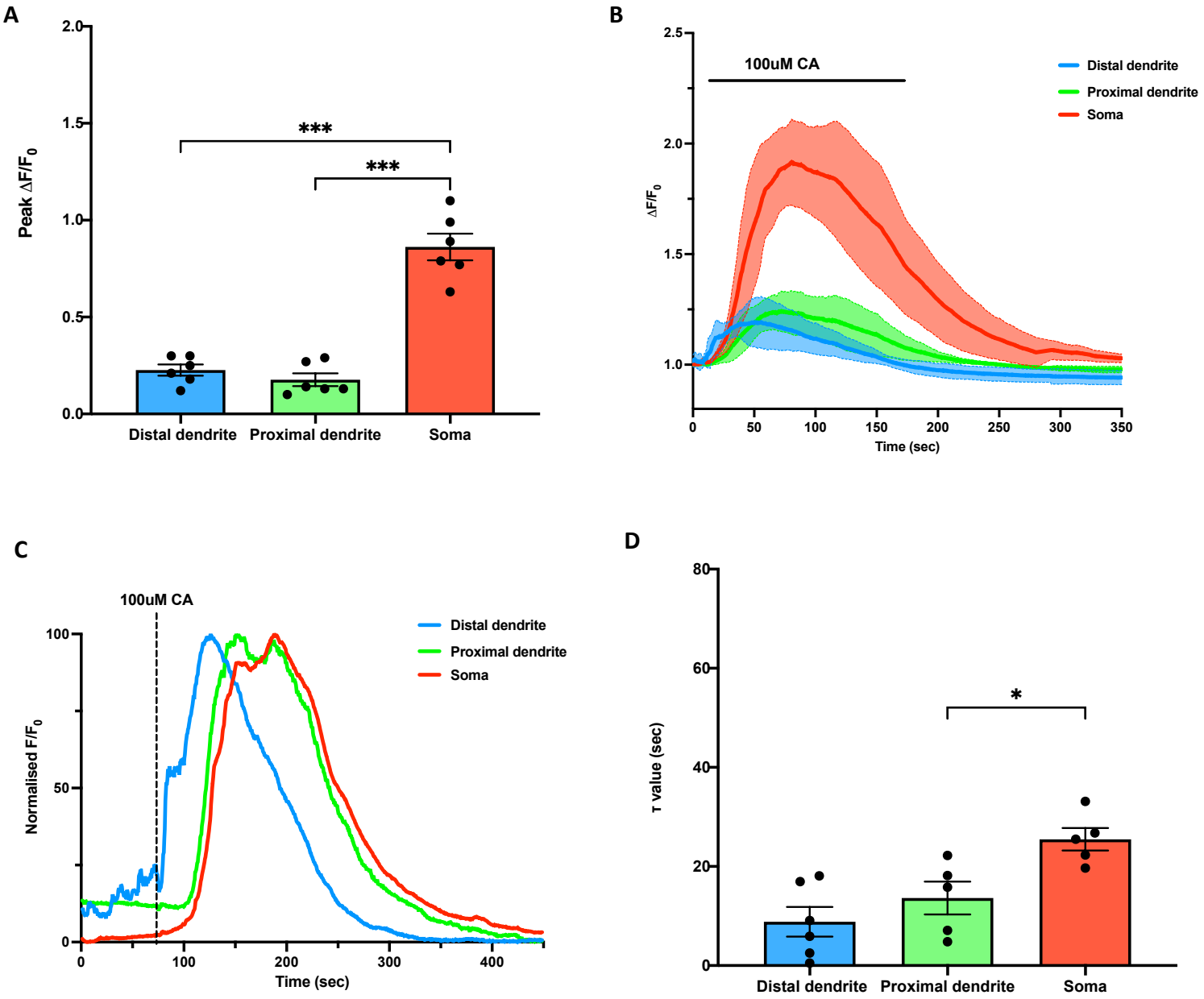


Figure 3.7: ***Spatiotemporal dynamics of the intracellular Ca^{2+} response to 100uM CA.*** *(A) Peak amplitude values of $\Delta F/F_0$ for C4da neurons stimulated with 100uM CA (n=6-7). (B) An average of individual Ca^{2+} responses to 100uM CA in the C4da soma and dendrites (n=5). (C) Representative Ca^{2+} trace obtained from different cellular compartments within the C4da neuron upon the addition of 100uM CA, $\Delta F/F_0$ is normalised for the peak amplitude. (D) Time constant (τ) of calcium increase, in different cellular compartments, induced by stimulation with 50uM CA (n=5-6). Data are shown as mean ± SEM.*

3.6 Effect of CA on the cAMP levels within C4da nociceptive neurons

Next, we wanted to investigate whether CA could induce cAMP production in the C4da nociceptive neurons. We performed four experiments in which the transgenic larvae were exposed to 100uM CA, followed by 50uM FSK and 100uM IBMX as positive controls. As previously stated, (Section 3.2), function of the control solutions was confirmed by running an initial experiment stimulating with FSK and IBMX alone. In 75% of C4da neurons, CA induced a decrease in the FRET ratio (CFP emission/YFP emission) which would be detected as a reduction in cAMP. We were able to obtain a positive FSK control response in two experiments, which can be observed as a subsequent increase in the FRET ratio (Figure 3.8). In the third experiment, which exhibited a slight decrease in the FRET ratio following stimulation with CA, the C4da neuron did not respond to 50uM FSK or 100uM IBMX. In the final experiment, CA induced no detectable change in the FRET ratio, but we were also unable to obtain a positive control response. Similar to the PGE2 cAMP experiments, we were unable to obtain control responses in half of the experiments conducted.

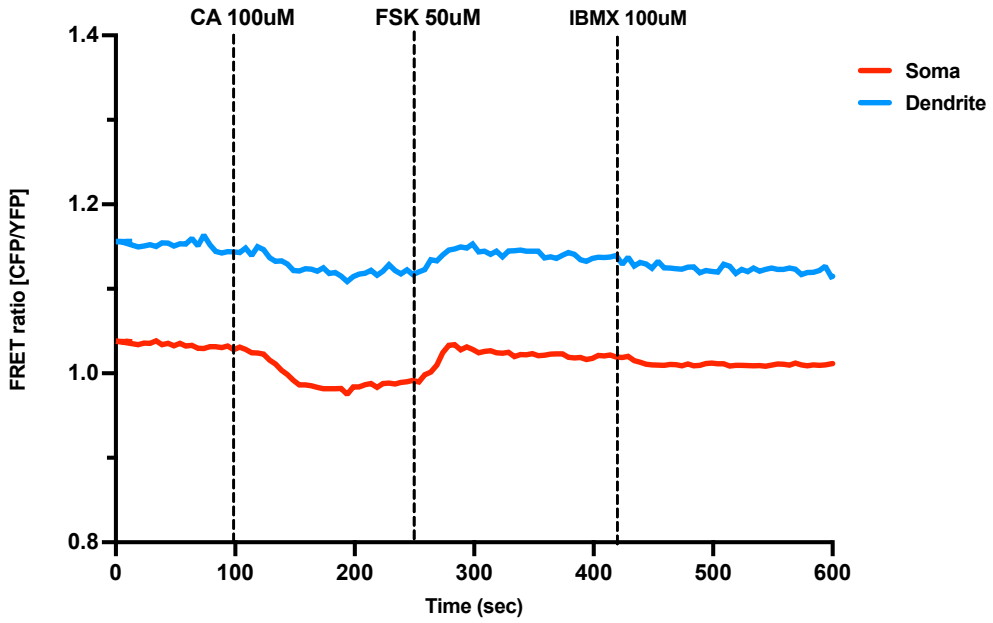


Figure 3.8: ***Monitoring the intracellular cAMP concentration in C4da nociceptive neurons stimulated with 100uM CA.*** *Representative FRET trace of C4da neuron stimulated with 100uM CA, followed by 50uM FSK and 100uM IBMX. CA induces a detectable decrease in the FRET ratio. Subsequent application of FSK induces an expected increase in the FRET ratio, detecting an increase in intracellular cAMP concentration.*

3.7 Capsaicin-induced Ca^{2+} response in C4da nociceptors

Within the pain field, TRPV1 has been studied extensively and implicated as a key component of inflammatory pain and hyperalgesia. In mammalian DRGs, TRPV1 expression is not exclusive to the nerve terminals and is expressed across the entire course of the sensory neuron (Caterina *et al.*, 1997; Julius, 2013). Therefore, TRPV1 is ideally positioned to be sensitised by proinflammatory mediators released by immune cells at the site of injury. We aimed to investigate whether the TRPV1 channel agonist capsaicin is able to stimulate a Ca^{2+} influx in the C4da nociceptive neurons. We hypothesised, based on the lack of evidence that capsaicin is a nociceptive stimulus for *Drosophila* (Al-Anzi, Tracey and Benzer, 2006; Marella *et al.*, 2006), a Ca^{2+} response would not be induced. Our previous data has confirmed CA is able to trigger an intracellular Ca^{2+} increase in these sensory neurons (Section 3.3). Thus, the preparations were stimulated with 100uM CA at the end of each experiment as a positive control. We performed three experiments to monitor the cytosolic Ca^{2+} concentration, within the C4da nociceptive neurons, following the application of 20uM capsaicin. For two experiments, capsaicin alone triggered Ca^{2+} spiking activity in the dendrites of the C4da neurons (Figure 3.9A). However, no change in intracellular Ca^{2+} was observed in the C4da soma. In these experiments, the capsaicin remained in the imaging chamber whilst we stimulated with 100uM CA.

Following this, we conducted an experiment where capsaicin was washed out prior to the application of CA. The observed response was not exclusive to the dendrites in this C4da neuron, with capsaicin also triggering a noticeable increase in the Ca^{2+} concentration within cell body (Figure 3.9B). Perfusing, at a flow rate of 1ml/min, resulted in the initial reduction of the capsaicin induced dendritic Ca^{2+} response followed by the soma. The pH of the HL3 bathing solution did not fluctuate following the addition of capsaicin, which eliminated the possibility that this observed response was due the solution being more acidic at this capsaicin concentration. However, more experiments will need to be conducted to report with confidence that capsaicin is able to induce a nociceptive response in the C4da sensory neurons.

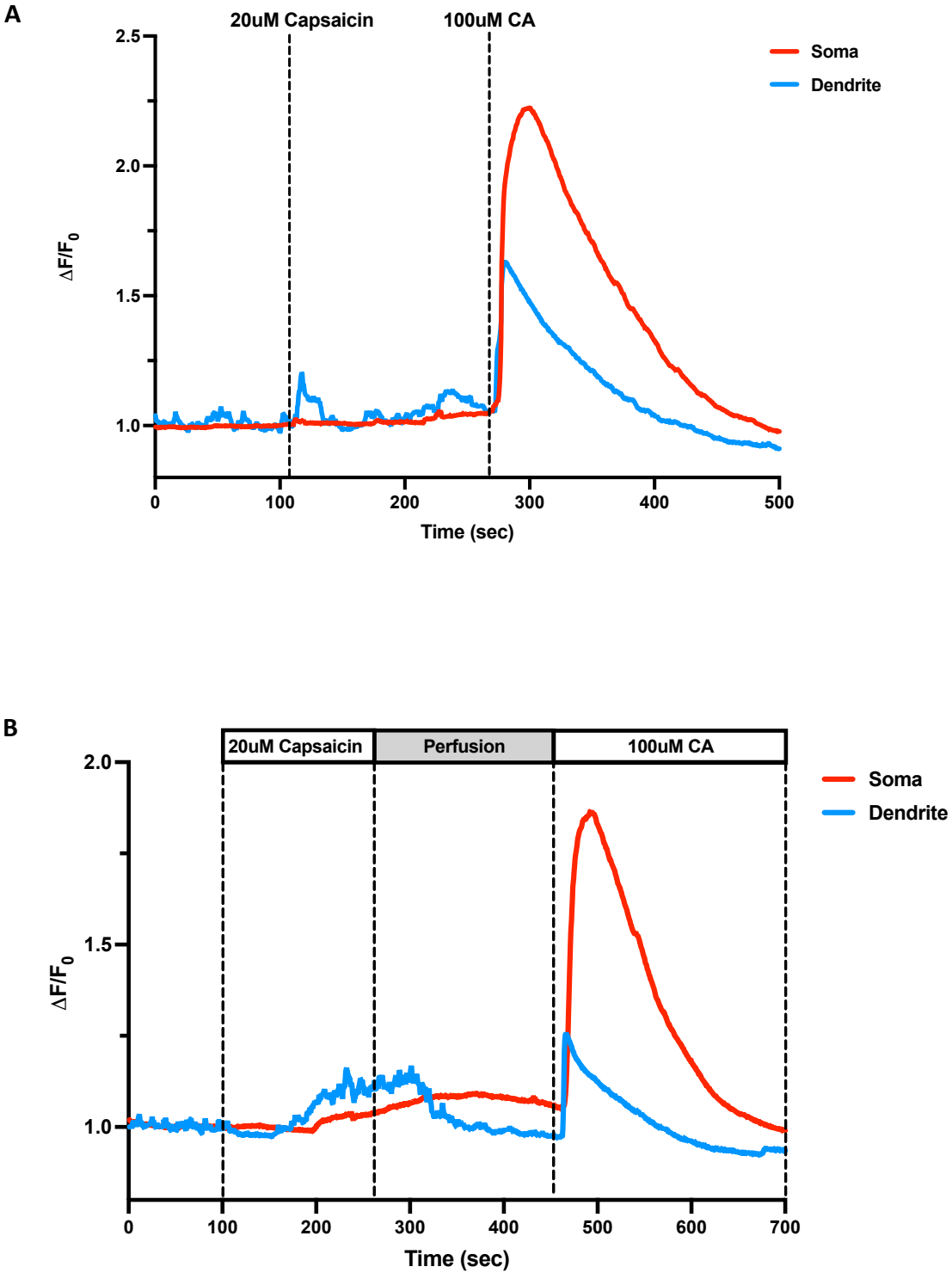


Figure 3.9: ***Stimulation of 20uM capsaicin induces a minor intracellular Ca^{2+} response.*** *(A) Representative Ca^{2+} trace obtained upon the addition of 20uM capsaicin, followed by 100uM CA. (B) Experiment ran that stimulated with 20uM capsaicin, which was then perfused out of the imaging chamber, followed by subsequent stimulation with 100uM CA.*

4 Discussion

The principal aim of this project was to provide the experimental tools necessary to advance mechanistic understanding of nociception and accelerate the pipeline of analgesic drug discovery, by establishing a novel robust invertebrate model.

4.1 Changes in intracellular cAMP can be monitored in C4da nociceptive neurons

In this project, we genetically expressed the Epac1-camps sensor in the *Drosophila* larval C4da nociceptive neurons, to determine whether we are able to monitor cAMP dynamics with high temporal and spatial resolution. Our results demonstrate that Epac1-camps is able to be expressed in these sensory neurons and stimulation with the adenylate cyclase activator FSK induces an intracellular cAMP increase. However, the dynamic range of the Epac1-camps sensor was lower than the reported *in vivo* data (Section 3.1). It is not unusual for *in vivo* dynamic range values to be reduced compared to *in vitro* measurements (Abraham *et al.*, 2015). However, in our case the reduced dynamic range seems to be specific to the C4da nociceptive neurons. Our results show the Epac1-camps sensor has a higher dynamic range in motor neurons (31%) compared to transfected HEK cells (25%), suggesting that the lower dynamic range could have been caused by a biological effect. Specifically, nociceptive neurons could have high basal levels of cAMP that is caused by constitutively active receptors; thus, the dynamic range is smaller. We could test this hypothesis by employing inhibitors of adenylyl cyclase. To an extent, the small dynamic range of our sensor in the C4da nociceptive neurons limits its application when the intracellular cAMP responses are subtle or transient. Despite this, we have shown we are still able to monitor changes in intracellular cAMP concentration within these sensory neurons.

To further optimise the model, we could select a different genetically encoded cAMP sensor to exclusively express in the C4da nociceptive neurons. One approach would be to amplify and clone a brighter FRET-based cAMP sensor, to allow better detection of the induced responses. The fluorescent protein mTurquiose2 is the current brightest FRET donor, with a significantly increased brightness over other donor variants reported both *in vitro* and *in vivo* (Goedhart *et al.*, 2012). This group reported the improved performance of mTurquiose2 as a donor for FRET, which was attributed to this being a brighter cyan variant with a faster maturation. Studies employing the FRET-based cAMP biosensor $^{T}Epac^{VV}$, which uses the mTurquoise2 donor in combination with

the YFP derivative Venus (Klarenbeek *et al.*, 2011), have reported the sensors efficacy in living mammalian cells and mice (Stangherlin *et al.*, 2014; Muntean *et al.*, 2018). Although this cAMP sensor has not been expressed before in *Drosophila*, it would be valuable to investigate whether the improved brightness of this CFP variant will optimise the cAMP FRET protocol we have developed. Following amplification of the DNA fragment containing the coding sequence of the brighter cAMP sensor, the plasmid obtained would be sent to BestGene (California, USA) to generate a new reporter fly line. Selective expression in the C4da nociceptive neurons would be achieved as previously described (Figure 2.1).

Alternatively, we could employ a biosensor with a different affinity for cAMP. If the basal cAMP concentration within these C4da nociceptive neurons is high, we would need a sensor with a lower affinity, so saturation is not achieved as fast. The Epac1-camps sensor we utilised for our experiments has a dynamic range of 24% and a dissociation constant (K_D) value of 2.4uM (Nikolaev *et al.*, 2004; Jiang *et al.*, 2017). This denotes the cAMP concentration in which the cyclic nucleotide binding domain (CNBD) sites within the sensor are occupied, a measure of the cAMP binding affinity. Currently, the sensor with the lowest reported affinity for cAMP is the first generation FRET sensor CFP-Epac-YFP (Ponsioen *et al.*, 2004). When compared to our Epac1-camps sensor, this CFP-Epac-YFP has a significantly reduced affinity for cAMP (K_D= 50uM) and a slightly higher dynamic range (30%). However, there are more recent reporters for cAMP that have been developed. The fourth generation Epac-based FRET sensor Epac-S^{H126} displays an even wider dynamic range (80.4%) with its affinity for cAMP nearly four times lower than Epac1-camps (K_D= 9.5uM) (Klarenbeek *et al.*, 2015). This sensor also incorporates the donor mTurquioise2 alongside the FRET acceptor Venus, which displays significantly increased brightness. Employing the Epac-S^{H126} cAMP sensor will therefore combine our two proposed approaches to optimise the invertebrate model, by employing a sensor that is both brighter and has a lower affinity for cAMP.

Kawata et al. (2022) recently developed a genetically encoded cAMP indicator "gCarvi", based on the cAMP-binding domain of a bacterial cAMP receptor protein, to monitor cAMP kinetics. This would have many advantages over the current Epac-based FRET sensor, Epac1-camps. Extension of gCarvi to a ratiometric cAMP indicator, via fusing the monomeric red fluorescent protein mCherry to the gCarvi N terminus, allows the quantitative imaging of neuronal intracellular cAMP concentration. We propose *Drosophila* C4da nociceptive

neurons have high basal levels of cAMP, based on the small dynamic range when we selectively expressed our Epac1-camps sensor. By exploiting ratiometric gCarvi we could quantify the basal cAMP concentrations of these sensory neurons, assuming this is within the concertation range detected by the sensor (0.2 to 20uM). Ratiometric gCarvi can quantity intracellular cAMP with a wide dynamic range that spans the physiological concentration range reported in mammalian neurons, both at basal and peak elevated levels (Neves *et al.*, 2008; Mironov *et al.*, 2009; Muntean *et al.*, 2018). Kawata et al. (2022) adopted the mCherry-fused ratiometric probe to measure the basal intracellular cAMP concentration in cultured hippocampal neurons. Quantitative measurement of elevations in intracellular cAMP concentration, through G_s-dependent receptors, report an increase of up to 20uM (Jiang *et al.*, 2007; Salonikidis *et al.*, 2008; Violin *et al.*, 2008). This concentration is within the dynamic range of gCarvi, which further validates this indicator as a powerful tool for monitoring cAMP dynamics.

Importantly, this sensor also enables simultaneous cAMP and Ca^{2+} imaging to occur within subcellular compartments. Monomeric gCarvi, coexpressed with the sensitive red protein Ca^{2+} calcium indicator jRCaMP1b (Dana *et al.*, 2016), was able to measure intracellular Ca^{2+} concentrations in cultured hippocampal neurons during cAMP elevations (Kawata *et al.*, 2022). Identical to our *in vivo* cAMP experiments, the authors applied FSK and IBMX to elevate the intracellular cAMP concentration. Future studies could utilise the monomeric gCarvi sensor to combine the cAMP and Ca^{2+} experiments we have conducted in this thesis. Besides accelerating the throughput of the model, this will allow us to directly visualise and quantify the cross-regulation of cAMP and Ca^{2+} in living nociceptive neurons. One approach would be to coexpress gCarvi with the genetically encoded Ca^{2+} indicator GCaMP6m, a sensor that we have already established can be selectively expressed in the C4da nociceptive neurons and can function to detect changes cytosolic Ca^{2+} concentration that are induced by nociceptive stimuli (Section 3.3). Alternatively, jRCaMP1b could be employed in experiments where cAMP and Ca^{2+} are simultaneously monitored. In addition to the function of this red fluorescent probe alongside gCarvi already confirmed, jRCaMP1b has been previously coexpressed at the *Drosophila* larval NMJ (Dana *et al.*, 2016).

4.2 Does PGE2-sensitisation of sensory neurons occur in *Drosophila*?

In invertebrates, PGs regulate various physiological processes such as reproduction, fluid secretion, ion transport and the immune response (Stanley and Kim, 2011). PG synthesis and signalling are conserved in *Drosophila* (Sparcklen and Tootle, 2015). However Arachidonic acid (AA), an essential precursor in the synthesis of PGs, is not found in *Drosophila*. Nevertheless, the AA lipid precursor linoleic acid is present at low levels within the organism (Pages *et al.*, 1986; Shen *et al.*, 2010). The COX-like enzyme Pxt was identified in *Drosophila* that functions upstream of PGs in follicle maturation, with critical residues being conserved between COX-1 and Pxt (Tootle and Spradling, 2008). Studies investigating the roles of PGs in *Drosophila* have primarily focused on oogenesis and follicle development (Kobayashi and Narumiya, 2002; Tootle and Spradling, 2008; Stanley and Kim, 2011). Despite the literature strongly supporting the high conservation of PG function in *Drosophila*, the role of PGE2 in the regulation of nociceptive hypersensitivity has not been investigated in this organism.

We reported an inhibition of the FSK control response in 71% of C4da nociceptive neurons that were pre-stimulated with PGE2. One potential explanation is that in these sensory neurons, PGE2 could inhibit the FSK-induced cAMP increase via the activation of a Gi pathway. Activation of isoforms of the prostaglandin receptor EP3, reduces the cAMP level by inhibiting adenylyl cyclase (Jabbour and Sales, 2004; Jang, Kim and Hwang, 2020). However, if our hypothesis that these C4da nociceptive neurons have high basal cAMP levels is correct, we would expect that activation of Gi-coupled EP receptors would lead to a decrease in the basal FRET signal. Our results show PGE2 alone had no effect on the FRET ratio in all seven experiments. As FSK is able to directly activate all adenylyl cyclases, not exclusively those inhibited by activation of a Gi pathway, we should also still observe an FSK-induced cAMP response irrespective any inhibition. Therefore, we conclude it is unlikely that PGE2 is binding to a Gi-coupled EP receptor subtype and additional experiments are needed to determine the reason behind the abolition of the FSK control response.

Based on our results, we are not able to conclude if the C4da nociceptive neurons express any orthologues of the four G protein-coupled receptors (GPCRs) that are activated by PGE2 (EP1-EP4). The EP3C isoform and EP4 receptor subtype mediate PGE2-induced sensitisation in mammals (Section 1.3.2). Although, it is possible that

in *Drosophila* that other PGE2 receptor subtypes are implicated in peripheral sensitisation. The *Drosophila* orthologue of the genes that encode EP1-EP4 (PTGER1-PTGER4) is CG7497, an integral component of the plasma cell membrane (FlyBase, 2022). Based on sequence and structural similarity, this gene is predicted to be involved in an adenylyl cyclase activating GPCR coupled receptor signalling pathway (Brody and Cravchik, 2000; Gaudet *et al.*, 2011). Gaudet et al. (2011) also proposed an involvement of CG7497 in the positive regulation of cytosolic Ca^{2+} concentration. Integrative orthologue prediction tools facilitate the identification of orthologues, serving as a measure of confidence in the ortholog relationship. It is predicted with moderate confidence that CG7497 is an orthologue of PTGER1, PTGER2 and PTGER3. Additionally, the pair PTGER4 (encoding the EP4 receptor) and CG7497 are highly predicted to be an orthologue match. When we examine the pairwise protein alignment of PTGER4 alongside CG7497, CG7497 is 39% similar to its mammalian ortholog by amino acid identity. Specifically, the domains of PTGER4 with the highest similarity (31%) between the two genes are transmembrane (TM) helix 2 and TM helix 3. To date, expression of CG7497 has only been confirmed in the adult head (FlyBase, 2022). There is no evidence that this gene is expressed in the C4da nociceptive neurons, the cellular model of study in this project.

We expect that if we are able to induce sensitisation of the C4da nociceptive neurons in this model, this would likely be achieved through a TNF-α mediated mechanism instead. Expression of the TNF receptor, Wengen, has been confirmed in these nociceptive neurons. Eiger, the *Drosophila* homologue of TNF-α, is released from UV-damaged epidermal cells and is able to act directly on its receptor, Wengen (Section 1.3.2) (Daniel T. Babcock, Landry and Galko, 2009). Potentially, convergence of signalling cascades could occur in *Drosophila* that allows separate signalling cascades to modulate a single component of nociceptive sensitisation. This would be comparable to what is observed in mammalian models of thermal hyperalgesia, where TRPV1 function is modulated by multiple mechanisms (Bhave *et al.*, 2002, 2003; Jung *et al.*, 2004; Planells-Cases *et al.*, 2005).

4.3 The nociceptive effects of cinnamaldehyde (CA) are conserved in *Drosophila*

Our primary finding is that CA is able to activate the *Drosophila* TRPA1 homologues (dTRPA1 and/or Painless) and stimulate a Ca^{2+} response within the C4da nociceptive neurons. This result supports the high conservation of the peripheral mechanisms of nociception, given that a mammalian nociceptive stimulus is also able to elicit a response in *Drosophila* sensory neurons. We advocate the adoption of this invertebrate model in pain research programmes, with our data able to demonstrate the powerful toolkit of *Drosophila* genetics that is not fully utilised within this field. We expect that CA is more likely to activate dTRPA1, expressed on the C4da nociceptive neurons, due to this homologue being significantly more analogous to the mammalian TRPA1 channel than Painless. It is predicted with high confidence that dTRPA1 is a homologue of TRPA1 (FlyBase, 2022). When we examine the pairwise protein alignment of dTRPA1 alongside TRPA1, the amino acid sequence is 35% identical and 57% similar. Whereas it is predicted with low confidence that Painless is a TRPA1 homologue, which is attributable to the poor similarity in the amino acid sequence (18% identical, 33% similar). To confirm whether activation of dTRPA1 is necessary for the Ca^{2+} response induced by CA, we could exclusively express the GCaMP6m sensor in the C4da nociceptive neurons of dTRPA1 mutant *Drosophila*. This mutant line can be purchased from the Bloomington Drosophila Stock Centre (Bloomington, USA), stock number 67131.

The CA-induced Ca^{2+} response observed could be attributed to Ca^{2+} influx and/or Ca^{2+} release from intracellular stores. Basak, Sutradhar and Howard (2021) conducted Ca^{2+} imaging within the axons, dendrites, and soma of the C4da nociceptive neurons. Direct stimulation, to model penetration of the larval cuticle during a parasitoid wasp attack, was achieved through a focused laser beam. Using the genetically encoded Ca^{2+} indicator, this group observed an intracellular Ca^{2+} response to the highly localised tissue damage. This group attributed the change in cytosolic Ca^{2+}, at least in part, to Ca^{2+} influx through the L-type voltage gated calcium channel (VGCC). When RNAi mediated knockdown of the Ca-α1D subunit within the VGCC was performed, smaller changes in fluorescence were observed within the sensory neurons. This finding also replicates a previous study that explored the molecular basis of the Ca^{2+} response to noxious thermal stimulation (Terada *et al.*, 2016). Experiments were performed in various VGCC loss-of-function mutants and wild-type larvae in the presence of the selective L-type VGCC inhibitor, Nimodipine. The authors suggested

dTRPA1 is activated by noxious heat stimulation, which results in the local Ca^{2+} influx in the C4da dendrite. Following this, an increase in the Ca^{2+} conductance of Painless causes a large depolarisation and subsequent activation of L-type VGCCs. Based on these studies, it could also be that the CA-induced Ca^{2+} response we have observed in these sensory neurons is also dependent on L-type VGCCs, alongside direct binding of CA to the TRP channel. In *Drosophila,* the *straightjacket (stj)* gene, encoding an $\alpha 2\delta$ subunit of the VGCCs, has not only been implicated in acute nociception but in nociceptive sensitisation (Section 1.7, 1.9). We expect this $\alpha 2\delta$ subunit, alongside the Ca-α1D subunit, assemble to form a functional VGCC within the C4da nociceptive neurons.

Terada et al. (2016) proposed a negligible role of Ca^{2+} induced Ca^{2+} release (CICR) from intracellular stores in the Ca^{2+} response induced by noxious heat stimulation. Inositol Triphosphate Receptors and Ryanodine receptors are intracellular Ca^{2+} channels, located in the endoplasmic reticulum, that are responsible for the release of Ca^{2+} from intracellular stores. Both the occurrence and amplitude of the Ca^{2+} were unchanged in mutants for the genes encoding these channels (Casas-Tinto *et al.*, 2011; Gao *et al.*, 2013). Thus, we expect that CICR does not significantly contribute to the CA-induced Ca^{2+} response we observed in the C4da nociceptive neurons. To confirm this, we could adapt the experiments conducted by Terada et al. (2016) or alternatively employ a selective Ryanodine receptor inhibitor.

The potent and selective TRPA1 inhibitor, AP18, was unable to inhibit the peak soma Ca^{2+} response in C4da nociceptive neurons to 50uM CA. Although this did not come out as significant, interestingly the peak soma Ca^{2+} response was noticeably higher in neurons incubated with the antagonist. There has been a lack of detailed comparative analysis to reveal the specific binding site of AP18 within mammalian TRPA1. The amino acid residues that constitute the binding site of AP18 may not be conserved in the *Drosophila* TRPA1 homologues dTRPA1 and/or Painless. The mammalian TRPA1 antagonist A-967079, developed for use as a potential analgesic, is structurally related to AP18 and exerts similar pharmacological effects (Chen *et al.*, 2011; Banzawa *et al.*, 2014). A-967079 does not inhibit TRPA1 in non-mammalian vertebrates (Nakatsuka *et al.*, 2013; Banzawa *et al.*, 2014). This finding could explain why we are unable to observe an AP18 inhibitory effect in *Drosophila*, due to the mammalian specific action of these antagonists. A-967079 has a unique effect

on TRPA1, where it is able to act as an agonist for chicken and frog TRPA1s. A single amino acid within TRPA1, present within the fifth TM domain, is responsible for inhibitory/stimulatory actions of A-967079 (Banzawa *et al.*, 2014). A mutation from leucine to isoleucine (L881I) within the A-967079 binding pocket drastically changes the properties of the TRPA1 channel. Additional residues within this domain, S873 and T874, have also been reported to be implicated in A-967079 TRPA1 inhibition (Paulsen *et al.*, 2015). When we perform pairwise alignment of human TRPA1 and dTRPA1, the amino acid sequences of the TM domains (region 767-997) are 35% identical and 57% similar. The residues previously discussed to be implicated in A-967079 mediated TRPA1 inhibition (S873, T874 and L881), are not conserved in dTRPA1 (FlyBase, 2022).

Alternatively, a higher concentration of AP18 might be needed to block the nociceptive response to CA within these sensory neurons. We selected 20uM as an appropriate concentration of AP18 in these experiments, due to the IC_{50} of the inhibitor being 3.1uM and 4.5uM at human and mouse TRPA1, respectively. However, future experiments could trial different concentrations of AP18. Potentially AP18 could exert a significant effect at higher concentrations, whether this be inhibition or stimulation.

Using the more potent TRPA1 antagonist AM0902, we were able to pharmacologically manipulate the system. Following optimisation of the protocol and the selection of an appropriate antagonist, we had limited time to replicate a complete concentration-response curve in the presence of AM0902. Therefore, we examined two concentrations of CA. Given that we were able to significantly inhibit the nociceptive response to CA at both concentrations, our work further validates the translational utility of this invertebrate model to specifically study nociception.

4.4 Spatiotemporal dynamics of the CA-induced Ca^{2+} response

Importantly, we have also demonstrated data can be obtained from different neuronal compartments of the C4da nociceptive neurons (distal dendrites, proximal dendrites, and soma). When examining the CA-induced Ca^{2+} response, we found the speed of the accumulation of Ca^{2+} is faster within the C4da dendrites but the peak amplitude is higher in the soma. This finding replicates a previous study that investigated the Ca^{2+} response to noxious thermal stimulation within these sensory neurons (Terada *et al.*, 2016). The authors selectively expressed the intramolecular FRET-based Ca^{2+} probe, TN-XXL, within the C4da nociceptive neurons. A heat probe was utilised for localised noxious heat stimulation, whereas we stimulated with generalised application of the TRPA1 agonist CA. Despite differences in nociceptive stimulus and application, we both report the induced Ca^{2+} response emerges within the distal and proximal dendrites, prior to the soma. Without localised stimulation, we still observe a difference between the neuronal compartments. Likely, other parameters such as receptor density of the TRPA1 homologues and localised Ca^{2+} are contributing to this response profile. Information regarding the subcellular localisation of dTRPA1 and Painless in the C4da nociceptive neurons is limited, so we are unable to conclude whether uneven receptor distribution has a contribution (Hwang, Stearns and Tracey, 2012). Our data show the peak amplitude of the Ca^{2+} response is higher within the C4da soma, which is likely attributable to this neuronal compartment being larger and therefore expression of the GCaMP6m sensor is greater.

Limited studies have investigated the effect of CA on cAMP production. Iwaoka et al*.* (2010) exposed human adrenal cells for 24 hours to various concentrations of CA, to measure progesterone secretion from these cells. This group found cinnamaldehyde exposure increase the cAMP concentration in the cultured medium. An indirect increase of cAMP was proposed, where progesterone production that was stimulated by CA subsequently increased the cAMP levels. We found application of CA reduces the basal cAMP levels within the C4da nociceptive neurons. One explanation that needs to be further substantiated is that these sensory neurons express components of the cAMP cascade, such as adenylyl cyclases and phosphodiesterases, that can be modulated by Ca^{2+}. An example of a calcium-sensitive dependent adenylyl cyclase expressed in *Drosophila* is Rutabaga, implicated in learning and memory (Livingstone, Sziber and Quinn, 1984; Levin *et al.*, 1992). In our case, the robust Ca^{2+} response stimulated by CA could induce the reduction of cAMP observed.

4.5 Is capsaicin a nociceptive stimulus for *Drosophila*?

We hypothesised the TRPV1 channel agonist capsaicin could not induce a Ca^{2+} response in the C4da nociceptive neurons. The two TRPV related channels in *Drosophila*, IAV and NAN, are highly expressed in the chordotonal organs (Kim *et al.*, 2003), not the C4da nociceptive neurons. Additionally, there is no evidence that the TRPV1 homologues expressed in *Drosophila* are gated by capsaicin. Although birds, mammals, nematodes, and flies express functional TRPV channels, the effects of capsaicin on TRPV1 are specific to the mammalian channel (Al-Anzi, Tracey and Benzer, 2006). Mammalian species including humans, rodents and dogs are highly sensitive to capsaicin. Whereas TRPV1 expressed in rabbits, chickens, tropical clawed frogs, and zebrafish are considered insensitive to capsaicin (Saito and Tominaga, 2015, 2017). Chimeric studies, selectively expressing regions of rat or human TRPV1 into rabbit TRPV1, identified Y511 and S512 within the third TM domain as key residues for capsaicin sensitivity (Gavva *et al.*, 2004). Based on our data, we are unable to conclude whether capsaicin is a nociceptive stimulus for *Drosophila*. The capsaicin data presented in this thesis were obtained from three individual preparations using two different protocols and therefore were not quantified, due to being underpowered. In all three experiments, a small Ca^{2+} response was induced in the dendrites of the sensory neurons. Capsaicin only induced a noticeable change in the cytosolic Ca^{2+} concentration within the soma in a single neuron. The capsaicin-induced Ca^{2+} response in rat DRG neurons has an estimated EC_{50} value of 63 nM (Millns, Chapman and Kendall, 2001). Based on this value for mammalian TRPV1, we expect the concentration of capsaicin we were stimulating the transgenic larvae with (20uM) was too high. Therefore, additional experiments need to be conducted for us to fully understand if capsaicin has an effect on *Drosophila* nociceptive neurons.

One explanation for the noticeable Ca^{2+} response within the C4da dendrites is that the *Drosophila* TRPA1 homologues, known to be expressed on these nociceptive neurons, are also partially gated by capsaicin. Functional overlap of the TRP channels could exist due to the *Drosophila* genome encoding 13 TRP channels and the mammalian TRP superfamily consisting of 28 members. Studies investigating the nociceptive effects of capsaicin in *Drosophila* are limited. Marella *et al.* (2006) reported capsaicin is a neutral substance for *Drosophila*, with adult flies exhibiting no preference for roaming on agarose containing capsaicin compared to plain agarose. Ca^{2+} imaging experiments performed by this group, where the genetically encoded indicator

GCamp is expressed in different taste cell populations, do not display capsaicin-induced fluorescent changes. This finding was contradicted by a later group who reported a positive preference for capsaicin when adult flies were given a choice between sucrose containing capsaicin and sucrose alone (Al-Anzi, Tracey and Benzer, 2006). The difference is likely due to this assay measuring the digestion of food, as opposed to roaming. Adult flies mutant for the *painless* gene still display a positive gustatory preference for capsaicin (Al-Anzi, Tracey and Benzer, 2006). Based on this evidence, Painless is not required for capsaicin detection in *Drosophila*. The effects of capsaicin on TRPV1 are very specific to mammalian channel. Thus, the positive preference of capsaicin in adult flies is unlikely to be caused by direct binding of capsaicin to a TRP channel.

It is interesting that this group found a feeding preference for capsaicin in *Drosophila*, considering this compound has been shown to impede feeding in many invertebrates (Richmond and Gerking, 1979). This observation is likely a result of the concentration of capsaicin that was used. Li et al. (2020) hypothesised that a low concentration of capsaicin could provoke excitement in *Drosophila*, based on capsaicin affecting behavioural thermoregulation of insects (Olszewska and Tęgowska, 2011). Whereas exposure to higher concentrations of capsaicin would trigger aversion. A behavioural assay performed in third instar larvae found an increase in the frequency of writhing behaviour, a nociceptive response characterised by body twisting, with increasing concentrations of capsaicin. Female *Drosophila* exhibit also exhibit robust aversion to laying eggs on capsaicin-containing sites (Li *et al.*, 2020). Genetic analysis found this oppositional aversion is mediated by activation of nociceptive neurons expressing the *painless* gene, leading to the authors proposing capsaicin is able to bind to TRPA channels. Painless and dTRPA1 levels were also elevated in the guts of flies that had ingested capsaicin-containing food compared to controls. With our results, it is therefore possible that capsaicin is able to partially bind to the TRPA1 homologues in *Drosophila* and elicit a minimal Ca^{2+} response within the dendrites of the C4da nociceptive neurons.

4.6 Future directions

Following on from the data presented in this thesis, we aim to better define the CA-induced Ca^{2+} response observed in the C4da nociceptive neurons. Additional experiments could examine the CA-induced Ca^{2+} response at the level of the ventral nerve cord (VNC). Using the same imaging protocol (Section 2.4), we would

expose the VNC and record the Ca^{2+} response following generalised application of CA. It would be interesting to see if we can observe a nociceptive response at the central terminal, this will allow us to follow the Ca^{2+} signal from the peripheral terminal to second order neurons within the VNC. To dissect the molecular mechanism of this nociceptive response, we would confirm whether the Ca^{2+} response observed is exclusively attributed to CA binding to the *Drosophila* TRPA1 homologue(s), or if there is a contribution of CICR. To investigate the subcellular localisation of dTRPA1 and Painless in the C4da nociceptive neurons, we would utilise a *Drosophila* Minos Mediated Integration Cassette (MiMIC) line to tag the TRPA1 homologues endogenously (Nagarkar-Jaiswal *et al.*, 2015). Further experiments could also investigate whether stimulation with other TRPA1 agonists, such as allyl isothiocyanate, induces a Ca^{2+} response within *Drosophila* sensory neurons that is comparable to what we have observed with CA.

We are also adapting an established protocol by Lopez-Bellido and Galko (2021) to measure mechanical nociception in *Drosophila* larvae. The aim is to perform this behavioural assay prior to the neuronal Ca^{2+} imaging, within the same larvae. The integration of the behavioural assessment and imaging approach will allow us to have a system where we can directly correlate nociceptive behaviour with neuronal signalling, further validating the utility of this model organism to study acute nociception and sensitisation.

The involvement of conserved genes that underlie human pain can be investigated by identifying the corresponding *Drosophila* orthologue(s). In a context that normally requires rodent models, *Drosophila* can be utilised as a high throughput *in vivo* screening model organism. Importantly, there are no ethical issues concerning the use of this invertebrate organism. The selective expression of mutated/silenced genes in the C4da nociceptive neurons, alongside control experiments, will further substantiate the role of this target gene in mammalian nociception. We can either approach this by conducting individual gene manipulations, or gene family manipulations, to observe the effect on nociception. If this is successful, our findings can then be translated into mammalian species for further development. Incorporating this invertebrate model to streamline the analgesic discovery pipeline will increase the probability of experimental success in rodent models. Thus, reducing the cost of experimentation and complying with the 3Rs (replacement, reduction, and refinement).

As previously discussed, there is strong evidence for the conservation of nociception between invertebrates and vertebrates. However, it remains unclear whether invertebrates experience the emotional components of pain. Neuroanatomical and neurobiological evidence has supported CNS control of nocifensive behaviour in insects (Gibbons, Sarlak and Chittka, 2022), which is consistent with mammalian pain (Section 1.3). Ohashi and Sakai (2018) found food deprivation in flies reduces responses to noxious heat. This behavioural change was not observed in decapitated flies, demonstrating the importance of the insect brain in heat avoidance. The existence of descending controls in invertebrates, such as *Drosophila*, argues the possibility that a form of pain perception is present in these organisms. Recently, the Birch *et al.* (2021) framework for evaluating evidence of sentience was used to assess whether the nervous system of different insects could support pain (Gibbons, Crump and Chittka, 2022). The same framework, consisting of eight criteria, led to the UK government including two invertebrate groups (decapod crustaceans and cephalopods) in the Animal Welfare (Sentience) Act 2022. This legally recognised their capacity to experience pain, distress and/or harm. Using this approach, *Drosophila* show "strong evidence" for pain by satisfying six criteria. In comparison, octopuses satisfy seven of these criteria and are now protected under the UK law. This evidence outlines the potential implications for this invertebrate model to study pain, in addition to nociception.

5 Conclusion

In conclusion, we have established the expression of fluorescent sensors in *Drosophila* nociceptive neurons for the real-time imaging of the signalling molecules underlying nociception (cAMP and Ca^{2+}). We have characterised the CA-induced Ca^{2+} response and pharmacologically manipulated the system to observe the impact on this nociceptive response. The results reported in this thesis strongly support our hypothesis that *Drosophila melanogaster* are an appropriate model organism to be adopted into pre-clinical pain research, demonstrating the high conservation of the fundamental physiology and molecular biology of nociception. Further optimisation of the cAMP protocol, through employing an alternative cAMP sensor, is needed to allow us to fully investigate intracellular signalling dynamics of this secondary messenger.

Acknowledgements

I would like to thank my supervisors for always believing in me, I could not have submitted this thesis without their constant support.

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