Drug Delivery Systems for Advanced Treatment of Pain and Inflammation

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&

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Abstract

G protein-coupled receptors (GPCRs) contribute to nociception and are considered to be effective therapeutic targets to achieve analgesia. Recent findings indicate that stimulation of GPCRs by extracellular ligands does not exclusively lead to plasma membrane-delimited signalling. Instead, many receptors undergo endocytosis to mediate receptor recycling or degradation and mounting evidence suggests that during these trafficking events, internalised receptors can also signal from subcellular compartments. The main focus of this thesis is to understand how the spatiotemporal signalling of GPCRs can influence cellular processes relevant to pain and inflammation.

The metabotropic glutamate receptor mGlu5 is essential for neuronal development and synaptic transmission. mGlu5 dysregulation also plays important roles in pathophysiological processes such as schizophrenia, fragile syndrome X and pain. It has been recently demonstrated that $mGlu_5$ pools located to intracellular locations such as the nucleus is required for pain transmission on second order spinal neurons. Once activated, $mGlu_5$ can also redistribute from the plasma membrane to endosomes, yet the importance of this trafficking phenomenon to pain transmission is unclear. We therefore investigated if $mGlu_5$ located on endosomes contributes to signalling from this location and whether $mGlu_5$ endosomal signalling is a potential therapeutic target to modulate pain. With a focus on $mGlu_5$ and also the nociceptive trafficking of the GPCR NK_1R, this thesis utilises genetic encoded biosensors, pharmacological tools and polymeric nanoparticles for selective, location-specific drug delivery, to explore the importance of recruiting receptors to endosomes for signalling, neuronal excitability and pain transmission.

In addition, the broader goals of the thesis were to investigate how GPCRs can achieve their complete signalling repertoire by also functionally interacting with TRP channels. This particular study focussed on functional interactions between the 5-HT receptor subtype 2A GPCR and the Transient Potential Receptor Vanilloid 4 (TRPV4) ion channel to further understand the role of their crosstalk in increasing vascular permeability (oedema) in airway and gastrointestinal tissue in mice. This investigation provided relevant evidence that GPCRs not only signal within the plasma membrane and intracellular locations, but also can influence on other receptor through downstream pathways, affecting cell or tissue-specific signalling such as oedema.

To summarise, this thesis includes 4 results chapters and 3 manuscripts published in peer reviewed journals. The main project of this thesis, is an unpublished chapter prepared in manuscript format, characterising and controlling spatiotemporal signalling of the $mGlu_5$ in the context of pain.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Date: 19 December 2021

Publications during enrolment

Retamal, J. S., Ramírez-García, P. D., Shenoy, P. A., Poole, D. P., and Veldhuis, N. A. (2019). Internalized GPCRs as Potential Therapeutic Targets for the Management of Pain. Front Mol Neurosci 12, 273. doi:10.3389/fnmol.2019.00273.

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Ramírez-García, P. D., ***Retamal, J. S***., Shenoy, P., Imlach, W., Sykes, M., Truong, N., et al. (2019). A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain. Nat Nanotechnol 14, 1150–1159. doi:10.1038/s41565-019-0568-x.

Peng, S., Grace, M. S., Gondin, A. B., ***Retamal, J. S***., Dill, L., Darby, W., et al. (2020). The transient receptor potential vanilloid 4 (TRPV4) ion channel mediates protease activated receptor 1 (PAR1)-induced vascular hyperpermeability. Lab. Invest. 5, 4–11. doi:10.1038/s41374-020-0430-7.

Mai, Q. N., Shenoy, P., Quach, T., ***Retamal, J. S***., Gondin, A. B., Yeatman, H. R., et al. (2021). A lipid-anchored neurokinin 1 receptor antagonist prolongs pain relief by a three-pronged mechanism of action targeting the receptor at the plasma membrane and in endosomes. J. Biol. Chem., 100345. doi:10.1016/j.jbc.2021.100345.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes three original papers published in peer reviewed journals and one unpublished work under preparation. The ideas, development, and writing of all the papers presented in this thesis were the principal responsibility of myself, working within the Drug Delivery, Disposition and Dynamics Theme, under the supervision of Dr. Nicholas Veldhuis and Professor Thomas Davis, and Dr. Daniel Poole and Dr. Karen Gregory from the Drug Discovery Biology Theme, at the Monash University Faculty of Pharmacy and Pharmaceutical Sciences.

The thesis also includes a *pre-chapter 3* that corresponds to my contribution towards the manuscript "*A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain*" published in *Nature Nanotechnology* in 2019 (See Appendix 1 and 2) of which I was 2nd author. It provides clear evidence of my experimental design and contribution to the study, and includes my rationale, results and a discussion related to the approach and studies performed.

The inclusion of co-authors for the following listed publications reflects my contribution to several manuscripts that were published as active collaborations, and acknowledges the contribution of other researchers from Monash University that were a part of the broader team.

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Chapter 2	*Internalized GPCRs as Potential Therapeutic Targets for the Management of Pain*	*Published*	*50%. Concept and collecting data and writing manuscript*	1. Paulina Ramirez 10%, 2. Priyank Shenoy 10%. *3.* Daniel P. Poole. 10%. *4.* Nicholas A. Veldhuis 20%.	*1. No* *2. No* *3. No* *4. No*

Chapter 5	*Serotonin-induced vascular permeability is mediated by Transient Receptor Potential Vanilloid 4 in the airway and upper gastrointestinal tract of mice*	*Published*	*50%. Concept and collecting data and writing manuscript*	1. Megan S. Grace, 10%. *2.* Larissa K. Dill, 5%. *3.* Paulina Ramirez, 5%. *4.* Arisbel B. Gondin, 2%. *5.* Sadia Alvi, 2%. *6.* Nigel W. Bunnett, 2%. *7. Thomas P. Davis*, 2% *8. Daniel P. Poole.* 10%. 9. *Nicholas A. Veldhuis* 12%	1. No 2. No 3. No 4. No 5. Yes 6. No 7. No 8. No 9. No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

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Main Supervisor signature:  **Date: 19 December 2021**

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I would also like to give special thanks to my loving, caring and supporting partner Paulina Ramirez for her company, support and understanding during these difficult years, this is an achievement that I could not do without you.

I would like to dedicate this new achievement to my parents, Erika Santibanez and Hector Retamal, for their continuous support and understanding when undertaking this long trip to complete my research and studies. Your prayer for me was what sustained me this far.

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Chapter 1:

Introduction

1. Overview of Fundamental Pain Biology

According to the International Association for the Study of Pain (IASP), **pain** is defined as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage*". Pain is a physiological protective mechanism, that is essential for survival and wellbeing. It provides awareness of noxious stimuli, initiates avoidance behaviours to prevent further injury and promotes wound healing processes (Baron, 2006). This type of pain is known as **acute pain or nociception**. Acute pain is a valuable alarm system that is usually activated due to an illness or injury, and resolves after the noxious stimulus is withdrawn or the injured tissue is repaired (Feizerfan and Sheh, 2015). However, in some cases pain can persist after the damage has been repaired, or the sensation of pain can appear in the absence of injury. In both cases, pain is no longer a physiological function and transforms into a pathological state, known as **pathological pain** or **chronic pain** (Feizerfan and Sheh, 2015; Steeds, 2016). Pathological pain is characterised by an enhanced response to a nociceptive stimulus (**hyperalgesia**) and a painful response against a tactile or non-painful stimulus (**allodynia**) (Basbaum and Fields, 1978; Merskey, 1982) **(Figure 1A**) lasting for an extended period (**Figure 1B)**, typically more than two months. Examples of chronic pain include headache, postsurgical pain, post-trauma pain, lower back pain, cancer pain, arthritis pain, and neurogenic pain (pain caused by nerve damage) (Mills et al., 2019).

Pain is one of the principal reasons for seeking medical care (Johannes et al., 2010), affecting up to 30% of adults worldwide, and it is estimated that each year the number of patients newly diagnosed with any condition of pathological pain such as neuropathic pain or arthritis will increase by 10% (Goldberg and McGee, 2011). Thus, more effective treatments options are required to manage pathological pain.

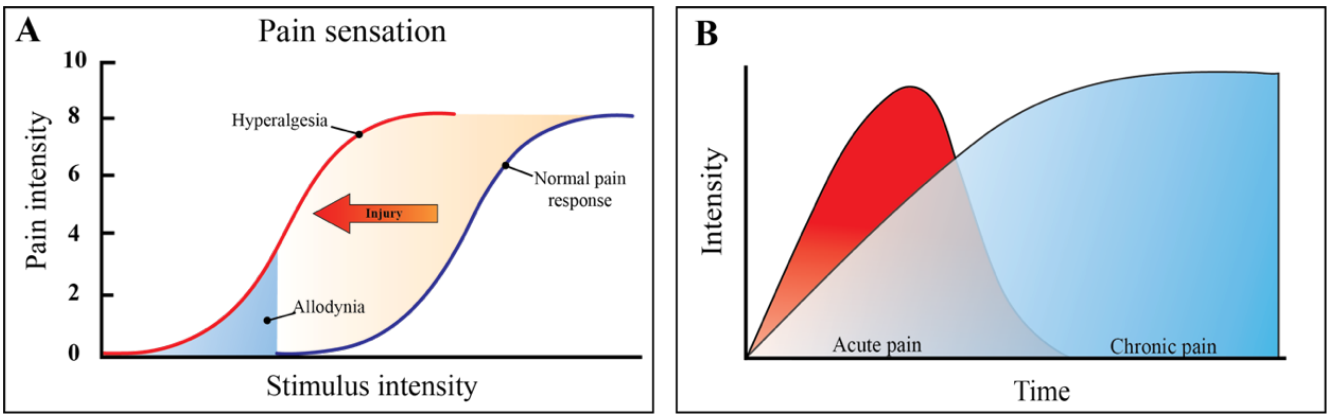

Figure 1. Pain response and pain latency. **A)** Scheme showing a normal pain response blue curve) vs. the pathological pain response following injury (red curve). The pathological pain response presents two phenomena known as hyperalgesia and allodynia (Gottschalk and Smith, 2001). **B)** Comparative scheme between acute and pathological pain in terms of time. Acute pain is characterized as a transient phenomenon. However, chronic pain is described as a pathology, and it can be initiated by damage or in the absence of injury, in both cases, pathological pain persists over time (Walker et al., 2014).

1.1. Pain Transmission: Overview

Pain sensation or **nociception**, is the capability of a specialised subset of neurons (**nociceptive neurons**), to transduce and transmit nociceptive information from the periphery to the brain (Basbaum et al., 2009). Nociceptive neurons are equipped with specialised receptors known as **nociceptors**, that sense a painful stimulus and integrate the signal into an electrical response. The transmission of the painful stimulus occurs in three major steps (**Figure 2**), described briefly here and in further detail below.

In the initiation of pain, the stimulus is sensed by nociceptors (cell surface receptors or ion channels) located in the peripheral nerve endings of primary afferent neurons (nerves that innervate e.g., muscle, organs, head, face, eyes, nose) that transduce painful stimuli into an electrical signal. Next, the electrical signal is sent along nociceptive fibres to the dorsal horn of the spinal cord. These nociceptive fibres are known as Aδ and C fibres (**Figure 2**, **step 1**).

In the spinal cord, the electrical signal promotes the release of neurotransmitters from primary sensory neurons into the synaptic cleft, being the first synapse in the pain pathway. These neurotransmitters then activate their specific receptors on the surface of secondary neurons, creating a **postsynaptic action potential,** that is sent towards the brainstem (**Figure 2, step 2)**. Thus, receptors located in the spinal cord are one of the key pharmacological targets in pain management.

In the thalamus, a third neuron (second synapse) is projected to the somatosensory cortex S1 and insular cortex in the brain (**Figure 1, step 3**). The brain then determines the location and intensity of the stimulus, and a painful response will be then produced.

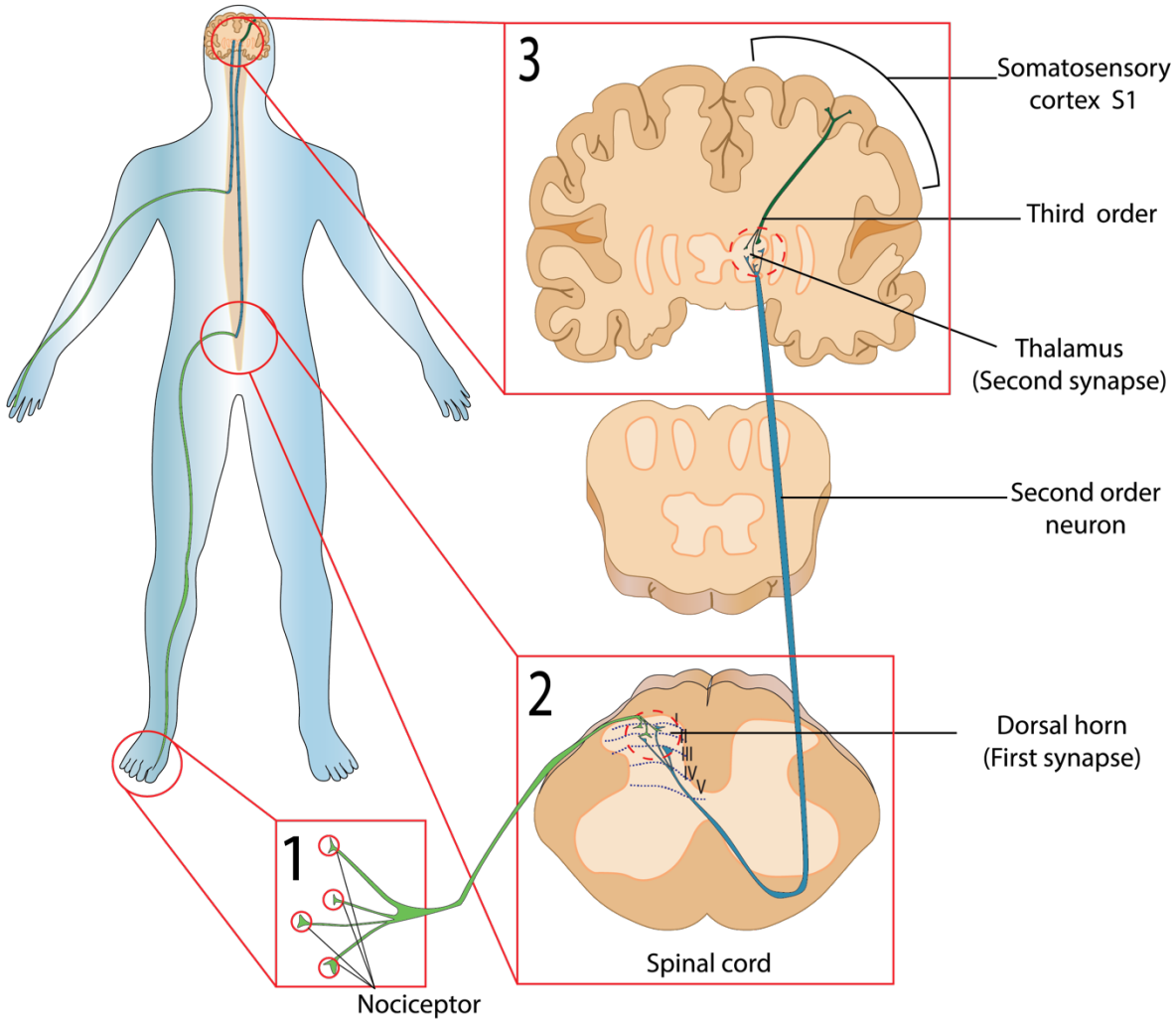

Figure 2. The anatomical and functional organisation of the somatosensory system. Sensory information from the periphery is sensed by nociceptors located on the primary afferent neurons (1). The pain information is transduced into an electrical signal, and the electrical signal is sent to the dorsal root ganglia (DRG), and then to the spinal cord (2). In the spinal cord, the peripheral neurons synapse with ascending secondary neurons to send the information toward the thalamus and cerebral cortex, where the brain will determine the location and intensity of the stimulus (3).

1.2. Initiation of pain: Neurons and fibres involved in pain transmission

Pain stimulus is detected by nociceptors located in peripheral nerve endings of nociceptive neurons (Kakigi et al., 1991; Obi et al., 2007) **(Figure 2**). Nociceptive neurons are classified as pseudo unipolar, which are characterised by a cell body located in the **Dorsal Root Ganglion (DRG)** (Kakigi

et al., 1991; Le Bars and Chitour, 1983; Obi et al., 2007) **(Figure 4**), and a short axon that is split into two branches or projections. This morphological specialisation allows nociceptive neurons to send and receive information from either end. The terminals of the peripheral projections sense chemical (e.g., pro-inflammatory mediators, inflammatory neuropeptides, protons, ATP, proteases), thermal, and mechanical stimuli. The central projection transmits the information collected in the periphery to the central nervous system in the spinal cord. The central projection of nociceptive neurons will then synapse with ascending secondary neurons (**Figure 2 & 3** (Obi et al., 2007)).

Nociceptive neurons in the periphery are organised in groups to form fibres. These fibres are classified into three major groups known as A, B and C fibres. A and B fibres comprise the major myelinated neurons in the body, and unmyelinated neurons are known as **C fibres**. A fibres are further subdivided into A alpha (Aα), A beta (Aβ) and **A delta (Aδ),** where α and β A-fibres are known to carry non-painful somatosensory stimuli such as proprioception and touch, meanwhile nociceptive information is transmitted exclusively through Aδ and C fibres. The type of fibre transmitting the pain information depends on the frequency and intensity of the stimulus (**Figure 3 &4)** (Craig, 2003; Ikeda et al., 2003).

Aδ fibres transmit information from peripheral mechanoreceptors and thermoreceptors to the dorsal horn of the spinal cord. These fibres are lightly myelinated, their cell bodies have a medium-sized diameter (1-5 μm), and transmit information at a relatively fast speed (from 9 to 18 m/s) (Kakigi et al., 1991; Obi et al., 2007). Aδ fibres are activated by stimuli of low and medium frequency and intensity and transmit nociceptive and tactile information to the spinal cord, where they synapse with secondary neurons (ascending neurons) in the laminae III to V region of the dorsal horn (Kakigi et al., 1991; Le Bars and Chitour, 1983; Obi et al., 2007). The neurons forming these fibres are known as wide dynamic range neurons due to their ability to transmit painful and non-painful stimuli (**Figure 3)** (Thomas Cheng, 2010)).

C fibres are known exclusively as nociceptive fibres, responsible for the slow and long-lasting sensation of pain thermal and chemical. C fibres are unmyelinated, smaller in diameter (0.2-1 μm), with the slower speed transmission of 0.4 to 4 m/s (Obi et al., 2007). C fibres are activated exclusively by high frequency and intensity stimulus and transmit nociceptive information toward the superficial regions of the spinal cord dorsal horn, synapsing with projection neurons in laminae I and II (Craig, 2003; Ikeda et al., 2003) (Error! Reference source not found.).

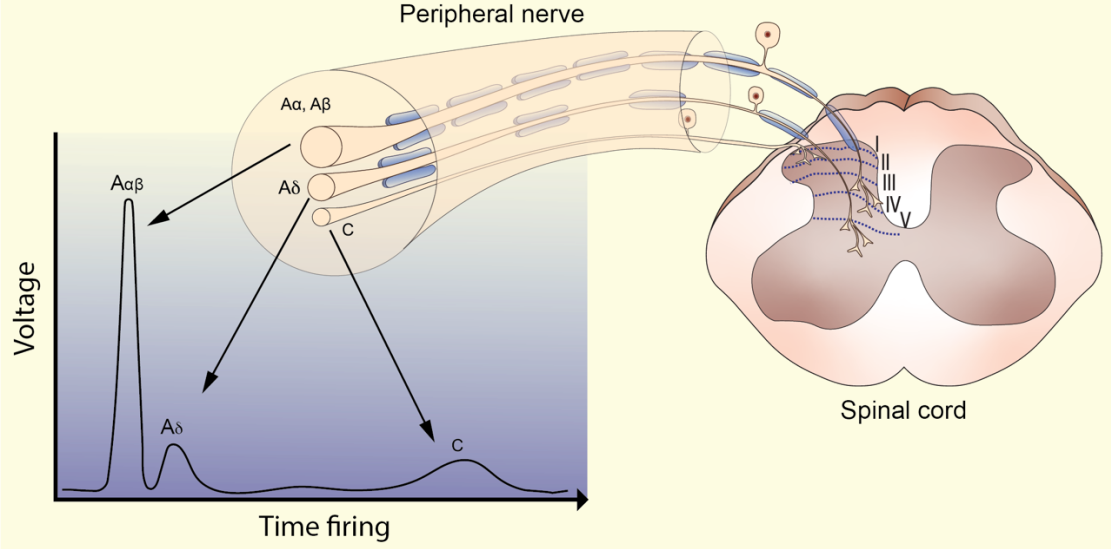

Figure 3. Transmission of peripheral information to the central nervous system. Illustration of the differences between Aα, Aß, Aδ and C fibres in peripheral nerves. Nociceptive Aδ fibres are lightly myelinated whereas C fibres are unmyelinated which influences the speed transmission along fibres.

1.3. Pain transduction and transmission

Under specific circumstances, such as an injury or infection, damaged cells release a variety of mediators such as H^+ and ATP, which can activate peripheral nociceptive neurons to promote pain (Liptom, 1994). Activation of peripheral nociceptive neurons generate a rapid and localised response through local release of neuropeptides such as substance P and calcitonin gene related peptide (CGRP). This promotes swelling, temperature increase, tenderness and pain, all of which are hallmarks of **neurogenic inflammation.** The activation of nociceptive neurons independently of the type of stimuli, causes increased cation influx resulting in a rapid neuronal response (Thomas Cheng, 2010) and triggering of action potentials. Specifically, the action potential is triggered by the influx and increase of cations in the intracellular space of neurons, creating changes in the membrane potential. Generation of an action potential enables transmission of the signal to the CNS.

In normal pain condition, the resting membrane potential of sensory neurons oscillates between -50 and -70mV, and these values depend on the types of ion channels that are open and the concentration of Na^+ and K^+ in the resting state (Liu and Sandkühler, 1997)**B, green current**). Once a stimulus activates nociceptors in the peripheral free nerve endings, an action potential begins at the axon creating changes in the membrane potential, known as depolarisation. If the difference in the membrane potential exceeds the threshold, it triggers the activation of voltage-gated sodium channels (VGSCs or Na_v channels), leading to neuron depolarisation. Voltage-gated sodium channels remain open until reaching a peak of +40mV. Once the peak is reached, sodium channels close and potassium

voltage-gated channels open, decreasing the intracellular concentration of potassium, repolarising axons and returning the membrane potential to its resting state of -50 to -70 mV.

The action potential triggered in the peripheral ending is then propagated along the axon of these neurons via local currents, mainly through two types of neurons, myelinated and unmyelinated neurons. The myelin present on the neurons creates an insulating layer on the axon, with periodic gaps of myelin along the axon known as Nodes of Ranvier (Lubetzki et al., 2020). In the areas where the axons are covered with myelin, the axons lack voltage-gated ion channels. Meanwhile, the nodes of Ranvier are highly dense on voltage-gated ion channels. The myelin surrounding the axon accelerates the conduction of the electrical signal by increasing the membrane resistance and reducing the membrane capacitance, allowing the electrical signals to be rapidly conducted from one node to the next, where it causes depolarization of adjacent axonal membranes triggering and reaching the activation threshold of voltage-gated ion channels that allows the action potential to travel (Giuliodori and Zuccolilli, 2004). In this manner, an action potential is rapidly conducted through a neuron, known as saltatory conduction (**Figure 4**), as is the case of A∂ fibers for fast pain transmission.

Myelination creates an insulating layer around the axon, which accelerates the conduction of the electrical signal by increasing the membrane resistance and reducing the membrane capacitance, allowing the electrical signals to be rapidly conducted (Bleazard et al., 1994; Brown et al., 1995; Littlewood et al., 1995; Liu et al., 1994; Nakaya et al., 1994) (**Figure 4A**), as is the case of Aδ fibres for fast pain transmission (Lubetzki et al., 2020). The absence of myelin, as in C-fibres, the action potential would propagate actively through the activation threshold of voltage-gated ion channels (Giuliodori and Zuccolilli, 2004). Thus, the action potential is propagated at slower speeds through the axon ((Barber and Vasko, 1996; Bonnet et al., 2015; Scuteri et al., 2019; Yarwood et al., 2017) **(Figure 4A**), which underlies the role of C fibres in the slow and lasting sensation of pain.

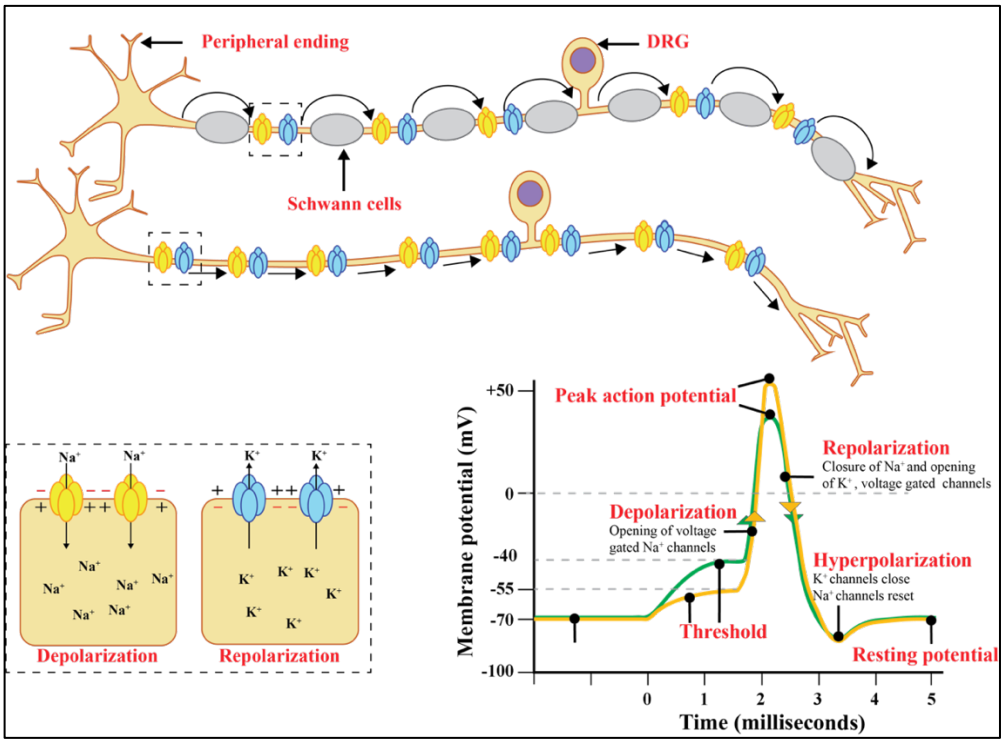

Figure 4. Action potentials in Aδ and C fibres. The rapid influx of Na^+ ions generates an action potential through the opening of voltage-gated Na^+ channels (yellow and green), which generate a brief change in the membrane potential (depolarisation). After reaching maximal depolarisation (peak action potential), voltage-gated Na^+ channels are closed, followed by a slower efflux of K^+ ions due to the opening of voltage-gated K^+ channels (light blue) to restore the membrane potential (repolarisation). The action potential is generated at the initial segment of the axon and is propagated throughout the axon to communicate (synapse) with other neurons.

In pathological pain conditions, the firing threshold decreases, making neurons more susceptible to depolarisation (**Figure 4B, yellow current**). The alterations in the threshold in pathological pain are due to higher activity of different membrane receptors such as VGSCs or TRP channels. Changes in the activity of Na_v channels also occur, making nociceptive neurons susceptible to depolarisation by low-intensity stimulus (no painful stimulus), reaching a higher amplitude of action potential (**peak action potential**). A higher amplitude *stimulus active a vast number of receptors generating a widespread action potential and, consequently, Ca^{2+} channel* that favours the release of a higher amount of neurotransmitter in the spinal cord, amplifying the initial pain stimulus, leading to an enhanced synaptic transmission.

1.4. Transmission in the central nervous system

Once the peripheral nociceptive nerve endings are activated and the action potential is generated, the information is transmitted toward the spinal cord through Aδ and C fibres that synapse with projection neurons in the dorsal horn. The dorsal horn is divided into six physical references or laminae (Bars and Chitour, 1983; Falinower et al., 1994). Laminae I and II are the physical references of the most superficial laminae and the principal site of C fibre innervation, whereas Aδ fibres synapse with secondary neurons in laminae III to V (Bleazard et al., 1994; Brown et al., 1995; Littlewood et al., 1995; Liu et al., 1994; Nakaya et al., 1994).

In the spinal cord, peripheral nociceptive neurons promote the release of neurotransmitters such as substance P (SP), Calcitonin Gene-Related Peptide (CGRP) and glutamate, in the synaptic space (dorsal horn), leading to activation of secondary neurons (Brierley and Linden, 2014). SP and CGRP activate the neurokinin 1 receptor (NK_1R) (Sakurada et al., 1992; 1995), and calcitonin receptor-like receptor (CALCRL/RAMP1), respectively (Barber and Vasko, 1996; Bonnet et al., 2015; Scuteri et al., 2019; Yarwood et al., 2017). Glutamate activates the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and metabotropic glutamate receptors (mGlu 1 & 5) (Lax et al., 2014).

Activation of these receptors on the second order neuron leads to an excitatory postsynaptic potential (EPSP) (**Figure 5)** (Baccus, 1998; Jackman and Regehr, 2017). If this EPSP is greater than threshold it triggers an action potential that travels through the anterolateral funiculus (spinothalamic tract) towards the brainstem (reticular formation, nucleus coeruleus, tectum and periaqueductal grey matter) and the thalamus (posterolateral and posteromedial ventral nucleus). From the thalamus, a third neuron (**second synapse**) is projected to the somatosensory cortex S1 and insular cortex (**Figure 2**) (Barber and Vasko, 1996; Kuner, 2010; Vergouts et al., 2017)**,** where the brain will determine the location and intensity of the stimulus. Additionally, the nociceptive information also travels to the superior centre (brain) via the amygdala and hypothalamic spinoparabrachial tract (Buritova et al., 1998), both of which are involved with reactions related to pain including fear, memory and emotional behaviour.

Pain transmission is also modulated by interneurons (also known as association neurons), which are found exclusively in the CNS. Interneurons acts as an intermediate between primary sensory and secondary neurons, modulating neuronal activity by releasing the neurotransmitter gamma-aminobutyric acid (GABA), which decrease the firing and restore the resting state of neurons. In

Addition, inhibitory interneurons in the spinal cord are considered as a new pharmacological target. Pharmacological modification of interneurons overall modifies the tone of activity of this subset of neurons that may gate of amplify outputs in the spinal cord leading to a much greater analgesic effect (Hughes and Todd, 2020).

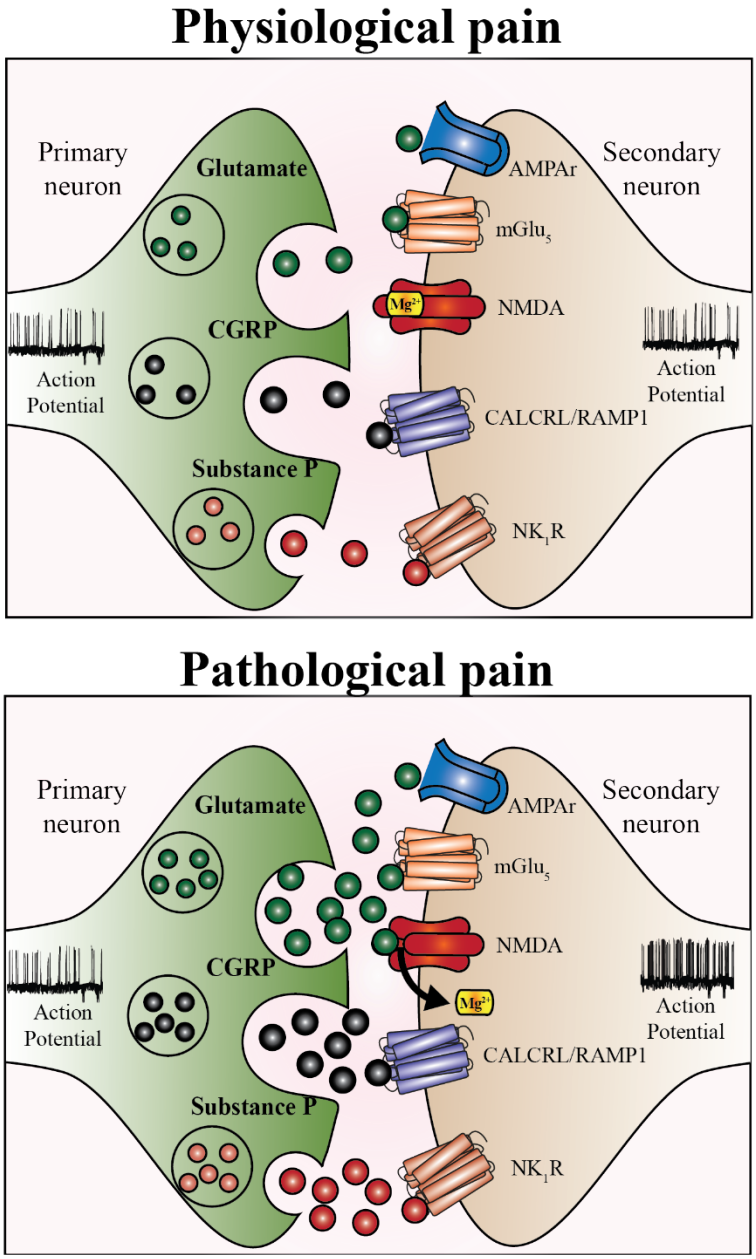

Figure 5. Pain Pathway, first synapse. The primary afferent neurons release glutamate, substance P and CGRP to activate second order neurons. Glutamate binds to the a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptor (AMPA), N-methyl-D-aspartate (NMDA), and G protein-coupled metabotropic receptors (mGlu). In acute pain, NMDAR remains inactive due to magnesium bind to the core. Pathological pain is characterised by the enhanced release of glutamate, substance P and CGRP. The maintained nociceptive stimulation triggers the release of magnesium from the NMDA core, enhancing a short burst of low-medium frequency stimuli signal, that results into an exacerbated long-lasting post-synaptic strength or increase in the intensity of the action potential.

2. Pathological pain: transition from acute to chronic pain

Chronic pain is associated with structural and functional changes to pathways leading from the periphery to the brain such as increases in long-term potentiation at synapses and neuronal hypersensitivity resulting in **persistent pain**. Persistent pain can be caused by permanent nerve injury (e.g., following significant physical injury such as occurs in a car crash), or chronic inflammatory insult such as occurs in osteoarthritis. The change from acute to pathological pain is explained by modifications that are known to occur at different levels of the nociceptive pathway that ultimately lead to enhanced conduction of nociceptive information. Also known as **synaptic facilitation**, the processes that lead to such pathological states are described below.

2.1. Dysregulation of pain transmission: the periphery

In the periphery, repetitive noxious stimulation can promote to prolonged inflammation through activation of immune cells and subsequent release of pro-inflammatory molecules like lipids and cytokines. Exposure to these mediators can lead to hyperexcitability of nociceptive fibres (peripheral sensitisation). This can create a series of changes in the periphery by increasing primary afferent receptor signalling, leading to changes such as increased activity of protein kinase A and C (**PKA**, **PKC)** and extracellular signal-regulated kinases (**ERK)** (Barber and Vasko, 1996; Kuner, 2010; Vergouts et al., 2017) and upregulation of voltage-gated sodium channels and **TRP channel** expression and activity (Honda et al., 2017; Lin et al., 2007; Patapoutian et al., 2009). Collectively, these changes ultimately lead to a reduced **threshold** for activation of nociceptive neurons and hyperalgesia as described above (Ji and Woolf, 2001; Woolf and Salter, 2000). Increased afferent activity can also lead to enhanced production and release of neurotransmitters such as SP and CGRP in the periphery and spinal cord to promote neurogenic inflammation and amplify pain signalling, respectively (Chapman et al., 2008; D'Mello and Dickenson, 2008; Hunt and Mantyh, 2001).

The modifications outlined above create a positive feedback loop where the release of neurotransmitters triggers the further release of inflammatory mediators. This results in sustained activation even in the absence of the original noxious stimulus and increases responsiveness and promotes spontaneous activation of peripheral nociceptive endings. Inflammatory mediators such as bradykinin, histamine, and prostaglandins activate their respective GPCRs, leading to TPR sensitisation of pain pathways {Zhang:2007kh, Shim:2007jz, Bandell:2004td, Poole:2013es}. In addition, associated downstream signalling through PKA and PKC can modulate the sensory neuron

activity by phosphorylation of voltage-gated sodium channels and **TRP channels** (discussed in detail in section 4.2**)**. This phosphorylation increases the excitability of the nociceptor by decreasing the threshold of sodium and TRP channels, leading to prolonged neuronal depolarisation and enhanced neuronal responses (Luo et al., 2008) **(Figure 6**).

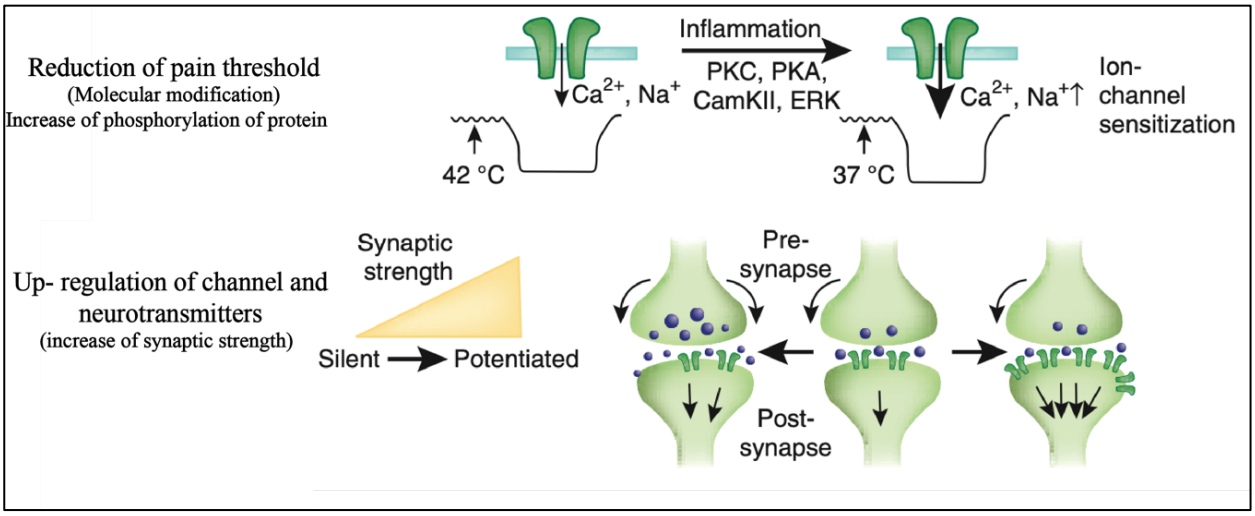

Figure 6. Molecular mechanisms underlying dysregulated pain transmission. A) Phosphorylation of membrane receptors (sensitisation) by PKC and PKA, leads to changes in receptor threshold. **B)** Changes in synaptic settings (upregulation of channels and neurotransmitter release), increases the synthesis of neurotransmitter in the pre-synaptic neurons and/or increase of receptor in the post-synaptic membrane. The increase of synaptic strength leads to chronic pain. Taken and modified from (Kuner, 2010, Permission License Number ; 5297640962247)

2.2. Central sensitisation: the spinal cord

In addition to the modification observed in the periphery, under chronic pain conditions the spinal cord undergoes extensive modifications such as **i)** increase in the release of classic neurotransmitters like glutamate and SP (Drdla and Sandkühler, 2008; Toyoda et al., 2009), **ii)** release of BDNF and ATP (Melemedjian et al., 2013; Panja and Bramham, 2014; Wang et al., 2016) **iii)** phosphorylation of membrane receptors (Kuner, 2010; Schröder et al., 2009; Tingley et al., 1997), **iv)** changes to the configuration of membrane receptors (Luo et al., 2008), increase in the synthesis of ionotropic receptors (Clapham, 2003; Hinman et al., 2006; Lin et al., 2007; Patapoutian et al., 2009), **vi)** increase of calcium in the post synaptic terminal (Luo et al., 2008), **vii)** synthesis and release of nitric oxide, **viii)** activation of microglia and astrocytes (Clark et al., 2007; Pascual et al., 2012; Trang et al., 2012; Wodarski et al., 2009), **iix)** changes in the regulatory activity of inhibitory interneurons (Hughes and Todd, 2020; Yam et al., 2018), and **ix)** the focus of this thesis, the capability of GPCRs to signal from intracellular compartments and participate in pain transmission (Jensen et al., 2017; Jimenez-Vargas

et al., 2018; Ramírez-García et al., 2019; Retamal et al., 2019; Vincent et al., 2016; 2017; Yarwood et al., 2017).

It is also essential to highlight the importance of the spinal N-methyl-D-aspartate receptor (NMDAR), given that its activity is associated with chronic pain states (Lipton, 2004; Paoletti and Neyton, 2007). NMDAR is an ionotropic glutamate receptor and nonselective ion channel that **remains inactive during acute pain states** due to magnesium binding to specific sites on the receptor, which blocks the passage of cations through this channel (Mayer et al., 1984; Nowak et al., 1984). However, maintained nociceptive stimulation causes a strong depolarisation of secondary neurons, leading to a massive influx of ions that create changes in the membrane potential, promoting the removal of magnesium from the NMDAR channel (**Figure 5**). Activated NMDAR enhances post-synaptic strength, turning a low intensity stimulus into an exacerbated long-lasting stimulus (days to months, (Constandil et al., 2011; Fang-Hu et al., 2015; Gao and Ji, 2010; Gomes et al., 2013; Guo et al., 2007; Hide et al., 2000; Suzuki, 2004; Wagner and Myers, 1996; Zhang et al., 2008) **(Figure 1 & Figure 5)**.

Neuroglial cells in the spinal cord (microglia and astrocytes) also play an essential role in developing and maintaining chronic pain. In the late 1990s, our knowledge about pain focused solely on the role and importance of neurons, while microglia were considered only resident macrophages of the CNS (Gosselin et al., 2010). However, many studies now indicate that neuroglial cells are critical players in the modulation of neuronal network excitability (Aldskogius and Kozlova, 1998; Eyo and Dailey, 2013; Gao and Ji, 2010; Gao et al., 2010b; Inoue, 2017; Kobayashi et al., 2008; Milligan and Watkins, 2009; Trang et al., 2012).

In normal conditions, microglia and astrocytes are in a resting or quiescent state and are present in all adult spinal cord and brain regions at relatively high density (Jinno et al., 2007; Long et al., 1998). At primary stages of inflammation, neuroglial cells can be converted to reactive states and participate in the pathogenesis of neurological disorders. Microglia and astrocytes express a wide range of membrane receptors, and as a consequence, glial cells can sense neuronal activity, allowing bidirectional communication with neurons (Harry and Kraft, 2012; Pocock and Kettenmann, 2007). Activated microglial cells release a wide range of substances such as small molecules (reactive oxygen species, nitric oxide), chemokines, prostaglandins, and neurotrophic factors (BDNF). Whereas activation of astrocytes promotes release of glutamate, d-serine and ATP as well as prostaglandins and neuropeptides (Constandil et al., 2011; Fang-Hu et al., 2015; Gao and Ji, 2010; Gomes et al.,

2013; Guo et al., 2007; Hide et al., 2000; Suzuki, 2004; Wagner and Myers, 1996; Zhang et al., 2012). All these molecules can control and or modify neuronal function, enhancing neuronal excitability, up-regulating NMDA receptors, and down-regulating inhibitory receptors such as GABA receptors.

The majority of drugs used in the clinic to manage and regulate chronic pain target the receptors expressed in the membrane of cells in the spinal cord. However, none of the currently available drugs effectively manage chronic pain and most have side-effects that limit their use.

2.3. Dysregulation in the interpretation of pain: processing in the brain and descending pathways

The modifications and dysregulations that occur in the brain under pain states are not fully understood. However, imaging analysis such as radiological, functional magnetic resonance imaging (fMRI) and positron emission tomography have revealed important centres and some changes that occur following onset of sensitisation. These changes are directly associated with clinical observations in the thalamus (Saab and Barrett, 2016), middle/anterior insula, anterior cingulate cortex and prefrontal cortex (Ong et al., 2019), periaqueductal grey (PAG) and rostral ventromedial medulla (RVM) (descending pathway) (Mills et al., 2018) and reticular formation (regulating descending pathways) (Martins and Tavares, 2017).

All these centres are associated with one or more pathophysiological changes in the brain. However, these centres are strongly correlated with dysregulation that can affect somatosensory discrimination, autonomic and sensory coordination, changes in affective and motivational components of pain, anxiety and depression-induced pain and psychological stressors that accentuate pain intensity (Becker et al., 2018). In addition, the most common and problematic issues associated with chronic pain is substance misuse and addiction to pain killers (Corbett et al., 2006; Devulder et al., 2005).

3. G Protein-Coupled Receptors in the synaptic facilitation of pain

3.1. G protein-coupled receptors: classification, structure and signalling

GPCRs are the largest superfamily of transmembrane receptors, characterised by seven transmembrane domains linked within three intracellular and extracellular loops of various lengths (Stevens et al., 2013). Genetic analysis found more than 800 GPCR sequences in humans (Fredriksson et al., 2003), and it is estimated that GPCRs are involved in almost every physiological and pathophysiological process. GPCRs are well-established therapeutic targets, with 475 drugs approved

by the FDA that act on 108 unique GPCR targets and more than 321 agents in clinical trials (Hauser et al., 2017; Lagerström and Schiöth, 2008).

GPCRs are involved in almost all physiological processes acting as mediators and/or modulators detecting a wide range of stimuli from physical stimuli to neurotransmitters, hormones, cytokines and chemical signals (Ca^{2+}, H^{+}) (Rosenbaum et al., 2009). The principal function of GPCRs is to sense these diverse extracellular stimuli and transduce these signals into a cellular response. These responses are mediated by binding or "coupling" to specific intracellular effector proteins known as heterotrimeric G proteins, leading to the modulation of downstream effector proteins (Downes and Gautam, 1999; Lagerström and Schiöth, 2008; Rosenbaum et al., 2009).

G proteins are comprised of three subunits; Gα, Gβ and Gγ, and according to genetic analysis, sixteen genes encode Gα subunits, five genes encode Gβ subunits, and twelve genes encode Gγ subunits. The Gα-subunits are formed by two domains: a GTPase domain that catalyses the hydrolysis of GTP and a helical domain that bind the GTP within the core of the protein. In the quiescent state, the α-subunit binds to guanosine diphosphate (GDP) in the core. Upon ligand binding to the GPCR, a conformational change occurs in the GPCR structure leading to G protein recruitment, resulting in the exchange of GDP by GTP. The exchange triggers the reorganisation and dissociation of the Gαβγ complex into Gα and Gβγ units, leading to subsequent stimulation of downstream effector proteins such as adenylyl cyclase, phospholipase C (PLC) and RhoGEF (Cabrera-Vera et al., 2003; Offermanns, 2003; Stevens et al., 2013). Each unit (Gα and Gβγ) can exert independent functions, eliciting a variety of signalling cascades (Oldham and Hamm, 2008; Tuteja, 2009) (**Figure 7**). Most of these processes are known to contribute to signalling pathways associated with sensory processes and pain transmission. G proteins are classified into four groups based on their similarity in the α subunits; $G\alpha_{12/13}$, $G\alpha_{s}$, $G\alpha_{i/o}$ and $G\alpha_{q/11}$ (Cabrera-Vera et al., 2003; Downes and Gautam, 1999; Offermanns, 2003; Rosenbaum et al., 2009).

Activation of GPCRs associated with Gαs proteins stimulates adenylyl cyclase activation, leading to increased intracellular production of **cyclic adenosine monophosphate (cAMP)** through ATP hydrolysis. cAMP binds and activates downstream effectors like protein kinase A (PKA), which can phosphorylate various transcriptional factors, such as cAMP element-binding protein (CREB), or ion channels. An examples of GPCRs coupled to Gαs involved in pain is the CLR/RAMP receptor, postulated as the key mediator in primary migraine and the bradykinin 1 and 2 (B1-2) receptors which

are associated with inflammatory pain and itch (Geppetti et al., 2015; Lennerz et al., 2008; Moore and Salvatore, 2012).

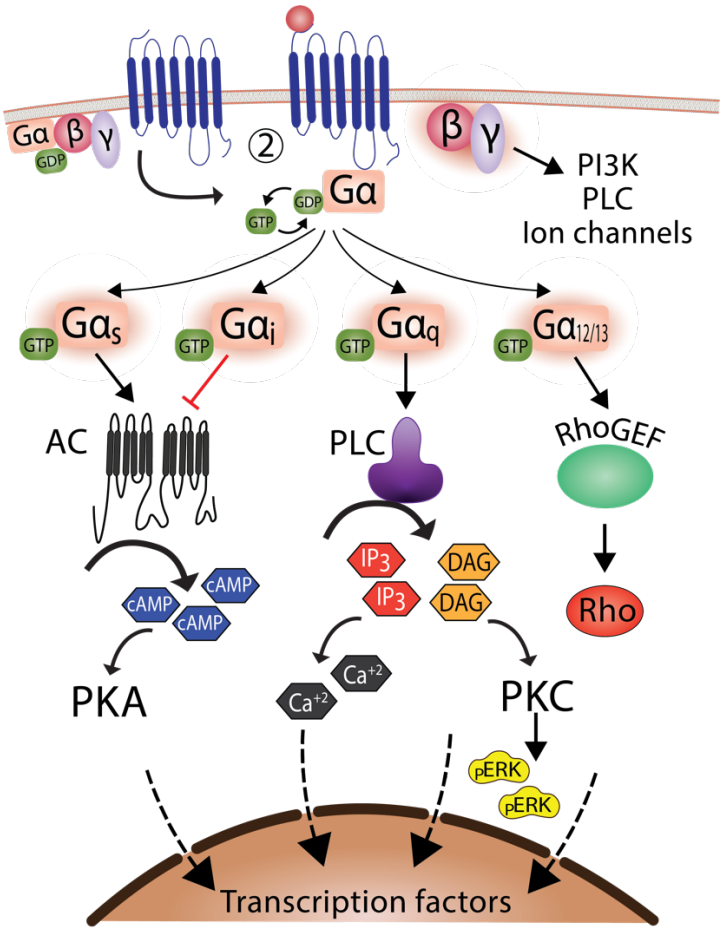

Figure 7. Classical G protein-dependent signalling by GPCRs. Agonist binding, G-protein exchange GDP by GTP, eliciting the dissociation of the G-protein trimeric complex into Gα and Gβγ leading to the formation of a complex formed by agonist bound GPCR and Gα subunit. Several effectors are activated depending on the Gα subunit recruited. Activation of effector results in the increase of second messenger molecules, such as cAMP, calcium and pERK. The final outcome is the activation of transcription factor in the nucleus.

GPCRs that activate Gαi/o protein, inhibit adenylate cyclase activity reducing cAMP production, and decreases PKC activity (Taussig et al., 1993). $G\alpha_i$ and $G\alpha_o$ family members regulate multiple physiological processes such as leukocyte chemotaxis and neuronal growth in inflammatory and sensory pathways. In terms of pain, GPCRs that mediate analgesia such as cannabinoid, opioid and $GABA_b$ receptors activate $G\alpha_{i/o}$ signalling (Glass and Northup, 1999; Padgett and Slesinger, 2010; Rios et al., 2006; Senese et al., 2020).

GPCRs coupled to $G\alpha_q$ directly activate **phospholipase C (PLC)**. PLC catalyses the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol

(DAG). IP3 acts on IP3 receptors found in the endoplasmic reticulum (ER) to mobilise Ca^{2+} and DAG activates PKC (Sweitzer et al., 2004; Tingley et al., 1997). Both IP3 and DAG are key players in regulating multiple physiological processes such as cell proliferation, differentiation, channel regulation and gene transcription. GPCRs coupled to $G\alpha_q$ are the biggest group of receptors that participate in pain, and include the NK_1R and mGlu 1&5 (Honda et al., 2017; Laird et al., 2001; Ramírez-García et al., 2019; Teodoro et al., 2013; Vincent et al., 2016; 2017). The P2Y receptor 1, 2 and 4 (P2Y1-2 & 4), and chemokine CXC motif receptors (CXCR) both associated with glial cell activation in neuropathic pain (Akgün et al., 2015; Foster et al., 2011; Kobayashi et al., 2008; Trang et al., 2012; Tressel et al., 2011; Tsuda et al., 2009; Zhou et al., 2016).

The $G\alpha_{12/13}$ family, known as gap proto-oncogenes, highly overexpressed in breast, prostate and liver cancers (Kelly et al., 2006a; 2006b; Suzuki et al., 2009; Yang et al., 2020) as well as with cell migration and invasion. GPCRs coupled to $G\alpha_{12/13}$ activate Rho GTPase nucleotide exchange factors (RhoGEFs) and phospholipase D (PLD) and mediate diverse cellular functions through RhoGEF-RhoA pathway (Kozasa et al., 1998; Siehler, 2009; Suzuki et al., 2009). $G\alpha_{12/13}$ activation is associated with cellular effectors like the Na^+/H^+ exchanger (Hooley et al., 1996). An example of GPCRs associated with $G\alpha_{12/13}$ in pain and inflammation are the Protease-Activated Receptor 1 and 2 subtypes (PAR1, 2), particularly PAR1 and 2 in inflammatory pain (Amadesi et al., 2004; Arora et al., 2007; Grant et al., 2007; Poole et al., 2013; Tillu et al., 2015).

Particularly in pain, GPCRs associated with G_s (CLR/RAMP1) and G_q (NK_1R, mGluR5) coupling are the most significant receptor reported to participate in pain. The main effectors are the activation of PKA and PKC, which phosphorylate key proteins that enhance the physiological activity of nociceptors such as TRP channels and other ionotropic receptors. This mechanism plays a critical role in nociceptive changes in peripheral and central sensitisation (Amadesi et al., 2006; Barber and Vasko, 1996; Decosterd and Woolf, 2000; Tingley et al., 1997; Vergouts et al., 2017; Willis, 2001; Zhang et al., 2007).

3.2. Trafficking of GPCRs

Trafficking of GPCRs is a crucial component of their activity cycle. GPCRs can trigger G protein-dependent or independent signalling. However, this signalling is not maintained indefinitely but is instead regulated by the trafficking-dependent processes of receptor endocytosis, degradation, recycling and resensitisation.

First, a ligand binds to its cognate GPCR in the extracellular space, then the GPCR bound to its ligand undergoes endocytosis mediated by clathrin/dynamin or caveolin/dynamin-dependent mechanisms or by clathrin and caveolin independent mechanisms (**Figure 8**). Once located in endosomes (pH 6.8-5.9), the receptor is dissociated from its agonist, neuropeptides are degraded by peptidases under the acidic condition of endosomes, interrupting the signalling. Dissociated receptors can follow two possible routes: targeting to lysosomes for degradation or recycling back to the plasma membrane, known as resensitisation. Resensitisation is a highly regulated system that plays an essential role in modulating GPCR signalling.

GPCRs can also be affected by the process of desensitisation. This process is described as the loss of response after prolonged or repeated administration of an agonist (Hausdorff et al., 1990; Kelly et al., 2008). Desensitisation can be classified as homologous and heterologous desensitisation.

Homologous desensitisation is characterised by the loss of response to agonists that act at a specific GPCR subtype (Kelly et al., 2008). This desensitisation can occur via phosphorylation of GPCRs on multiple residues by GRKs and favours β-arrestin recruitment and subsequent internalisation (Kelly et al., 2008; Magalhaes et al., 2012; Shukla et al., 2014). In contrast, heterologous desensitisation is a more generalised effect that involves loss of agonist responsiveness at multiple GPCR subtypes (Kelly et al., 2008). Heterologous desensitisation can happen when activating one GPCR desensitises a second GPCR through a phosphorylation-dependent mechanism or by auto-phosphorylation of the second messenger proteins PKA and PKC (Hausdorff et al., 1990; Kelly et al., 2008; Tuteja, 2009).

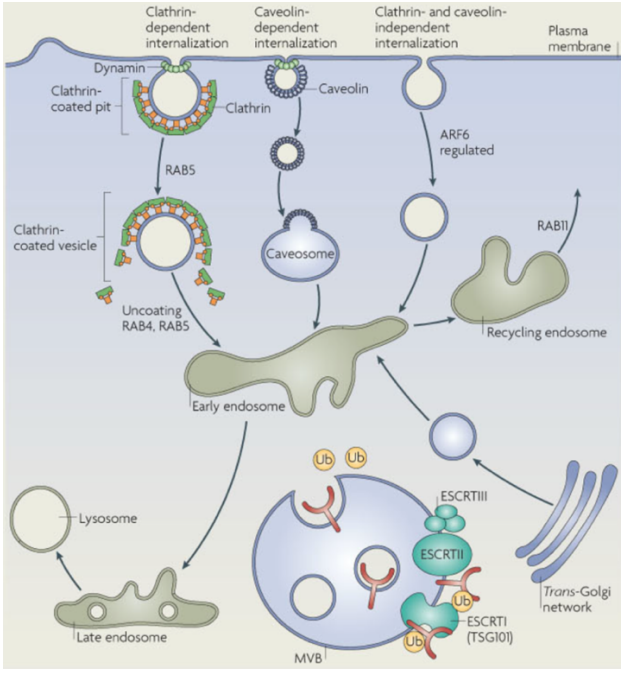

Figure 8. Endocytosis pathway. Scheme of endocytosis mediated by clathrin/dynamin and caveolin/ dynamin dependent internalisation and clathrin and caveolin independent internalisation. All these internalisation mechanisms may result in formation of early endosomes (EE). EE-associated receptors can be directed to either recycling or late endosomes (Gould and Lippincott-Schwartz, 2009. Permission License Number 5297640662952).

3.3. Endosomes: compartmentalised signalling by internalised GPCRs

The trafficking mechanisms described above suggest that all GPCR signalling terminates following endocytosis. For many years, GPCRs signalling was exclusively delimited to the plasma membrane, and internalisation was a mechanism through which GPCRs signalling was ended. However, it is now

accepted that endocytosis can also promote new and unique signalling by GPCRs.

Although the concept of GPCR signalling from the endosomal network is novel, the first receptor described to signal from endosomes was the receptor tyrosine kinases (RTKs), particularly the epidermal growth factor receptor (EGFR) and insulin receptor (IR) (Baass et al., 1995; Di Guglielmo

et al., 1994). Later, it was observed that upon nerve growth factor (NGF) stimulation, the tyrosine kinase A receptor (trkA) was able to signal from endosomes (Grimes et al., 1996, 1997; Beattie et al., 2000). Posterior work reinforced the concept of endosomal signalling by showing that another RTK, the platelet-derived growth factor receptor (PDGFR) was also found to elicit a biological response

from their endosomal location (Pennock and Wang, 2003).

Toll-like receptors such as TLR3, TLR4 and TLR9 are also shown to redistribute in endosomes for optimal innate immune responses (Johnsen et al., 2006; Kagan et al., 2008) and the importance of

redistribution is exemplified by the disruption of TLR4 signalling elicited by the inhibition of TLR4 endocytosis (Kagan et al., 2008).

Similarly, studies on the β2-adrenergic receptor (β_2AR) and thyroid-stimulating hormone receptor (TSHR) revealed that unique, sustained signalling profiles were associated with internalised GPCRs (Daaka et al., 1998; Calebiro et al., 2009; Werthmann et al., 2012). Later work demonstrated similar endosomal-mediated signalling responses in the parathyroid hormone receptor (PTHR) and V2 vasopressin receptor (V2R)(Ferrandon et al., 2009; Feinstein et al., 2013; Wehbi et al., 2013). The first evidence that endocytosis can promote acute GPCR-G protein signalling was demonstrated for the D1 dopamine receptor (DRD1) with a prompt, acute and reversible form of G protein activation in endosomes (Kotowski et al., 2011)

Upon agonist stimulation, the receptor stabilises in an active conformation by interacting with G proteins to induce signalling (described in the previous section). This signalling is transient and derived from the activation of GPCRs at the plasma membrane (**Figure 9, point 1-3**). The GPCRs are then phosphorylated by GPCR kinases (GRKs), leading to the recruitment of β-arrestin (βARRs). The association of βARRs with GPCRs initiates receptor internalisation through clathrin interaction with the endocytic machinery (**Figure 9, point 4 & 5**). Once the GPCR reaches the endosomes, associated neuropeptide ligands, such as SP, are degraded by peptidases under these acidic conditions (Roosterman et al. 2007, Padilla et al. 2008). Endosomal GPCRs can continue to signal, promoting activation of second messenger molecules such as PKC and ERK1/2 (**Figure 9**, point 5), which can then regulate nuclear events (Pavlos and Friedman, 2017; Thomsen et al., 2018).

For GPCRs, the first evidence that endocytosis can promote distinct GPCR signalling was demonstrated for the D1 dopamine receptor (DRD1) with a prompt, acute and reversible form of G protein activation in endosomes. In addition to the DRD1, parathyroid hormone receptor (PTHR), vasopressin type 2 receptor (V2R), thyroid-stimulating hormone receptor (TSHR), luteinizing hormone receptor (LHR), sphingosine-1-phosphate 1 receptor (S1PR1), neurokinin type 1 receptor (NK1R), calcitonin receptor-like receptor (CLR), and C-C chemokine receptor-1 (CCR1) have been reported to signal from the endosomal network upon ligand stimulation (Pavlos and Friedman, 2017; Thomsen et al., 2018).

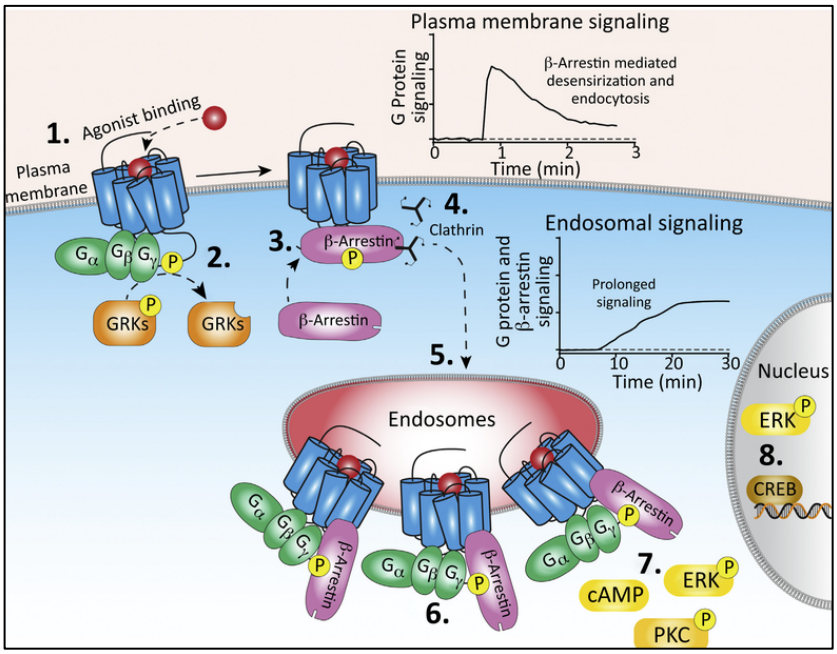

Figure 9. Compartmentalised signalling by GPCRs in endosomes. 1. Agonist binding at the plasma membrane stabilises GPCRs through interaction with heterotrimeric G proteins, triggering the plasma membrane signal. **2.** GPCR kinases phosphorylate GPCRs. **3.** Recruitment of βARRs that then bind the GPCR core, **4.** clathrin and AP2 bind to mediate GPCR endocytosis. **5** and **6**. GPCRs continue to signal in endosomes to promote prolonged endosomal signalling, which can regulate nuclear events (Thomsen et al., 2018).

The GPCRs known to contribute to pain signalling can also signal from endosomes are discussed extensively in Chapter 2 of this PhD thesis

3.4. GPCRs involved in nociception.

Immune and non-immune cells associated with sites of injury respond to tissue damage by releasing an inflammatory soup that is composed of monoamine transmitters, pro-inflammatory cytokines, and peptides. This inflammatory soup produced by infiltrating and resident immune cells can activate a variety of receptor such as ionotropic channels and GPCRs that sensitise primary afferent neurons and contribute to pain hypersensitivity. Many of these GPCRs have been investigated as potential therapeutic targets for pain and some of the most relevant GPCRs are summarised in **Table 1** below.

Table 1. Summary of key excitatory and inhibitory GPCRs involved in pain.

Receptor Family	Agonist	Mechanism	Localisation	Reference
	Excitatory receptors			
Prostaglandin Receptor 1 – 4 (EP_{1-4})	Prostaglandin (PGE1-2), Prostacyclin (PGI2)	↑ PLC/IP3, DAG/PKC	CNS, PNS	(Jang et al., 2020; Kanai et al., 2007; Lin et al., 2006; Sarkar et al., 2003)
Histamine Receptor 1 - 4 (HR_{1-4})	Histamine (Monoamine)	HR1: ↑ PLC/IP3, DAG/PKC	CNS, PNS	(Coruzzi et al., 2007; Khalilzadeh et al., 2018; Obara et al., 2020; Raffa, 2001; Rosa and Fantozzi, 2013)

Metabotropic glutamate Receptor 1 & 5 ($mGlu_{1/5}$)	Glutamate	↑ PLC/IP3, DAG/PKC	CNS, PNS	(Fisher and Coderre, 1996; Honda et al., 2017; Osikowicz et al., 2013; Vincent et al., 2016; 2017)
Protease-activated Receptor 2 (PAR_2)	Trypsin, Tryptase, Elastase, Cathepsin S	↑ GEF/PLD/Rho	PNS	(Amadesi et al., 2004; 2006; Bao et al., 2014; Chen et al., 2015; Grant et al., 2007; Poole et al., 2013; Tillu et al., 2015; Wei et al., 2016; Zhao et al., 2014)
Neurokinin 1 Receptor (NK_1R)	Substance P (SP), Neurokinin A/B (NKA, NKB)	↑ PLC/IP3, DAG/PKC	CNS, PNS	(Grimes et al., 1996; Howe et al., 2001b; Iadarola et al., 2017; Jensen et al., 2017; Laird et al., 2001; Lee and Kim, 2007; Mai et al., 2021; Mantyh, 2002; Marlin and Li, 2015; Ramírez-García et al., 2019; Yoshimura and Yonehara, 2006)
Calcitonin receptor-like Receptor (CRLR)	Calcitonin gene-related peptide (CGRP)	↑ AC/cAMP/PKA	CNS, PNS	(Bell, 2014; Moore and Salvatore, 2012; Scuteri et al., 2019; Yarwood et al., 2017)
Angiotensin Receptor 1 (AT_1R)	Angiotensin II	↑ PLC/IP3, DAG/PKC	PNS	(Anand et al., 2013; 2015; Danser and Anand, 2014; Shepherd et al., 2018a; 2018b; Smith et al., 2013a; 2013b)
Bradykinin Receptor 1 & 2 (B_1R & B_2R)	Bradykinin	↑ PLC/IP3, DAG/PKC	CNS, PNS	(Bandell et al., 2004; Hall, 1997; Huang and Player, 2010; Steranka et al., 1988)
5-Hydroxytryptamine Receptor 1 - 7 ($5\text{-}HT_{1\text{-}7}$)	Serotonin (5-HT)	$5\text{-}HT_{2a}$: ↑ PLC/IP3, DAG/PKC	CNS, PNS	(Ayme-Dietrich et al., 2017; Cortes-Altamirano et al., 2018; Koo and Balaban, 2006; Nascimento et al., 2011; Rocha-González et al., 2005; Sasaki et al., 2006)
Inhibitory receptors				
γ-Aminobutyric acid type B receptors (GABAB)	Gamma-aminobutyric acid (GABA)	↓ AC/cAMP/PKA	CNS & PNS	(Enna, 1997; Liu et al., 2018; Malcangio, 2018; Martins et al., 2015)
Mu, Delta and kappa Opioid Receptor (MOR, DOR, KOR)	MOR: enkephalins & endorphins, KOR: Dynorphins, DOR: Enkephalins	↓ AC/cAMP/PKA	CNS & PNS	(Cahill and Coderre, 2002; Chen et al., 2014; Corbett et al., 2006; Przewłocki and Przewłocka, 2001; Scherrer et al., 2009; Vanderah, 2010)
Cannabinoid 1 & 2 receptors (CB1R & CB2R)	Anandamide, 2-Arachidonoylglycerol	↓AC/cAMP/PKA	CNS & PNS	(Freeman et al., 2019; Korzh et al., 2008; Lossignol, 2019; Romero-Sandoval et al., 2017; Wang et al., 2008)

*CNS: central nervous system; *PNS: peripheral nervous system; *↑ increase; *↓ decrease; *PLC: phospholipase C, *IP3: inositol trisphosphate, *DAG: diacylglycerol, *PKC: Protein Kinase C, *AC: adenylyl cyclase, *cAMP: cyclic adenosine monophosphate, *PKA: Protein Kinase A, *GEF: Guanine nucleotide exchange factors, *PLD: phospholipase D, *Rho: Rho factor.

The research presented in this thesis will focus on signalling by NK_1R and $mGlu_5$, and more detailed information for these specific receptors is provided in the compartmentalised signalling section.

3.5. Compartmentalised signalling by nociceptive GPCRs

In the pain field, it has been recently described that the signalling and trafficking of GPCRs plays an important role in determining the pain signalling profile. For example, the protease-activated receptor 2 (PAR_2) (Defea et al., 2000), calcitonin receptor-like receptor (CLR){Padilla:2007cd} and the neurokinin 1 receptor (NK_1R) {Cattaruzza:2009fb, Pelayo:2011gt, Roosterman:2007gk} elicit a signal from the endosomes and is this endosomal signalling the one that mediates pain transmission (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Yarwood et al., 2017). In addition to PAR_2, CLR

and NK_1R, the metabotropic glutamate receptor 5 (mGluR5), have been recently shown to signal from the nuclear membrane in chronic pain models (O'Malley et al., 2003; Vincent et al., 2016; 2017).

The endosomal network is positioned both temporally and physically between the plasma membrane and other organelles such as the nucleus and lysosomes. In addition, their organised structural network of physically and biochemically distinct membranous domains favours compartmentalised signalling (**Figure 8 & Figure 9**). Temporal regulation enables different activation profiles, which are dependent on the trafficking kinetics of agonist-stimulated receptors. Upon agonist stimulation, some receptors are rapidly redistributed into endosomes and their signalling from this location is dependent on the proportion of receptors undergoing degradation compared to those being recycled.

We know that GPCR signalling is not plasma membrane delimited. Instead, it is a highly dynamic event that can also occur in subcellular compartments, where many internalised receptors can continue to signal from intracellular locations (Defea et al., 2000; Grimes et al., 1996; Irannejad et al., 2017; Jensen et al., 2014; 2017; Tsvetanova et al., 2015; Vilardaga et al., 2014; Vincent et al., 2016). Moreover, these signalling events are distinct from those originating at the plasma membrane and regulated by different mechanisms (Geppetti et al., 2015; Jensen et al., 2014; Murphy et al., 2009; Tsvetanova et al., 2015).

This PhD thesis will discuss the contribution of compartmentalised signalling by NK_1R and $mGlu_5$ receptors to pain transmission. Opportunities to therapeutically exploit these signalling and trafficking relationships will be reviewed in detail below.

3.6. The Neurokinin 1 Receptor (NK_1R) in pain

NK_1R is a GPCR with the highest affinity for the neuropeptide substance P (SP) (Rupniak et al., 2018). NK_1R is predominantly distributed in immune cells, endothelial cells, myenteric neurons and neurons of the PNS and CNS, where SP stimulation can induce plasma leakage, inflammation and pain transmission (Bleazard et al., 1994; Garcia-Recio and Gascón, 2015; King et al., 2005; Laird et al., 2001; Mantyh et al., 2002).

The importance of NK_1R in pain transmission has been studied *in vivo* by the intrathecal administration of various NK_1R antagonists. These studies demonstrated their ability to reduce hyperalgesia initiated by tissue/nerve injury or inflammation in different pain models such as intraplantar injection of carrageenan, formalin or capsaicin (King et al., 2005; Laird et al., 2001; Liu et al., 1994; Liu and Sandkühler, 1997; Mansikka et al., 1999; Santos and Calixto, 1997; Teodoro et

al., 2013). In addition, electrophysiological studies using spinal cord slices have shown that antagonists of the NK_1R can suppress long-term potentiation (LTP) of afferent nociceptive neurons, demonstrating that NK_1R is necessary for the induction of C-fibre evoked spinal facilitation (Coste et al., 2008; Liu and Sandkühler, 1997; Ramírez-García et al., 2019).

Electrophysiological studies indicate that inflammatory pain triggered by capsaicin or formalin increases intracellular levels of Ca^{2+} in DRG neurons, releasing SP in the spinal cord followed by the depolarization of secondary neurons through activation of non-selective cation channels and PKC (Ito et al., 2002). In addition, inhibition of the PKCε isoform decreased capsaicin-stimulated release of glutamate and CGRP in spinal cords and associated pain behaviour. In contrast, the PKCγ isoform contributes to formalin-induced nociception (Sweitzer et al., 2004).

The relevance of NK_1R in pain transmission has been studied using SP conjugated to the ribosome inactivating protein (Saporin; SP-SAP). SP selectively targets NK_1R+ spinal neurons that carry nociceptive signals, and saporin (effector molecule) destroys the targeted neuron. The administration into the intrathecal space, suggests that SP-SAP selectively destroys pain-transmitting neurons, reducing pain sensation in animal models (Nichols et al., 1999; Wiley and Lappi, 1997). Following the same strategy, SP conjugated to *Pseudomonas* exotoxin (SP-PE35) selectively ablates NK_1R expressing cells in the dorsal horn of the spinal cord but not NK_2R or NK_3R expressing neurons when administered in the subarachnoid space. The specific deletion of NK_1R using this approach robustly attenuated thermal and mechanical pain and inflammatory hyperalgesia (Iadarola et al., 2017).

Immunolabeling studies in the spinal cord indicated that the highest density of SP and NK_1R positive nociceptive neurons are found in lamina I and to a lesser extent in lamina II of the dorsal horn (Sakurada et al., 1992; 1995). In addition, Mantyh *et al*., described that acute noxious stimulation by SP or capsaicin induced the **internalisation of NK_1R** in the soma of neurons in lamina I and in dendrites located in lamina III and IV in the spinal cord of rats (Mantyh et al., 1995a; 1995b; 1997).

The cell surface metalloendopeptidase neprilysin degrades SP in the extracellular space, which limits NK_1R activation and terminates its actions at the cell surface (Sturiale et al., 1999). However, once SP bound to NK_1R, the activated receptor interacts with βARRs, which mediates receptor desensitisation and endocytosis (McConalogue et al., 1999). NK_1R recycling and resensitisation requires endosomal acidification and receptor dephosphorylation, promoting dissociation of βARRs to initiate its return to the plasma membrane (Garland et al., 1996). In the recycling and resensitisation process, the metalloendopeptidase endothelin-converting enzyme 1 (ECE-1) degrades SP-bound to

NK_1R in acidified early endosomes, disrupting SP-NK_1R-βARRs complex, resulting in rapid NK_1R recycling to the plasma membrane {Cattaruzza:2009fb, Cottrell:2009hk, Pelayo:2011gt, Jensen:2014ju, Roosterman:2007gk}.

Jensen *et al*., ({Jensen:2017et}) explored the signalling by NK_1R located in endosomes. Their findings suggest that the NK_1R can signal from the plasma membrane and endosomes. NK_1R located in endosomes increases nuclear ERK, cytosolic PKC, and cytosolic cAMP (Jensen et al., 2017). In addition, the administration of the dynamin GTPase activity inhibitor (Dyngo4A) prevented sustained SP-induced excitation of neurons in spinal cord slices, and reduced nociception in the capsaicin model of evoked pain. Furthermore, conjugation of cholestanol (a flexible PEG linker of 27 carbon atoms length) to the NK_1R antagonist Spantide blocked NK_1R endosomal signalling and prolonged the antinociceptive effect of spantide.

The synthesis of NK_1R antagonists started in 1990 due to the high expression of NK_1R and SP in the CNS and its association with several pathologies like depression, anxiety, emesis and pain (Duffy, 2004). Currently available in the market, aprepitant is the first antagonist of NK_1R approved by the FDA (2003, Emed®, Merck) to prevent postoperative and chemotherapy-induced nausea and vomiting (Quartara and Altamura, 2006; Quartara et al., 2009). Aprepitant was demonstrated to be efficacious as an analgesic drug in preclinical studies using animal models of pain. However, like many other NK_1R antagonists, aprepitant was ineffective in managing pain in humans (Quartara et al., 2009). It is postulated that the low efficacy of NK_1R antagonist for managing pain may be due to lack of access to the receptor population located in endosomes directly related to pain transmission.

These findings provide evidence to support the important role that endosomal signalling of the NK_1R plays in pain transmission and identifies endosomal NK_1R as a new target to develop strategies to deliver antinociceptive drugs into intracellular compartments to relieve pain.

3.7. The metabotropic glutamate receptor 5 ($mGlu_5$) in pain

Metabotropic glutamate receptor 5 ($mGlu_5$) is classified as a group I excitatory mGlu receptor, together with $mGlu_1$. $mGlu_5$ is mainly distributed in many regions of the brain, spinal cord, and sensory neurons (Alvarez et al., 2000; Vidnyánszky et al., 1994). $mGlu_5$ plays important roles in the brain such as synaptic plasticity, pain, learning and memory (Pin and Duvoisin, 1995). Dysregulations of $mGlu_5$ signalling is associated with several pathologies including bipolar disorder (Blacker et al.,

2017), neurodegenerative diseases (Akkus et al., 2017; Berry-Kravis et al., 2018; Ribeiro et al., 2017), and pain (Kotecha et al., 2003; Lax et al., 2014; Vincent et al., 2016; 2017).

The participation of $mGlu_5$ in pain has been studied using the selective group I mGluR agonist, (R,S)-3,5-dihydroxyphenylglycine (DHPG). The administration of DHPG causes instantaneous thermal and mechanical pain in control mice that is reduced by the $mGlu_5$ antagonist, 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP) (Fisher and Coderre, 1996). In addition to MPEP, Fenobam, a potent $mGlu_5$ **negative allosteric modulator (NAM)**, is reported to have a robust analgesic effect in models of sciatic nerve ligation and inflammatory pain (Fisher and Coderre, 1996; Honda et al., 2017; Lax et al., 2014; Neugebauer, 2002).

Recently, $mGlu_5$ was found in intracellular compartments, particularly in the inner **nuclear membrane and cytosol** in neuropathic and inflammatory pain model. The activation of nuclear $mGlu_5$ leads to sustained nuclear Ca^{2+} responses and increased phosphorylated-ERK1/2 (pERK) and c-Fos in the nucleus (Vincent et al., 2016; 2017). Furthermore, the blockade of intracellular, but not plasma membrane $mGlu_5$, reduced pERK and Ca^{2+} in the nucleus and suppressed pain behaviour (Vincent et al., 2016; 2017). In addition, the authors described that blocking the **neuronal glutamate transporter (EAAT3)** produced analgesia and decreased c-Fos expression, whereas blocking glial glutamate transporters (EAAT1 & 2) increased pain behaviour and c-fos expression (Vincent et al., 2016; 2017). Importantly, both studies demonstrated that internalised populations of $mGlu_5$ are involved in pain behaviours, supporting the concept that internalised GPCRs may play an important role in pain transmission.

Glutamate (endogenous orthosteric ligand of $mGlu_5$) is recognised by ionotropic, metabotropic receptors as well as transporters, and due to the high sequence similarity in the orthosteric binding site with other mGlu receptors, targeting allosteric binding pockets of $mGlu_5$ has emerged as a promising drug discovery strategy (Gregory and Goudet, 2021; Harpsøe et al., 2015; Leach and Gregory, 2017). Allosteric modulators interact with sites that are distinct from the orthosteric site, allowing simultaneously binding of both an orthosteric and an allosteric ligand to the receptor (Gregory and Goudet, 2021; Neubig et al., 2003). Allosteric modulators that enhance the affinity or efficacy of an orthosteric ligand are known as positive allosteric modulators (PAM), and those that inhibit are known as negative allosteric modulators (NAM) (Neubig et al., 2003).

Negative allosteric modulators (NAMs) of $mGlu_5$ diminish glutamate induced receptor responses in animal models, indicating that $mGlu_5$ inhibition is a viable therapeutic strategy for the treatment of

several pathologies, including pain (Akkus et al., 2017; Berry-Kravis et al., 2018; Matosin et al., 2017; Osikowicz et al., 2013; Pałucha-Poniewiera et al., 2013; Pereira and Goudet, 2018; Rook et al., 2015). Exciting advances in medical chemistry in the recent years have generated numerous allosteric modulators of $mGlu_5$. However, at the present fenobam is the best characterised and most commonly used $mGlu_5$ NAM with demonstrated anti-nociceptive effects in a broad range of models of inflammatory, neuropathic, and visceral pain in rodents (Crock et al., 2012a; 2012b; Lax et al., 2014; Montana et al., 2011; Pereira and Goudet, 2018; Roppe et al., 2004; Vincent et al., 2016). The anti-nociceptive effect of fenobam in pre-clinical models of pain provides strong support for the hypothesis that $mGlu_5$ modulates nociceptive sensitisation in humans.

Recently, a double-blind placebo-controlled study in humans utilising a model of cutaneous sensitisation by capsaicin cream evaluated pharmacokinetics and analgesic effects of fenobam (Cavallone et al., 2020). The study indicates that fenobam is highly tolerated after oral administration but present at highly variable plasma concentrations. In addition, fenobam was associated with a transient reduction in the area of hypersensitivity when plasma concentration were at their peak, but did not produce any persistent anti-hyperalgesic or anti-nociceptive effect compared to placebo (Cavallone et al., 2020). Thus, the variability in the effect of fenobam in human and rodent studies may be due to multiple factors, including the plasma concentration and receptor occupancy, reduced drug effect in humans relative to rodents or, as observed by Vincent and colleagues, $mGlu_5$ expression and signalling from intracellular location in pain conditions. However, a detailed investigation into the process of internalisation and signalling from intracellular locations such as the endosomes remain unexplored. Chapter 4 of this PhD thesis will explore the endosomal signalling of $mGlu_5$ that could open a new target alternative to design novel therapeutic agents.

4. GPCR- TRP channels interactions

Sensory nerves are equipped with receptors and ion channels to detect and respond to diverse thermal, mechanical, and chemical noxious stimuli. These receptors include GPCRs and TRP ion channels. GPCRs can modulate the activity of TRP that are involved in pain transmission (Chen et al., 2016; Peng et al., 2020; Poole et al., 2013; Veldhuis and Bunnett, 2014; Zhao et al., 2014). Thus, it is imperative to understand how GPCRs can influence the activity of TRP channels and how GPCRs may contribute to pain and inflammation through TRP sensitisation.

TRPs are a group of non-selective cation channels located generally in the plasma membrane, allowing ions to pass in and out of the cell. TRP channels are formed by four similar subunits, each with six transmembrane domains and intracellular N and C termini (Clapham, 2003). Some of the members of the TRP family are the vanilloid (TRPV1, 2, 3 & 4), melastatin (TRPM2 & 8) and ankyrin (TRPA1) channels (Sun and Dong, 2016; Veldhuis and Bunnett, 2014). TRP channels play critical roles in various sensory functions, such as thermal, mechanical, pain, itch, and chronic inflammation (Chai et al., 2017; Clapham, 2003; Sun and Dong, 2016; Veldhuis et al., 2015). For example, TRPV1 and TRPA1 are implicated in the transduction of GPCR activation into membrane depolarisation in nociceptive neurons (Ding et al., 2010; Honda et al., 2017).

TRP channels are a major downstream effector of GPCR signalling, and the GPCR-TRP axis is vital for pain, itch, cough, neurogenic inflammation and oedema (Basbaum et al., 2009; Bautista et al., 2006; Grace et al., 2014a; 2014b; Veldhuis and Bunnett, 2014). The signal emanated from activated-GPCRs (protein kinases) can alter TRP channel activity or even increase the expression of TRP channels at the cell surface. GPCR signalling can generate mediators that stimulate and directly activate TRP channels (GPCR-TRP channel coupling) or enhance their responsiveness to TRP activators, a process known as TRP sensitisation (Veldhuis and Bunnett, 2014).

The sensitivity to a ligand, and the magnitude and duration of TRP activation can be augmented by functional interactions (termed 'coupling') with GPCRs. These interactions are bidirectional, where the functional coupling of a GPCR to an ionotropic channel such as TRPs can lead to augmentation of GPCR signalling (Veldhuis and Bunnett, 2014). GPCRs alter TRP activity by two general mechanisms: G_{α}-mediated activation of phospholipases C and A2 (PLC, PLA2) to cleave fatty acids and generate endogenous activators of TRPs such as arachidonic acid (AA) and phosphatidylinositol 4,5-bisphosphate (PIP2). The second mechanism is through activation of PKC and PKA Serine/threonine kinases that phosphorylate the intracellular C-tail of the TRPs to increase cell surface expression and activity (Amadesi et al., 2004; 2006; Geppetti et al., 2015; Meents et al., 2017; Peng et al., 2020; Poole et al., 2013; Premkumar and Ahern, 2000; Veldhuis and Bunnett, 2014; Zhao et al., 2014).

The ability of GPCRs to alter TRP activity is illustrated through the interactions between PAR_1/PAR_2 and TRPV4 (Grant et al., 2007; Peng et al., 2020; Poole et al., 2013). PAR activation can sensitise TRPV4 through channel phosphorylation and enhance TRPV4 signalling through the production of endogenous TRPV4 activators (e.g., arachidonic acid and 5′,6′-EET) (Amadesi et al., 2006; Grant et

al., 2007; Poole et al., 2015). On the other hand, TRPV4 activity augments PAR1- and PAR2-dependent signalling, and this bidirectional PAR-TRPV4 relationship play an important role in PAR-evoked oedema (Grace et al., 2014a; Peng et al., 2020; Poole et al., 2013). Another example is the histamine receptor 1 (H_1R) that activates TRPV1 in sensory neurons by activating the PLC/PKC and PLA2/fatty acid-pathways, leading to scratching in mice (Kim et al., 2004; Shim et al., 2007).

Chapter 5 of this PhD thesis describes a novel mechanism through which TRPV4 contributes to 5-HT_{2A}-induced plasma extravasation in the airways and upper gastrointestinal tract, with evidence supporting a mechanism of action involving SP and CGRP release.

5. Current treatment for pain: Pain management

Worldwide, pain is one of the principal reasons for seeking medical care, where the main reasons are pain associated with surgery, severe illness, joint pain, osteoarthritis, migraine, trauma, childbirth, and burns (Dahlhamer et al., 2018; Mills et al., 2019). Surveys have identified that between 17-30% of adults suffer pain at any given time, with increasing prevalence with advancing age, where more than 40% of patients report insufficient relief of moderate to high levels of pain (Brennan et al., 2007; Dahlhamer et al., 2018; Goldberg and McGee, 2011; Johannes et al., 2010; Millan, 1999; Mills et al., 2019; Nahin, 2015; Vargas et al., 2018). In recent years, many significant advances in basic and clinical pain research have been made. However, the analgesic landscape has not changed dramatically due to the limited availability of effective analgesic agents and the potential abuse of routinely prescribed drugs (Blanch et al., 2014; Dowell et al., 2016; Goodman and Brett, 2017; Retamal et al., 2019). Thus, there is a critical need for more effective and safer analgesics to manage pain.

Pain affects more than 70 million adults in the USA alone (Becker et al., 2018; Gaskin and Richard, 2012; Johannes et al., 2010), with an estimated economic cost of between $560 and $635 billion annually (Gaskin and Richard, 2012). In Australia, the government spent more than $270 million in treatments related to opioid usage in 2012 only (Blanch et al., 2014). In recent years, the misuse of pain medications has grown (Schuchat et al., 2017), emphasizing the critical need to develop new pain medicines with greater efficacy and better safety profiles (Dowell et al., 2016; Goodman and Brett, 2017).

In the midst of a growing opioid crisis, the Centers for Disease Control and Prevention (CDC) has recommended that clinicians consider other medications before turning to opioids for patients with chronic non-cancer pain (Goodman and Brett, 2017). The CDC guidelines also recommended for

safety, and logistical reasons, approved drugs that are currently used for other indications to treat pain as co-adjuvant drugs (e.g., anti-depressants and gabapentinoids (Cooper et al., 2017; Kremer et al., 2016)) as first-line agents for neuropathic pain. However, these recommendations may also lead to safety concerns through excessive usage of gabapentinoids to manage pain (Goodman and Brett, 2017; Johansen, 2018), where their misuse is normally associated with euphoria (Schjerning et al., 2016). Thus, there is an urgent need for effective novel or alternative analgesic agents to advance beyond current mainstay therapies in the clinic.

The most utilised analgesics in the clinic involve non-steroidal anti-inflammatory drugs (NSAIDs), paracetamol, cyclooxygenase-2 inhibitors (coxibs) and opioids. Acute pain is largely and effectively managed by NSAIDs (Ibuprofen, naproxen, etc.) and low doses of opioids (morphine, tramadol). However, these drugs have low and poor efficacy or are associated with significant side-effect liabilities treating chronic pain, as demonstrated in many types of cancer, neuralgia and arthritis (Mantyh et al., 2002).

Nevertheless, the chronic usage of coxibs and NSAIDs have cardiovascular and gastrointestinal side effects (Blanch et al., 2014; Whelton, 2000a), while paracetamol carries a risk of hepatotoxicity with excessive use (Mahadevan et al., 2006). Opioids have well-known side effects, such as tolerance, dependence, nausea, vomiting, constipation, and respiratory depression. In chronic pain cases, the persistent sensation of pain requires long-term administration of analgesics, which exacerbates side effects and can lead to treatment suspension (Blanch et al., 2014; Gerra et al., 2004).

On the other hand, the development of new treatments for pain is challenging because animal pain models **i)** do not represent the complexity of clinical pain conditions and **ii)** do not assess subjective pain experiences; **iii)** preclinical data provide little assurance regarding the direction of new analgesic development; and **iv)** clinical trials routinely use specific population groups and fail to capture the multi-factorial nature of chronic pain (Mao, 2012).

Currently, access to proper treatments to modulate pain is still a major medical challenge. However, despite the advances in medicinal chemistry, with thousands of morphine analogues and structurally distinct opioids, there has not been an appreciable improvement in diminishing the undesired side effects of opioid analgesics (Che and Roth, 2021).

5.1. Paracetamol, NSAIDs and Coxibs

Paracetamol is a para-aminophenol used for the treatment of acute pain. In the clinic, paracetamol is normally administered in addition to opioids as a strategy to reduce opioid consumption and as a synergic combination to manage pain (Busse et al., 2020; Gatti et al., 2010). Additionally, its combination with NSAIDs or caffeine has been shown to be more effective than either paracetamol or NSAID alone (Maund et al., 2011).

NSAIDs are very effective analgesics in various acute pain states. For example coxibs are effective for postoperative pain, lower back pain and inflammatory pain (Bian et al., 2018; Maund et al., 2011; Ramachandran et al., 2012). When given in combination with opioids after surgery, coxibs and NSAIDs reduced opioid consumption more effectively than paracetamol (Gatti et al., 2010; Ramachandran et al., 2012). The primary concern with coxibs and NSAID use is the high dose and dosing frequency required, which can lead to potential renal, cardiovascular and gastrointestinal side effects (Mahadevan et al., 2006; Maund et al., 2011; Whelton, 2000b).

5.2. Local anaesthetics

Local anaesthetics are another strategy to achieve analgesia without a loss of consciousness. Local anaesthetics can be classified into ester (e.g. benzocaine, procaine) or amine (e.g. bupvicaine, lidocaine) anaesthetics, among many others. (Elliott et al., 2019). Local anaesthetics are effective for managing acute and some types of chronic pain, such as neuropathic pain (Elliott et al., 2019). For example, intravenous administration of lignocaine or lidocaine are effective in providing analgesia after a wide range of surgeries (Barreveld et al., 2013). Despite their effectiveness, all local anaesthetics require frequent administration, and they exhibit neurotoxicity when given in high concentrations for a prolonged period (Jevtovic-Todorovic, 2016).

5.3. Tricyclic antidepressant and gabapentinoids

Tricyclic antidepressants (TCAs) and gabapentinoids can be safe and effective alternatives to relieve pain. Since the 1980s, clinical trials have confirmed the benefit of TCA to relieve post-herpetic neuropathic pain (Watson et al., 1982) as well as painful diabetic polyneuropathy (Max et al., 1987; Micó et al., 2006; Perahia et al., 2006). The effectiveness of TCAs may be mediated by the upregulation and release of the anti-inflammatory cytokine IL-10 by peripheral monocytes and macrophages and by increasing inhibitory $GABA_b$ function (Liu et al., 2018; McCarson et al., 2006; Sud et al., 2008).

Gabapentinoids, which include gabapentin and pregabalin, have proved to be clinically effective in several neuropathic pain conditions (Finnerup et al., 2015). Gabapentin, which is FDA approved for post-herpetic neuropathic pain, was initially developed to be an anticonvulsant agent. Gabapentin binds selectively to the α2δ subunit of voltage-dependent calcium channels (VDCCs) (Li et al., 2011; Zamponi et al., 2015), which mediates Ca^{2+} channel influx and neuronal hyperexcitability (Field et al., 2006; Gee et al., 1996; Matsuzawa et al., 2014). In the clinic, antidepressants and gabapentinoids exhibit an efficient antiallodynic/antihyperalgesic effect in neuropathic pain, diabetic polyneuropathy, and arthritis {Martins:2015es, Goodman:2017dia, Liu:2018hc}.

5.4. NMDA receptor antagonists

NMDA is an ionotropic receptor activated by the excitatory neurotransmitter glutamate, which is released centrally after noxious peripheral stimuli (Chizh and Headley, 2005). The NMDA receptor has been associated with hyperalgesia, neuropathic pain, and reduced functionality of opioids (Brenner et al., 2004; Paoletti and Neyton, 2007; Willis, 2001). There are several drugs with NMDA receptor antagonist activity used in the clinic, including ketamine, memantine and amantadine (Hewitt, 2000; Kreutzwiser and Tawfic, 2019). Ketamine is the most well-established NMDA antagonist. It is routinely used in the treatment of pain and is effective in neuropathic and cancer-related pain (Mercadante et al., 2000; Visser and Schug, 2006). However, ketamine use remains a secondary option due to the significant side effects such as hallucinations, confusion, drowsiness and transient loss of consciousness (Allen and Ivester, 2017).

Table 2. Summary of key analgesic classes used in the clinic.

Type of analgesic	Examples	Mechanism of action
Opioids	Codeine, Fentanyl, Hydromorphone, Methadone, Morphine, Oxycodone, Tramadol	↑ MOR, DOR, KOR
Non-selective NSAIDS and Coxibs	Aspirin, Paracetamol, Celecoxib, parecoxib	↓ $COX_{1\text{-}2}$ ↓ prostaglandins and prostacyclin
Local anaesthetics	Lidocaine, procaine, mexiletine, Ropivacaine	↑ $GABA_A$, ↓ NMDA
NMDA-receptor antagonists	Ketamine, dextromethorphan, amantadine, memantine	↓ NMDA
Antidepressant medicines	Amitriptyline, Desipramine, duloxetine, venlafaxine, Milnacipran	↓ VDCCs, ↑ $GABA_b$
Anticonvulsant medicines	Gabapentin, pregabalin, sodium valproate, carbamazepine, oxcarbazepine	↓ VDCCs

cannabinoids and cannabimimetic	Nabiximols, Dronabinol, nabilone, Cannador	↑ CB_{1-2}
Corticosteroids	Dexamethasone, betamethasone, methylprednisolone, prednisolone, ketorolac	↓Prostaglandin, phospholipase

*↑ increase activity, *↓ decrease activity

5.5. Corticosteroids

In the clinic, dexamethasone, betamethasone, methylprednisolone, prednisolone, and ketorolac are the most utilised corticosteroids for pain management. Corticoids reduce pain by inhibiting prostaglandin synthesis and inhibiting phospholipase, which leads to reduction of the inflammation and vascular permeability that promote tissue oedema (Vyvey, 2010). Corticosteroids are particularly useful as adjuvant therapy for metastatic bone pain, neuropathic pain, and visceral pain (Koh et al., 2013). However, corticosteroid usage is associated with fluid retention and electrolyte imbalances, bone demineralisation, gastrointestinal disease, and impaired glucose metabolism (Grennan and Wang, 2019).

6. New pharmacological strategies to relieve pain.

Opioids are the most widely used and effective drugs available to manage pain. However, the chronic use of opioids is associated with a wide range of detrimental and debilitating side effects, leading to the need to develop or redesign currently available drugs to manage pain without side effects associated with their usage. Many new strategies and drug discovery programs have been initiated to identify novel targets and develop improved analgesics. Some of these new strategies will be discussed below.

6.1.1 Chemical modification to target the mu opioid receptor (MOR)

Pain associated with tissue damage or inflammation is often characterised by acidosis, decreasing the acidity in the injured area (pH < 7). The decrease of pH allows for redesigning agonists of the mu opioid receptor (MOR) to bind the receptor only under acid conditions. Protonation of the MOR agonist fentanyl is essential for binding to the MOR and its dissociation constant (pK_a) of 8.43, allows it to be in its protonated form at physiological pH. Fluorination of fentanyl lowered its pK_a to 6.8, allowing the protonation to occur at pH 6.8, hence leading to activity only in conditions of acidosis (Thurlkill et al., 2005). This new molecule, NFEPP, has been successfully used in animal models of peripheral nerve injury, sciatic nerve injury, and abdominal pain, where a decrease of side effects

associated with the usage of fentanyl was observed (Jiménez-Vargas et al., 2021; Machelska and Celik, 2018; Spahn et al., 2017).

6.1.2 Bivalent and Bifunctional ligands

Chemokine receptor 5 (CCR5), CB1, and mGluR5 are some examples of GPCRs co-expressed with MOR on nociceptive neurons (Maguire and France, 2014; Rios et al., 2006; Schröder et al., 2009).

Bivalent hybrid (bivalent ligands) compounds have emerged as a safe strategy to manage pain while decreasing opioid-related side effects (Cataldo et al., 2019; Foster and Conn, 2017; Le Naour et al., 2013; Machelska and Celik, 2018; Peterson et al., 2017). Based on the evidence of co-expression of different excitatory and inhibitory GPCRs on nociceptive neurons, bivalent ligands have been structurally designed to simultaneously deliver an opioid agonist with a GPCR antagonist. Bivalent ligands are comprised of pharmacophores of mu agonist (oxymorphamine) connected to $mGlu_5$ antagonist (MPEP), CB1 inverse agonist (Rimonabant) or CCR5 antagonist (TAK220) through a spacer linker of varying length (20-22 atoms) to target MOR heteromers (Akgün et al., 2013; 2015; 2019; Guillemyn et al., 2015; Le Naour et al., 2013; Peterson et al., 2017; Starnowska et al., 2017).

The effectiveness of MOR agonists is increased upon co-administration of an antagonist for another GPCR such as mGlu5 or CCR5, which is associated with decreased MOR phosphorylation, internalisation and desensitisation (Schröder et al., 2009). Bivalent hybrid compounds are effective at decreasing pain behaviour in models of inflammatory pain, bone cancer pain and chemotherapy-induced peripheral neuropathy, and decreasing side effects associated with opioid usage (Akgün et al., 2015; 2019; Cataldo et al., 2019; Guillemyn et al., 2015; Le Naour et al., 2013; Peterson et al., 2017; Starnowska et al., 2017).

6.1.3 Gene therapy

Gene therapy or gene transfer refers to introducing, expressing, silencing, or interfering DNA or RNA in a specific tissue or cell. There are three possible gene therapy approaches: i) cell-based therapies, such as transplantation of transformed cells, ii) delivery of plasmid or oligonucleotides, encapsulated in liposomes and, iii) packaging in viral-based vectors (Guedon et al., 2015; Li and Samulski, 2020).

Viral vectors, normally based on herpes simplex virus-1 (HSV-1), have been used to transfer the enkephalin precursor proenkephalin (PENK) gene in the dorsal root ganglia, leading to decreased pain in capsaicin, formalin and bone cancer pain models (Goss et al., 2001; 2002; Wilson et al., 1999). In addition, the HSV-1-vector has been used to deliver mRNA to express anti-inflammatory cytokines

such as IL-4, IL-10 and L-13 (Hao et al., 2003; Meunier et al., 2005). Recently, adeno-associated virus (AAV) vector was used to encode glutamate decarboxylase 65 (GAD65), glial cell-derived neurotrophic factor (GDNF) and IL-10 genes, showing a potent efficacy in spared nerve injury model by decreasing inflammation and fibre hyperexcitability that provides long-lasting analgesia (Kim et al., 2020).

7. Targeting endosomal signalling using nanoparticles

Over the last 20 years, nanomaterials and nanoparticles have been developed and investigated as drug carriers due to their ability to improve existing treatments by enhancing drug tolerability, circulation half-life and specificity (Ojea-Jiménez et al., 2013; Uhrich et al., 1999). Nanoparticles have been designed using a wide number of different materials, variable in chemical nature that can be classified into four categories; i) carbon nanostructures, ii) polymeric-based, iii) lipid-based and iv) inorganic nanocarriers (Hawkins et al., 2008).

When we think about targeting and blocking at specific locations, the first issue are the spatial aspects of delivery. With this concept in mind, nanoparticles and nanomaterials are designed and developed to interact in a biological environment and respond to specific stimuli such as changes in pH (e.g., reduced pH in endosomes or in tissues such as the gastrointestinal tract or solid tumours), temperature or oxygen concentration. Once nanoparticles interact with their stimuli, an environmentally-induced 'change to the properties of the material' typically leads to the release of the loaded drug at the target site (Gao et al., 2010a; Hawkins et al., 2008; Zhu et al., 2012).

To achieve spatially-directed delivery, nanocarriers must reach the bloodstream for sufficiently long periods to accumulate in the target tissue. However, nanocarriers are susceptible to opsonisation (capability of the immune system to recognise and coat antigens or exogenous bodies with antibodies for further elimination), retention and clearance, impeding accumulation. One strategy to overcome this difficulty is to coat nanocarriers with water-soluble polymers, increasing the molar mass and biocompatibility, reducing kidney clearance, and preventing opsonisation by the immune system (Owens and Peppas, 2006).

The use of nanocarriers offers an advantage of controlling and prolonging drug release and a stable drug concentration maintained in the therapeutic range for an extended period of time, decreasing drug toxicity. Overall, nanocarriers enhance the pharmacokinetics of drugs by maintaining a stable

drug concentration in the target tissue that avoids toxic peaks and side effects, reduces the frequency of administration, and protects the drug from degradation.

7.1.1 Nanomedicine and endosomal targeting to achieve analgesia pain

At present, there are over 50 FDA-approved nanomedicines for drug delivery, of which only five are indicated for the treatment of pain. SkyePharma Inc. develops DepoDur, the first FDA-approved liposome-based nanoparticles that increase drug release time, which are used for loading morphine sulphate. DepoDur is a liposome injection indicated to treat postoperative pain following major surgical procedures (Gambling et al., 2005).

Another example is Exparel, a liposome-based nanoparticle for prolonged-release of the local anaesthetic bupivacaine. The ensemble of bupivacaine into liposome increases the time-release effects by 72 hours (Richard et al., 2012). Exparel administration resulted in reduced length of hospitalisation, hospital cost per patient, opioid consumption and necessity for postoperative narcotic medications.

Transdermal or oral transmucosal systems for the prolonged release of fentanyl are also approved and are currently available marketed as DURAGESIC and Actiq, respectively. DURAGESIC is indicated for the management of moderate to severe chronic pain. Meanwhile, Actiq is indicated for breakthrough pain in cancer patients (Beiranvand and Sorori, 2019; Bulbake et al., 2017; Saraghi and Hersh, 2013).

In preclinical studies, Poly(lactic-co-glycolic acid) (PLGA), nanocarriers loading tramadol hydrochloride and functionalised with the glycoproteins transferrin and lactoferrin, enhanced the pharmacological effect and circulation times of tramadol (Lalani et al., 2013). Another key example is the use of PLGA-ketamine as an adjuvant for neuropathic pain, as the PLGA formulation increases the analgesia time of ketamine up to 34h (Han et al., 2018; 2015).

Polymeric nanocarriers (poly-e-caprolactone and polyethene glycol) loaded with minocycline (an antibiotic with microglial inhibition activity) were tested in a spinal cord injury model. Nanocarriers decreased the expression of IL-6 in the spinal cord, decreasing hyperalgesia and thermal allodynia in rodents (Papa et al., 2013).

Subcellular compartments such as endosomes and lysosomes can be targeted using nanocarriers to release and increase intracellular drug accumulation without disrupting endosomes. For example, pH-responsive nanoparticles targeting endosomes take advantage of the acidic pH of endosomes as a triggering stimulus to deliver a drug and effectively block endosomal signalling. Another example of

nanoparticles targeting organelles in the cells are LED light-responsive nanoparticles to target the nucleus (Liu et al., 2021; Peng et al., 2017; Zelmer et al., 2020).

The CLR/RAMP1, NK_1R and PAR_2 are demonstrated to mediate nociceptive signals from their endosomal location (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Yarwood et al., 2017). Ramírez-García *et al.,* showed that by selectively targeting endosomal receptors by pH-responsive nanoparticles, superior analgesia is achieved. Importantly, these studies repurpose clinically approved drugs for other conditions to target endosomal targeting selectively.

Nevertheless, several requisites need to be fulfilled in order to target endosomal signalling effectively. First, the drug needs to be able to cross the plasma membrane. Second, the drugs need to enter into the endosomes and remain for a sufficient period to effectively block the receptor to decrease its signalling without disrupting the endosome formation and structure.

One strategy to achieve this goal is to use lipidated antagonists to promote endosomal delivery and retention. Drugs are conjugated to membrane lipids, such as cholestanol that anchors to the plasma membrane allowing antagonist passive internalisation. An example is proposed by Jensen *et al*. (2017). Here, the authors used a cholestanol conjugated-NK_1R antagonist (Spantide) for endosomal delivery, promoting incorporation into endosomes to block pain transmission (Jensen et al., 2017). Additional studies by Yarwood *et al*. and Jimenez-Vargas *et al*. supported the utilisation of conjugated antagonists for the successful targeting of endosomal CLR/RAMP1 and PAR_2 receptors, respectively (Jimenez-Vargas et al., 2018; Yarwood et al., 2017). However, one of the disadvantages of this strategy is the slow internalisation rate of cholestanol conjugated-drugs that required at least 3 h for accumulation into endosomes *in vitro* (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Mai et al., 2021; Yarwood et al., 2017). A suitable alternative for selective drug delivery into endosomes is the use of nanoparticles. However, to successfully achieve endosomal delivery, nanoparticle disassembly must be fast, and it must also respond to subtle changes of pH encountered in the endosomes.

The studies presented in this PhD thesis provide proof of the concept that nanotechnology may have potential utility in targeting or redistributing drug cargo to specific location in the cells, such as endosomes, to achieve greater drug efficacy and kinetics. I have focused on delivery drugs to pain-transmitting GPCR targets in the central nervous system, which warrants some discussion about the challenges of administration routes and limitations of drugs or nanomaterials that can pass the BBB. Approximately more than 98% of small molecules and most large molecules cannot reach the brain through the blood-brain barrier (Arvanitis et al., 2020). While it is known that some small molecules

can pass the blood-brain barrier, including the NK1R antagonist Aprepitant that was studied in Chapter 3 (Ramírez-García et al., 2019), their ability to target specific cellular membranes is a potential limitation for achieving maximal efficacy and therefore poses a challenge for finding the balance between dose and safety. To address this, nanotechnology has emerged as a potential solution for increasing drug concentrations in specific cellular sites or tissues. Indeed, there are an increasing number of studies that support this concept, and this includes programs focused on developing new therapies via improved brain delivery (Teleanu et al., 2018; Asefy et al., 2021).

Drug targeting and delivery to the brain represent critical challenges due to the blood-brain barrier, being a tightly regulated series of cells that enable uptake of required nutrients while protecting the brain against foreign substances, including pathogens and therapeutic agents (Patel and Patel, 2017). BBB is formed by the brain microvascular endothelial cells, and the blood-cerebrospinal fluid barrier, comprised of the epithelial layer of choroid plexus, the cerebral ventricles, and the arachnoid mater covering the outer brain surface (Teleanu et al., 2018). Some of the main routes for nanoparticles delivery to the central nervous system includes nasal and systemic administration (Teleanu et al., 2018).

Nanomaterials can also be engineered in various ways to optimize BBB permeability. This includes chemical modification through addition of targeting ligands, for example, with conjugation to molecules such as albumin, or by making nanoparticles that a small enough to freely move across the BBB through junctions (between cells) or via transcellular pathways, typically following uptake into the endothelial endocytic network. In our studies, nanoparticles were injected direct to the spinal cord to avoid the low nanoparticles permeability through the BBB and enhance nanoparticles disposition on the CNS.

In this thesis, nanoparticles were administered directly into the spinal cord avoiding problem with the nanoparticles the crossing the BBB. However, if you were progress this further as a therapeutic, approaches such as these would be critical, given that intrathecal administration is a challenging drug administration route in the clinic, that is typically avoided and only used in extreme cases of chronic pain.

The pathways for delivering therapeutic agents to the brain can either be invasive or non-invasive. The non-invasive administration strategies are based on the anatomical structure of the brain capillaries, vessels that form a tight barrier as a part of a neurovascular unit, that includes pericytes and astrocytes, and extracellular environment [3,5,](Bernardo-Castro et al., 2020)

Nanotechnology is an emerging field that encompasses knowledge from multiple disciplines and represents the capacity to manipulate and control matter at atomic levels. Therefore, the implication of nanotechnology for the development of non-invasive drug delivery strategies could lead to the design of novel and improved formulations to enhance the delivery of therapeutic agents across the blood-brain barrier (Dong, 2018; Wu et al., 2019). However, further research is needed to understand and mediate the blood-brain barrier crossing mechanisms and improve brain delivery methods' efficiency using nanotechnology.

Chapter 4 demonstrates that mGlu5 signals from endosomes, and that this signalling depends on the nature of the agonist used and their availability in the cytosol. Additionally, we demonstrated that pH-responsive nanoparticles loaded with a mGlu5 NAM are a potent tool to modulate pain transmission by decreasing neuronal excitability and nociceptive neuron firing compared to free drug *in vivo*.

Lastly, Chapter 5 expands our understanding of GPCR-TRP interactions, particularly how the serotonin subtype 2A receptor ($5HT_{2A}$) interacts with TRPV4 to enhance vascular permeability in the airways and upper gastrointestinal tract *in vivo*.

This thesis demonstrates the importance of endosomal signalling in pain transmission and supports the utilisation of pH-responsive nanoparticles as a promising tool for endosomal targeting of receptors. Additionally, endosomal signalling by receptors may explain the failure of many drugs at the clinical level. Finally, this thesis also investigates the GPCR-TRP axis, a crucial interaction in pain and inflammation, where the interaction of TRPV4 contributes to $5\text{-}HT_{2A}$-induced plasma extravasation, with evidence supporting a neurogenic mechanism of action. Our novel findings open new opportunities for therapeutic drugs to decrease inflammation.

8. References

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Chapter 2:

Internalized GPCRs as Potential Therapeutic Targets for the Management of Pain

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Internalized GPCRs as Potential Therapeutic Targets for the Management of Pain

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Peripheral and central neurons in the pain pathway are well equipped to detect and respond to extracellular stimuli such as pro-inflammatory mediators and neurotransmitters through the cell surface expression of receptors that can mediate rapid intracellular signaling. Following injury or infection, activation of cell surface G protein-coupled receptors (GPCRs) initiates cell signaling processes that lead to the generation of action potentials in neurons or inflammatory responses such as cytokine secretion by immune cells. However, it is now appreciated that cell surface events alone may not be sufficient for all receptors to generate their complete signaling repertoire. Following an initial wave of signaling at the cell surface, active GPCRs can engage with endocytic proteins such as the adaptor protein β-arrestin (βArr) to promote clathrin-mediated internalization. Classically, βArr-mediated internalization of GPCRs was hypothesized to terminate signaling, yet for multiple GPCRs known to contribute to pain, it has been demonstrated that endocytosis can also promote a unique "second wave" of signaling from intracellular membranes, including those of endosomes and the Golgi, that is spatiotemporally distinct from initial cell-surface events. In the context of pain, understanding the cellular and molecular mechanisms that drive spatiotemporal signaling of GPCRs is invaluable for understanding how pain occurs and persists, and how current analgesics achieve efficacy or promote side-effects. This review article discusses the importance of receptor localization for signaling outcomes of pro- and anti-nociceptive GPCRs, and new analgesic opportunities emerging through the development of "location-biased" ligands that favor binding with intracellular GPCR populations.

Keywords: pain, analgesia, GPCR, trafficking, endosome, drug delivery, signal transduction

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Abbreviations: GPCRs, G protein couple receptors; βArr, adaptor protein β-arrestin; FDA, Food and Drug Administration; NSAIDs, non-steroidal anti-inflammatory drugs; Coxibs, cyclooxygenase-2 inhibitors; CB1-2, Cannabinoid 1-2 receptors; SP, Substance P; NK_1R, Neurokinin receptor 1; CGRP, Calcitonin gene-related peptide; CLR, Calcitonin receptor-like receptor; RAMP1, Receptor activity-modifying protein; MOR, Mu-opioid receptor; DOR, Delta-opioid receptor; mGluR5, Metabotropic glutamate 5 receptor; PAR_2, Protease-activated receptor-2; 5-HT, serotoninergic; CCR5, Chemokine receptor 5; GRKs, GPCR kinases; PTHR, parathyroid; TSHR, thyroid-stimulating hormone; β_1AR, β_1 Adrenergic; NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide; ERK, Extracellular signal-regulated kinases; PKC, Protein kinase C; cAMP, Cyclic adenosine monophosphate; FRET, Resonance Energy Transfer; BRET, Bioluminescence Resonance Energy Transfer; ER, endoplasmic reticulum; TRPV1, TRPV4, Transient receptor potential cation channel subfamily V member 1-4.

INTRODUCTION

The sensation and transmission of pain are essential physiological processes that allow us to detect and react to harmful stimuli and initiate inflammatory responses to protect damaged tissue and promote wound healing. Peripheral and central processes that lead to pain transmission are highly adaptive, and the pain experienced is usually proportional to the extent of the injury. As a part of this adaptive physiological response, a heightened sensitivity to pain occurs to provide awareness of damaged tissue and maintain protective behavior for the duration of an injury.

As healing occurs, this sensitization typically reduces over time. In contrast, in chronic inflammatory and neuropathic pain conditions such as arthritis, fibromyalgia or diabetic-related neuropathy, where damaged tissue is unable to heal or inflammatory mediators continue to be produced, this sensitization fails to diminish and can cause significant discomfort and loss of function over extended time periods (Scholz and Woolf, 2002). This is typically described through two phenomena: (a) *allodynia*, where one feels pain in response to a normally non-painful stimulus; and (b) *hyperalgesia*, where one experiences an exacerbated pain sensation to a moderately painful stimulus (Baron, 2006; Steeds, 2016). Due to the complexity of chronic pain and significant limitations with safety and compliance for available analgesics, these conditions are extremely difficult to manage, thus impacting the quality of life for many patients.

Despite many advances in basic research and in the clinic, the analgesic landscape in recent decades has seen few changes, due to the limited availability of effective analgesic agents and the potential for abuse of routinely prescribed drugs (Dowell et al., 2016; Goodman and Brett, 2017). In the midst of a growing opioid crisis (Schuchat et al., 2017), the development of new pain medicines is becoming increasingly important. For safety and logistical reasons, the most obvious gains can be made by repurposing Food and Drug Administration (FDA)-approved drugs that are currently used for other indications (e.g., anti-depressants; Kremer et al., 2016; Cooper et al., 2017) or re-formulating established analgesics such as opioids to improve pharmacokinetic profiles (Saraghi and Hersh, 2013). However, new and effective therapeutic approaches may also be gained through greater characterization of the underlying cellular and molecular mechanisms that lead to pain, as a means to identify new molecular targets and further define how analgesic side-effects occur and can be avoided.

G protein-coupled receptors (GPCRs) are important mediators of pain or analgesia and many of these receptors participate in dynamic trafficking processes such as endocytosis, as a part of their activity cycle. It is now evident that receptor trafficking is also critical for the initiation of spatially and temporally distinct signaling events, and importantly, some of these location-specific or compartmentalized processes are associated with greater modulation of pain (Geppetti et al., 2015; Irannejad et al., 2017; Stoeber et al., 2018; Thomsen et al., 2018). Here, we address limitations of the current analgesic landscape and look to new drug discovery studies focused on GPCRs that participate in dynamic trafficking processes in neurons. New biophysical tools that have been used to characterize compartmentalized signaling reveal how the membrane partitioning properties of drugs influence their functional selectivity for location-specific processes. This knowledge has been exploited through the use of lipid-anchored drug conjugates that increase GPCR targeting in specific subcellular domains, to enhance analgesic outcomes through the inhibition of endosomal signaling.

CHALLENGES AND LIMITATIONS OF CURRENT ANALGESICS

Chronic or persistent pain incorporates a complex range of disorders that requires a combination of non-pharmacological and pharmacological approaches for treatment. From a pharmacological perspective, treatment is possible by administering one or more therapeutic agents such as paracetamol/acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase-2 inhibitors (Coxibs) followed by careful use of opioids for elevated pain (e.g., morphine or oxycodone). Unfortunately, each of these drugs has associated side-effects that limit their use. NSAIDs and Coxibs have potential cardiovascular and gastrointestinal side effects (Whelton, 2000), and should be used more sparingly than paracetamol/acetaminophen, which carries a risk of hepatotoxicity with excessive use (Mahadevan et al., 2006). While opioids remain some of the most effective analgesics available in the clinic, they have a high abuse potential due to their euphoric or addictive properties, and where repeated use leads to receptor desensitization and tolerance. To overcome tolerance, patients with chronic pain can be subjected to sustained increases in dosing or switching to other more potent opioids to improve analgesia, which often provides only temporary gains in pain relief. However, this approach may increase the risk of tolerance and addiction over time, in addition to increasing the likelihood of debilitating side-effects such as constipation and respiratory depression (Corbett et al., 2006; Boudreau et al., 2009; Volkow et al., 2011).

Alternative GPCR targets have been identified to reduce reliance on opioid analgesics. Cannabinoids, which are proposed as effective opioid alternatives, reduce pain through activation of $G_{i/o}$-coupled cannabinoid receptors (primarily CB_1), which leads to the downregulation of excitatory processes, and modulation of serotoninergic (5-HT) and noradrenergic pathways. Although widely available and used for millennia, we are yet to see the outcomes of systematic use in the clinic for treating pain, and it is also acknowledged to lead to behavioral risks that require further investigation (Mendiguren et al., 2018). Gabapentinoids such as gabapentin or pregabalin, target the α2δ subunit of voltage-gated calcium channels and have been approved as first-line medications to manage neuropathic pain (e.g., postherpetic neuralgia, fibromyalgia). These were initially used for the treatment of epilepsy, and in some cases for anxiety disorders. Although regarded as relatively safe drugs, safety concerns for gabapentinoids have grown and include excessive usage and behavioral risks such as suicidal behavior

(Johansen, 2018; Molero et al., 2019). Together, this provides a small insight into established and emerging risks associated with common analgesics. This raises the question of whether any of these compounds can be modified to improve their safety profiles and if new or emerging targets are available. We discuss these points below in the context of receptor trafficking, which is a critical component of the activity cycle for many molecular pain targets.

TARGETING GPCRs FOR THE TREATMENT OF PAIN

Members of the GPCR superfamily are considered to be druggable targets due to high levels of cell surface expression and their ability to contribute to all pathophysiological processes, including pain. Accordingly, GPCR-selective drugs represent more than one-third of all FDA-approved medicines (Hauser et al., 2017). There are at least 40 members of the GPCR family that are considered to be potential therapeutic targets for the regulation of pain (Stone and Molliver, 2009). Yet despite advanced drug discovery programs for multiple receptors, and abuse concerns for opioid receptors, very few targets have clinically succeeded beyond opioids in the past decade, with notable exceptions being the recent approval of Fremanezumab, Eptinezumab, Galcanezumab, and Erenumab for treatment of migraine, being monoclonal antibodies that target the neuropeptide calcitonin gene-related peptide (CGRP) or its receptor, Calcitonin Receptor-Like Receptor/Receptor Activity-Modifying Protein 1 (CLR/RAMP1; see review by Scuteri et al., 2019).

There are a number of challenges in the early phase of analgesic drug discovery for GPCRs. This includes safety concerns for targets that have overlapping functions in other tissues, and inaccurate evaluations of efficacy when using relatively simplified rodent-based pre-clinical pain models to represent the complexity of clinical pain conditions or characterize human-selective compounds (Mao, 2012). Furthermore, the localization of receptors in pre-synaptic and post-synaptic neurons is critical for the activity cycle and nociceptive outputs of several GPCRs (**Figure 1**). On a cellular level, considerations for the intracellular disposition of analgesics and their ability to regulate receptor trafficking and localization have also recently been proposed to be an important part of the drug discovery process (Jensen et al., 2017; Yarwood et al., 2017; Jimenez-Vargas et al., 2018; Stoeber et al., 2018).

RECEPTOR TRAFFICKING LEADS TO SPATIOTEMPORALLY DISTINCT SIGNALING PROCESSES

GPCRs are highly dynamic proteins that achieve distinct signaling outcomes by adopting different conformational states (Rasmussen et al., 2011; Latorraca et al., 2017). Extracellular ligands that bind cell surface GPCRs promote receptor conformations that activate heterotrimeric G proteins to transduce downstream signaling and also favor phosphorylation by GPCR kinases (GRKs). This phosphorylation occurs primarily at the C-terminus to enhance engagement with β-arrestins (βArrs), which can function as adaptor proteins to mediate distinct signaling processes such as MAPK activity, and also facilitate interactions with clathrin-coated membranes to promote endocytosis into endosomes (Ferguson et al., 1996). This was historically considered to facilitate termination of signaling by targeting receptors to degradative pathways, or rapid receptor recycling to reset the activity cycle during the internalization process, and increase the potential for sustained signaling once the receptor is recovered at the plasma membrane (PM; Ferguson et al., 1996; Shukla et al., 2014).

A more recent theory has emerged, suggesting that a third trafficking possibility exists, whereby receptors can remain on intracellular membranes such as endosomes for sustained periods of time, to facilitate distinct signaling processes in a βArr- or a G protein-dependent manner. This paradigm shift was initially revealed by studies on Gs-coupled receptors such as the parathyroid (PTHR), thyroid-stimulating hormone (TSHR) and β2 adrenergic receptors to demonstrate that endosomal-mediated sustained cyclic adenosine monophosphate (cAMP) production could be observed after endocytosis has occurred (reviewed in detail by Vilardaga et al., 2014; Tsvetanova et al., 2015; Thomsen et al., 2018).

The development of genetically encoded tools such as conformation-selective nanobodies, Förster/Fluorescence Resonance Energy Transfer (FRET) or Bioluminescence Resonance Energy Transfer (BRET) biosensors, have provide highly sensitive approaches for observing and measuring dynamic activation states and spatiotemporal signaling [e.g., compartmentalized cAMP production, Protein kinase C (PKC) and Extracellular signal-regulated kinases (ERK) activity] of GPCRs in real-time (Irannejad et al., 2017; Halls and Canals, 2018). Given the prevalence and importance of trafficking GPCRs in neurons, the internalization and location-specific signaling of several GPCRs with established roles in pain have been described, including but not limited to the Neurokinin 1 Receptor (NK_1R), CLR/RAMP1, metabotropic glutamate receptor 5 (mGluR5), chemokine receptor (CCR1), Protease-Activated Receptor 2 (PAR_2) and the Mu Opioid Receptor (MOR; Mantyh et al., 1995a; O'Malley et al., 2003; Gilliland et al., 2013; Poole et al., 2015; Jensen et al., 2017; Yarwood et al., 2017; Stoeber et al., 2018). An overview of these trafficking outcomes is summarized in **Table 1**, to reveal how stimulation with endogenous ligands alters receptor localization *in vitro*, or in pre-clinical pain models.

LIGANDS EXERT LOCATION BIASED EFFECTS BY ACCESSING DIFFERENT RECEPTOR POOLS

More recently, conformation-selective single-domain camelid antibodies (nanobodies) that can detect and bind active-state receptors have been instrumental for advancing this concept to other organelles. Distinct nanobody clones that are known to engage with the β_1 Adrenergic Receptor (β_1AR) or MOR

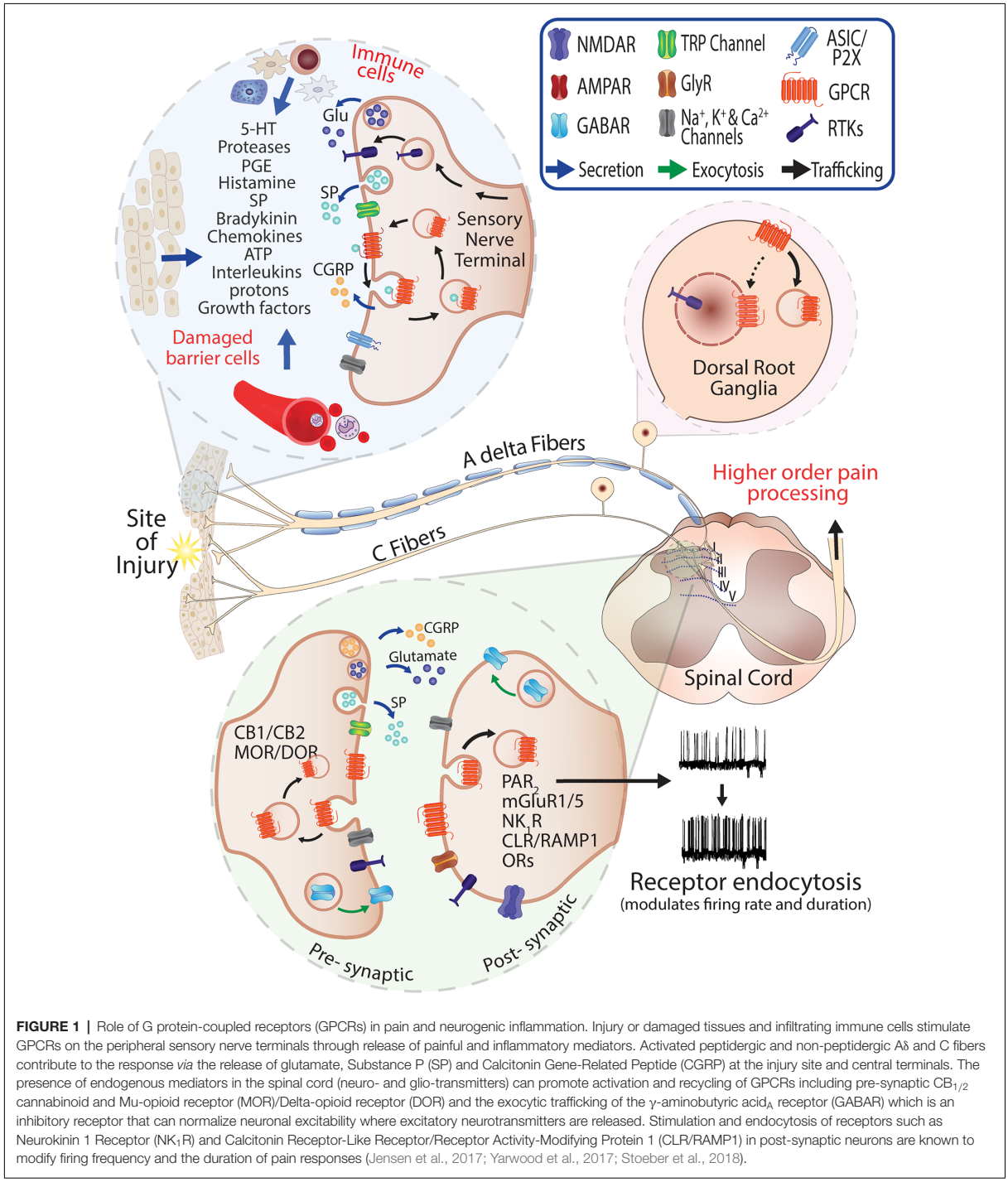

FIGURE 1 | Role of G protein-coupled receptors (GPCRs) in pain and neurogenic inflammation. Injury or damaged tissues and infiltrating immune cells stimulate GPCRs on the peripheral sensory nerve terminals through release of painful and inflammatory mediators. Activated peptidergic and non-peptidergic Aδ and C fibers contribute to the response *via* the release of glutamate, Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP) at the injury site and central terminals. The presence of endogenous mediators in the spinal cord (neuro- and glio-transmitters) can promote activation and recycling of GPCRs including pre-synaptic $CB_{1/2}$ cannabinoid and Mu-opioid receptor (MOR)/Delta-opioid receptor (DOR) and the exocytic trafficking of the γ-aminobutyric $acid_A$ receptor (GABAR) which is an inhibitory receptor that can normalize neuronal excitability where excitatory neurotransmitters are released. Stimulation and endocytosis of receptors such as Neurokinin 1 Receptor (NK_1R) and Calcitonin Receptor-Like Receptor/Receptor Activity-Modifying Protein 1 (CLR/RAMP1) in post-synaptic neurons are known to modify firing frequency and the duration of pain responses (Jensen et al., 2017; Yarwood et al., 2017; Stoeber et al., 2018).

have been shown to be recruited to the Golgi apparatus in a GPCR activity-dependent manner independently from initial stimulation at the cell surface. Specifically, this is achieved using relatively lipophilic ligands that can freely diffuse throughout the cell, or hydrophilic compounds that are proposed to access Golgi pools *via* transporters (Irannejad et al., 2017; Stoeber et al., 2018).

These important pharmacological insights have significant implications for understanding how drugs may exert their

TABLE 1 | Receptors in pain pathways that undergo stimulation-induced endocytosis.

Receptor family	Endogenous stimuli	Localization (unstimulated)	Pain/Stimulus-induced trafficking	Reference
Mu and Delta Opioid Receptors (MOR, DOR)	Enkephalins Dynorphins	PM TGN	PM → Endosomes Direct activation on TGN by morphine	Sternini et al. (1996), Haberstock-Debic et al. (2005) and Stoeber et al. (2018)
Endocannabinoid Receptors (CB1, CB2)	AEA 2-AG	PM	PM, Endosomes	Rozenfeld and Devi (2008), Lever et al. (2009) and Flores-Otero et al. (2014)
Metabotropic Glutamate Receptor 5 (mGluR5)	Glutamate	PM ER Nucleus	PM Direct activation on Nuclear inner membrane	O'Malley et al. (2003) and Vincent et al. (2016, 2017)
Protease-Activated Receptor 2 (PAR_2)	Trypsin, Tryptase, Elastase, Cathepsin S	PM TGN	PM → Endosomes PM → Lysosomes	DeFea et al. (2000), Ricks and Trejo (2009) and Jimenez-Vargas et al. (2018)
Neurokinin 1 Receptor (NK_1R)	Substance P Neurokinin A/B	PM	PM → Endosomes	Mantyh et al. (1995a,b) and Jensen et al. (2017)
Calcitonin Receptor-Like Receptor; Receptor Activity-Modifying Protein 1 (CLR/RAMP1)	CGRP Amylin	PM	PM → Endosomes	Padilla et al. (2007) and Yarwood et al. (2017)
Angiotensin Receptor 1 (AT_1R)	Angiotensin II	PM	PM → Endosomes	Hein et al. (1997)
5-Hydroxytryptamine Receptor (5-HT2A)	Serotonin	PM	PM → Endosomes	Bhattacharyya et al. (2002) and Freeman et al. (2006)

PM, plasma membrane; ER, endoplasmic reticulum, TGN, trans-Golgi Network; → denotes direction of receptor trafficking, from unstimulated receptor location to stimulated receptor location.

effects (or side-effects) and are consistent with other receptors that contribute to pain transmission. For example, endogenous peptide-based enkephalins can stimulate MOR and Delta-Opioid Receptor (DOR) to activate rapid signaling processes in micro-domains of the cell surface and sustained signaling from endosomes (Finn and Whistler, 2001; Groer et al., 2011; Halls et al., 2016; Stoeber et al., 2018), whereas non-peptide opioids such as morphine can freely diffuse through cells to stimulate Golgi pools of the MOR, and initiate a spatiotemporally distinct wave of signaling. The importance of opioid-induced Golgi signaling for analgesia and its association with safety outcomes remains to be determined *in vivo* (Stoeber et al., 2018).

Under pathological pain conditions, the excitatory mGluR5 has been detected in intracellular locations, including the inner nuclear membrane and endoplasmic reticulum (ER; Jong et al., 2014; Purgert et al., 2014; Vincent et al., 2016, 2017). Stimulated mGluR5 couples with $G\alpha_q$ to evoke cytoplasmic and nuclear calcium mobilization (Jong et al., 2009). Furthermore, in models of spared-nerve injury (Vincent et al., 2016) and inflammatory pain (Vincent et al., 2017), 60% of the mGluR5 receptor population was shown to be localized to the inner nuclear membrane in spinal dorsal horn neurons (Vincent et al., 2016). Importantly, activation of nuclear mGluR5 leads to sustained nuclear Ca^{2+} signaling, phosphorylation of ERK1/2 and induction of c-fos expression, leading to increased nociceptive hypersensitivity (Lee et al., 2008; Jong et al., 2009; Purgert et al., 2014; Vincent et al., 2016, 2017). Blockade of cell surface mGluR5 by the impermeable antagonist LY393053 resulted in limited analgesia and modest reductions in second messenger coupling. In contrast, the membrane-permeable antagonist fenobam significantly reduced mechanical allodynia, MAP kinase (ERK1/2) phosphorylation and c-fos expression in a spared-nerve injury pain model. Although these differences may be caused by a range of factors including drug disposition and differences in potencies, it may also provide indirect evidence for the initiation of distinct mGluR5-dependent pain responses from different cellular locations (Lax et al., 2014; Vincent et al., 2016, 2017). Focused drug discovery around cell-permeant compounds biased toward intracellular mGluR5 pools is warranted and may lead to new opportunities for targeting glutamate signaling for analgesia.

MODIFYING INTRINSIC DRUG PROPERTIES TO INFLUENCE LOCATION BIAS

The studies above suggest that GPCRs that undergo endocytosis may be modulated more effectively by ligands that can diffuse to intracellular sites. This raises questions about whether the intrinsic properties of analgesic agents can be enhanced by chemical modification, to increase activity or partitioning into membranes where GPCRs are known to initiate signals associated with pain.

Lipid-Anchored Ligands for Increased Endosomal Accumulation

The NK_1R, has an established role in pain transmission and is well known to internalize when stimulated by the neurotransmitter, Substance P (SP). Peripheral inflammation-induced either acutely with intraplantar capsaicin or over sustained periods with Complete Freund's Adjuvant, leads to pre-synaptic release of SP from primary afferent terminals onto the dorsal horn, and evokes robust NK_1R internalization in Lamina I and II neurons of the spinal cord (Mantyh et al., 1995a; Abbadie et al., 1996, 1997; Jensen et al., 2017). Analogous to the endosomal signaling phenomena

described above, it has also recently been reported that NK_1R can mediate compartmentalized signaling processes including sustained PKC, nuclear ERK activity and cAMP production, in a clathrin/dynamin and βArr-dependent manner (Jensen et al., 2014, 2017; Poole et al., 2015). Similarly, CLR/RAMP1 which has an established role in central pain transmission and migraine pain (Lee and Kim, 2007; Bell, 2014), can undergo a CGRP-mediated redistribution into endosomes in HEK cells (Padilla et al., 2007) and in spinal cord sections (Yarwood et al., 2017). *In vitro* studies to clarify CLR/RAMP1-mediated compartmentalized signaling also showed that endocytosed receptor is associated with sustained nuclear ERK activity, cytosolic PKC activity and cytosolic cAMP production in HEK cells, and mediates sustained neuronal excitation in electrophysiological studies on rat spinal cord slices (Yarwood et al., 2017).

To demonstrate a similar potential for targeting endosomal receptor populations in peripheral neurons, PAR_2 expressed on primary afferents is proposed to mediate inflammatory pain responses and its activity is strongly associated with irritable bowel syndrome (IBS). PAR_2 signaling is also a stimulation-dependent process, where cleavage by different proteases can lead to distinct trafficking and location-based signaling outcomes. Trypsin proteolytically cleaves the extracellular amino terminus to activate PAR_2 and promote PAR_2 internalization into endosomes (DeFea et al., 2000; Ricks and Trejo, 2009). Endosomal PAR_2 continues to signal through nuclear ERK and cytosolic PKC (Jimenez-Vargas et al., 2018). In contrast, elastase and cathepsin S mediated cleavage of the N-terminus activates PAR_2 but does not stimulate PAR_2 endocytosis (Zhao et al., 2014, 2015). Consequently, PM-delimited PAR_2 signaling is relatively transient and is proposed to only mediate sustained signaling *via* activation of downstream effectors such as TRPV1 and TRPV4 ion channels (Poole et al., 2013; Jimenez-Vargas et al., 2018).

These data indicate that the internalization of excitatory GPCRs into endosomes may be associated with the generation of spatiotemporally distinct signaling profiles (Jensen et al., 2017; Yarwood et al., 2017; Jimenez-Vargas et al., 2018). Paradoxically, these internalized signaling processes are associated with persistent hyper-excitability of nociceptors and enhanced pain transmission through mechanisms that are not entirely clear, but require sustained kinase activity (Thomsen et al., 2018).

Pharmacological strategies have been employed to understand the importance of location bias of these receptors in pain transmission. Chemical modification by conjugation to the sterol cholestanol has previously been used by Simons and colleagues as a strategy to increase membrane affinity and the endosomal accumulation of a β-secretase transition state inhibitor (Rajendran et al., 2008). Using a similar lipid-anchor approach, antagonists for NK_1R, CLR/RAMP1, and PAR_2 were functionalized with the sterol moiety cholestanol, separated by a flexible polyethylene glycol (PEG_{12}) linker. Focusing on the NK_1R peptide antagonist spantide I (Jensen et al., 2017), the CLR/RAMP1 peptide antagonist $CGRP_{8-37}$ (Yarwood et al., 2017) and I-343, a small molecule PAR_2 antagonist (Jimenez-Vargas et al., 2018), the lipid anchor increased efficacy at the PM for all three compounds, and promoted incorporation and accumulation into endosomes, and is proposed to be maintained on the outer leaflet of membranes to target extracellular GPCR binding pockets, that are also accessible within the lumen of endosomes. This resulted in greater antagonism of endosomal-delimited signaling processes and more effective analgesia relative to unlipidated control compounds.

Alternative membrane-targeted antagonists have been developed for GPCRs, and the best studied of these are pepducins. Using peptides antagonists based on the sequences of GPCR intracellular domains to competitively bind G protein coupling, pepducins are anchored to membranes by chemical modification with palmitic acid (Covic et al., 2002), and these palmitoylated peptides have been proposed to flip to the inner leaflet of the PM to provide cell surface-delimited signaling inhibition. Pepducins are efficacious in inflammatory models (edema, osteoarthritis, sepsis) by selectively targeting GPCRs including PARs (PAR1, 2, 4) and chemokine receptors (CXCR1, 2, 4; Tressel et al., 2011; Tsuji et al., 2013).

Together, these studies support the use of lipid conjugation as a strategy for modifying the location biased profiles of drugs. The lipophilic properties of the anchor dominate the membrane partitioning of ligands, even hydrophobic small molecules, and are therefore a critical determinant for achieving unique membrane distributions, to improve ligand efficacy at specific subcellular locations (**Figure 2**). While pepducins have entered clinical trials (Gurbel et al., 2016), cholestanol conjugates that lead to the accumulation of ligands in endosomes have not advanced beyond pre-clinical pain models, but suggest that targeting endosomes through drug delivery strategies may be a useful therapeutic approach for the management of pathological pain.

Modifying pH-Sensitivity of MOR-Opioid Interactions

Increasing ligand selectivity for GPCR binding under acidic conditions is a potential alternative strategy for favoring the modulation of GPCRs in endosomes. Relative to the physiological pH of the extracellular environment, trafficking proteins are exposed to an increasingly acidic gradient, as cargo is sorted deeper into the endosomal network. The reduction in pH increases proteolytic activity, which is essential for lysosomal protein degradation, and also for modulating the activity and presence of peptides such as SP or CGRP in endosomal compartments (Padilla et al., 2007).

With a need to reduce opioid-MOR interactions that lead to on-target side effects such as sedation, addiction and constipation, Stein and colleagues recently explored the potential for a pH-sensitive analog of the MOR agonist fentanyl to selectively engage with MOR only in pathological conditions, where acidosis is likely to occur (Spahn et al., 2017). The acid dissociation constant (pK_a) of fentanyl is >8 and can activate MOR in physiological conditions (pH 7.4) and between pH 5 and 7, being the expected pH range within the microenvironment of inflamed tissue (Ludwig et al., 2003; Thurlkill et al., 2005). It was therefore hypothesized that reducing the pKa of fentanyl >7 by

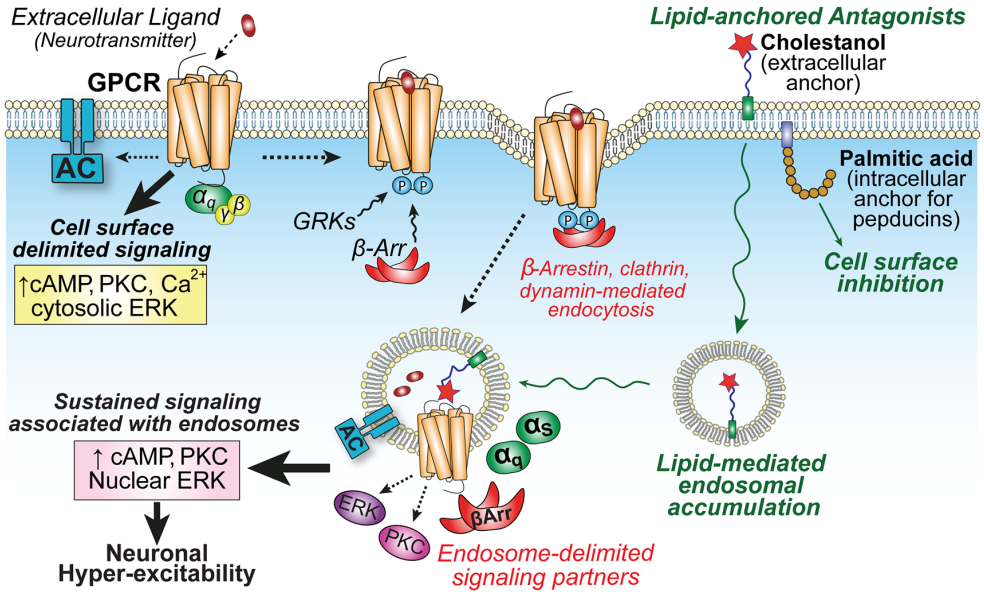

FIGURE 2 | GPCR localization influences compartmentalized signaling and neuronal hyper-excitability. Activation of GPCRs on central neurons by extracellular neuropeptides (e.g., NK_1R or CL/RAMP1) initiates cell surface-delimited G protein-dependent signaling events. This is followed by GPCR kinase (GRK) phosphorylation, arrestin-binding, and clathrin-mediated endocytosis into endosomes to promote the recruitment of unique signaling complexes and drive spatiotemporally distinct signaling that is associated with sustained excitability of neurons in spinal cord slice preparations (Jensen et al., 2017; Yarwood et al., 2017). Lipid conjugation can influence membrane partitioning of antagonists. Palmitoylated pepducins are proposed to inhibit G protein-mediated inflammatory processes on the cytoplasmic interface of the plasma membrane (PM); whereas the sterol moiety cholestanol increases drug accumulation in endosomes to enhance inhibition of sustained endosomal-delimited signaling.

replacement of side-chain hydrogens would favor binding exclusively in pathological conditions.

Utilizing atomic-level structural information for MOR (Manglik et al., 2012) hydrogen replacement fentanyl analogs were designed and binding energies were measured in computational simulations, to identify candidates for further *in vitro* testing and assessment in pain models. The substitution of hydrogen by fluorine resulted in the development of (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP) with a pK_a of 6.8 (Spahn et al., 2017). NFEPP and fentanyl were intravenously administered and compared using two models of persistent or acute inflammatory pain (Spahn et al., 2017) and more recently in neuropathic and abdominal pain in rats (Rodriguez-Gaztelumendi et al., 2018). Fentanyl produced analgesia in both injured and non-injured tissue. However, NFEPP analgesia was restricted to inflamed, acidic tissues. High doses of fentanyl induced respiratory depression, sedation and CNS-associated side-effects such as decrease of defecation, heart rate, and blood oxygen saturation, whereas NFEPP did not (Spahn et al., 2017; Rodriguez-Gaztelumendi et al., 2018).

These studies demonstrate the importance of protonation of ligands for receptor binding and activation, and the potential to modulate receptor affinity at pathological pH, thus limiting on-target side effects and unwanted MOR interactions in healthy tissues. The pH range of endosomes is comparable to inflamed tissue and hence, further *in vitro* studies may be useful to determine if the properties of NFEPP also enhance binding with endosomal receptor pools. Furthermore, if NFEPP maintains its ability to partition into membranes to access and activate the Golgi pool of MOR, this may suggest that MOR activation in the Golgi is favorable for analgesia, rather than being associated with poor safety outcomes.

CONCLUDING REMARKS

The signaling and trafficking of GPCRs is important for mediating physiological processes at the PM and can also drive distinct, compartmentalized signaling events from intracellular sites. In the context of pain, defining this relationship may provide significant opportunities for neuropharmacology and analgesic drug discovery. However, while this may provide important insights that pinpoint discrete signaling outcomes most closely associated with modulating pain behaviors, or favorable drug properties that achieve analgesia while avoiding safety issues, it also critical to translate these proof of concept studies to human tissues and diseases. It remains unknown (and very challenging), for example, to demonstrate how the Golgi-specific MOR-signaling component influences analgesia or other side-effects in animals or humans, or if pH-sensitive fentanyl analogs provide genuine advantages over the parent compound in humans with chronic pain.

Although a relatively new phenomenon, ligands that have been identified or modified to possess unique location-biased properties have provided both interesting and valuable proof of concept findings that warrant further investigation. This includes receptors discussed in this review article and many others that contribute to pain in both neurons and non-neuronal cells that drive signaling processes that lead to sustained pain. With the availability of powerful new technologies and biophysical tools, it is predicted that further in-depth compartmentalized signaling-focused drug discovery studies on other trafficking receptors will provide many more valuable insights and other location-specific drug targets.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Pre-Chapter 3:

Behavioural assessment of pH-responsive nanoparticles for targeting endosomal NK_1R as an alternative approach for pain management

Pre-chapter statement

The pre-chapter titled "Behavioural assessment of pH-responsive nanoparticles for targeting endosomal NK_1R as an alternative for pain management" explains in detail my contribution to the manuscript "*A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain*" published in *Nature Nanotechnology* in 2019, included in this thesis as appendix 1 and 2.

In this publication, we explored the concept of endosomal signalling as a potential therapeutic target for pain management. We demonstrate that polymeric nanoparticles that selectively deliver the NK_1R antagonist Aprepitant into endosomes decreased NK_1R-mediated signalling pathways associated with pain transmission without disrupting physiological cell function.

My contribution to this work was the assessment of the analgesic potency of nanoparticles in three rodent models of pain, examination of the biodistribution of nanoparticles, and the electrophysiological assessment of the effects of nanoparticles in the sciatic nerve ligation model of neuropathic pain in rats. These studies will be described in this pre-chapter.

My contribution to this work is presented in the following figures in the original manuscript (Appendix 1 and 2):

Figure 3. Panels A, B and D

1. Quantitative assessment of the biodistribution of DIPMA- Cy5 nanoparticles in the spinal cord.
2. Quantification of Aprepitant in the spinal cord.
3. Administration of nanoparticles and preparation of samples presented in panel C

Figure 4. Panels B, C, D, E, G and H

Design, interpretation and assessment of the analgesic potency of nanoparticles using von Frey filaments to assess mechanical allodynia in pain models: Capsaicin induced pain (acute inflammatory pain), CFA induced chronic inflammatory pain and Randall Selitto test for Neuropathic pain model (sural nerve sparing model).

Figure 5- Panels A - F

Design, interpretation and assessment of the analgesic efficacy of nanoparticles in the sciatic nerve ligation model of neuropathic pain in rats using electrophysiology to assess C- reflex and wind up.

Figure S6. Supplementary material

1. Introduction

GPCRs are conventionally seen as cell surface receptors that detect extracellular stimuli and couple to G proteins, which lead to plasma membrane signalling events in certain tissue (Defea et al., 2000; Vilardaga et al., 2014; Geppetti et al., 2015). However, growing evidence indicates that GPCRs can also signal from intracellular locations such as endosomes (Roosterman et al., 2007; Cattaruzza et al., 2009; Murphy et al., 2009; Pelayo et al., 2011; Vilardaga et al., 2014; Poole et al., 2015; Tsvetanova et al., 2015; Irannejad et al., 2017; Jensen et al., 2017; Yarwood et al., 2017; Stoeber et al., 2018; Retamal et al., 2019; Jimenez-Vargas et al., 2020).

The neurokinin 1 receptor (NK_1R), a GPCR with a high affinity for the neuropeptide substance P (SP) (McConalogue et al., 1999), is predominantly expressed in immune, endothelial, neurons of the peripheral (PNS) and central nervous systems (CNS) (Bleazard et al., 1994; Mantyh et al., 1997; Mantyh, 2002; King et al., 2005), and dysregulation of its signalling is associated with autoimmune diseases, inflammation and pain (Coudoré-Civiale et al., 1998; Lieb et al., 2002; Mantyh, 2002; Duffy, 2004; Johnson et al., 2017; Mishra and Lal, 2021).

Substance P is the first discovered peptide neurotransmitter (Euler and Gaddum, 1931; Lembeck and Donnerer, 2004), and the involvement of NK_1R in pain transmission is well-established, and its role has been studied by the central or peripheral administration of NK_1R antagonists in both chronic and acute models of pain (Liu and Sandkühler, 1997; Mansikka et al., 1999; Laird et al., 2001; King et al., 2005; Teodoro et al., 2013). The capability of NK_1R antagonists to decrease pain is correlated with electrophysiological studies on spinal cord slices, where NK_1R antagonists suppress long-term potentiation (LTP) of afferent nociceptive spinal neurons, demonstrating that NK_1R is an essential receptor for the activation of C-fibres in pain transmission (Liu and Sandkühler, 1997; Coste et al., 2008; Jensen et al., 2017). Furthermore, the requirement for NK_1R in pain transmission has been demonstrated using the selective ablation of NK_1R+ spinal neurons using SP conjugated to the ribosome-inactivating protein, Saporin (SP-SAP). SP-SAP is internalised and selectively depleted cells in the spinal cord that express NK_1R, leading to decreased pain-transmitting afferent neurons of the laminae I and II. NK_1R+ spinal cord neurons depletion reduced capsaicin-induced mechanical and thermal hyperalgesia, providing further support that NK_1R is a central player in the pain transmission pathway (Mantyh et al., 1997; Wiley and Lappi, 1997; Nichols et al., 1999; Iadarola et al., 2017).

Mantyh *et al*., described that acute noxious stimulation by SP or capsaicin-induced NK_1R internalisation in the soma of neurons in lamina I and in dendrites located in lamina III & IV in the

spinal cord of rats. It was assumed that pre-synaptic SP release had occurred following a pain stimulus, and upon activation of NK_1R, the observed internalisation was hypothesised to be involved in neuronal plasticity, particularly in pain transmission (Mantyh et al., 1995a, 1995b; Abbadie et al., 1997; Nichols et al., 1999).

Following this observation, some years later, others explored the trafficking mechanism for NK_1R and related the concept of internalisation to unique signalling outcomes. Initially, using microscopy and western blot to measure kinase activity, studies in model cells and neurons demonstrated that NK_1R activation by SP promotes receptor interaction with βARRs, which mediates clathrin-mediated endocytosis (McConalogue et al., 1999). The metalloendopeptidase endothelin converting enzyme 1 (ECE-1) degrades SP-bound to NK_1R in acidified early endosomes, disrupting SP-NK_1R-βARRs complex, resulting in rapid NK_1R recycling to the plasma membrane (Padilla et al., 2007; Roosterman et al., 2007; Cattaruzza et al., 2009; Cottrell et al., 2009; Pelayo et al., 2011; Jensen et al., 2014, 2017; Poole et al., 2015). However, when ECE-1 is inhibited, NK_1R remains in endosomes for extended periods (Padilla et al., 2007; Roosterman et al., 2007; Cottrell et al., 2009).

Together, their findings suggest that the NK_1R can signal from the plasma membrane and endosomes, this hypothesis was substantially supported in 2017 through a study that utilised a series of compartmentalised FRET-based signalling biosensors and endocytosis inhibitors to confirm how NK_1R internalisation is associated with specific signalling events. NK_1R in endosomes was specifically associated with sustained increases in nuclear ERK, cytosolic PKC, and cytosolic cAMP (Jensen et al., 2017). In addition, the dynamin GTPase activity inhibitor (Dyngo4A) prevented sustained SP-induced excitation of neurons in spinal cord slices, and reduced nociception of the capsaicin-evoked pain. However, this inhibitor was considered a potent inhibitor of multiple neuronal processes, including synaptic turnover. It provided further indirect support for a role for NK_1R in promoting pain transmission from an endosomal location (Jensen et al., 2017).

The NK_1R antagonist Aprepitant, currently used to treat emesis, showed promising analgesic effects in preclinical models. However, Aprepitant failed in subsequent clinical trials to treat pain (Kramer et al., 1998; Quartara et al., 2009). The clinical failure of Aprepitant for pain treatment might relate to its inability to effectively target and antagonise the NK_1R within acidified endosomes. Initial efforts to address this utilised an endosomal targeting technique, whereby the low potency NK_1R peptide antagonist spantide I, was conjugated to a cholestanol-based lipid anchor to enhance membrane association and increase the accumulation of the antagonist in endosomal membranes. This was shown

to be effective and selective for inhibiting the FRET-based, endosomal-associated NK_1R signalling events described above. However, related studies have characterised this lipidated compound in greater detail using a range of quantitative imaging and signalling approaches, to show that the lipid-anchored antagonists may be effective for pain inhibition, by targeting NK1R in endosomes, but also by blocking arresting mediated endocytosis at the cell surface (Mai et al., 2021).

As an alternative delivery approach to the lipid conjugates, we describe a new delivery system, pH-responsive nanoparticles (DIPMA Np), that were utilised to alter the distribution and efficacy of the antagonist Aprepitant *in vivo*. It was hypothesised to be superior to lipid-conjugates due to its ability to deliver a drug to specific cellular sites without chemically modifying it and bypass cell surface receptors to achieve selective, enhanced delivery of Aprepitant to endosomes where SP-stimulated NK_1R is known to reside during pain. In this broader study, the Np-mediated delivery was more effective than "free" non-formulated Aprepitant, decreasing ERK signalling *in vitro* and nerve firing as measured by electrophysiology. In addition, this delivery method enhanced the antinociceptive capability of Aprepitant in clinical pain models, and the delivery of nanoparticles within endosomes decreased the concentration required to achieve antinociception. The efficacy of DIPMA nanoparticles to suppress nociception could be due to the antagonism of endosomal NK_1R that is directly related to C-fibre activation.

Nanoparticles have potential use to direct drugs to subcellular compartments, such as endosomes, from where GPCRs can generate disease-relevant signals. Here, we demonstrated that nanoparticle encapsulation enhances and prolongs analgesia and provides proof-of-concept and new opportunities for developing new alternatives for non-opioid therapies for pain.

2. Material and method

Animals. Male C57BL/6 mice (6-10 weeks) were obtained from the Monash Animal Research Platform, and male Sprague–Dawley rats (225-250 g) were obtained from the Faculty of Medicine of the University of Chile. Mice and rats were housed in groups of four, in a temperature (22 ± 4 °C) and humidity-controlled environment with a 12 h light/dark cycle and food and water *ad libitum*. All studies were performed following the ARRIVE guideline and the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983; Kilkenny et al., 2010). Studies were approved by the Animal Ethics Committee of the Monash Institute of Pharmaceutical Sciences and the Bioethics Committee of the University of Santiago of Chile. In behavioural tests, animals were randomly assigned to treatment groups, investigators were blinded to the treatment groups, and the studies were performed during the light cycle. At the end of the experiments, animals were euthanised by anaesthetic overdose and thoracotomy.

Drug administration. The following drugs were administered by intrathecal injection into the lumbar intervertebral space (L4/L5) in a volume of 5µL for mice and 10µL for rats: Aprepitant (100 and 300nM), DIPMA nanoparticles delivering an equivalent dose of Aprepitant (DIPMA-AP, 100 or 300nM), non-pH-responsive nanoparticles (BMA-AP, 100 or 300nM), controls (empty DIPMA-Ø, or a mixture of DIPMA-Ø and Aprepitant 100nM) or vehicle (artificial cerebrospinal fluid, aCSF). Drugs treatments were administered 30 min before the induction of acute nociceptive pain (capsaicin), 48 h after the establishment of inflammatory nociception (CFA), or 10 days post neuropathic sural nerve sparing (SNS) surgery. Morphine was used as the gold standard pain treatment (3 mg/kg intraperitoneal) to compare the antinociceptive efficacy of DIPMA nanoparticles in the inflammatory and neuropathic model of pain.

Biodistribution studies. Sedated mice (2% isoflurane) were placed in an *in vivo* imaging system (IVIS spectrum Lumina II, Perkin Elmer). Control images were obtained before DIPMA conjugated to Cy5 administration (time 0). Followed by the intrathecal administration of DIPMA or BMA-Cy5 (5µL, 50µg/ml). Images were obtained at 1 min and at 1, 1.5, 2, 4, 8 and 24 h post nanoparticle administration.

Uptake of nanoparticles in the spinal cord. DIPMA-Cy5 nanoparticles were administered intrathecally in mice under sedation (2% isoflurane) to mimic the therapeutic situation where nanoparticles might be used to treat pain. After 30 min, capsaicin (5µg) was administered (10µL, intraplantar) into the left hind paw. After 1 h post-nanoparticle administration, mice were

transcardially perfused with 50 ml of PBS followed by 50 ml of ice-cold 4% paraformaldehyde (PFA). The spinal cord (L3-L6) was removed, post-fixed in 4% PFA (2 h, 4 °C) and cryoprotected in PBS containing 30% (w/v) sucrose (24 h, 4 °C). The spinal cord was embedded in tissue freezing medium (TFM, General Data), and 30 µm serial coronal sections were cut. Sections were blocked in PBS containing 0.2% Triton X-100 and 10% normal horse serum (NHS; 1 h, room temperature). Sections were incubated with rabbit anti-PGP9.5 (1:500, Abcam ab27053) in PBS containing 0.2% Triton X-100 and 3% NHS (overnight at 4 ºC). Sections were washed four times in PBS and incubated with donkey anti-rabbit Alexa488 (1:1,000, ThermoFisher Scientific; 30 min, room temperature). Sections were imaged on a Leica SP8 confocal microscope with HC PLAPO ×40 or ×63 oil objectives.

Determination of Aprepitant concentrations in the spinal cord. Free Aprepitant (100nM) or DIPMA-AP nanoparticles delivering the same Aprepitant concentration (10µg/ml − 100nM Aprepitant) was administered by intrathecal injection. Mice were killed at 1 h or 4 h post-treatment. The spinal cord (L2–L6) was removed to determine the tissue concentration of Aprepitant by Liquid Chromatography-Mass Spectrometry (LC-MS), as described in the Supplementary Methods (Appendix 2).

Acute and inflammatory nociception in mice. Acute inflammatory pain was induced by the intraplantar administration of Capsaicin (5µg) or vehicle (0.9% NaCl, control) into the left hind paw of sedated mice (2% isoflurane). Chronic inflammatory pain was induced by the administration of Complete Freund's Adjuvant (CFA) (0.5 mg/m1) or vehicle (0.9% NaCl) by intraplantar injection (10µL) into the left hind paw of sedated mice (2% isoflurane) (Jensen et al., 2017). Drugs were administered by intrathecal injection 48 h after CFA.

Pain assessment. Mice were acclimatised to the experimental apparatus and environment for 2 h on two successive days. Calibrated Von Frey Filaments, VVF (Jensen et al., 2017) were used to assess mechanical allodynia in acute and chronic inflammatory models of pain by measuring withdrawal thresholds to stimulation of the plantar surfaces of hind paws using an up-down regiment. VFF withdrawal thresholds were measured in triplicate to establish a baseline for each mouse. In the capsaicin model, VFF withdrawal was measured every 30 min for the first 2 h after drug administration, then at 60 min intervals for the next 2 h, and finally after 24 h. In the CFA model, VFF withdrawal was measured every 30 min for the first 3 h after drug administration, then at 60 min intervals for the next 5 h, and finally after 24 h. Results were normalised to the baseline withdrawal thresholds of each mouse and expressed as a percentage of baseline.

Neuropathic pain. Neuropathic nociception was induced in rats using a variation of the SNS injury model, which induces rapid onset and sustained mechanical and thermal hyperalgesia (Decosterd and Woolf, 2000). Briefly, rats were anaesthetised (2% isoflurane), the right hind leg was shaved to the level of the pelvic origin of the sciatic nerve, then a skin incision (~10 mm in length) was made. The subcutaneous tissue and the *biceps femoris* muscle were dissected to expose the sciatic nerve, the three-terminal distal branches of the sciatic nerve (tibial, common peroneal and sural nerves) were identified, and the sural nerve was transected (Bravo et al., 2014). Control (sham) rats underwent a similar surgery but without transection of the sural nerve. The animals were given daily 3 mg/kg of ketoprofen and 5 mg/kg of enrofloxacin during the two days following surgery.

Neuropathic pain assessment. Nociceptive behaviour (mechanical hyperalgesia) was assessed in rats by measuring hind paw withdrawal pressure thresholds using an algesimeter (Ugo Basile) with a cut-off value of 570 g/cm^2 to prevent injury (Hargreaves et al., 1988; Bravo et al., 2014). Briefly, the right hind paw of the rat was subjected to increasing pressure until the paw withdrawal occurred, and the threshold obtained was recorded. Mechanical hyperalgesia was evaluated before (basal) and 5, 9 and 10 days after surgery. On day 10, drugs were administered by intrathecal injection, and withdrawal thresholds were recorded every 30 min for 7 h.

Electrophysiological recording of the nociceptive C-reflex (electromyography). The electromyographic activity (EMG) of nociceptive C fibres (C-reflex) were recorded from the biceps femoris muscle of neuropathic rats (Bravo et al., 2014). Rats were under 1.8% isoflurane and placed on a regulated thermal pad (37 ± 0.5°C) throughout this procedure. Briefly, low-frequency (0.1 Hz, for basal C-reflex) or high-frequency (1 Hz, for C-reflex wind-up) rectangular electrical pulses of supramaximal strength (double C-fiber threshold) were applied every 10 seconds in the receptive field of the right common peroneal and tibial nerves utilising two stainless steel needle electrodes inserted under the skin of the second and third toes of the right hind paw. Using another stainless steel needle electrode, the C-reflex was recorded from the ipsilateral biceps femoris muscle. The EMG activity was sampled at 100 kHz and integrated into a 150 to 450 ms time window after the stimulus. Wind-up is a potentiation of the C-reflex response when the stimulating frequency is increased to 1 Hz. The wind-up score corresponds to the slope of the first seven consecutive C-reflex recordings obtained at 1 Hz stimulation.

Statistical analysis. Data were analysed using GraphPad Prism 8 (GraphPad Software). Data are presented as mean ± s.e.m. For multiple comparisons, results were compared using one- or two-way

ANOVA followed by posthoc multiple comparison tests, as described in the figure legends. $P<0.05$ was considered significantly different to the null hypothesis at the 95% confidence level.

3. Results

3.1. Biodistribution and delivery of nanoparticle cargo in vivo.

Initial studies of nanoparticle uptake *in vitro* showed that DIPMA-Cy5 nanoparticles are localised to early (RAB5) and late (RAB11) endosomes after 30 to 60 min (*Figure 2a, appendix 1. Figure 3a, appendix 2*). HEK cells expressing NK_1R-GFP were similarly treated with DIPMA-Cy5 and stimulated with SP (30-60 min) to evoke NK_1R endocytosis. DIPMA-Cy5 nanoparticles in these cells were codistributed with NK_1R-GFP in endosomes (*Figure 2b, appendix 1. Figure 3b, appendix 2*). The internalisation mechanism through which nanoparticles entered cells was determined using inhibitors of clathrin (PitStop2,(Robertson et al., 2014)) and dynamin (Dyngo4a, dependent endocytosis. Both PitStop2 and Dyngo4a decreased the cellular uptake of DIPMA-Cy5 nanoparticles, consistent with clathrin- and dynamin-dependent endocytosis (*Figure 2d–f, appendix 1*). These studies indicated that DIPMA nanoparticles are internalised into NK_1R containing endosomes *in vitro*.

We next examined nanoparticle biodistribution *in vivo*. DIPMA-Cy5 or BMA-Cy5 nanoparticles were administered intrathecally (L4/L5) in mice. Cy5 fluorescence associated to DIPMA nanoparticles was detectable after 1 min post-nanoparticle administration, and the fluorescence was retained at the injection site for up to 24 h (**Figure I A, B**. *Figure 3 a, b, appendix 1*). We used confocal imaging of spinal cord slices to explore the cellular uptake *in vivo*. DIPMA-Cy5 and BMA-Cy5 nanoparticles were found within laminae I-III of the dorsal horn overlapping with the neuronal marker PGP9.5 (*Figure 3c, appendix 1*).

To evaluate nanoparticles as a drug delivery system, free Aprepitant, DIPMA or BMA nanoparticles loaded with Aprepitant were injected intrathecally to mice. After 1 or 4 h post-administration, the spinal cord was harvested, and LC-MS was used to quantify the concentration of Aprepitant. After 1h post-drug administration, the Aprepitant concentration was two-fold higher for DIPMA-Ap than BMA-Ap and approximately four-fold higher than free Aprepitant (**Figure I C**. *Figure 3d, appendix 1*). LC-MS analysis of spinal cord samples after 4 h post-administration showed that the Aprepitant concentration for DIPMA-Ap and BMA-Ap were similar, while almost undetectable for free Aprepitant. These results indicate that encapsulation in nanoparticles causes retention of Aprepitant in the spinal cord compared to the free drug, which was almost undetectable at 4 h.

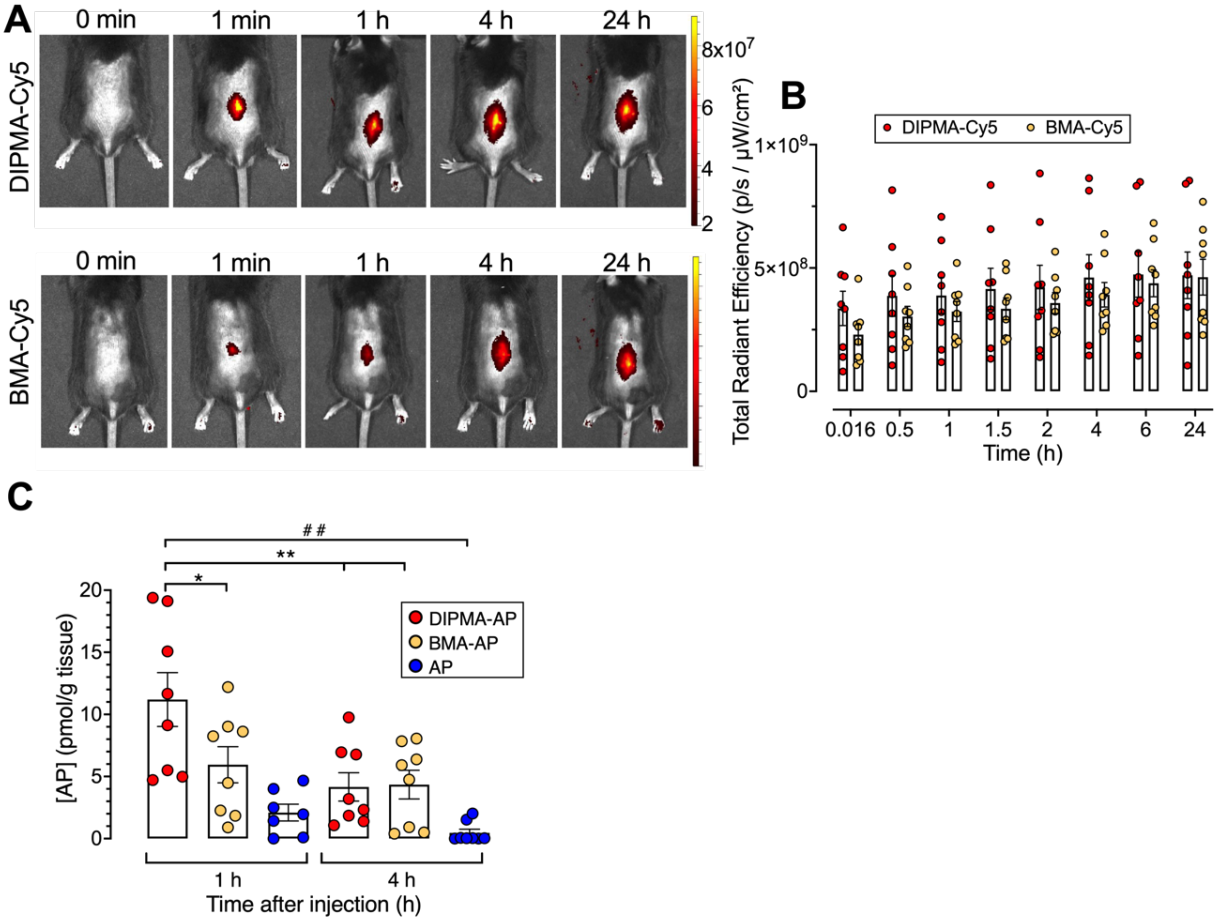

Figure 10. ***In vivo*** **biodistribution nanoparticles and Aprepitant in the spinal cord. A)** Distribution of DIPMA-Cy5 and BMA-Cy5 nanoparticles at different time points after intrathecal injection. Representative images of n= 8 mice. The Y axis is expressed as Cy5 fluorescence intensity measured as radiant efficiency with units psµ/Wcm^2. (p, photons. s, seconds. W, watts). **B)** Quantification of DIPMA-Cy5 and BMA-Cy5-nanoparticles after intrathecal administration. Data are expressed as mean ± s.e.m., n= 8 mice. **C)** Aprepitant concentrations in the spinal cord measured 1 h and 4 h after intrathecal administration. Data are presented as mean ± s.e.m.; n= 7 mice. *P< 0.05, **P< 0.01, #P< 0.001, ##P< 0.0001. Two-way ANOVA, Tukey's posthoc test.

3.2. Aprepitant loaded in nanoparticles decrease nociception.

We examined the hypothesis that incorporating Aprepitant into nanoparticles enhances its antinociceptive actions by selectively inhibiting endosomal NK_1R *in spinal neurons*.

The antinociceptive efficacy of free or nanoparticle-encapsulated Aprepitant was evaluated in preclinical pain models (nociceptive, inflammatory and neuropathic pain) (**Figure 11-14**. *Figure 4d, appendix 1*). For this purpose, nanoparticles, free Aprepitant or vehicle was injected intrathecally, and mechanical nociception was studied as an indicator of nociception by measuring hind paw withdrawal responses to stimulation of the plantar surface. Hindpaw withdrawal in mice was induced by calibrated

von Frey filaments (VFFs) and in rats by applying sufficient pressure to induce withdrawal of the hind paw (Randall-Selitto test).

Nociceptive model of pain (Capsaicin). The intraplantar injection of capsaicin activates the transient receptor potential vanilloid 1 (TRPV1) on primary sensory neurons to enhance release SP in the dorsal horn, which evokes NK_1R endocytosis in spinal neurons and development of allodynia (Mantyh et al., 1995b; Jensen et al., 2017).

In vehicle and DIPMA-Ø (empty) pre-treated mice (veh, black circle or Ø, open grey circle), intraplantar capsaicin injection significantly decreased the VFF threshold from 0.5 to 4h, which returned to baseline values after 24h (**Figure 11 B. Figure 4b, appendix 1**). Free Aprepitant (100nM) or DIPMA-Ø mixed with free Aprepitant (100nM) caused modest antinociception after 1 h (16 ± 4 and 15 ± 3% inhibition, respectively). BMA-Ap (100nM Aprepitant) had a similar effect to free Aprepitant. However, DIPMA-Ap (100nM Aprepitant) caused a marked antinociception effect observed at 0.5 h (1 h, 34 ± 3% inhibition). This antinociceptive effect was sustained for at least 4 h (35 ± 2% inhibition).

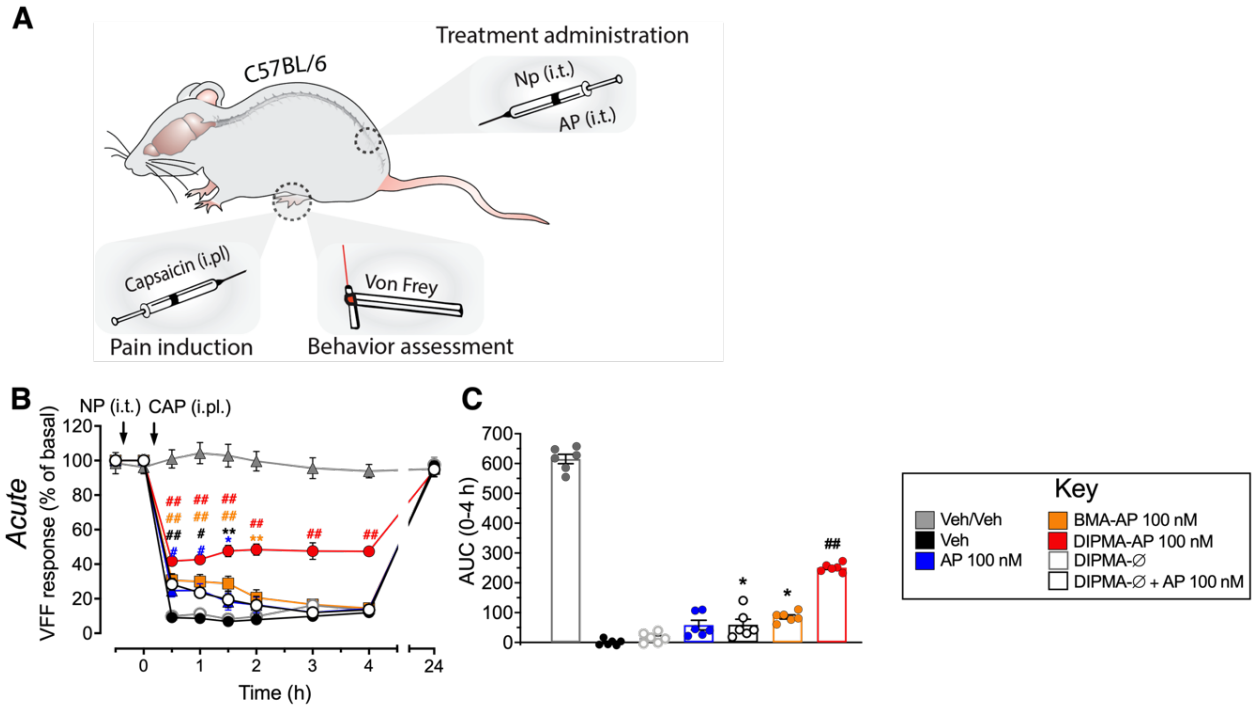

Figure 11. Effects of nanoparticles on preclinical models of capsaicin-evoked acute nociceptive pain in mice. A) Aprepitant, nanoparticles (NPs) or vehicle (Veh) was injected intrathecally (i.t. 5 µl) 30 min before intraplantar (i.pl.) injection of capsaicin (CAP). **B)** Withdrawal responses were measured to stimulation of the plantar surface of the injected hind paw with Von Frey Filaments. **C)** Integrated response as the area under the curve (AUC). Data are presented as mean ± s.e.m., n= 6 animals for all experiments. *P< 0.05, **P< 0.005, ***, #P< 0.001, ##P< 0.0001 compared to vehicle. Two-way ANOVA, Dunnett's post-hoc test.

DIPMA-Ap reduces nociception on CFA-induced inflammatory pain. Intraplantar administration of CFA causes sustained mechanical allodynia and NK_1R endocytosis in spinal neurons (Stein et al., 1988; Jensen et al., 2017), allowing assessment of the capacity of encapsulated Aprepitant to reverse inflammatory pain. CFA injection after 48 h induced a marked decrease in VFF threshold in vehicle-treated animals (Veh) (**Figure 12 A**. *Figure 4d–f, appendix 1*). Aprepitant (100 and 300nM) decreased hyperalgesia induced by CFA for approximately 2h. BMA nanoparticles loaded with Aprepitant (100nM) had similar effects to free Aprepitant. Meanwhile, DIPMA nanoparticles loading the lowest Aprepitant concentration (100nM) produced significantly greater allodynia inhibition than the same dose of free Aprepitant (54 ± 4%), and this inhibition was maintained for 6 h. The analgesic effects of DIPMA nanoparticles was compared against the gold standard pain treatment morphine (3 mg/kg, intraperitoneal). Morphine fully reversed the mechanical allodynia after 0.5 h. However, the morphine effect diminished rapidly following peak analgesia after 3 h.

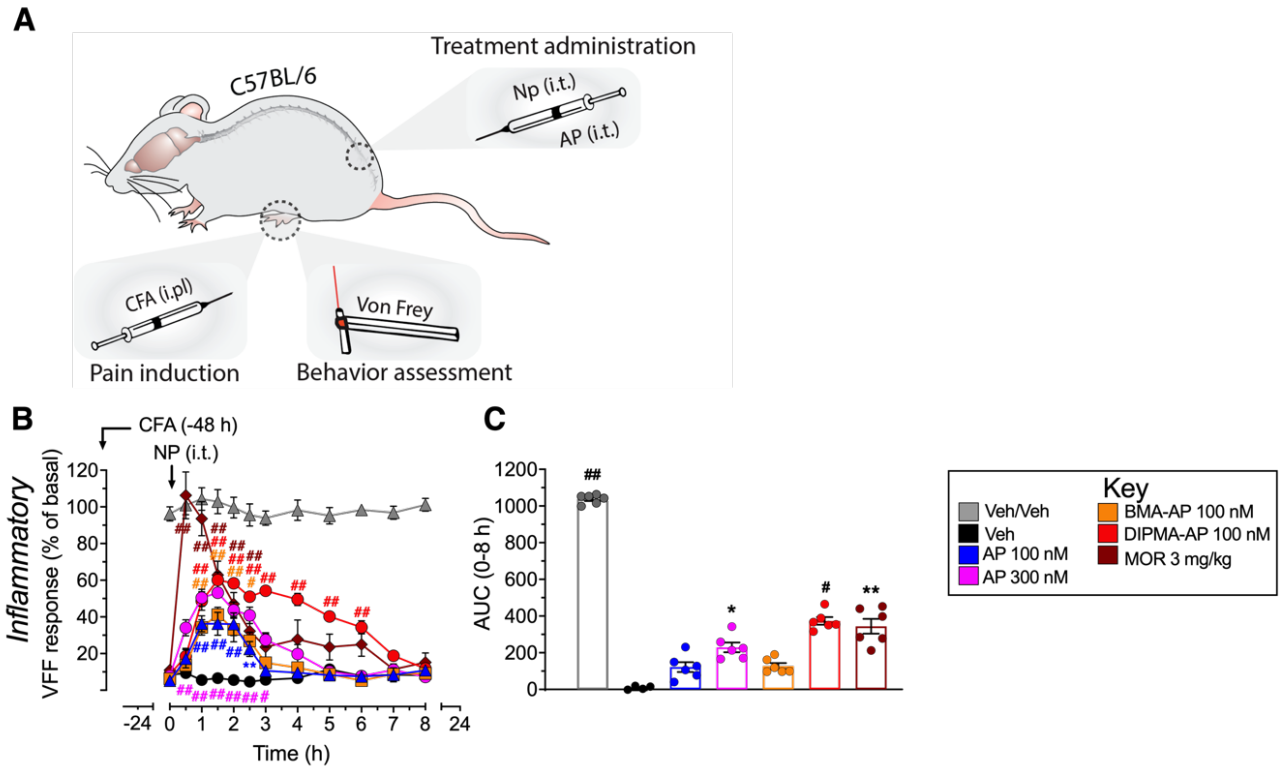

Figure 12. Effects of nanoparticles in the CFA-model of inflammatory pain. A) CFA-evoked sustained inflammatory nociception in mice. **B)** AP, NP or Veh was administered by i.t. injection (5 µl) 48 h after CFA intraplantar injection. Withdrawal responses were measured to VFF stimulation of the plantar surface of the injected hind paw. C) Integrated response as the area under the curve (AUC). Data are presented as mean ± s.e.m., n= 6 animals for all experiments. *P< 0.05, **P< 0.005, ***, #P< 0.001, ##P< 0.0001 compared to vehicle. Two-way ANOVA, Dunnett's post-hoc test.

NPs are effective at reducing mechanical hyperalgesia in the sural nerve spreading model. The analgesic effects of DIPMA-Ap was assessed in the spared of the sural nerve (SNS) model of neuropathic pain. This well-established model produces mechanical hyperalgesia in rats for >50 days (Decosterd and Woolf, 2000; Bravo et al., 2014).

Aprepitant 300nM transiently decreased withdrawal thresholds after 0.5 h to a maximum of 40 ± 2% inhibition after 1 h, whereas 100nm did not affect the withdrawal threshold (**Figure 13 A**. *Figure 4, appendix 2*). A higher dose of free Aprepitant (1μM) decreased significantly hyperalgesia after 1 h (75 ± 4% inhibition), returning to baseline values after 3 h (**Figure 14,** *Figure 4, appendix 2*). BMA nanoparticles loaded with Aprepitant (300 and 500nM) decreased hyperalgesia to a similar level to that of free Aprepitant (**Figure 13 B**. *Figure 4, appendix 2*). DIPMA-Ap (100 and 300nM) enhanced the antinociceptive effect of Aprepitant compared to the same concentration of free Aprepitant. These effects were observed for 4.5 h when none of the other treatments was effective (**Figure 13 B**. *Figure 4g appendix 1*).

In addition, DIPMA loading a higher concentration of Aprepitant (500 nM) completely reversed hyperalgesia for a period of 4.5 h (**Figure 14 A**. *Figure 6a, appendix 2*).

The preclinical pain models assessed in this study revealed that nanoparticle encapsulation and endosomal delivery enhance and prolong the analgesic effects of Aprepitant and validate the potential use of nanoparticles to enhance the antinociceptive effect drugs that had failed in the clinic.

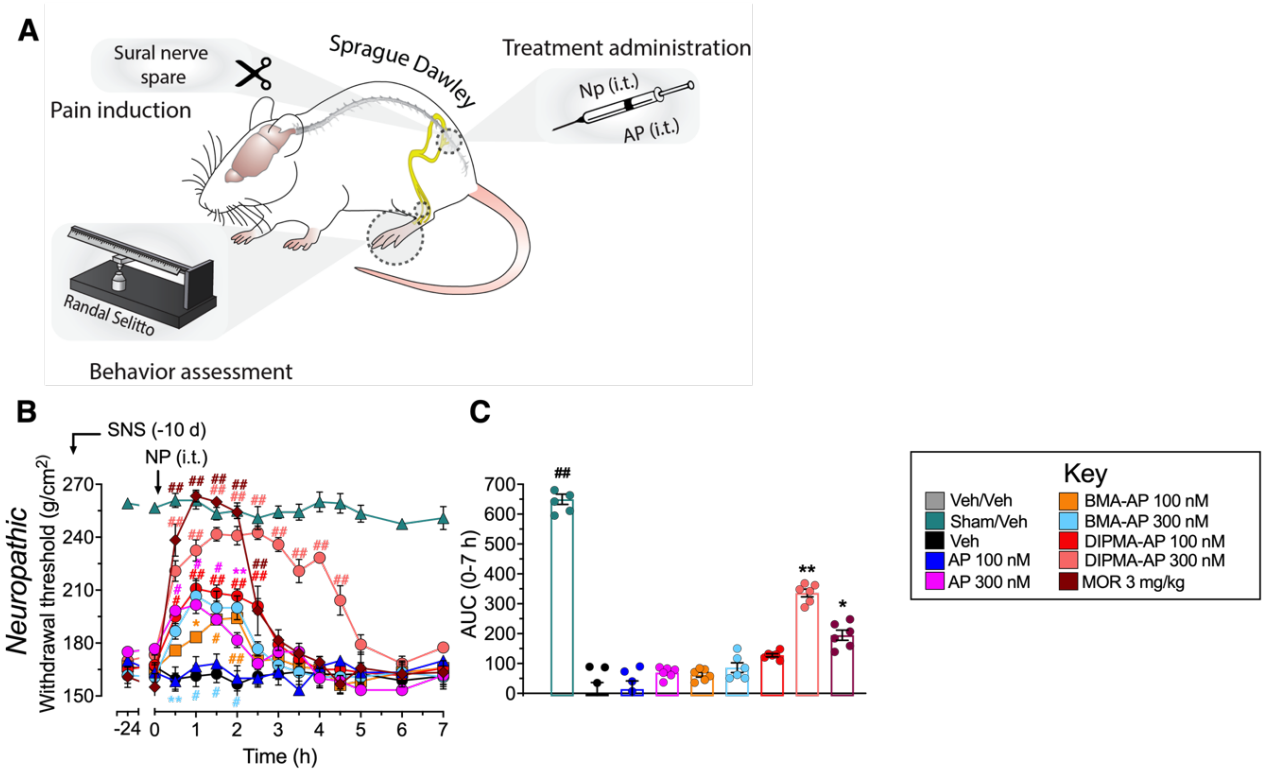

Figure 13. Analgesic effect of nanoparticles in the SNS model of neuropathic pain. A) AP, NP or Veh (10µl, i.t.) was injected 10 days after the establishment of the sural nerve spared (SNS) model. **B)** Withdrawal responses to pressure stimulus were assessed using the Randall-Selitto test. **C)** Integrated responses expressed as AUC. Data are presented as mean ± s.e.m., n= 6 animals for all experiments. *P< 0.05, **P< 0.005, ***, #P< 0.001, ##P< 0.0001 compared to vehicle. Two-way ANOVA, Dunnett's post-hoc test.

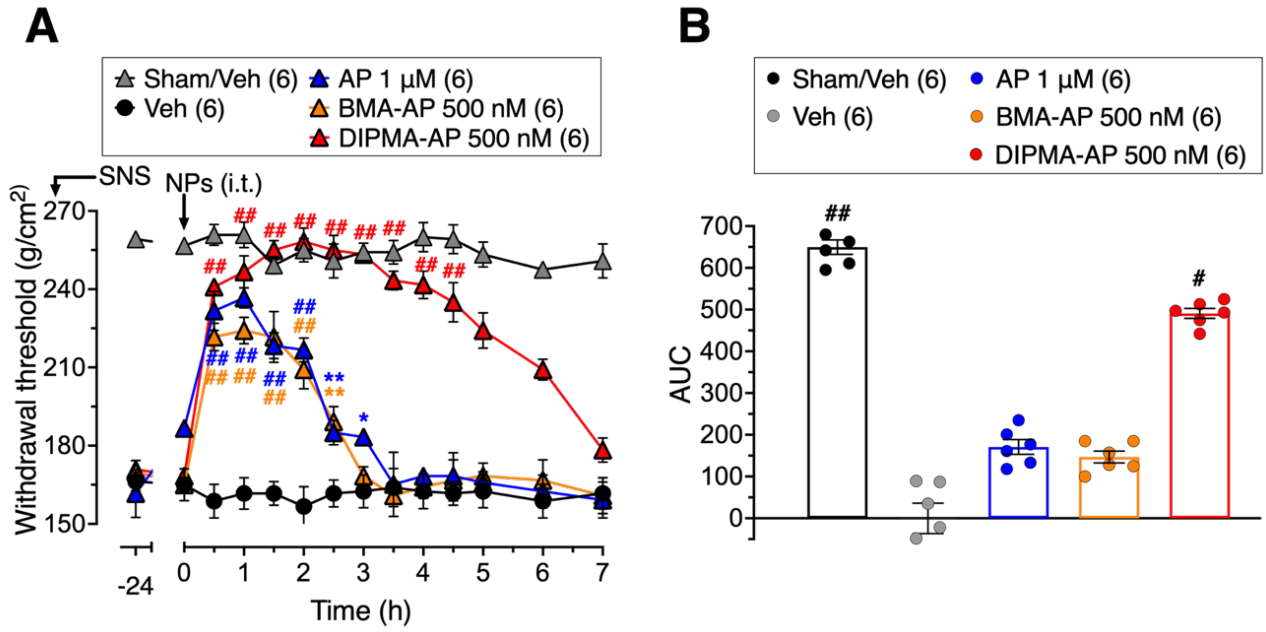

Figure 14. Effects of nanoparticles on neuropathic nociception. A) Vehicle (Veh), free Aprepitant (1µM), DIPMA (500nM) or BMA nanoparticles (500nM) loaded with Aprepitant were administered by intrathecal

injection 10 days after SNS or sham surgery. Paw withdrawal responses were assessed using Randall-Selitto test. **B)** Integrated responses were expressed as area under the curve (AUC) from 0-7 h. Data are presented as mean ± SEM from n= 6 rats. *P<0.05, **P<0.005, #P<0.001, ##P<0.0001 compared to vehicle. Two-way ANOVA, Dunnett's post-hoc test.

3.3. NK_1R endosomal inhibition decrease neuronal activity.

C-nociceptive fibres transmit painful stimuli toward the CNS by releasing SP, CGRP and glutamate in the dorsal horn (Geppetti et al., 2015). Chronic pain is the hallmark of central sensitisation, which is an amplified nociceptive transmission that decreases nociceptive threshold. We assessed the effects of DIPMA-Ap on the C-fiber reflex in the sural nerve spare model of neuropathic pain.

First, we measured the threshold current required to activate C-fibre reflexes in sham control and neuropathic rats to examine central sensitisation. Our findings indicate that an electrical current of 10.3 ± 1.2 mA is required to trigger C-fibre reflex in sham control rats, whereas 3.2 ± 2.8 mA was necessary to activate C-fibre. In addition, repeated low frequency electrical stimuli (0.1 Hz) caused a constant and stable C-reflex activity over time (**Figure 15 A-C**. Figure 5a-c, Appendix 1), while high repeated frequency stimuli (1.0 Hz) evoked a progressive increase in C-reflex activity, known as wind-up (**Figure 15 D-F**. Figure 5a-f, Appendix 1).

In rats with neuropathic pain, intrathecal administration of Aprepitant (1μM) decreased C-reflex only at 30 min but did not affect wind-up activity at any time. In contrast, DIPMA nanoparticles loaded with 3 times less Aprepitant (300nM) significantly decreased C-reflex activity at 45 min and wind-up activity at 15 min post-administration. The decrease of C-reflex and wind-up activity was inhibited for the total duration of the experiments (120 min) (**Figure 15 B and E**). The effectiveness of DIPMA-AP to suppress nociception could be due to antagonism of sustained SP-induced excitation of spinal neurons, which requires NK_1R signalling from endosomes (Jensen et al., 2017).

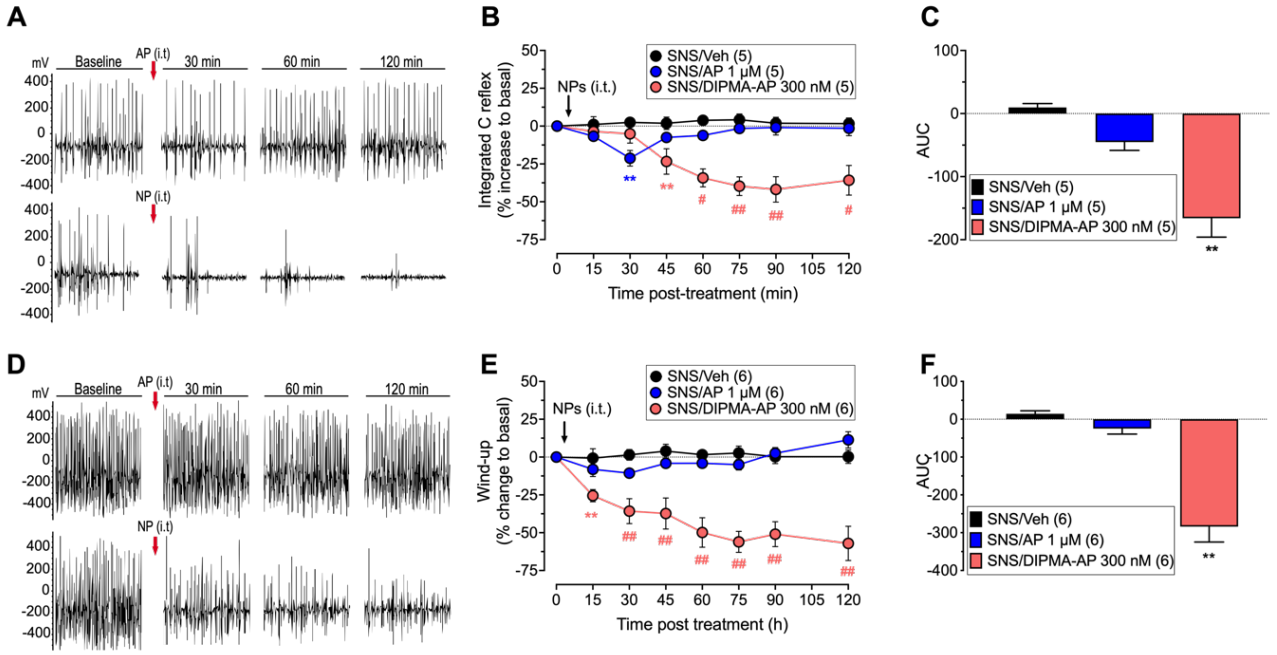

Figure 15. Effect of nanoparticles on C-fiber activity *in vivo*. C-fibre and wind-up activity were studied in rats10 days post-SNS. **A-C)** C-fibre reflexes and **D-F)** wind-up responses were measured after AP, DIPMA-AP, or Veh administered by i.t. injection (10 µl). **A and D**) representative recordings comparing AP and DIPMA-AP. **B and E**) Time course effects of treatments. **C and F)** integrated responses expressed as AUC. Data are presented as mean ± s.e.m., n= 5 rats per group. **P< 0.005, #P< 0.001, ##P< 0.0001 compared to vehicle. Two-way ANOVA, Dunn's posthoc test.

4. Discussion

This study was developed based on the knowledge that painful stimuli can evoke NK_1R endocytosis in spinal neurons (Mantyh et al., 1995a, 1995b; Abbadie et al., 1996; Jensen et al., 2017), leading to the hypothesis that endosomal NK_1R signalling can mediate neuronal excitation and enhanced nociception (Jensen et al., 2017). Clathrin and dynamin are essential proteins required for endosomal NK_1R signalling, and endocytosis inhibitors significantly decrease NK_1R signalling *in vitro* and nociception *in vivo* (Jensen et al., 2017; Yarwood et al., 2017; Jimenez-Vargas et al., 2018). However, dynamin and clathrin inhibitors may affect the trafficking of many other receptors and the normal function of the cells. Thus, considerable efforts are required to advance these compounds to the clinic to manage pain.

Lipid-conjugated antagonists are a potent alternative to target receptors located into endosomes as shown for NK_1R, PAR_2 and CLR, where lipid conjugation improved endosomal targeting, leading to a decrease of nociception (Jensen et al., 2017; Yarwood et al., 2017; Jimenez-Vargas et al., 2018; Mai et al., 2021). However, lipid conjugated antagonists may lose selectivity due to the wide membrane distribution (Jensen et al., 2017; Mai et al., 2021). Furthermore, these compounds are large and require chemical modification of an antagonist to enable attachment the lipid anchor via a PEG-based spacer. While limited studies have indicated that Aprepitant modification is possible (Halik et al., 2020), this has yet to be investigated further.

Here, we developed a pH-responsive nanoparticle incorporating the polymeric unit DIPMA into the hydrophobic core. The specific details of this synthesis are described in greater detail in the associated broader study (Appendix 1 and 2), with *in vitro* and cellular characterisation, it was shown to provide fast and selective distribution of aprepitant into the endosomal network. Functional tests using the FRET-based ERK sensor demonstrated that DIPMA-mediated delivery of aprepitant into endosomes decreased endosomal-associated NK_1R-mediated ERK activity *in vitro* more effectively than Aprepitant. Additional studies to track a fluorescently-labelled variant of the DIPMA-Cy5 Np showed that the administration remains in the injection site (spinal cord), and confocal microscopy studies indicated that DIPMA-Cy5 are incorporated into neurons when looking more closely at spinal cord sections. We subsequently tested the hypothesis that the encapsulation of Aprepitant into pH-responsive nanoparticles enhances its antinociceptive actions by selectively inhibiting endosomal NK_1R *in spinal neurons.*

In this pre-chapter, which provided key data to contribute to a large, international collaborative publication, we demonstrated that DIPMA-Cy5 nanoparticles are detectable within 1 min following administration of DIPMA-Cy5 nanoparticles and the fluorescence remained within the injection site for up to 24 h. In addition, LC-MS analysis showed that encapsulation of Aprepitant in DIPMA nanoparticles causes drug retention in the spinal cord. We examined the hypothesis that incorporating Aprepitant into nanoparticles enhances antinociceptive actions by selectively inhibiting endosomal NK_1R *signalling in spinal neurons*. The antinociceptive efficacy of targeting endosomal NK_1R utilising DIPMA-Ap was evaluated in nociceptive pain, inflammatory and neuropathic pain. Nanoparticle encapsulation enhanced the antinociceptive actions of Aprepitant in preclinical models of pain. These findings were consistent with the capacity of DIPMA-AP to inhibit sustained endosomal signalling *in vitro* (Appendix 1). Finally, we examined the effect of DIPMA-Ap in central sensitisation through the study of C-nociceptive fibre excitability. DIPMA-Ap significantly decreased the C-nociceptive fibres activity and wind-up activity in the SNS model of neuropathic pain, where free Aprepitant had no effect. These findings indicate that inhibiting endosomal NK_1R signalling decreases C-nociceptive fibres excitability, leading to nociceptive decrease, consistent with the capacity of DIPMA-Ap to inhibit SP-induced excitation of spinal neurons in vitro (Jensen et al., 2017; Ramírez-García et al., 2019).

The potential mechanisms involved may be inhibition of the endosomal signalling that affects ERK activity and promotes neuronal hyperexcitability (Schmidlin et al., 2001; Woolf, 2011; Geppetti et al., 2015; Gegelashvili and Bjerrum, 2017; Jensen et al., 2017; Yarwood et al., 2017; Stoeber et al., 2018; Ramírez-García et al., 2019; Mai et al., 2021). However, the precise mechanisms require further characterisation. For example, while ERK activity is associated with enhanced pain transmission and may promote transcriptional changes and therefore affect neuroplastic events during chronic pain states, the immediate signalling processes that enable a GPCR within an endosome to drive a largely cell-surface membrane depolarisation event, remain unknown. PKC is one candidate that is known to have a significant effect on neuronal excitability and pain (Barber and Vasko, 1996; Tingley et al., 1997; Déry et al., 2001; Brenner et al., 2004; Sweitzer et al., 2004; Kelly et al., 2008; Lee et al., 2008; Vergouts et al., 2017) and further studies into how NK1R-mediated upregulation of PKC related to direct changes in neuronal excitability would be valuable to investigate.

The enhanced effects of DIPMA-AP in the preclinical pain model could be related to delivery and retention of Aprepitant in endosomes of spinal neurons and the continued release of drug as nanoparticles encounter increasingly acidified endosomal compartments. The electrophysiological studies support the

antinociceptive enhancement of Aprepitant indicates that inhibiting the endosomal signalling of NK_1R decreases pain transmission by reducing the activity of C-fibres and decreasing the excitation of spinal neurons. However, further studies are required to fully understand the mechanism of action and interactions of these nanoparticles in the spinal cord.

In this study, Aprepitant was loaded at a maximum of 50% into DIPMA nanoparticles, and further modification of the chemistry may improve the encapsulation ratio of the drugs, including pharmacokinetics and pharmacodynamic studies PK/PD. Indeed, similar studies using related micelle-based drug delivery systems have been used to deliver anticancer cargo into a murine cancer model. These studies found that nanoparticles with lower loading present a superior PK/PD profile and enhanced efficacy in the murine cancer model, reducing cytotoxicity (Chu et al., 2013; Rodallec et al., 2018; Lin et al., 2020).

The therapeutic efficacy of nanoparticles such as these could also be improved by testing other antagonists for GPCRs known to participate in pain transmission such as NK_1R, CLR and PAR2 (Jensen et al., 2017; Yarwood et al., 2017; Jimenez-Vargas et al., 2018). This could be valuable for delivering a large drug payload, containing other drugs alone, and even delivering other antagonists as a co-loaded system with aprepitant in the same nanoparticles. While these Nps were administered directly into the spine, the current expectation is that peripheral administration would not be able to achieve the same therapeutic effect due to the challenges of nanoparticles crossing the blood-brain barrier (Zhou et al., 2018; Feng et al., 2019), while this remains to be tested, other strategies may aid this approach. In addition, these particles may be optimised for drug delivery to specific tissues, for example, by incorporating targeted molecules such as antibodies or agonists to the exterior of the particles to increase cell selectivity (MacKay et al., 2014; Kaur et al., 2016; Chamseddine and Kokkolaras, 2018; Mitchell et al., 2020). This has been investigated for related nanoparticle systems, for example, antibody conjugation to gold nanoparticles resulting in a sensitive bioconjugation to detect specific bacteria (Arruebo et al., 2009; Busch et al., 2019) or by functionalisation that incorporate glutathione (GSH) to the outer face of NPs. GSH on the surface enhances neuronal uptake of nanoparticles and avoid the lysosomal degradation (Paka and Ramassamy, 2017). These studies demonstrated that choosing the ideal molecule to decorate the exterior of nanoparticles is a crucial step to improving and innovating cells targeting through the use of nanomaterials.

Finally, GPCRs target most clinically approved drugs, representing more than 30% of all available therapeutics. However, many drugs for many GPCR targets have failed in the clinic. Indeed, those

drugs that have succeeded only target 15% of all GPCRs in this superfamily (Purcell and Hall, 2017; Congreve et al., 2020). In some cases, this may be for unknown reasons, however suboptimal formulation, leading to limited absorption, a lack of targeting and safety issues are major causes. In addition, a substantial explanation of the failure may be due to the wide intracellular distribution of GPCRs -many GPCRs are known to undergo internalisation processes and hence, in some cases at least, drugs may not be able to reach the concentration required to bind and modulate these internalised receptor pools effectively. While this is a proof-of-concept study that may have limited therapeutic potential in its current form, nanoparticle encapsulation more broadly may advance the development of drugs to treat multiple diseases by altering their intracellular distribution to fine-tune signalling processes of pathophysiological importance.

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Chapter 4:

Endosomal metabotropic glutamate receptor 5 mediates location-specific signalling and pain transmission

1. Abstract

The metabotropic glutamate receptor 5 ($mGlu_5$), a GPCR essential for biological process such as neuronal development and synaptic transmission. However, mGlu5 signalling dysregulation plays important roles in pathophysiological processes such as schizophrenia, fragile syndrome X and pain. Previous studies in neurons have shown that $mGlu_5$ is expressed on the plasma membrane, within the endoplasmic reticulum and can also undergo endocytosis. Recent studies investigating $mGlu_5$ in neuropathic and inflammatory animal pain models have also revealed that sustained stimulation promotes recruitment to the inner nuclear membrane. These data suggest that the intracellular distribution of $mGlu_5$ is dynamic and that trafficking between the plasma membrane and intracellular sites such as the nucleus can modulate gene expression and signalling pathways of importance to pain. Although $mGlu_5$ endocytosis is known, investigations into the role of mGlu5 trafficking to endosomes and whether this contributes to pain are yet to be explored. Furthermore, many potent, selective mGlu5 antagonists and allosteric modulators have been optimised to achieve superior pharmaco-kinetic properties and minimise off-target effects, but to date have led to limited success in preclinical development, potentially due to a lack of targeting or absorption into intracellular locations.

Consistent with other trafficking receptors, we hypothesised that $mGlu_5$ internalisation to endosomes promotes location-specific, sustained signalling, to contribute to physiological processes such as pain transmission. Using genetic encoded signalling biosensors and pharmacological tools, we report that cell surface stimulation leads to $mGlu_5$ recruitment of $G\alpha_q$ and $G\alpha_s$ protein subunits at the plasma membrane. Following endocytosis $mGlu_5$ continue to recruit $G\alpha_q$ in Rab5-positive membranes, to promote sustained intracellular Ca^{2+} mobilisation and ERK activity. The kinetics and amplitude of this signalling is reduced with inhibition of dynamin or the excitatory amino acid transporters (EAAT), suggesting that dynamin-dependent endocytosis and intracellular glutamate transport are required for trafficking and the complete $mGlu_5$ signalling response. To further demonstrate the importance of signalling from endosomes, we utilised pH-responsive micellar nanoparticles to selectively load and release the $mGlu_5$ negative allosteric modulator VU058 (DIPMA-VU058) within the acidified environment of endosomes. Biasing drug action toward endosomal $mGlu_5$ pools improved the efficacy of VU-058 for inhibiting sustained intracellular Ca^{2+} mobilisation and ERK activity *in vitro*. Furthermore, intrathecal administration of VU-058 nanoparticles achieved superior analgesia in acute and chronic models of pain when compared to VU-058 alone. Together, these findings suggest that $mGlu_5$ can signal from endosomes and can lead to more effective pain inhibition when biasing drug

toward these sites. It highlights the importance of profiling trafficking-signalling relationships and the need for inhibitors that modulate mGlu5 at multiple locations, when assessing drug efficacy and formulation.

2. Introduction

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system (CNS), exerting its action by activating ionotropic (iGlu) and metabotropic glutamate (mGlu) receptors. iGlu receptors are ligand-gated ion channels and are involved in fast excitatory neurotransmission (Traynelis et al., 2010). The three iGlu receptor subclasses are the alpha amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainic acid (KAr) and N-methyl-D-aspartate (NMDA) receptors Bonnet:2015hx, Paoletti:2007je, Traynelis:2010hi, Pollok:2020ee}. mGlu receptors are GPCRs responsible for the slow neuromodulatory response to glutamate, classified into three subgroups (I-III) based on their signalling pathway and sequence homology (Niswender and Conn, 2010; Pereira and Goudet, 2018).

The group I ($mGlu_{1}$ & $_{5}$) are highly expressed in sensory neurons, and are excitatory receptors that canonically couple $G\alpha_{q/11}$ leading to activation of phospholipase C (PLC), followed by activation of protein kinase C (PKC) and mitogen activated protein kinase (MAPK) pathways (Alvarez et al., 2000; Hubert et al., 2001; Rush et al., 2002; Vidnyánszky et al., 1994). The group II ($mGlu_{2}$ & $_{3}$) and III ($mGlu_{4}$, $_{6}$, $_{7}$ & $_{8}$) are involved in presynaptic inhibition and modulation through adenylate cyclase (AC) inhibition, which leads cAMP reduction and inactivation of protein kinase A (PKA) (Crupi et al., 2019; Pereira and Goudet, 2018).

mGlu receptor activity is directly affected by the availability of free glutamate, where an extracellular imbalance of glutamate is associated with pathogenesis of many neurological disorders (Dong et al., 2009; Olloquequi et al., 2018; Osikowicz et al., 2013; Petrenko and Shimoji, 2001). For example, high glutamate concentrations overstimulate glutamate receptors to trigger excitotoxicity, leading to apoptosis and necrosis, mainly as a consequence of the massive Ca^{2+} influx ($_{i}[Ca^{2+}]$) from extracellular sources (Dong et al., 2009; Olloquequi et al., 2018). Furthermore, there are no known extracellular enzymes that degrade glutamate. Thus, a tight regulation of the extracellular glutamate levels is essential for avoid neurotoxicity (Gegelashvili and Bjerrum, 2017; Pereira and Goudet, 2018).

Extracellular glutamate is highly regulated by uptake through five different excitatory amino acid transporter (EAATs). EAAT1 and EAAT2 are expressed predominantly in glial cells (microglia and astrocytes), whereas EAAT3 (or EAAC1) is neuron-specific. In pain, EAAT1-3 activity regulate the extracellular glutamate concentration, where an increased extracellular glutamate concentration induces pain-related behaviours (Liaw et al., 2005; Magi et al., 2019; Weng et al., 2006). Prior investigation indicates that EAAT3 undergoes constitutive clathrin-dependent endocytosis trafficking,

where a minor proportion of EAAT3 is at the cell surface, and the vast majority is found in intracellular compartments (endosomes) (He et al., 2000; Holmseth et al., 2012). However, EAAT3 trafficking is regulated by the levels of activated PKC, where high levels block constitutive exocytosis and promote endocytosis of EAAT3, causing its accumulation in Rab5 and Rab11 endosomes (Padovano et al., 2009). Furthermore, EAAT pharmacological inhibition elevates extracellular glutamate concentrations in the spinal cord inducing hypersensitivity, thermal and mechanical hyperalgesia through mGlu and iGlu receptors activation (Liaw et al., 2005; Scimemi et al., 2009). Nonetheless, EAAT3 inhibition decreases nuclear $mGlu_5$ signalling and plays an important role in neuronal excitation that reduce pain behaviour in CFA-induced pain and neuropathic mice (Jong et al., 2014; Vincent et al., 2016; 2017). While a detailed mechanism of how EAAT3 affects $mGlu_5$ signalling remains unclear, these findings suggest that iGlu receptors may be highly sensitive to extracellular glutamate whereas $mGlu_5$ signalling rely on glutamate uptake into intracellular sites for promoting pain.

$mGlu_5$ is widely expressed and distributed in many regions in the brain and spinal cord (Alvarez et al., 2000; Honda et al., 2017; Lax et al., 2014; Neugebauer, 2002; Vidnyánszky et al., 1994; Vincent et al., 2016), and is a crucial modulator of synaptic plasticity and neuronal development (Eng et al., 2016; Niu et al., 2020; Xiao et al., 2013; Xie et al., 2017). Indeed, dysregulation of $mGlu_5$ signalling is associated with neurological disorders including psychosis and schizophrenia (Kryszkowski and Boczek, 2021; Nicoletti et al., 2019), addiction (Ceccarini et al., 2020; Peters et al., 2016), fragile syndrome X (Hessl et al., 2019; Stoppel et al., 2017; Youssef et al., 2018) and pain (Peterson et al., 2017; Vincent et al., 2016; 2017; Yu et al., 2018) and is therefore considered an important therapeutic target in the CNS. This is also consistent with pain-focussed studies, where $mGlu_5$ antagonists have shown promise for decreasing mechanical and thermal hyperalgesia in a broad range of pain conditions, including pre-clinical inflammatory pain and long neuropathic pain models (Lax et al., 2014; Montana et al., 2011; Vincent et al., 2016; 2017). Unfortunately, for those ligands that have progressed to clinical safety and efficacy studies, $mGlu_5$ antagonists have resulted in increased opioid tolerance, locomotor deficits and minimal effect decreasing pain (Abou Farha et al., 2014; Cavallone et al., 2020; Schröder et al., 2009; Sevostianova and Danysz, 2006). Thus, better drug formulation and drug delivery system are required as well as understanding of the receptor biology at intracellular location could be the key to improve outcomes.

While most research effort is focused on $mGlu_5$ in the plasma membrane, the localisation of $mGlu_5$ is dynamic, and requires trafficking between the plasma membrane and intracellular membranes (Francesconi et al., 2009; Trivedi and Bhattacharyya, 2012; Vincent et al., 2016). Activated $mGlu_5$ can rapidly internalise via clathrin-mediated processes, as well as via caveolin mediated endocytosis (Dhami and Ferguson, 2006; Fourgeaud et al., 2003). Both internalisation mechanisms require the GTPase dynamin for scission of newly formed vesicles budding from the cell surface. Studies in other trafficking GPCRs in spinal neurons, such as the Neurokinin 1 receptor (NK_1R) and the Calcitonin receptor-like receptor (CLR), have shown that dynamin-mediated endocytosis is critical for these receptors to also undergo endocytosis and achieve a complete signalling repertoire that can stimulate neuronal excitability and pain transmission (Jensen et al., 2017; Ramírez-García et al., 2019; Yarwood et al., 2017). Several studies indicate that $mGlu_5$ receptors undergoing endocytosis via these pathways are initially recruited to Rab5-positive early endosomes (Francesconi et al., 2009; Jong et al., 2009a; Lee et al., 2008; Stoppel et al., 2017; Trivedi and Bhattacharyya, 2012). As for most GPCRs, including those that undergo beta-arrestin mediated endocytosis, the canonical view is that directing receptors to endosomes is required for termination of cell surface signalling. However, growing evidence indicates GPCRs in endosomes can generate persistent signals from intracellular sites and many of these are critical for the control of nociceptive neurons (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Jiménez-Vargas et al., 2021; Ramírez-García et al., 2019; Yarwood et al., 2017).

In spinal neurons, $mGlu_5$ is expressed on the plasma membrane and endoplasmic reticulum (Jong et al., 2009b; O'Malley et al., 2003), and it was recently shown that $mGlu_5$ distribution or translocate from these sites to the inner nuclear membrane and cytosol after nerve injury and CFA-induced pain (Vincent et al., 2016; 2017). Intracellular $mGlu_5$ is associated with upregulation of Jun and c-Fos transcription factors to enhance pain behaviour. In addition, EAAT3 is also proposed to play an important role, by supplying glutamate to intracellular $mGlu_5$ enhancing its signalling repertoire (Jong et al., 2009a; O'Malley et al., 2003; Vincent et al., 2016; 2017).

Here, using genetic-encoded signalling biosensors, we show $mGlu_5$ activation leads $G\alpha_{q/11}$ recruitment into Rab5-positive endosomes in an agonist-dependent manner, which promotes sustained intracellular Ca^{2+} mobilisation and ERK activity. In addition, EAAT3 contributes as an intracellular supplier of glutamate, modulating $mGlu_5$ intracellular signalling. Furthermore, we used a recently described drug delivery system to show that endosomal delivery is more effective at blocking $mGlu_5$ intracellular signal *in vitro* and *in vivo* using acute and chronic pain models.

3. Methods

Drugs. Glutamate as L-Glutamic acid, (S)-3,5-dihydroxyphenylglycine (DHPG) were obtained from (Sigma Aldrich, NSW, Australia). DL-threo-β-benzyloxyaspartic acid (DL-TBOA), JNJ16259685 obtained from (Tocris, Bristol, United Kingdom). pH-responsive DIPMA and DIPMA-Cy5 block copolymers used for assembling nanoparticles were synthesized previously (Ramírez-García et al., 2019) and provided as a gift from Michael Whittaker (Monash University). Dyngo4A (Abcam, Cambridge, United Kingdom), VU0366058 (VU058; Medkoo Biosciences, North Carolina, USA).

Cell lines. HEK293A cells stably expressing wild-type rat $mGlu_5$ (HEK-$mGlu_5$), $mGlu_{5\text{-venus}}$ or $mGlu_{5\text{-Rluc}}$ (a gift from Professor Kevin Pfleger, University of Western Australia) were maintained at 37ºC and 5% CO_2 in Dulbecco's modified eagle medium (DMEM) with 5% (vol/vol) fetal bovine serum (FBS) and 500µg/mL geneticin (G418) (Sigma Aldrich, Darmstadt, Germany) for cell line selection.

Intracellular calcium assay. HEK-$mGlu_5$ cells were seeded at a density of 30,000 cells/well onto poly-D-lysine coated clear-bottom 96 well plates (Corning, NY, USA) using assay media (glutamine-free DMEM supplemented with 5% dialyzed FBS and 20mM HEPES) for 24h. Cells were then loaded with Fura2-AM ester (1µM. Sigma Aldrich, NSW, Australia) in HEPES-buffered saline (150mM NaCl, 2.6mM KCl, 0.1mM $CaCl_2$, 1.18mM $MgCl_2$, 10mM D-glucose, 10mM HEPES and 0.5% BSA, pH 7.4) supplemented with 4 mM probenecid and pluronic F127 (0.02%; Sigma Aldrich, NSW, Australia) for 45min at 37ºC. Fluorescence was measured at 340nm and 380nm excitation and 530nm emission using a FlexStation III Microplate Reader (Molecular Devices, Sunnyvale, CA) as previously described (Peng et al., 2020; Retamal et al., 2021). A baseline was recorded for 20 s before agonist addition. Agonist and antagonist treatments are as indicated in each experiment. Results are expressed as a percentage of the maximum i[Ca^{2+}] response to ionomycin (1µM).

Cell transfection. HEK cells expressing stable wild type $mGlu_5$ or $mGlu_5$-RLuc8 (700.000 cells/dish) were seeded onto 10 cm petri dishes (Corning™, USA) in DMEM (5% FBS-G418) and incubated for 24h (37°C, 5% CO_2). Prior to the transfection, media was changed to fresh DMEM supplemented with 5% FBS and 5 µg/ml G418. Plasmid DNA-encoding cytosolic or nuclear ERK sensor (nucEKAR, cytoEKAR; (Harvey et al., 2008), NES-venus-mGα or NES-NLuc-mGα ($mG\alpha_s$, $mG\alpha_{q/11}$, $mG\alpha_{i/0}$ or $mG\alpha_{12/13}$) (gift from Prof. Nevin A. Lambert, Augusta University (Wan et al., 2018), or mutant

dynamin K44E plasmid (as indicated in relevant sections(Schmidlin et al., 2001)). Cells were transfected (2.5µg DNA per dish) using PEI at a 1:6 ratio and plated 24 h post-transfection.

Förster resonance energy transfer (FRET) measurements. HEK-mGlu$_5$ cells transfected with nucEKAR and cytoEKAR sensor were seeded onto poly-D-lysine coated black 96 well culture plates (Perkin Elmer, USA) and incubated for 24h (37°C, 5% CO_2). Cells were serum-starved for 8-12h prior to use. On the day of the assay, cells were equilibrated in Hanks' balanced salt solution (HBSS) supplemented with 12mM HEPES at 37°C in a CO_2-free incubator. FRET signals were measured using a PHERAstar FS (BMG LABTECH, Germany) with an optic module FI 430/530/480 every 1min. Baseline readings were obtained for 5min followed by stimulation with either agonist or the positive control, phorbol 12,13-dibutyrate (PDBu, 1µM). Measurements were then taken for a further 30min. The half-maximal effective concentration (EC_{50}) was determined by plotting the area under the curve (AUC) of the response for cells challenged with a concentration range of the agonist.

Bioluminescence resonance energy transfer (BRET). HEK-mGlu$_{5\text{-Rluc8}}$ cells transfected with miniG$\alpha_{\text{-Venus}}$, KRas$_{\text{-GFP}}$ or RAB5$_{\text{-GFP}}$ were seeded onto poly-D-lysine coated white 96-well plates and incubated for 24h. On the day of the assay, cells were equilibrated in HBSS supplemented with 12mM HEPES at 37°C in a CO_2-free incubator. BRET experiments were performed using coelenterazine-H (NanoLight Technologies, AZ) for *Renilla* luciferase (RLuc8) or furimazine (NLuc). BRET signal was measured using a PHERAstar FS and calculated as the emission ratio at 530nm by Venus over the emission at 430nm by RLuc8 (optic module FI 535 480). Baseline was measured for 5min followed by stimulation with agonist.

pH responsive nanoparticles (NPs). Polymer synthesis, nanoparticle assembly, and characterisation are described in detail (Ramírez-García et al., 2019). Briefly, amphipathic block copolymers were synthesised with units of Di-isopropyl methacrylate (DIPMA) units in the hydrophobic portion to DIPMA co-assembling nanoparticles were made using a of DIPMA copolymer (1mg/mL) and 1mM of VU0366058 in tetrahydrofuran (THF). The mixture was added into PBS (pH 7.4) under vigorous stirring. Finally, nanoparticles were dialyzed against PBS using dialysis membrane MWCO 3500 (Thermo Fisher Scientific, USA) for 24h. DIPMA-Cy5 nanoparticles for imaging studies were assembled following the same methodology without addition of drug.

mGlu$_5$ trafficking and immunofluorescence. HEK-mGlu$_{5\text{-Venus}}$ cells were seeded onto poly-D-lysine coated glass coverslips in DMEM supplemented with 5% FBS for 24h. Cells were challenged with agonist (1µM) for 5, 15, 30min or 1h. Cells were washed with phosphate-buffered saline (PBS)

before fixation (4% paraformaldehyde, 15min on ice), then incubated in permeabilizing/blocking buffer (5% normal horse serum, 0.1% saponin in PBS containing 0.1% sodium azide) for 1h at room temperature. Cells were incubated with a monoclonal purified anti-EEA1 (mouse, # 610457, BD bioscience, RRID: AB_397830, 1:500) in blocking buffer overnight at 4°C. Cells were then washed and incubated with secondary antibody (donkey anti-mouse Alexa 546, 1:500; ThermoFisher) in PBS for 2h at room temperature. Coverslips were mounted using ProLong Diamond Antifade Mountant (ThermoFisher).

Nanoparticle trafficking in HEK-mGlu$_5$ cells. HEK-mGlu$_5$ or HEK-mGlu$_{5\text{-Venus}}$ cells were plated on poly-D-lysine coated glass coverslips in DMEM supplemented with 5% FBS for 24h. Cells were incubated with DIPMA-Cy5 nanoparticles (20μg ml−1, 30 min, 37°C) or vehicle, followed by the addition of agonist (10μM). Endosomes were identified by immunofluorescence using a monoclonal purified anti-EEA1 as described above using secondary antibody (donkey anti-mouse Alexa 546, 1:500; ThermoFisher). HEK-mGlu$_5$ cells were used to identify the interaction of nanoparticles with endosomes after mGlu$_5$ internalisation. At the same time, HEK-mGlu$_{5\text{-Venus}}$ was used to identify the interaction of nanoparticles with mGlu$_5$ after agonist addition.

Confocal microscopy. Cells were imaged using a Leica TCS- SP8 Lightning confocal microscope equipped with HCX PL APO ×10 and 63 (NA 1.40) glycerol objective. Three regions for each treatment were captured at 16-bit depth and 1024 × 1024- pixel resolution in six independent experiments. Images were analysed using the FIJI distribution of ImageJ, and co-localisation was evaluated by determining the Manders overlap coefficient (Manders et al., 1993).

Analysis of behavioural responses to pain. Male C57BL/6 mice (6-10 weeks) were sourced from the Monash Animal Research Platform and the New York University Langone Medical Center. This study was approved by the respective Animal Ethics Committees at Monash Institute of Pharmaceutical Sciences, Monash University and New York University Langone Medical Center. Animals were maintained at 22 ± 3°C in a controlled environment of 12h light/dark cycle with food and water *ad libitum*. Studies were performed following the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and adhered to the ARRIVE guidelines (Kilkenny et al., 2010). Investigators were double blinded to the treatment groups, and animals were randomly assigned to treatments and studied during the light cycle. At the end of the study, animals were euthanised by anaesthetic overdose.

Drug administration. Drugs were administered by intrathecal injection (5µl) into the intervertebral space (L4/L5) 30min before capsaicin administration, 48h after complete Freund's adjuvant (CFA) administration, or 10 days post sciatic nerve injury. The following compounds were tested: Fenobam (200nM), VU0366058 (300nM) or nanoparticles delivering an equivalent dose of VU0366058 (DIPMA-VU058, 30µg ml-1, 300nM) or vehicle (artificial cerebrospinal fluid, aCSF).

Acute inflammatory pain model. The acute nociceptive pain model was assessed as previously described (Jensen et al., 2017; Ramírez-García et al., 2019). Briefly, capsaicin (5µg, 80% NaCl 0.9%, 20% tween20) or vehicle (0.9% NaCl) (10µl) was subcutaneously administered to the plantar left hind paw of sedated mice (3% isoflurane). After 30 min post-capsaicin injection drugs were administered intrathecally.

Chronic inflammatory pain model: The inflammatory chronic pain model was induced as previously described (Ramírez-García et al., 2019). Briefly, CFA (200µL of *Mycobacterium* 1 mg/mL) was mixed with an equal volume (200µL) of saline solution (0.9 % NaCl) and vortexed to form an emulsion. This emulsion (10µL) was subcutaneously administered in the plantar left hind paw of sedated mice (3% isoflurane), and drugs or nanoparticles were administered intrathecally after 48 h post CFA administration.

Spared Nerve Injury Model. The spared nerve injury (SNI) procedure was performed as previously described (Cichon et al., 2018), with notable adaptations. Briefly, animals were anesthetised with 5% inhaled isoflurane, the left thigh was shaved, and the area sterilised using isopropyl alcohol and iodine wipes. a 1cm skin incision was made on the lateral surface of the left thigh, the muscle bluntly dissected to identify the sciatic nerve and its 3 terminal branches. The common peroneal and tibial nerves were ligated using 6-0 silk (DemeTECH, USA), and a 1mm segment of the two nerves was removed just distal to the ligature (SNI). For sham procedures, the trifurcation was identified, but the nerves left untouched. The muscle layer was closed using 5-0 vicryl (Ethicon, USA), and the skin closed with 5-0 surgipro (Ethicon, USA). Animals were given buprenorphine (2.2 mg/kg i.p) prior to cessation of anaesthesia and then dosed every 12h for 3 days post-surgery. Investigators were blinded to the surgery (SNI or Sham) and treatments (nanoparticles or free drug). Mice were randomly assigned to treatments and the experiments were performed during the light cycle.

Mechanical allodynia. Mechanical nociception was assessed by measuring withdrawal thresholds of the ipsilateral and contralateral hind paw using calibrated Von Frey Filaments (VFF) as previously described (Jensen et al., 2017; Mai et al., 2021; Ramírez-García et al., 2019). Briefly, before

experiments, animals were acclimatised for 2h in individual acrylic boxes. A baseline of withdrawal thresholds was measured before administration of drugs or nanoparticles to establish a normal response for each mouse. After capsaicin administration, withdrawal thresholds were measured every 30min for the first 2h, then every 60min for the next 3h, and finally after 24h. Results are normalised and expressed as a percentage of baseline.

Statistical analysis. Data were analysed using GraphPad Prism 8 (GraphPad Software). Data are presented as mean ± s.e.m., unless noted otherwise. For multiple comparisons, results were compared using one- or two- way ANOVA followed by post-hoc multiple comparison tests, as described in the figure legends. P values < 0.05 were considered statistically significant.

4. Results

4.1. mGlu5 internalisation process and G-protein coupling.

Upon activation by glutamate or DHPG $mGlu_5$ undergoes Clathrin and caveolin-mediated endocytosis and once internalised into endosomes. Previous studies have shown it can recycle or be recruited to lysosomes to terminate signalling (Fourgeaud et al., 2003; Francesconi et al., 2009; Mahato et al., 2015; Trivedi and Bhattacharyya, 2012). To support prior imaging studies, we used BRET to quantify the endocytosis of $mGlu_5$, in response to the orthosteric endogenous agonist glutamate and the non-permeable and non-transported orthosteric agonist DHPG. To quantify $mGlu_5$ trafficking over time, HEK293 cells expressing stable recombinant mGlu5 with C-terminal fusion to *Renilla* luciferase 8 (HEK- $mGlu_{5\text{-}RLuc8}$; BRET donor) were transiently transfected with GFP-fusion proteins that reside the plasma membrane (KRas; $KRas_{\text{-}GFP}$) or early endosomes (Rab5 GTPase; $Rab5_{\text{-}GFP}$) (Bery et al., 2018; Khamlichi et al., 2019). Agonist-induced changes in proximity-dependent BRET energy transfer between $mGlu_{5\text{-}RLuc8}$ and $KRas_{\text{-}GFP}$ or $Rab5_{\text{-}GFP}$, were measured in the presence of luciferase substrate Coelanterazine H (**Figure 16 A**). A rapid decrease in the $mGlu_{5\text{-}RLuc8}$ -$Kras_{\text{-}GFP}$ BRET signal was observed upon stimulation with glutamate or DHPG, indicating rapid translocation of $mGlu_5$ away from the plasma membrane (**Figure 16 B-C, Supplementary Figure 1B**). These findings were correlated with an increased BRET ratio between $mGlu_5$ and Rab5 after glutamate or DHPG addition. Together, these findings indicate that $mGlu_5$ activation leads to rapid receptor internalisation into Rab5 positive endosomes (**Figure 16 C, Supplementary Figure 1D**). Interestingly, within the 30 min period of measurement, glutamate induced sustained $mGlu_5$ dissociation from the plasma membrane and a sustained association with positive Rab5-positive early endosomes. In contrast, DHPG induced a transient $mGlu_5$ internalisation, and a return of the BRET ratio to baseline values within 20min, suggesting that DHPG may promote greater association with rapid recycling pathways (**Figure 16 C, Supplementary Figure 1B, D**).

$mGlu_5$ endocytosis has previously been shown to participate in clathrin-mediated and caveolin-dependent processes (Fourgeaud et al., 2003; Francesconi et al., 2009; Trivedi and Bhattacharyya, 2012). Both mechanisms require activity of the GTPase dynamin for vesicle scission from the plasma membrane. Thus, we used the dynamin inhibitor Dyngo4A to assess if blocking dynamin-mediated endocytosis could prevent the internalisation measured in this BRET system. Pre-treatment with Dyngo4A prevented the glutamate and DHPG-induced dissociation of $mGlu_5$ from the plasma membrane and association with the endosomal marker Rab5 (**Figure 16B-D, Supplementary Figure**

1C, D). Confocal microscopy imaging of HEK cells expressing $mGlu_{5\text{-venus}}$ was used to confirm these BRET-based observations (**Figure 16E, F**). In absence of agonist, $mGlu_5$ distribution is predominantly at the cell surface and shows minimal overlap with early endosome antigen 1 (EEA1) staining (**Figure 16E,** Vehicle treatment). In cells challenged with glutamate or DHPG (EC_{80}, 30 min treatment shown) minimal $mGlu_5$ remained at the cell surface and internalised receptor was highly consistent with EEA1-positive early endosome membranes (white arrows, (**Figure 16E**). Association with early endosomes in fixed cells over time was also quantified by Manders' coefficient analysis, and indicated that glutamate induces a significant increase in overlap between $mGlu_5$ and EEA1 fluorescence between 15 min and 1h, whereas DHPG caused a significant interaction 15min post addition and subsequently decreased at 30 and 60 min (**Figure 16F**).

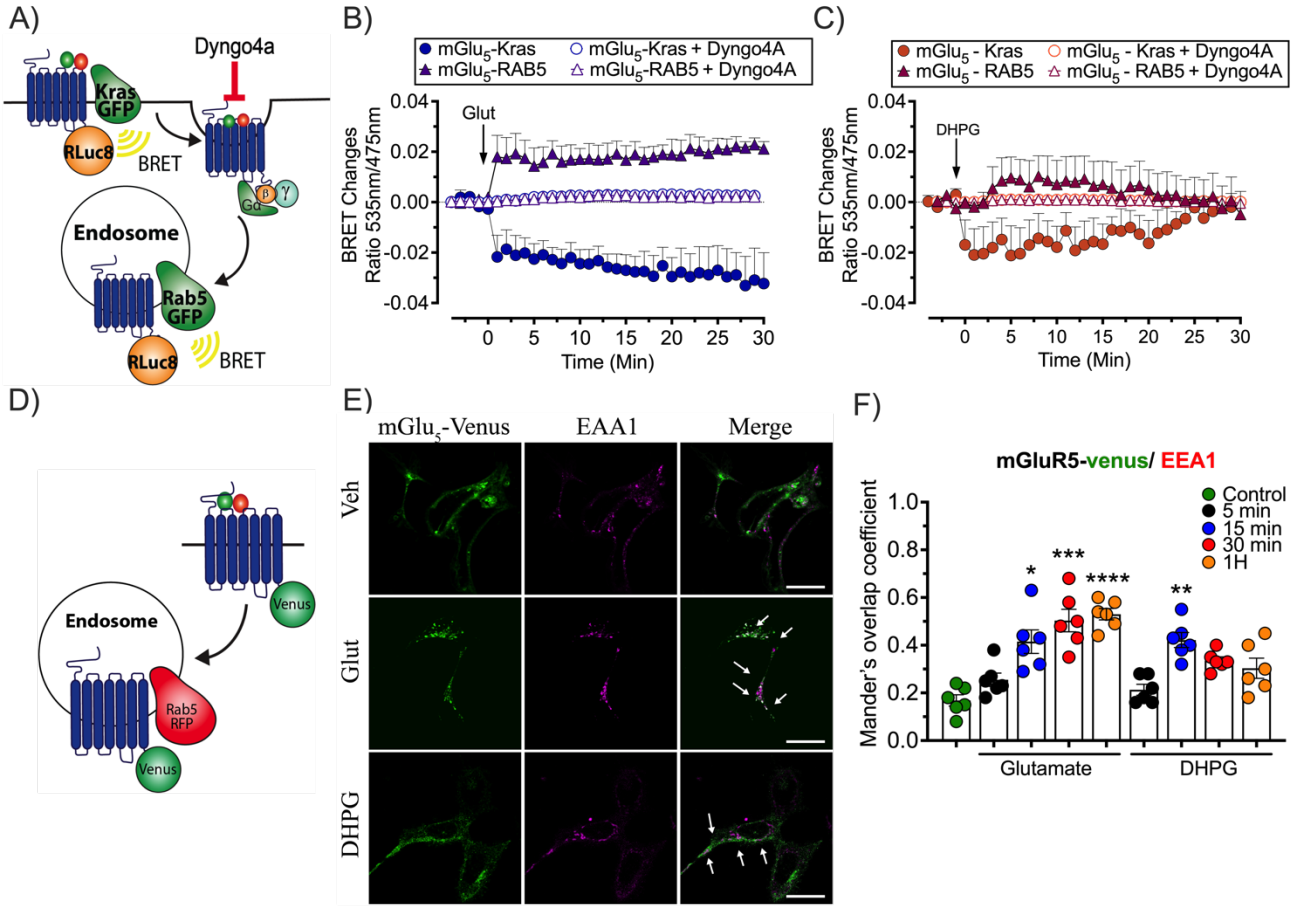


Figure 16. **$mGlu_5$ internalisation**. **A)** and **D)** Representative diagram for BRET and confocal microscopy assay utilised for $mGlu_5$ internalisation. **B)** and **C)** Representative time trace of BRET assay for $mGlu_5$ internalisation in association with Kras or Rab5 upon glutamate (10µM) or DHPG (10µM) addition in presence or absence of Dyngo4a. Data are presented as mean ± s.e.m., n = 6 independent experiments. **E)** Representative confocal images of vehicle, glutamate (1µM) and DHPG (1µM) treatment, 15 min post agonist addition**. F)** Analysis of Mander's overlapping coefficient after glutamate or DHPG-induced $mGlu_5$ internalisation into endosomes. Data are expressed as mean ± SEM of 20 cells, n = 6 independent experiments. *P < 0.05, **P < 0.005, ***P < 0.001, ****P < 0.0001 compared to control. one-way ANOVA, Dunnett's post-hoc test.

4.2. Assessing location-specific mGlu5 coupling to Gq and Gs protein subunits

GPCRs signal by coupling to heterotrimeric Gα proteins, which in turn activates kinases and other effector proteins to regulate nearly every aspect of cell function (Latorraca et al., 2017; Pavlos and Friedman, 2017; Tsvetanova et al., 2015). $mGlu_5$ is reported to couple to Gs and Gq proteins, and with knowledge that mGlu5 undergoes robust endocytosis. We hypothesised that $mGlu_5$ recruitment to early endosomes influences the magnitude and duration of Gα protein coupling, as observed for other nociceptive receptors (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Ramírez-García et al., 2019; Yarwood et al., 2017). $mGlu_5$ Gα recruitment was investigated in HEK-293 cells expressing stable $mGlu_5$-$_{Rluc8}$, by transfecting and transiently-expressing truncated, stabilised, catalytically inactive forms of G alpha protein subunits, known as "miniG" (mG) proteins, that contain a nuclear export sequence (NES) and are fused to a range of biophysical tags including Venus fluorescent protein (Wan et al., 2018). Established to investigate receptor engagement, a variety of mG fusions were developed, based on $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/0}$ or $G\alpha_{12/13}$. mGq (consisting of mGs scaffold with Gq loop that is required receptor binding) and mGs were applied here in microscopy and BRET-based biophysical assays, to assess ligand engagement and location-specific coupling (**Figure 17A**). Upon glutamate or DHPG stimulation, we observed an increase in BRET ratio for mGs and mGq proteins (**Figure 17B-D, supplementary Figure 2B-C**). Notably, glutamate induced a fast recruitment of mGq and mGs protein that was maintained over time. Two waves of mGq recruitment were also observed. While this may be an assay artefact, it was not evident for mGs recruitment and therefore may suggest rapid coupling at the cell surface, followed by enhanced recruitment with a second pool of receptor, being either additional cell surface coupling with recycling mGlu5 or recruitment of mGq to intracellular membranes (**Figure 17B, Supplementary Figure 2B**). In contrast, DHPG induced a slow increase in mGq and mGs protein recruitment compared to glutamate (**Figure 17C, supplementary Figure 2C**). With use of Dyngo4A pre-treatment to inhibit $mGlu_5$ internalisation, mGq recruitment in glutamate and DHPG-stimulated cells was abolished. mGs recruitment was also diminished with glutamate treatment, therefore suggesting that glutamate has the potential to promote robust G coupling from intracellular membranes (**Figure 17B-C, Supplementary Figure 2B-C**).

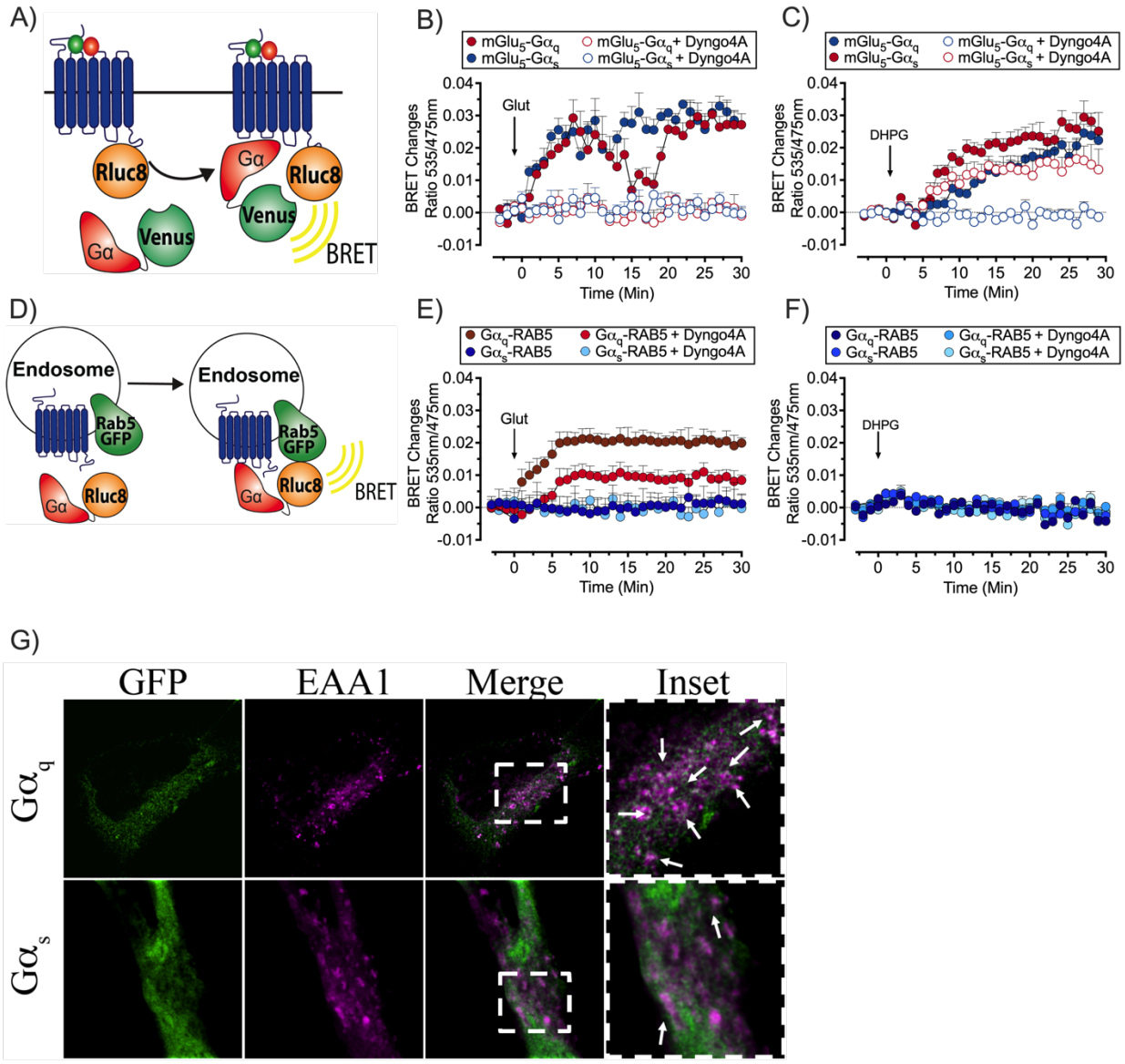


Figure 17. Measurement of mGlu5-dependent global and early endosome-specific Gα protein recruitment. A) and D) Illustrations of BRET tools applied to investigate global (B) or early endosome Gα protein recruitment (D). B) and C) Representative time trace of BRET assay for mGlu5-induced Gαq or Gαs recruitment upon glutamate or DHPG addition. E) and F) Representative time trace of BRET assay for mGlu5-induced Gαq or Gαs recruitment into endosomes after glutamate or DHPG addition in presence of absence of dyngo4a. G) Confocal microscopy of recombinant HEK-mGlu5 expressing Gαq or Gαs recruited to endosomes, 20 min post glutamate addition. Data are presented as mean ± s.e.m., n = 6 independent experiments.

4.3. $mGlu_5$ recruits $mG\alpha_{q/11}$ but not $mG\alpha_s$ in endosomes.

Our previous results indicate that $mGlu_5$ internalisation and Gα protein coupling is dependent on the nature of the agonists examined. Notably, glutamate induces a fast and sustained internalisation of $mGlu_5$ into endosomes and Gα protein recruitment. To examine whether $mGlu_5$ signals from the endosomes, we examined recruitment of Gα proteins into early endosomes using a location-specific

BRET assay. HEK-293 cells expressing wild type $mGlu_5$ were co-transfected with $Rab5_{-venus}$ and NES-NLuc-ms or mGq (**Figure 17D**).

An increase in the BRET ratio between Rab5-$_{Venus}$ and mGq-NLuc but not $mG\alpha_s$-NLuc8/Rab5-$_{Venus}$ was observed in cells stimulated with glutamate, which is indicative of $mG\alpha_{q/11}$ protein recruitment to early endosomes that contain internalised $mGlu_5$ (**Figure 17E, Supplementary Figure 3B**). To confirm this observation, Dyngo4A pre-treatment revealed a significant reduction in Rab5-mGq BRET ratio in response to glutamate. In contrast, stimulating cells with DHPG did not increase Rab5-mGq or Rab5-mGs BRET ratios, indicating that DHPG does not induce $mG\alpha_s$ or $mG\alpha_q$ recruitment into endosomes that contain $mGlu_5$ (**Figure 17F, Supplementary Figure 3B-C**). To confirm the recruitment of mGα proteins into EAA1 positive endosome, confocal microscopy was used to assess mG localisation in cultured HEK293 cells stably expressing untagged $mGlu_5$ and transfected with Venus-tagged mGq or mGs. In cells treated with 1μM glutamate for 15 min, mGq, but not mGs, showed a distribution that was consistent with staining using EEA1 antibody (resident protein of early endosomes; (**Figure 17G**).

Together, these data suggest that in the presence of glutamate, stimulated internalised $mGlu_5$ has the potential to recruit mGq on endosomal membranes and may therefore signal in $G\alpha_{q/11}$ protein-dependent manner from this location. DHPG-induced coupling to mGq is reduced with Dyngo4A treatment, but did not promote Rab5-specific mGq recruitment, further suggesting that DHPG can promote coupling on intracellular membranes, consistent with rapid recycling via a Rab5-independent mechanism.

4.4. Assessing endosome-mediated $mGlu_5$ signalling

Recent studies have indicated that the internalisation process is required for $mGlu_5$ to induce changes in intracellular Ca^{2+} concentrations ($_i[Ca^{2+}]$) via G protein-coupling dependent pathways (Honda et al., 2017; Jong et al., 2018; Neugebauer, 2002; Scheefhals et al., 2019; Vincent et al., 2016; 2017). However, how the internalisation process and EAAT3 may affect global calcium signalling by $mGlu_5$ has not been systematically investigated. Here, $_i[Ca^{2+}]$ was characterised by assessing glutamate- and DHPG-induced Ca^{2+} signalling in the presence of Dyngo4A or the EAAT3 inhibitor, DL-TBOA (TBOA). Glutamate triggered a rapid increase in $_i[Ca^{2+}]$ (first phase, 0 - 50sec) with an Emax of 52.9 ± 3.5% (relative to ionomycin control), followed by a second response (second phase 50-250sec), characterised by an Emax of 27.9 ± 2.9% (**Figure 18B;** EC_{50} reported in **Table 1**). To understand the

importance of $mGlu_5$ internalisation for glutamate-induced $_i[Ca^{2+}]$, we pre-treated the cells with Dyngo4A (30 µM) for 30 min. Inhibition of $mGlu_5$ internalisation decreased both phases of glutamate-induced $_i[Ca^{2+}]$ compared to the normal response (**Figure 18D, E**; **Table 1**).

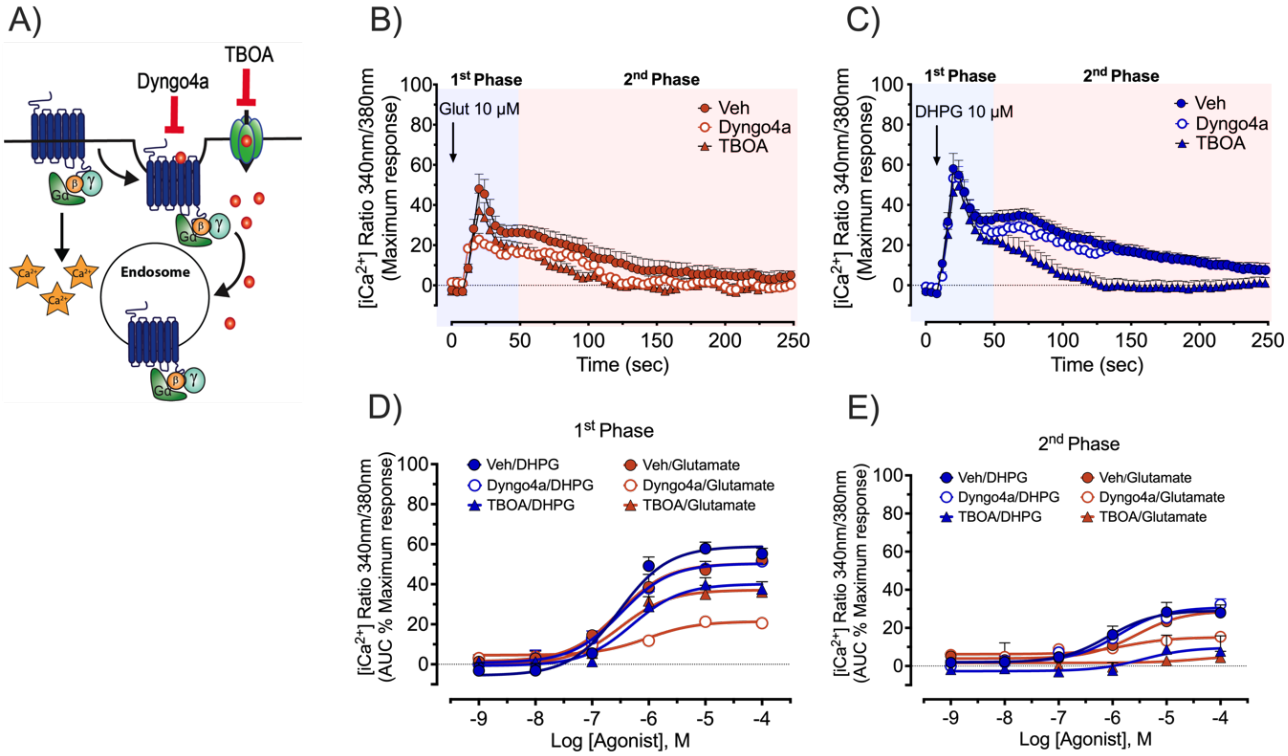


Figure 18. $mGlu_5$-induced calcium signalling in recombinant HEK-293 cells. Glutamate-induced calcium influx is dependent of internalisation process and EAATs activity. **A)** Representative diagram of $mGlu_5$ activation induced calcium influx. Representative time trace of **B)** glutamate and **C)** DHPG-induced calcium mobilisation in presence or absence of DL-TBOA (50 µM) or Dyngo4a (30 µM). Concentration response curve of normalised glutamate or DHPG-induced calcium influx **D)** First phase (0-50 sec) and **E)** Second phase (50-250 sec). Data are expressed as mean ± SEM and normalised to ionomycin (1µM). n = 6 independent experiments.

While EEAT transporters are critical for neuronal glutamate homeostasis, HEK293 cells endogenously express EAAT3, enabling assessment of the relative importance of EAAT3-dependent glutamate uptake in agonist-induced $_i[Ca^{2+}]$(Dunlop et al., 1999). To address this, we used the pharmacological inhibitor of EAATs, TBOA (50µM, 30 min). TBOA significantly decreased the second phase of the $_i[Ca^{2+}]$ response to glutamate (**Figure 18B**). The modification in the second phase of the response indicates that glutamate-induced $_i[Ca^{2+}]$ is mediated by the availability of glutamate in the cytosol and signalling by intracellular $mGlu_5$.

DHPG was used to enable study of the specific $_i[Ca^{2+}]$ signalling initiated by $mGlu_5$ at the plasma membrane and in rapid recycling pools. Consistent with observations in cells stimulated with

glutamate, DHPG promoted two-phases of $_i[Ca^{2+}]$ signalling. The first phase was characterised by a rapid increase of $_i[Ca^{2+}]$ with an Emax of 57.9 ± 3.1% (**Figure 18C-F**. **Table 1**), and a second phase with an Emax of 28.2 ± 2.4%. In contrast to glutamate-induced $_i[Ca^{2+}]$, yet in agreement with G protein-coupling experiments, inhibition of $mGlu_5$ internalisation showed minimal effects on any phase of DHPG-induced $_i[Ca^{2+}]$ (**Figure 18C-F**). Furthermore, inhibition of EAAT3 decreased the second phase of DHPG- induced $_i[Ca^{2+}]$ in a manner consistent with glutamate $_i[Ca^{2+}]$, where the Ca^{2+} signalling returns to baseline 2 min post-agonist addition.

4.5. $mGlu_5$ endocytosis mediates glutamate signalling in subcellular compartments.

$mGlu_5$ activation increases ERK activation and nuclear translocation to promote transcription and expression of proteins that enhance neuronal activity (Osterweil et al., 2010; Vincent et al., 2016; 2017). However, a detailed study of location-driven signalling remains unexplored. Here, we studied the link between $mGlu_5$ endocytosis and signalling from intracellular membranes. To explore the downstream signalling of $mGlu_5$, we assessed cytosolic and nuclear ERK activity using FRET sensors for cytosolic (CytoEKAR) or nuclear (NucEKAR) ERK activity.

Glutamate induced a rapid and sustained increase in cytosolic ERK activity at concentrations above 100nM (**Figure 19B. Table 1; Supplementary figure 4**). To understand the importance of $mGlu_5$ internalisation for cytosolic ERK activity, we transfected the dominant negative mutant of dynamin (K44E, prevents dynamin activity via loss of GTP binding) to inhibit $mGlu_5$ endocytosis. The inhibition of dynamin-mediated endocytosis by K44E decreased the second phase of cytosolic ERK activity (15 – 30min) and was associated with an increased EC_{50} (**Figure 19B, D-F. Table 1**). The non-permeable and non-transportable agonist DHPG induced a gradual increase in cytosolic ERK activity over time (**Figure 19C-F. Table 1**), which was not affected by K44E expression (**Figure 19C-F**). Collectively, these findings suggest that activation of cytosolic ERK by mGlu5 is independent of receptor trafficking or endocytosis.

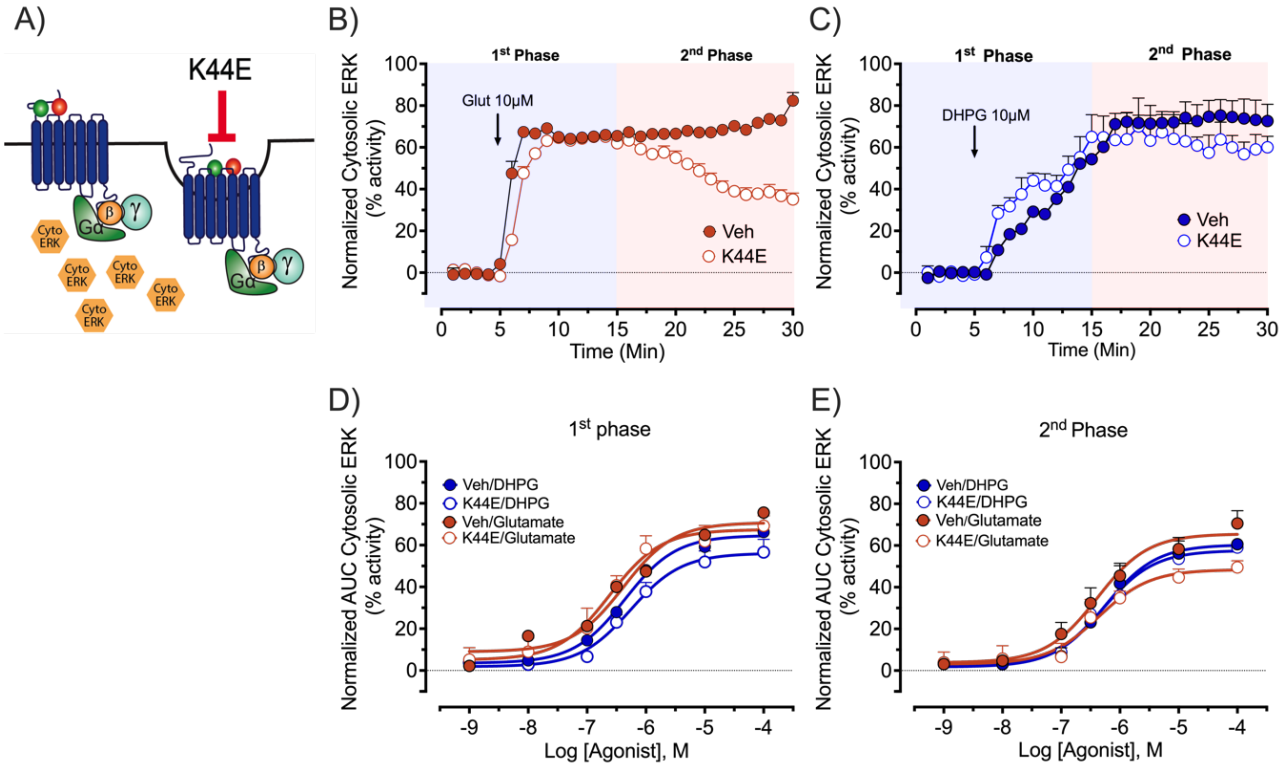


Figure 19. $mGlu_5$ activation induced cytosolic ERK activation. $mGlu_5$-induced cytosolic ERK activity is not affected by inhibition of $mGlu_5$ internalisation. **A)** Representative diagram of $mGlu_5$ activation induced cytosolic ERK activity. Representative time trace of **B)** glutamate and **C)** DHPG-induced cytosolic ERK activity in the presence or absence of K44E. Concentration response curve showing normalised glutamate and DHPG induced cytosolic ERK activity **D)** First phase (0 - 15min) and **E)** Second phase (15 - 30min). Data are expressed as mean ± SEM and normalised to PDBu (1 μM), n = 6 independent experiments.

4.6. Glutamate induced nuclear ERK activity is dependent of $mGlu_5$ internalisation and EAAT.

As an indicator of intracellular signalling, we used a nuclear-localised FRET biosensor to assess glutamate or DHPG-induced nuclear ERK. This approach is supported by previous studies that have shown ERK translocation and activity is enhanced by GPCR recruited to endosomes (Irannejad et al., 2017; Jensen et al., 2017; Jimenez-Vargas et al., 2018; Ramírez-García et al., 2019; Yarwood et al., 2017). Cell stimulation with glutamate promoted a rapid increase in nuclear ERK activity. Robust responses were observed with 100nM glutamate, and a maximum response was observed 10 min post glutamate addition (**Figure 20B**). In cells expressing the K44E variant of Dynamin, Nuclear ERK activity was still sustained but the maximal response was significantly decreased, suggesting that the internalisation of $mGlu_5$ is critical for the complete ERK signalling output (**Figure 20C-E**). AUC analysis of the first and second phase (10 min and 15 min post-drug addition, respectively) indicated

inhibition of $mGlu_5$ endocytosis affects both phases of glutamate-induced nuclear ERK (**Figure 20D-E. Table 1**).

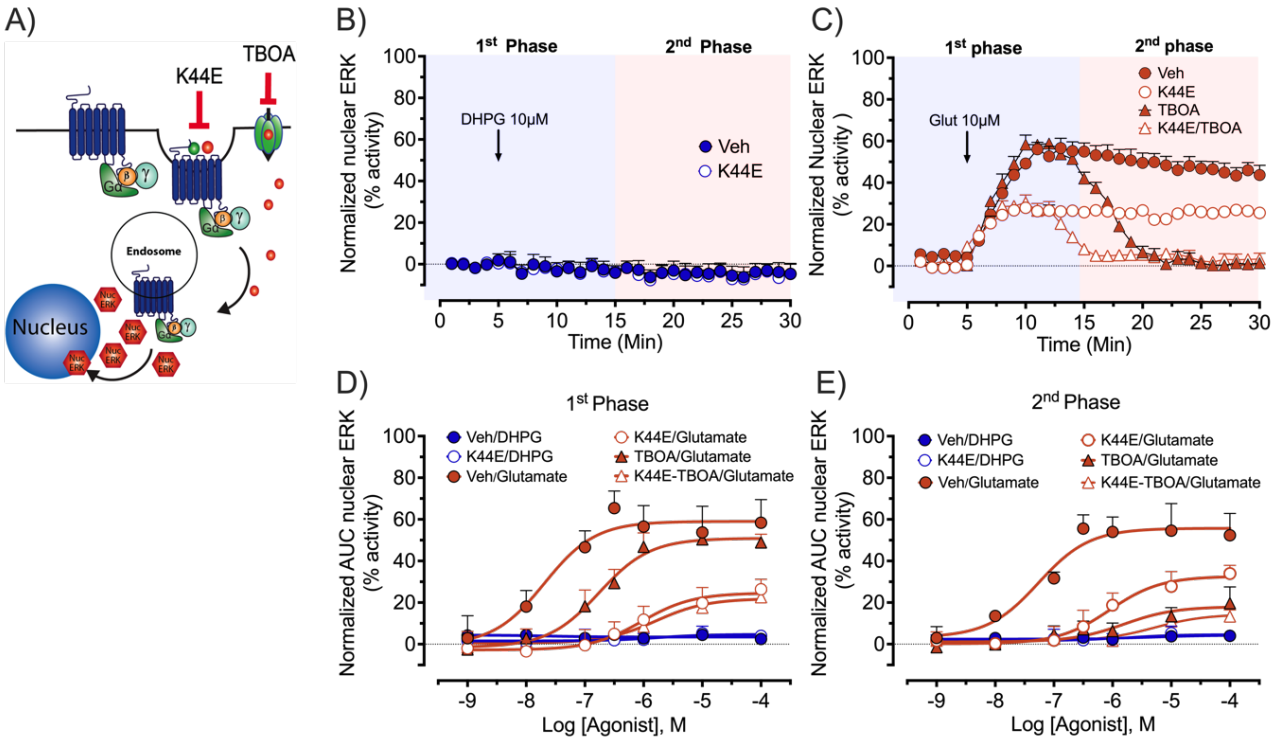


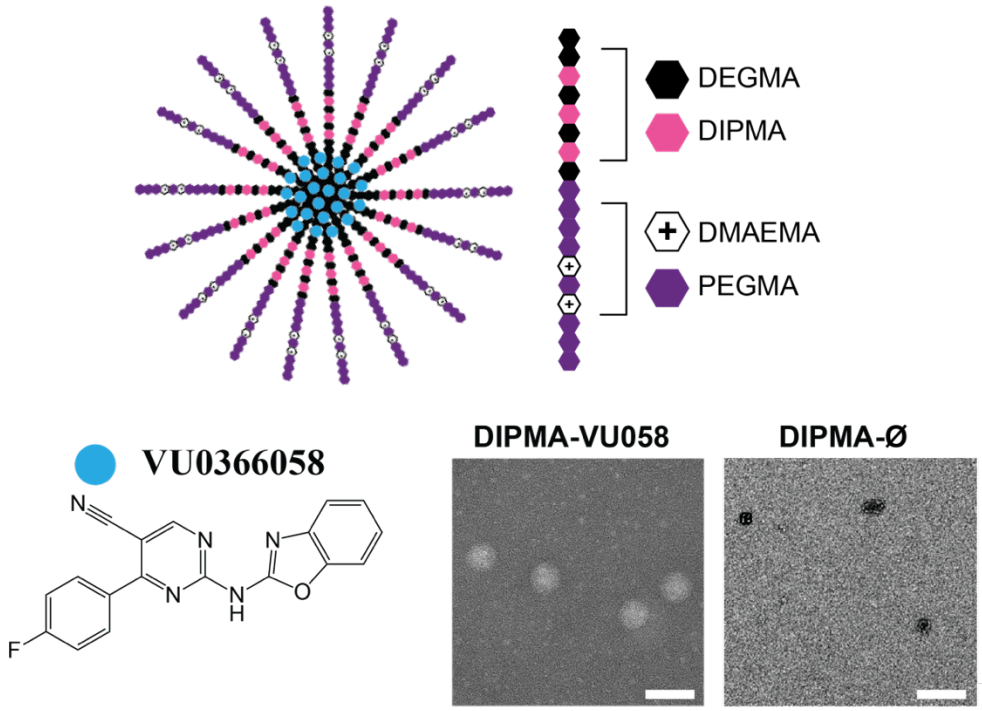
Figure 20. $mGlu_5$-mediated nuclear ERK activity. $mGlu_5$-induced nuclear ERK activity is dependent on the agonist, receptor internalisation process and EAAT activity. **A)** Representative diagram of $mGlu_5$-induced nuclear ERK activity, proposed to be activated in the cytosol, followed by translocation into the nucleus. Representative time trace of **B)** glutamate and **C)** DHPG-induced nuclear ERK activity in presence or absence of K44E or TBOA (50μM). Concentration response curve of normalised DHPG and glutamate induced nuclear ERK activity **D)** First phase (0 - 15min) and **E)** second phase (15 - 30 min). Data expressed as mean ± SEM and normalised to PDBu (1μM), n = 6.

Glutamate levels can alter $mGlu_5$ signalling, and glutamate can be transported from the extracellular space to the cytosol by EAAT transporter proteins, leading activation of intracellular $mGlu_5$ pools (Gegelashvili and Bjerrum, 2017; Purgert et al., 2014; Vincent et al., 2016; 2017). We characterised the importance of EAATs for location-specific nuclear ERK signalling mediated by $mGlu_5$. TBOA exclusively abolished the second phase of nuclear ERK activity observed after the addition of glutamate (**Figure 20C. Table1**). AUC analysis indicated inhibition of EAAT3 significantly reduced the sustained phase of glutamate-induced nuclear ERK activity (**Figure 20D-F**). In addition, when both endocytosis and EAATs were inhibited in combination, a significant decrease was observed in both phases of nuclear ERK activity induced by glutamate (**Figure 20C-F. Table 1**).

Together, these results indicate that $mGlu_5$ endocytosis and glutamate levels in the cytosol are important for glutamate-induced $mGlu_5$ to achieve its complete repertoire. Importantly, while the precise role of EEAT3 and other transporters in distributing glutamate into the lumen of endosomes remains unclear and has not been tested in this study, cytosolic glutamate levels are shown to be an important determinant of the sustained phase of intracellular signalling, as measured in $_i[Ca^{2+}]$ and nuclear ERK assays. In addition, none of the concentrations of DHPG induced nuclear ERK activity (**Figure 20D**). DHPG cannot be transported or diffuse through the plasma membrane and therefore does not activate existing intracellular $mGlu_5$ pools (Vincent et al., 2016; 2017). We hypothesise that the lack of nuclear ERK activation by DHPG is also due to the potential for limited internalisation into rapid recycling pools.

4.7. Characterisation of pH responsive nanoparticles for endosomal-specific drug delivery.

Our previous results indicated that $mGlu_5$ signalling is dependent on its location and the nature of the agonist examined. Recently, we developed a pH-responsive nanoparticle that consists of assembling amphipathic polymers in aqueous solutions, to form soft nanoparticles or micelles approximately 30-50nm in size, for passive uptake into cells and release of drug cargo when exposed to the acidic environment within the lumen of endosomes. In prior investigations (pre-chapter 3) nanoparticles were loaded with a hydrophobic antagonist for the NK_1R receptor, and due to the presence of unit were shown to enhance antagonism of endosomal-delimited NK_1R signalling, and decreasing pain behaviour relative to non-formulated drug. Here, we used DIPMA nanoparticles as a delivery tool for bypassing ligand-binding at the cell surface and directing $mGlu_5$-selective inhibitors to receptors that have been located and shown to be active in endosomes.



Nanoparticles	Diameter (nm)	Z-Potential (mV)	Drug loading (μM)
DIPMA-VU058	44.4 ± 1.5	-0.41 ± 0.05	3.85± 1.45 (40 %)
DIPMA-Ø	38.6 ± 3.4	-0.5 ± 0.02	NA

Figure 21. Characterisation of DIPMA nanoparticles. **A)** Structure of pH responsive (DIPMA) nanoparticles, pH responsive nanoparticles spontaneously assemble when using polymers comprised of a hydrophilic shell of P(PEGMA-co-DMAEMA) and hydrophobic core due to the presence of units of P(DIPMA-co-DEGMA). The lipophilic negative allosteric modulator VU0366058 (VU058) was chosen for drug loading. **B)** Representative transmission electron microscopy images of DIPMA-VU058 (100 nM VU-058) and DIPMA-Ø nanoparticles. **C)** Properties of DIPMA loaded VU058 (100 nM VU058) and DIPMA-Ø. Data are presented as mean ± s.d. Values in parentheses indicate the percentage of the initial drug incorporated into 1mg/ml DIPMA copolymer (% initial VU058 (mean ± s.d), n = 9 experiments. 100nm.

DIPMA nanoparticles were synthesised by as diblock copolymers, as previously described (Ramírez-García et al., 2019). The block copolymers used to generate these nanoparticles are comprised of a non-fouling hydrophilic portion with units of 2(p(PEGMA-co-DMAEMA)) and a hydrophobic portion with units of 1p(DIPMA-co-DEGMA). The DIPMA units within the core were chosen due to its intrinsic pKa (6.1). When exposed to the acidic environment of endosomes (pH 7-5.5) these units within the nanoparticle become protonated and promote charge-charge repulsion within the core, as a mechanism to achieve pH-sensitive drug release (**Figure 21A**) (Ramírez-García et al., 2019). Polymers solubilised in organic solvent were self-assembled by rapid co-injection into saline with VU0366058, a hydrophobic $mGlu_5$ negative allosteric modulator (NAM), to form micellar nanoparticles (described herein as DIPMA-VU058). Imaging by transmission electron microscopy indicated that nanoparticles loaded with VU0366058 present as a uniform spherical shape (**Figure**

21B). Dynamic light scattering studies (DLS) indicated that DIPMA-VU058 nanoparticles had a diameter of 44.4 ± 1.5nm with a slight negative ζ potential (-0.41 ± 0.05mV), intended to minimise interactions with phospolipid bilayers. Liquid chromatography-mass spectrometry (LC-MS) analyses indicated that DIPMA particles loaded approximately 40% of the initial drug added (**Figure 21C**). Empty particles were spherical in shape and had a smaller diameter of 38.6 ± 3.4nm and a similar ζ potential (-0.5 ± 0.02mV).

4.8. Assessing trafficking and endosomal uptake of nanoparticles on HEK-293 cells.

As previously described, trafficking of DIPMA particles into endosomes was assessed using nanoparticle assembled from DIPMA copolymers with cyanine 5 conjugated to the hydrophobic portion (DIPMA-Cy5) and incubated in recombinant HEK-$mGlu_5$ expressing $Rab5_{-GFP}$ (Ramírez-García et al., 2019). To assess the if DIPMA nanoparticles can internalise into $mGlu_5$-positive endosomes, HEK-293 cells expressing $mGlu_{5\text{-}Venus}$ or Rab5-GFP were incubated with DIPMA-Cy5 and imaged by confocal microscopy in live cells. DIPMA-Cy5 particles were detectable 5 min post-nanoparticle addition, but there was minimal overlap of these particles with mGlu5-$_{Venus}$ in unstimulated cells(**Figure 22A-B**). When cells were challenged with glutamate, the DIPMA-Cy5 particle distribution was punctate and remained in endosomes consistent with the localisation of mGlu5-$_{Venus}$. Subsequent pixel densitometry and Mander's overlapping analyses further indicated an increase in uptake of Cy5-DIPMA into $mGlu_5$-positive endosomes over time, peaking at 15 min post glutamate exposure (**Figure 22B**). Under the same conditions, DIPMA-Cy5 nanoparticles also showed increasing co-distribution with the early endosome resident protein $Rab5_{-GFP}$ over time(**Figure 22C**) (Ramírez-García et al., 2019). These results indicate that once activated by glutamate, $mGlu_5$ is internalised into Rab5-positive endosomes where DIPMA nanoparticles accumulate. This observation supports the use of DIPMA particles as a delivery system to target endosomal $mGlu_5$ to modulate their location-specific signalling.

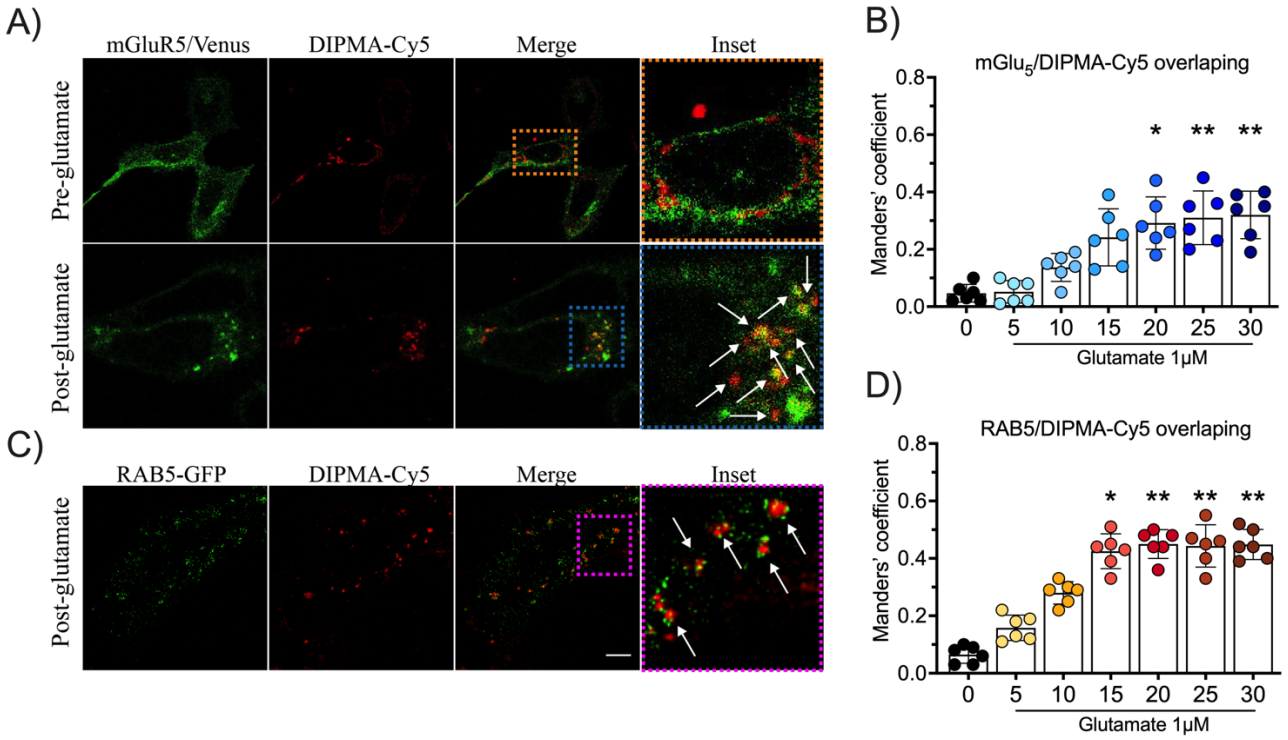


Figure 22. DIPMA-Cy5 nanoparticles overlap with $mGlu_5$ in recombinant HEK-293 cells. To determine whether nanoparticles traffic to endosomes containing the $mGlu_5$, DIPMA-Cy5 nanoparticles were incubated with HEK-293 cells expressing **A)** $mGlu_{5\text{-}Venus}$ or **C)** $RAB5_{\text{-}GFP}$, after 15min, cells were challenged with 1µM glutamate to promote internalisation of $mGlu_5$. Confocal show overlapping of DIPMA-Cy5 nanoparticles and $mGlu_{5\text{-}GFP}$ at 15 min after stimulation with glutamate. Manders' coefficient was used to assess the degree of colocalisation of DIPMA-Cy5 with **B)** $mGlu_{5\text{-}venus}$ and **D)** $RAB5_{\text{-}GFP}$. Data are presented as mean ± s.e.m., n = 5 independent experiments. *P < 0.05, **P < 0.005, ***P < 0.001, ****P < 0.0001 compared to time 0. One-way ANOVA, Dunnett's post-hoc test

4.9. Effects of nanoparticles on $mGlu_5$ endosomal signalling.

We demonstrated endosomal $mGlu_5$ induced calcium influx $_i[Ca^{2+}]$ and nuclear ERK, consistent with previous ERK studies that have shown that stimulated $mGlu_5$ is associated with excitation of spinal neurons (Rook et al., 2015). To assess the efficacy of our drug release system, we compared the ability of free drug or nanoparticles encapsulating VU058 to prevent $_i[Ca^{2+}]$ and nuclear ERK signalling in $mGlu_5$-expressing cells. Previously, empty DIPMA (DIPMA-Ø) particles showed no ability to indirectly activate nuclear ERK activity in recombinant HEK-293 cells. DIPMA-Ø particles also had no effect on agonist-stimulated activation of nuclear ERK (Ramírez-García et al., 2019). Extending our previous data indicating that endosomal mGlu5 contributes to glutamate induced $_i[Ca^{2+}]$ (**Figure 18**), we pre-incubated HEK-$mGlu_5$ cells with vehicle, free VU0366058 or DIPMA loaded with VU058 (100 and 300nM) and stimulated the cells with increasing concentrations of glutamate (1nM to 100µM), to assess if endosomal drug delivery could inhibit the $mGlu_5$ Ca^{2+} response. In vehicle treated cells, glutamate induced a rapid and sustained increase in $_i[Ca^{2+}]$ (**Figure 23B**). In cells treated

with free VU0366058 (100 and 300nM), we observed a moderate, concentration-dependent decrease in $_{i}[Ca^{2+}]$. In DIPMA-VU058 (100nM) treated cells, we observed a significant decrease in glutamate-induced $_{i}[Ca^{2+}]$ in both signalling phases, that was super-imposable with concentration response curves of cells pre-treated with 300nM free drug (**Figure 23C-D, Table 1**). In addition, DIPMA-VU058 (300nM) treated cells showed the greatest capacity to inhibit glutamate-induced $_{i}[Ca^{2+}]$, and almost abolished the sustained phase.

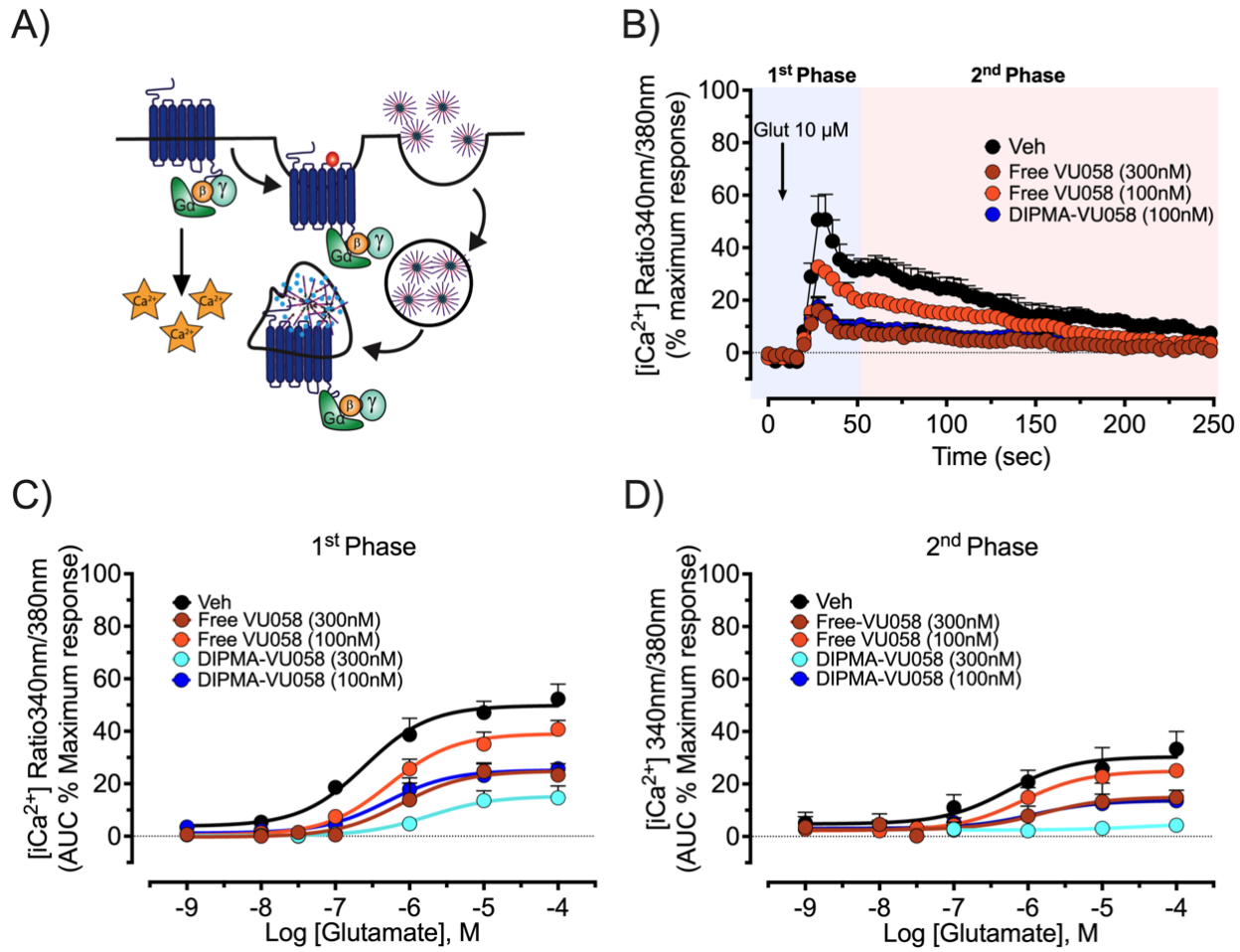


Figure 23. Modulation of glutamate-induced $_{i}[Ca^{2+}]$ signalling in endosomes. **A)** Representative diagram of $mGlu_5$ activation induced $_{i}[Ca^{2+}]$. **B)** Effect of DIPMA loaded with VU058 (100 and 300nM) on glutamate-induced $_{i}[Ca^{2+}]$. AUC analysis of glutamate induced $[Ca^{2+}]$ **C)** first phase (0-30 sec post-agonist) and **D)** second phase (50 - 250 sec post-agonist). Data are expressed as mean ± SEM and normalised to ionomycin (1µM, $i[Ca^{2+}]$). n = 6 independent experiments.

To compare the capacity of free VU0366058 and DIPMA-VU058 to modulate $mGlu_5$-dependent nuclear ERK activity, we measured glutamate-induced activation of nuclear ERK in recombinant HEK-$mGlu_5$ cells. Cells were pre-incubated with vehicle, free VU0366058 or DIPMA-VU058 (100

or 300nM) for 30min then challenged with glutamate (1nM - 100µM). In vehicle-treated cells, glutamate stimulated a rapid and sustained activation of nuclear ERK (**Figure 24B**).

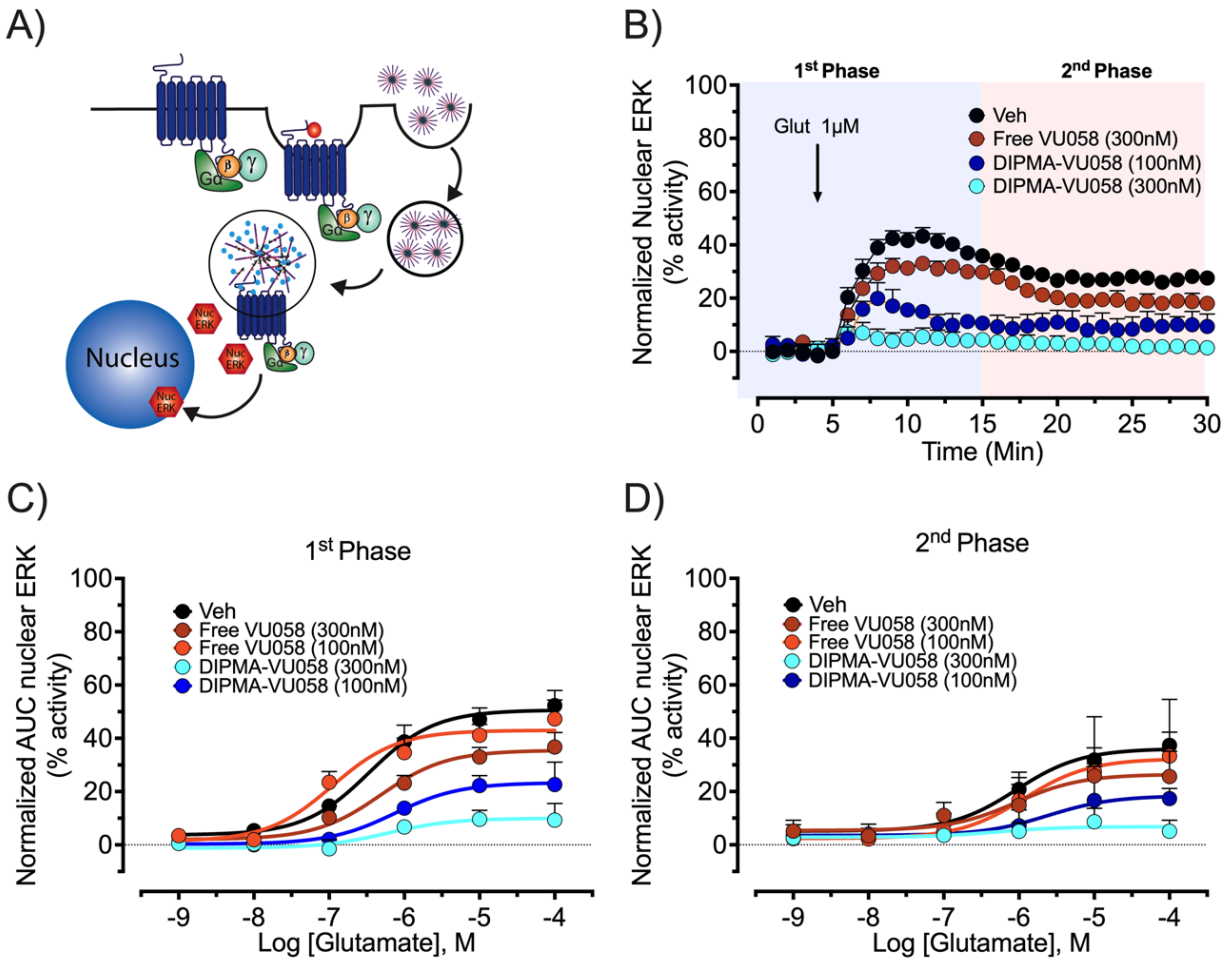


Figure 24. Modulation of glutamate induced nuclear ERK signalling in endosomes. **A)** Representative diagram of $mGlu_5$ activation induced nuclear ERK activity in HEK-293 cells. **B)** Effect of DIPMA loading VU058 (100 and 300nM) in glutamate induced nuclear ERK activity. AUC analysis of glutamate induced nuclear ERK **C)** First phase (0 - 15 min) and **D)** Second phase (15 - 30min). Data are expressed as mean ± SEM and normalised to PDBu (1µM), n = 6 independent experiments.

DIPMA-VU058 (100nM) significantly decreased nuclear ERK induced by glutamate at all concentrations, where a higher concentration of DIPMA-VU058 (300nM) completely inhibited glutamate response. Analysis of integrated AUC indicated that DIPMA-loading VU058 decreased glutamate-induced nuclear ERK activity at both concentrations in the first and second phase signalling of nuclear ERK. In contrast, free VU058 did not significantly decrease glutamate-induced nuclear ERK at any signalling phase (**Figure 24B-D**).

Table 3. Potency (pEC_{50}) parameters for $mGlu_5$ wild type receptor in signalling assays

Inhibition of dynamin and EEAT										
	1st phase					**2nd phase**				
	Vehicle	**Dy4a**	**K44E**	**TBOA**	**TBOA + K44E**	**Vehicle**	**Dy4a**	**K44E**	**TBOA**	**TBO + K44E**
Calcium mobilisation assay (pEC_{50})										
Glutamate	6.52 ± 0.06	6.03 ± 0.19	N/A	5.45 ± 0.23	N/A	5.55 ± 0.12	-5.75 ± 0.03	N/A	4.37 ± 0.51	N/A
DHPG	6.52 ± 0.03	6.49 ± 0.06	N/A	6.50 ± 0.04	N/A	6.08 ± 0.08	5.98 ± 0.12	N/A	5.41 ± 0.32	N/A
Cytosolic ERK (pEC_{50})										
Glutamate	6.38 ± 0.06	N/A	6.61 ± 0.07	N/A	N/A	6.39 ± 0.06	N/A	6.34 ± 0.09	N/A	N/A
DHPG	6.35 ± 0.07	N/A	6.26 ± 0.11	N/A	N/A	6.25 ± 0.14	N/A	6.28 ± 0.06	N/A	N/A
Nuclear ERK (pEC_{50})										
Glutamate	7.69 ± 0.07	N/A	6.03 ± 0.17	6.76 ± 0.09	5.78 ± 0.18	7.26 ± 0.13	N/A	6.05 ± 0.12	5.81 ± 0.18	5.28 ± 0.22
DHPG	N/D	N/A	N/D	N/A	N/A	N/D	N/A	N/D	N/A	N/A

Nanoparticle – free VU058 vs DIPMA-VU058										
Agonist	**1st phase**					**2nd phase**				
	Vehicle	**Free VU058**		**DIPMA-VU058**		**Vehicle**	**Free VU058**		**DIPMA-VU058**	
		100nM	**300nM**	**100nM**	**300nM**		**100nM**	**300nM**	**100nM**	**300nM**
Calcium mobilisation assay (pEC_{50})										
Glutamate	6.60 ± 0.05	6.27 ± 0.12	6.12 ± 0.08	6.70 ± 0.07	5.74 ±0.12	6.16 ± 0.20	6.07 ± 0.07	5.81 ± 0.15	5.92 ± 0.11	4.52 ± 0.23
Nuclear ERK (pEC_{50})										
Glutamate	7.46 ± 0.03	6.95 ± 0.07	6.33 ± 0.05	5.89 ± 0.04	5.22 ± 0.06	6.09 ± 0.09	6.03 ± 0.14	5.93 ± 0.07	5.61 ±0.12	4.30 ±0.32

N/A; not tested, N/D; not detected, data expressed as negative $LogEC_{50}$ ± s.e.m

4.10. Effect of nanoparticles on nociceptive models of pain

We hypothesised that targeting $mGlu_5$ located into endosomes by nanoparticles would enhance the antinociceptive actions of VU0366058 when delivered to spinal neurons. The efficacy of free or nanoparticle encapsulated VU0366058 was evaluated in preclinical models of acute (capsaicin), inflammatory (complete Freund's adjuvant) and neuropathic pain (sciatic nerve innervation) (**Figure 25**). Nanoparticles (DIPMA-VU058), free VU0366058, fenobam (an established antinociceptive $mGlu_5$ NAM) or vehicle (aCSF) were injected intrathecally before or after intraplanar injection or nerve injury. Mechanical nociception was studied by measuring withdrawal responses to stimulation of the plantar surface of the hindpaw using von Frey filaments (VVF).

Acute model of pain. Intraplantar injection of capsaicin activates transient receptor potential vanilloid 1 (TRPV1) on primary sensory neurons leading to release of glutamate and other neurotransmitters in the dorsal horn, which evokes glutamate receptor activation and $mGlu_5$ endocytosis in spinal neurons and causes transient-reversible acute pain (Mahato et al., 2015; Vidnyánszky et al., 1994; Yu et al., 2018). In vehicle pre-treated mice (aCSF, i.t.), capsaicin significantly decreased the VFF threshold from 0.5 to 5h, which returned to baseline after 24h (**Figure 25A**). The negative allosteric modulator fenobam (300nM, i.t.) resulted in significant antinociception after 0.5h (41 ± 14% inhibition), although the effect was decreased after 1.5h. Free VU0366058 (100nM, i.t.) had a similar effect after 0.5h (35 ± 5% inhibition) compared to fenobam. However, DIPMA-VU058 (100nM, i.t) caused marked anti-nociception at 1h (54 ± 11% inhibition) that was sustained for 5h (46 ± 7% inhibition). The use of DIPMA nanoparticles as a carrier to target endosomal $mGlu_5$ enhanced the antinociceptive effect of VU058 in an acute model of pain, whereas the free VU058 has not efficacy decreasing capsaicin-induced acute pain.

Inflammatory model of pain. Intraplantar injection of complete Freund's adjuvant (CFA) is an established model of sustained inflammatory mechanical allodynia. This model was used to assess the analgesic capacity of DIPMA-VU058 to reverse inflammatory nociception. There was a marked decrease in VVF responses when assessed at 48h after intraplantar CFA injection (**Figure 25C**). The intrathecal administration of vehicle (aCSF, i.t.) did not affect mechanical hyperalgesia, which persisted for 24h. Fenobam administration (200µM, i.t.) transiently decreased mechanical hyperalgesia-induced by CFA at 1.5h (28 ± 5% inhibition). Whereas, the administration of free VU0366058 (300nM, i.t.) did not reverse hyperalgesia at any time. In contrast, DIPMA-VU058 (300nM, i.t.) induced a potent inhibition of mechanical hyperalgesia (49 ± 11% inhibition) with a

maximal potency at 1.5h post NPs administration, where the inhibition was maintained for 4h, when other treatments were ineffective.

Neuropathic model of pain. The sciatic nerve innervation (SNI) model of neuropathic pain produces mechanical hyperalgesia that is sustained for >50 days (Bravo et al., 2014; Decosterd and Woolf, 2000). This model was used to examine the analgesic efficacy of DIPMA-VU058 to relieve chronic neuropathic nociception. After 10 days, SNI reduced the VVF of the ipsilateral hindpaw when compared to sham-operated mice, indicative of mechanical hyperalgesia (**Figure 25E**). Intrathecal administration of vehicle (aCSF, i.t.) did not affect mechanical hyperalgesia, which persisted for 5h. Fenobam administration (200µM, i.t.) inhibited withdrawal thresholds after 0.5h to a maximum of 36 ± 8% inhibition and returned to baseline after 2.5h. Free VU0366058 (300nM, i.t.) reversed hyperalgesia after 0.5h (32 ± 5% inhibition), although hyperalgesia returned to baseline after 3h. DIPMA-VU058 (300nM, i.t.) strongly reversed hyperalgesia, reaching the highest analgesia after 1h (72 ± 6% inhibition) and analgesia was maintained for 4.5h.

These data demonstrate nanoparticle encapsulation and endosomal delivery enhances and prolongs the analgesic effects of VU0366058. Potential mechanisms involved may be endosomal signalling inhibition that affect ERK activity and neuronal hyperexcitability. The enhanced effects of DIPMA-VU058 in each clinical model of pain could be related to delivery and retention of VU058 in endosomes of spinal neurons, and the continued release of drug as nanoparticles encounter increasingly acidified endosomal compartments (Ramírez-García et al., 2019).

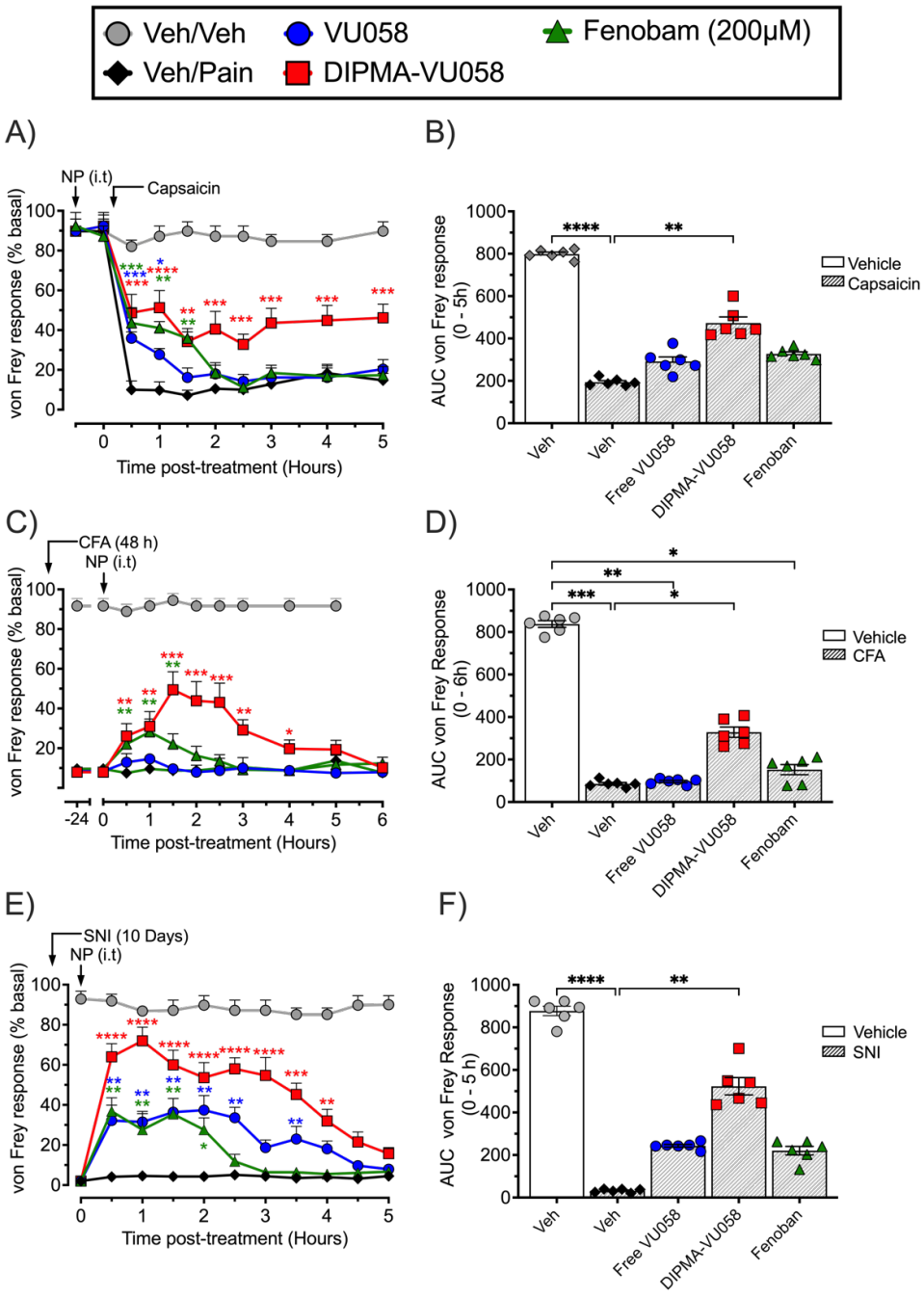


Figure 25. Assessing antinociceptive efficacy of DIPMA-formulated Vu058 in mice. A) Capsaicin-evoked acute nociception model in mice; Free VU0366058 (100nM), DIPMA-VU058 (100nM), fenobam (200µM) or vehicle (veh) (5µl) were injected intrathecally (i.t.) 30 min before intraplantar (i.pl.) injection of capsaicin (5µg/paw). **C)** CFA-evoked sustained inflammatory model; Free VU0366058 (300nM), DIPMA-VU058 (300nM), fenobam (200µM) or veh administered i.t. (5µl) 48 h post intraplantar CFA. **E)** SNI model was induced 10 day before i.t administration of drugs at same concentrations described for CFA. Integrated response presented as the area under the curve (AUC), **B)** Capsaicin, **D)** CFA and **F)** SNI-induced pain models. Data are presented as mean ± s.e.m., n = 6 animals for all experiments. *P < 0.05, **P < 0.005, ***P < 0.001, ****P < 0.0001 compared to veh/pain. one-way ANOVA, Dunnett's post-hoc test.

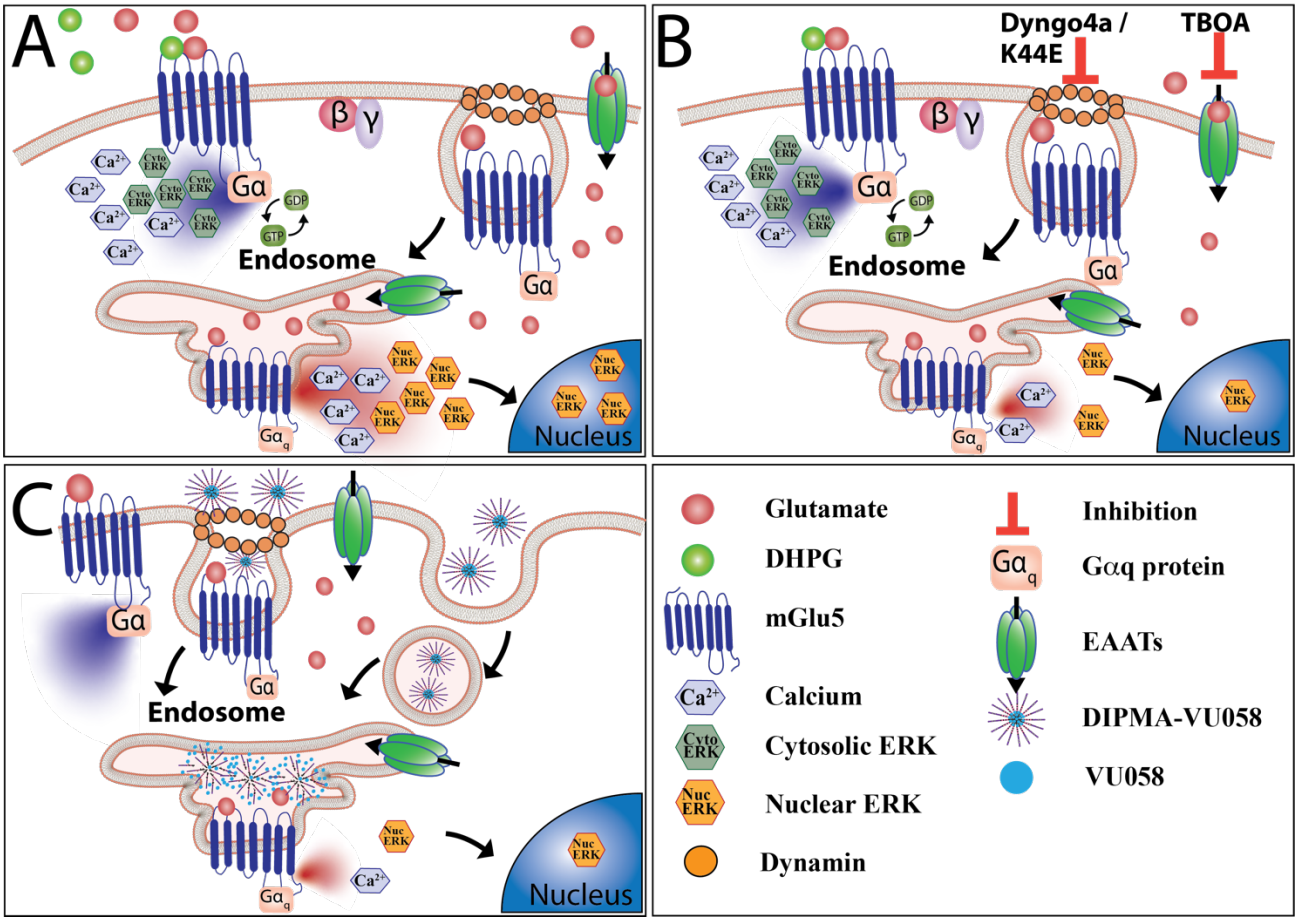


Figure 26. Summary of data investigating endosomal $mGlu_5$ signalling. A) Glutamate and DHPG-induced $mGlu_5$ signalling at the plasma membrane generating Ca^{2+} mobilisation and cytosolic ERK in agreement with G protein coupling. Whereas only glutamate activates endosomal $mGlu_5$, leading to Gαq coupling into endosomes inducing Ca^{2+} mobilisation and nuclear ERK activity dependent of EAATs. **B)** Pharmacological and genetic dynamin inhibitor (dyngo4a or K44E), and EAATs inhibitor (DL-TBOA) decrease endosomal $mGlu_5$ signalling. **C)** The pH responsive nanoparticles, a novel drug delivery system that target endosomes, release the negative allosteric modulator VU0366058, decreasing $mGlu_5$ endosomal signalling *in vitro* and nociceptive behaviour.

5. Discussion

Growing evidence indicates GPCR signalling is not confined to the plasma membrane and that intracellular GPCRs can signal from endosomes or other organelles such as the nucleus or endoplasmic reticulum (Jong et al., 2014; Vincent et al., 2016; 2017). For example, the endosomal signalling of the neurokinin 1 receptor (NK_1R), calcitonin receptor-like receptor (CLR) and protease-activated receptor-2 (PAR_2) in primary sensory and spinal neurons mediates nociception, generating persistent signalling in subcellular compartment that control gene transcript leading to neuronal excitation (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Ramírez-García et al., 2019; Retamal et al., 2019; Yarwood et al., 2017). Here we report that both $mGlu_5$ internalisation and the downstream signalling profile are highly dependent on the properties of the agonist examined. The endogenous membrane transportable agonist glutamate induces sustained $mGlu_5$ internalisation, recruitment of $G\alpha_q$ and $G\alpha_s$ protein to the plasma membrane, and $G\alpha_q$ protein recruitment into the endosomes. In contrast, the impermeable, non-transported agonist DHPG promoted transient recruitment of $G\alpha_q$ and $G\alpha_s$ proteins to the plasma membrane and limited recruitment of $G\alpha_q$ to endosomes. These differences in endosomal $G\alpha$ protein recruitment is consistent with the agonist-dependent differences in signalling profiles for $mGlu_5$, and the ability for glutamate to achieve a complete signalling response. It further provides indirect evidence that the pharmacological tool DHPG is promoting rapid recycling or entry into 'very early endosomes', and warrants further investigation using specific markers to these trafficking pathways.

$G\alpha_q$ protein recruitment to endosomes by $mGlu_5$ is associated with sustained intracellular Ca^{2+} responses and nuclear ERK activity. To our knowledge, these are the first experiments demonstrating $G\alpha_q$ protein recruitment by $mGlu_5$ into endosomes. Intracellular signalling induced by glutamate is directly associated with $mGlu_5$ internalisation, where both pharmacological or genetic inhibition of dynamin decrease Ca^{2+} responses and nuclear ERK activity. In concordance with previous studies, inhibition of EAAT by TBOA significantly decreases glutamate induced Ca^{2+} responses and nuclear ERK activity and therefore supports a role for these transporters in $mGlu_5$ intracellular signalling (Liaw et al., 2005; Vincent et al., 2016; Weng et al., 2006). In combination, dynamin and EAAT inhibition abolished glutamate induced Ca^{2+} and nuclear ERK responses. Indeed, we confirmed that responses to DHPG, which only activates $mGlu_5$ at or close to the plasma membrane, are not affected by the inhibition of dynamin or EAATs. In addition, DHPG does not induce nuclear ERK activity or intracellular Ca^{2+} signalling.

To further demonstrate that $mGlu_5$ can signal from endosomes, we used pH-responsive nanoparticles (DIPMA NPs), that were recently developed and utilised as a drug delivery system to target NK_1R located on endosomes, and associated with sustained neuronal excitability and pain transmission (Ramírez-García et al., 2019). Consistent with these studies, we confirmed that DIPMA-Cy5 NPs colocalised with early endosomes within 10 min, and showed an intracellular distribution that was consistent with stimulated $mGlu_5$ in endosomes after 20 min, thus indicating that DIPMA NPs may a useful tool for directing ligands to endosomal $mGlu_5$. DIPMA NPs were loaded with the $mGlu_5$ negative allosteric modulator VU0366058, loading 40% of the initial drug added to form DIPMA-VU058 NPs (3.8μM). The effects of DIPMA-VU058 on glutamate-induced Ca^{2+} and nuclear ERK activity were tested in HEK expressing $mGlu_5$ and compared against free VU058 at the same concentrations. Free VU058 significantly decreased glutamate induced Ca^{2+} and nuclear ERK activity only at 300nM but not at 100nM. DIPMA-VU058 (100nM) significantly inhibited glutamate-induced Ca^{2+} and nuclear ERK activity, whereas DIPMA-VU058 at 300nM abolished completely Ca^{2+} and nuclear ERK activity. The efficacy of free or DIPMA- encapsulated VU058 and fenobam on nocifensive behaviour was assessed in acute nociceptive, inflammatory and neuropathic pain models. Intrathecal administration was utilised for localised delivery (avoids systemic clearance and need to overcome the blood-brain barrier), and showed that fenobam had a modest effect on all three models and free VU058 had a modest effect on nociceptive and neuropathic pain, but did not reduce CFA-induced pain. In contrast, DIPMA NPs markedly enhanced the antinociceptive efficacy (maximum achievable analgesia and duration) of VU058 in nociceptive, inflammatory and neuropathic pain. when compared to free VU58. These findings are consistent with the improved capacity of nanoparticle-encapsulated VU058 to inhibit endosome-associated, sustained glutamate-dependent Ca^{2+} and nuclear ERK activity.

Here, all the signalling pathway assessed in this study are intracellular signalling. Thus, we used pharmacological and genetic inhibitors of dynamin to demonstrate the importance of $mGlu_5$ internalisation for signalling. Dynamin inhibitors disrupt trafficking of many receptors and channels that control nociception and it had showed significant reduction of pain behaviour (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Mai et al., 2021; Yarwood et al., 2017). However, considerable efforts are required to advance these compounds to the clinic, as dynamin inhibitors disrupt trafficking, degradation, recycling and fundamental cellular processes.

Consistent with our study, $mGlu_5$ activation in spinal cord dorsal horn neurons by glutamate produced sustained Ca^{2+} responses, whereas DHPG induced only transient Ca^{2+} responses (Vincent et al., 2016). Activation of intracellular $mGlu_5$ is Gq-dependent and is likely to require canonical PLC/IP_3R signalling to play a dynamic role in mobilising Ca^{2+} in a localised manner (Jong et al., 2014; Purgert et al., 2014; Vincent et al., 2016; 2017). Blocking only cell-surface $mGlu_5$ *in vivo* with a membrane impermeable antagonist had little effect on the severity of neuropathic pain, whereas intracellular $mGlu_5$ inhibition using a membrane permeable antagonist markedly reduced pain behaviour and pain protein markers such as pERK, Arc and c-fos expression (Vincent et al., 2016; 2017). Contrary to the idea that intracellular GPCRs are non-functional and merely constitute an internal reserve of receptors waiting to translocate to the cell surface, Vincent *et al.* demonstrated that $mGlu_5$ located on the inner nuclear membrane plays a dynamic role in signal transduction in response to CFA induced inflammatory and neuropathic pain, by signalling from this site to generate unique Ca^{2+} and ERK that is associated with neuronal hyperexcitability (Vincent et al., 2016; 2017). The importance of intracellular $mGlu_5$ is supported by the significant reduction of glutamate-induced pain behaviour following EAAT3 inhibition but not by EAAT1 or 2. Indeed, EAAT1 and 2 inhibition in sham animals was pronociceptive, with similar effects to glutamate-induced pain and mechanical allodynia (Vincent et al., 2016). Consistent with intracellular $mGlu_5$ driving pain behaviour, blocking glutamate entry into spinal cord neurons by inhibiting EAAT3 produced analgesia, whereas blocking glial glutamate transporters (EAAT1-2) increased pain behaviours and c-fos expression in the spinal cord (O'Malley et al., 2003; Vincent et al., 2016; 2017).

Previous studies have shown that HEK cells endogenously express the EAAT3 glutamate transporter subtype (Dunlop et al., 1999). In our study, one potential glutamate source for endosomal $mGlu_5$ receptors is extracellular glutamate uptake via the EAAT3, explaining the lack of intracellular signalling by DHPG. In addition, Group 1 mGlu receptors exhibit overlapping distributions with glutamate transporters in the cerebellum and hippocampus, and it is conceivable that glutamate uptake serves many purposes including signal termination, modulation of firing, as well as activation of extra- and intracellular mGlu receptors (Jong et al., 2009a; O'Malley et al., 2003; Vincent et al., 2016; 2017). Furthermore, *in vivo* studies demonstrated that specific EAAT1 or 2 inhibitors increases spinal extracellular glutamate concentration in control rats, increasing glutamate availability and generates spontaneous pain. However, EAAT3 inhibition achieved analgesia by blocking the transporters responsible for ligand uptake into spinal cord neurons(Vincent et al., 2016).

It is established that $mGlu_5$-dependent activation increases MEK and ERK protein synthesis and synaptic plasticity in pyramidal CA1 and cortical neurons (Huber et al., 2001; Mukherjee and Manahan-Vaughan, 2013; Niu et al., 2020; Osterweil et al., 2010; Purgert et al., 2014; Yu et al., 2018). However, $mGlu_5$ activity is unaffected by inhibition of protein kinase C (PKC) and phospholipase C (PLC) (Fitzjohn et al., 2001; Ireland and Abraham, 2002; Rush et al., 2002; Stoppel et al., 2017; Vergouts et al., 2017). In contrast to our observations of endosome-specific recruitment Gα sunits and subsequent signalling, $mGlu_5$ in fragile X syndrome can recruit β-arrestin2 (βarr2) and stimulates ERK activation, promoting protein synthesis independent of $G\alpha_q$ recruitment and PLC activation (Eng et al., 2016; Stoppel et al., 2017). While these differences may be cell type specific, further studies are required to determine the precise role of PLC and βarr2 in our system.

Given that $mGlu_5$ is an important target in many diseases, $mGlu_5$ drugs have been optimised for selectivity, affinity, and pharmaco-kinetic parameters as well as overcoming off-target effects and short-half lives of the drugs (Gregory and Goudet, 2021; Sengmany and Gregory, 2016) (Lea et al., 2005; Montana et al., 2011). However, little emphasis has been placed on targeting the intracellular receptor pool (Gregory and Goudet, 2021; Sengmany and Gregory, 2016). Lipid-conjugated antagonist are a potential strategy to target intracellular locations, but lipid-conjugation can reduce antagonist potency due to significant chemical modifications required during the conjugation process. However, the lipid-conjugation strategy has shown potential advantages in drug retention in membranes to increase 'local drug potency' and due to the ability to block both cell surface and endosomal receptor populations (Jensen et al., 2017; Mai et al., 2021). Recently, pH-responsive nanoparticles (DIPMA NPs) were developed as a novel strategy to exploit the acidic environment of endosomes for bypassing cell surface receptors and releasing drug cargo into the endosomal network. By directing greater drug quantities to this specific location, DIPMA NPs decrease the concentration of total drug required for blocking endosome-delimited events. For studies into the NK1R, this resulted in sustained inhibition of endosomal signalling of NK_1R compared to cell treatment with an equivalent concentration of free drug prepared in standard formulation. Furthermore, DIPMA NPs enhanced the anti-nociceptive actions of NK_1R antagonist in preclinical models of pain by decreasing SP-induced excitation of spinal neurons (Ramírez-García et al., 2019).

Similar to these studies, further experiments are required to more comprehensively explore the advantages and mechanism for endosomal drug delivery with $mGlu_5$. The importance of $mGlu_5$ internalisation for excitation and firing of neurons, for example, should be explored by

electrophysiology. Prior electrophysiological data indicate that the inhibition of dynamin by dyngo4a in spinal cord slices decreases firing and excitation of nociceptive neurons in the laminae I & II of the spinal cord of neuropathic rats (Jensen et al., 2017; Ramírez-García et al., 2019). Furthermore, we have performed preliminary electrophysiology studies for this $mGlu_5$ system and although not shown, these findings indicate that DIPMA-VU058 NPs significantly decreases glutamate-induced firing and excitation of neurons in the laminae I & II compared to pre-treatment with free VU058.

Previous studies demonstrated that pH responsive nanoparticles improved the anti-nociceptive actions of the NK_1R antagonist aprepitant and here were demonstrated that nanoparticle encapsulation enhanced the anti-nociceptive action of VU-058. While these pH-responsive nanoparticles have limitations due to their capacity to only encapsulate limited quantities of highly hydrophobic drugs such as VU058 or aprepitant, due to their simple assembly process , future opportunities also include testing if nanoparticles loaded with multiple antagonists for different nociceptive GPCRs may have the potential to generate synergistic anti-nociceptive effects. A further limitation of our study is the use of pre-clinical models of pain, which analyse induced nociception rather than pain perception. Examination of nanoparticle action on primary neurons is also required to further explore the signalling mechanisms involved in a native system, including important studies into the functional interactions between EAAT3 and $mGlu_5$ located on endosomes, which was briefly investigated here and may provide important new insights into how glutamate transport may influence location-specific $mGlu_5$-signalling pathways

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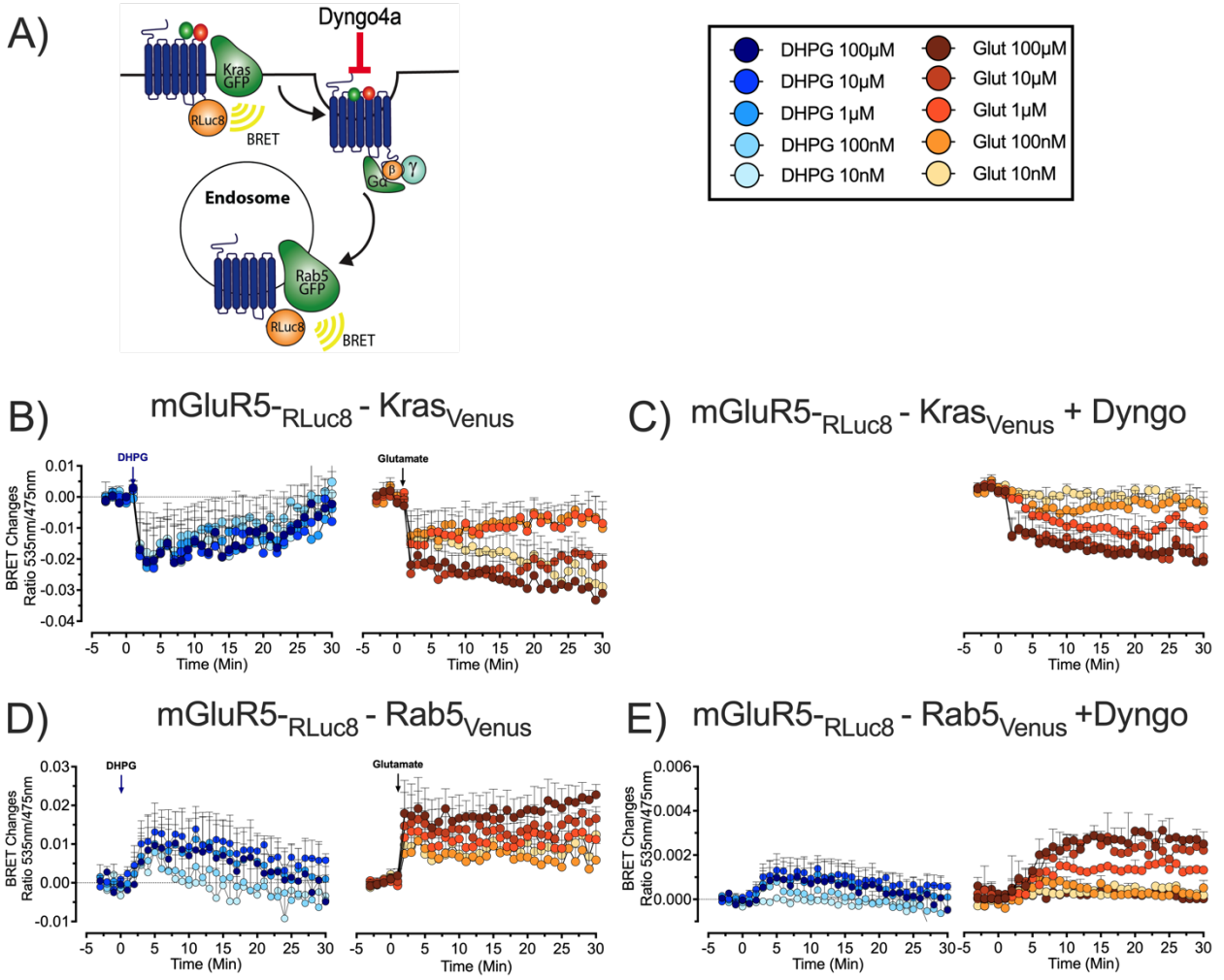
Conflict of interest

The authors declare no competing interests.

6. Supplementary figure



Supplementary Figure 1. $mGlu_5$ internalisation in HEK cells. **A)** Representative diagram for BRET assay utilised for $mGlu_5$ internalisation. **B)** and **C)** Time trace of BRET assay for $mGlu_5$ internalisation, measured by association with Kras. **D)** and **E)** association with Rab5 upon glutamate or DHPG addition in presence or absence of Dyngo4a. Data are expressed as mean ± SEM, n = 6 independent experiments.

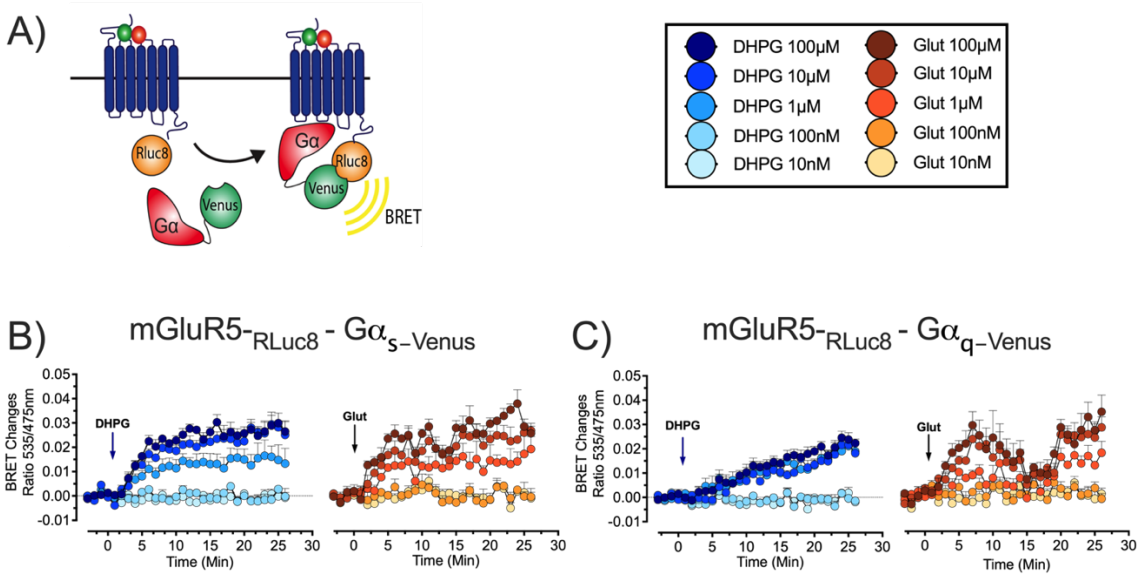
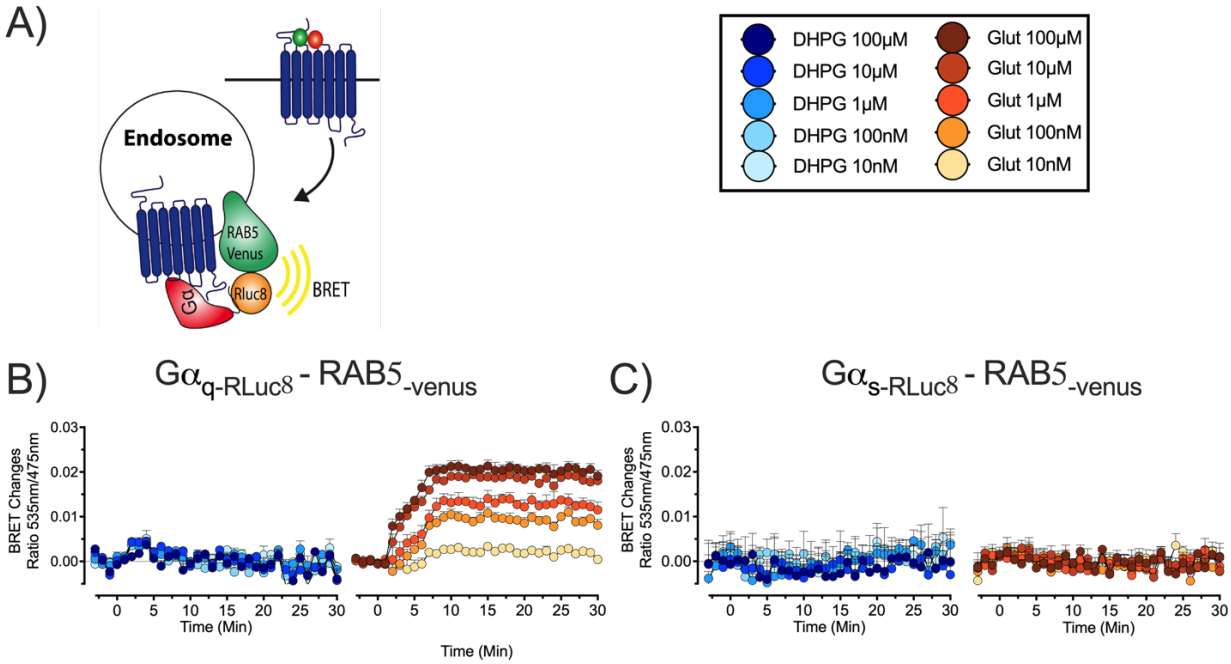


Figure supplementary 2. G-protein recruitment upon $mGlu_5$ activation. **A)** Representative diagram for BRET assay utilised for G-protein recruitment. **B)** and **C)** Time trace of BRET assay for $mGlu_5$-induced $G\alpha_q$ or $G\alpha_s$ recruitment upon glutamate or DHPG addition. Data are presented as mean ± s.e.m., n = 6 independent experiments.



Supplementary Figure 3. G protein recruitment upon $mGlu_5$ activation. **A)** Representative diagram for the BRET-based assay employing miniG constructs to measuring recruitment of Gα protein subunits. **B)** and **C)** Time trace of BRET assay for $mGlu_5$-induced $G\alpha_q$ or $G\alpha_s$ recruitment upon glutamate or DHPG addition. Data are presented as mean ± s.e.m., n = 6 independent experiments.

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Chapter 5:

5HT-induced vascular permeability is mediated by TRPV4 in the airways and upper GI tract

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ARTICLE

Serotonin-induced vascular permeability is mediated by transient receptor potential vanilloid 4 in the airways and upper gastrointestinal tract of mice

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Abstract

Endothelial and epithelial cells form physical barriers that modulate the exchange of fluid and molecules. The integrity of these barriers can be influenced by signaling through G protein-coupled receptors (GPCRs) and ion channels. Serotonin (5-HT) is an important vasoactive mediator of tissue edema and inflammation. However, the mechanisms that drive 5-HT-induced plasma extravasation are poorly defined. The Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel is an established enhancer of signaling by GPCRs that promote inflammation and endothelial barrier disruption. Here, we investigated the role of TRPV4 in 5-HT-induced plasma extravasation using pharmacological and genetic approaches. Activation of either TRPV4 or 5-HT receptors promoted significant plasma extravasation in the airway and upper gastrointestinal tract of mice. 5-HT-mediated extravasation was significantly reduced by pharmacological inhibition of the 5-HT_{2A} receptor subtype, or with antagonism or deletion of TRPV4, consistent with functional interaction between 5-HT receptors and TRPV4. Inhibition of receptors for the neuropeptides substance P (SP) or calcitonin gene-related peptide (CGRP) diminished 5-HT-induced plasma extravasation. Supporting studies assessing treatment of HUVEC with 5-HT, CGRP, or SP was associated with ERK phosphorylation. Exposure to the TRPV4 activator GSK1016790A, but not 5-HT, increased intracellular Ca^{2+} in these cells. However, 5-HT pre-treatment enhanced GSK1016790A-mediated Ca^{2+} signaling, consistent with sensitization of TRPV4. The functional interaction was further characterized in HEK293 cells expressing 5-HT_{2A} to reveal that TRPV4 enhances the duration of 5-HT-evoked Ca^{2+} signaling through a PLA_2 and PKC-dependent mechanism. In summary, this study demonstrates that TRPV4 contributes to 5-HT_{2A}-induced plasma extravasation in the airways and upper GI tract, with evidence supporting a mechanism of action involving SP and CGRP release.

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Introduction

The vasculature performs several important functions that are essential for maintaining fluid homeostasis. Endothelial cells make up the physical barrier in blood vessels that enables the control of fluid and molecule exchange from the circulation to the surrounding tissues. Physiological regulation of these barriers controls the extravasation of plasma proteins through inter-endothelial gaps, where cytoskeletal reorganization and disassembly of VE-cadherin junctions are essential regulators of endothelial permeability [1]. Disruption of these processes, as occurs in disease, is associated with unregulated movement and accumulation of fluids, leading to tissue edema.

Several inflammatory mediators, including proteases (e.g., thrombin), histamine, substance P (SP), and serotonin (5-HT) can activate specific receptors on vascular endothelial cells to promote changes in endothelial permeability. These changes can be mediated by an increase in intracellular calcium ($[Ca^{2+}]_i$) and activation of signaling pathways that regulate the contractile apparatus of cells, leading to cytoskeletal remodeling and disassembly of VE-Cadherin junctions [2]. This, in turn, causes endothelial cell contraction and cell junction disruption, resulting in increased endothelial permeability and tissue edema [2–6]. For example, increased endothelial $[Ca^{2+}]_i$ in pulmonary blood vessels leads to their constriction and to subsequent edema formation [3, 7, 8].

It has been demonstrated that systemic administration of 5-HT produces detrimental effects on the integrity of the endothelial barrier, leading to plasma extravasation into the surrounding tissue [9]. The biological actions of 5-HT are mediated through specific serotonin receptors ($5\text{-}HT_{1-7}$) [10], all of which are G protein-coupled receptors (GPCRs), with the notable exception of the $5\text{-}HT_3$ ligand-gated ion channel [11]. In addition to their well-defined roles in neurotransmission and clinical association with the pathogenesis of neurological diseases and neuropsychiatric disorders, 5-HT receptors are also key regulators of the homeostatic control of vasoconstriction and vascular permeability [12–14].

As previously described, elevated $[Ca^{2+}]_i$ in endothelial cells is required to cause barrier dysfunction. This elevation in $[Ca^{2+}]_i$ is triggered by mechanical stimuli or by activation of GPCRs and occurs in two phases, initiated by the release of Ca^{2+} from ER-stores and followed by entry of extracellular Ca^{2+} through cation channels [15]. Additionally, GPCR activation can also promote Ca^{2+} entry by activating cation channels, including transient receptor potential channels (TRP), which are the main non-selective cation channels in endothelial cells [1, 16]. The principal TRP channels that mediate endothelial cell permeability are TRPM2, TRPC1, 4 and 6 and vanilloid family members TRPV1 and 4 [17].

Transient Receptor Potential Vanilloid 4 (TRPV4) is an established enhancer of vascular permeability and edema that is expressed by a variety of cells including endothelia, peripheral sensory neurons, and immune cells [18–21]. TRPV4 is also a recognized promoter of neurogenic inflammation through enhanced release of neuropeptides, including SP and calcitonin gene-related peptide (CGRP), from peptidergic peripheral nerve endings [22, 23]. The sensitivity to ligand or mechanical activation, as well as the magnitude and duration of TRPV4 activity can be augmented by functional interactions (termed 'coupling') with GPCRs. These interactions are also known to be reciprocal, where functional coupling of a GPCR to an ion channel such as TRPV4 can lead to augmentation of GPCR signaling outputs. Furthermore, coupling between GPCRs and TRPV4 is proposed to contribute to disease-associated processes, including neurogenic inflammation and pain [24].

A well-characterized example of reciprocal coupling is illustrated through functional interactions between protease-activated receptors 1 and 2 (PAR1 and PAR2) and TRPV4 [20, 25, 26]. PAR activation can "sensitize" or reduce the activation threshold through channel phosphorylation and enhance TRPV4 signaling through the production of endogenous TRPV4 activators (e.g., arachidonic acid and 5′,6′-EET) [25–27]. Conversely, TRPV4 activity augments PAR1- and PAR2-dependent signaling, and this bidirectional PAR-TRPV4 relationship drives a significant component of PAR-evoked edema [20, 26, 27].

A variety of cell types co-express 5-HT receptors and TRPV4, highlighting their broader potential to functionally interact. Indeed, studies have demonstrated an important role for TRPV4 as an enhancer of 5-HT signaling associated with arterial smooth muscle proliferation [28, 29], pulmonary artery smooth muscle contraction [8, 19], itch [30], and visceral pain [31]. Immunohistochemistry and in situ hybridization studies have demonstrated that nociceptive dorsal root ganglion neurons mainly express 5-HT receptor subtypes 2A and 3 [32, 33]. Activation of $5\text{-}HT_{2A}$ expressed by these neurons promotes 5-HT-induced nociception and the release of SP and CGRP from peripheral nerve terminals, leading to a sustained increase in vascular permeability [34–38]. Neurogenic inflammation is initiated by the release of these neuropeptides [36, 39] and further studies have supported the involvement of neurogenic inflammation in 5-HT-evoked plasma extravasation by demonstrating significant inhibition of plasma protein extravasation with antimigraine drugs [40, 41].

Although there is strong evidence to support the importance of TRPV4 as an amplifier of 5-HT receptor signaling, the relative contribution of TRPV4 to 5-HT-induced edema

has not been defined in detail. We hypothesized that 5-HT-induced plasma extravasation is augmented by TRPV4 activity and is mediated, in part, through release of SP and CGRP from nerve fibers associated with microvasculature. In the present study, we found that the systemic administration of 5-HT-induced plasma extravasation in the airway and upper GI tract, particularly by the activation of $5HT_{2A}$, where the pharmacological inhibition or genetic deletion of TRPV4 attenuates 5-HT-induced plasma extravasation in the airways and upper GI tract, indicating a reciprocal coupling between 5-HT_{2A} and TRPV4 in vivo. In addition, in vitro studies indicated that 5-HT_{2A} interact with TRPV4 through the activation of PLA_2 and PKC. Moreover, we establish that inhibitors of NK_1R (SR140333) or the CGRP receptor (Olcegepant; BIBN4096) block 5-HT- and TRPV4-induced plasma extravasation in mice. These observations provide further mechanistic understanding of the important contribution that GPCR-TRP channel interactions have in fundamental biological processes, including the control of vascular permeability.

Materials and methods

Drugs and reagents

Evans Blue dye and GSK1016790A were purchased from Sigma-Aldrich (St. Louis, MO); 5-HT, HC067047, SR140333, GF 109203X (GFX), and BIBN 4096 (Olcegepant) were purchased from Tocris Bioscience (Bristol, UK); YM 26734 was from Cayman Chemical; WAY-100635 Maleate, GR 55562 dihydrochloride, GR113808 and SB 269970 hydrochloride were purchased from Abcam Australia (Melbourne, VIC Australia). Ketanserin and RS-127445 were purchased from Selleck Chemicals (Houston, TX. USA); Evans Blue was dissolved in sterile 0.9% saline. All drugs administered to mice were prepared on the day of experimentation in sterile 1% dimethyl sulfoxide (DMSO) in 0.9% saline.

Animals

All animal experiments adhered to the ARRIVE guidelines [42] and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Ethics Committees of RMIT and Monash Institute of Pharmaceutical Sciences. Wild-type C57Bl/6J and $TRPV4^{-/-}$ (kindly provided by Dr. W Liedtke, Duke University) (6–12 weeks, male) were obtained from the Animal Resources Center (Canning Vale, WA), or from Monash Animal Research Platform, Monash University. All animals were maintained in a temperature-controlled (24 °C) environment with a 12 h light/dark cycle and with access to food and water ad libitum.

Measurement of plasma extravasation

Mice were anaesthetized with a combination of Ketamine (100 mg/kg i.p.) and Xylazine (10 mg/kg i.p.) and kept on a warming pad. The skin at the throat was removed to expose the jugular veins. Substances were i.v. administered by passing a needle through the *pectoralis major* muscle to prevent bleeding on withdrawal. Evans Blue dye (20 mg/kg) or 0.9% saline were administered into the jugular vein, 1 min before injection of agonist (5-HT or GSK1016790A, dosing as indicated in relevant sections) or vehicle (1% DMSO in 0.9% saline). Mice were killed (5 min post-agonist administration) by exsanguination and perfused with saline solution. Tissue samples were collected, weighed, and placed in formamide (≥18 h at 37 °C) to facilitate dye extraction. Absorbance of the extracts was determined against standard concentrations of Evans Blue at 620 nm using a FlexStation III plate reader (Molecular Devices, Sunnyvale, CA). Antagonists of 5-HT_{1A} (WAY-100635, 80 µg/kg) [43], 5-HT_{1B} (GR 55562, 300 µg/kg) [44], 5-HT_{2A} (ketanserin, 2 mg/kg) [45], 5-HT_{2B} (RS-127445, 300 µg/kg) [46], 5-HT_4 (GR 113808, 1 mg/kg) [47], 5-HT_7 (SB269973, 300 µg/kg) [46], TRPV4 (HC0670471, 10 mg/kg), NK_1R (SR140333, 1 mg/kg), or CGRP receptor (Olcegepant, 1 mg/kg) were i.p. injected 60 min prior to anesthetics. Results were expressed as the amount of Evans Blue dye per wet weight tissue (ng of EB/mg of tissue).

Human umbilical vein endothelial cell (HUVEC) culture

HUVEC were grown in endothelial growth medium (EGM, Lonza, Mount Waverley, VIC, Australia) containing 2% fetal bovine serum and a SingleQuots Supplement Pack (Lonza) as described [20].

Transient transfection

Constructs of pcDNA3.1$^+$ human 5-HT receptors subtype 1A, 1B, 2A, 2B, 4, and 7 (hHT_{1A-7}) plasmids were purchased from the cDNA Resource Center (Bloomsburg, PA, USA). Human Embryonic Kidney 293 cell line with tetracycline-inducible (T-Rex™ 293) TRPV4 overexpression (HEK-TRPV4) was grown at 37 °C in 5% CO_2 in DMEM containing 10% FBS (5 µg/mL blasticidin S). Cells were transiently transfected with hHT_{1A-7} plasmids (75 ng DNA/well, HEK-5-HT_{1A-7}) using the standard protocol for the FuGENE reagent system (Promega

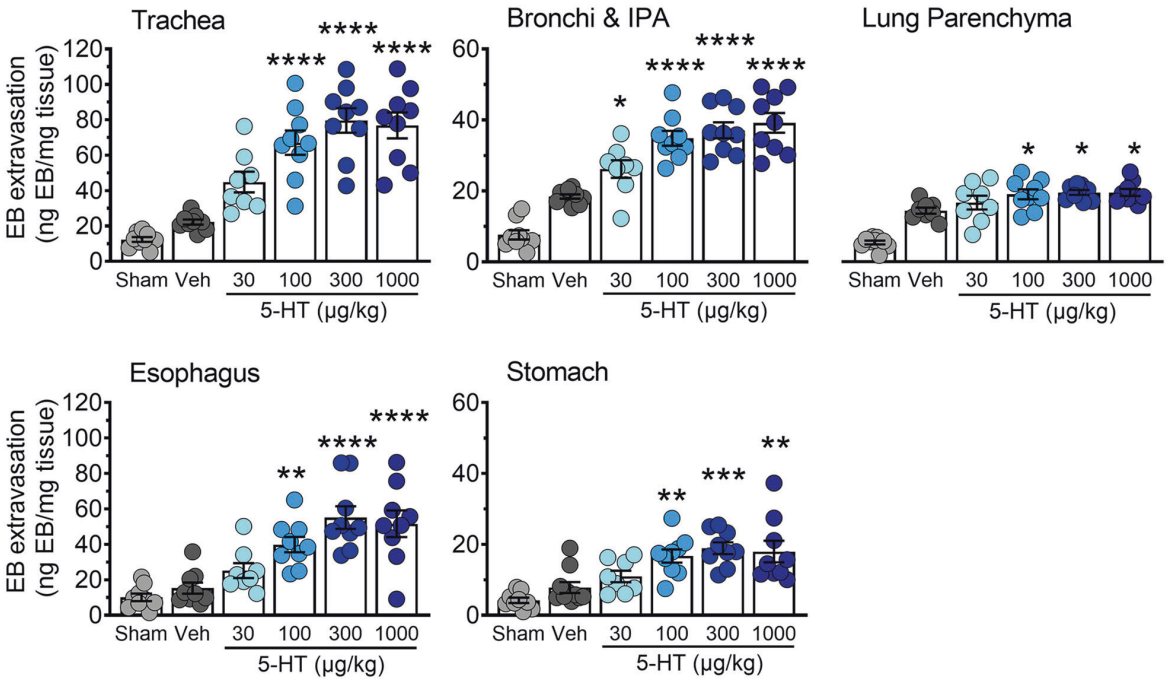

Fig. 1 5-HT causes vascular hyperpermeability in the airways and upper GI tract. Vascular hyperpermeability was assessed by the presence of Evans Blue in tissues of the airways, esophagus, and stomach following the intravenous injection of increasing concentrations of 5-HT (30–1000 μg/kg). Data are expressed as mean ± S.E.M., $n = 6$–9 mice per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; significantly different compared to vehicle treatment (1% DMSO in 0.9% saline); one-way ANOVA and Dunnett's multiple comparisons test.

Corporation Madison, WI USA). Expression of TRPV4 was induced overnight with 0.1 μg/mL tetracycline.

Ca^{2+} signaling assays

HUVEC or HEK cells were seeded onto poly-D-lysine coated 96-well plates (15,000 cells/well) and cultured for 48 h. Cells were loaded with Fura2-AM ester (1 μM) in Hank's Balanced Salt Solution (HBSS) supplemented with probenecid (2 mM) and pluronic acid (0.5 μM) for 45 min at 37 °C. Fluorescence was measured at 340/380 nm excitation and 530 nm emission wavelengths using a FlexStation III plate reader. Baseline measurements were recorded for 20 s prior to agonist addition. Responses to agonists were recorded for 200 s post-addition. For the PKC and PLA_2 inhibition assay, cells were incubated 30 min prior to 5-HT addition, as previously described with GF 109203X (GFX, 100 nM) [26] or YM26734 (30 μM) [48].

ERK phosphorylation assays

HUVEC were seeded onto non-coated 96-well plates (15,000 cells/well) and cultured for 48 h. Cells were serum starved for 6 h and treated as described in the results section. Phospho-ERK 1/2 (pERK1/2) was measured using the AlphaScreen SureFire p-ERK 1/2 (Thr202/Tyr204) Assay Kit (PerkinElmer, USA), according to the manufacturer's specifications. Fluorescence was measured using the EnVision multilabel plate reader (PerkinElmer). Data were normalized to the positive control (PDBu, 1 μM).

Statistical analysis

Data were analyzed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). All treatments were analyzed using one-way ANOVA with Dunnett's post-test. All data are presented as mean ± S.E.M., with a p value < 0.05 considered to be significantly different to the null hypothesis at the 95% confidence level.

Results

5-HT induces plasma extravasation in the airways and upper gastrointestinal tract

Evans Blue dye is commonly used as an indicator of altered vascular permeability to macromolecules due to its high

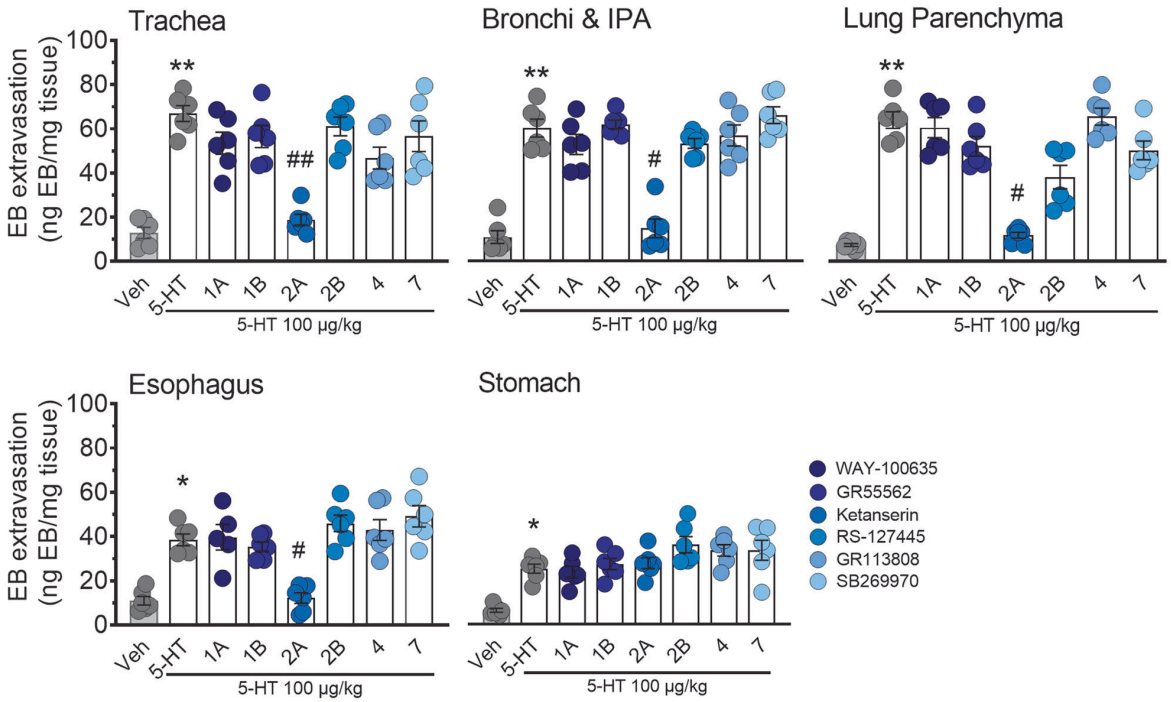

Fig. 2 5-HT-induced vascular hyperpermeability is inhibited in the airways and upper GI tract by the 5-HT$_{2A}$ selective antagonist ketanserin. Effect of WAY-100635 (5-HT$_{1A}$ antagonist, 80 µg/kg), GR 55562 (5-HT$_{1B}$ antagonist, 300 µg/kg), RS-127445 (5-HT$_{2B}$ antagonist, 300 µg/kg), GR 113808 (5-HT$_4$ antagonist, 1 mg/kg), or SB269973 (5-HT$_7$ antagonist, 300 µg/kg) in the airways and upper GI tract. Data are expressed as mean ± SEM for $n = 5$–6 experiments. *$p < 0.05$; **$p < 0.01$; One-way ANOVA and Dunnett's multiple comparisons test. * Indicates statistical significance compared to vehicle treatment, # indicates statistical significance compared to 5-HT 100 µg/kg treatment.

affinity for albumin. Under normal conditions, the vascular endothelium is impermeable to albumin, restricting albumin-bound Evans Blue to blood vessels. When inflammation occurs, albumin-bound Evans Blue is able to diffuse into surrounding tissues under conditions due to regulated, increased permeability of the vascular endothelium. Known as plasma extravasation, this process is important for promoting leukocyte infiltration, to initiate wound healing processes and subsequent swelling can also physically protect affected tissue [49].

To determine the effect of 5-HT on plasma extravasation, we examined the tissue distribution of Evans Blue following the administration of either vehicle (1% DMSO in 0.9% saline) or 5-HT (30–1000 µg/kg). For assessment of the natural absorbance of each tissue, an additional control group received an injection of saline solution without Evans Blue, followed by vehicle treatment. The vehicle treatment group did not exhibit significant basal leakiness of Evans Blue in the airways (trachea, bronchi and lung parenchyma) and upper gastrointestinal (GI) tract (esophagus and stomach) (Fig. 1). In contrast, the systemic administration of 5-HT elicited a dose-dependent increase in the amount of Evans Blue in tissues of the airways and upper GI tract, indicative of plasma extravasation (Fig. 1). A submaximal dose of 5-HT (100 µg/kg) was used in all subsequent experiments.

The pharmacological inhibition of 5-HT$_{2A}$ attenuates plasma extravasation in the airways and esophagus

To study the specific subtype of 5-HT receptor that is involved in 5-HT-induced plasma extravasation, mice were pre-treated with selective antagonists for 5-HT subtypes 1A (WAY-100635), 1B (GR 55562), 2A (ketanserin), 2B (RS-127445), 4 (GR 113808), or 7 (SB269973). The inhibition of 5-HT$_{2A}$ by ketanserin significantly attenuated plasma extravasation compared with vehicle pre-treated mice in the airways and esophagus (Fig. 2). However, ketanserin did not attenuate 5-HT-induced plasma extravasation in the stomach (Fig. 2). The inhibition of the 5-HT receptor subtypes 1A, 1B, 2B, 4, and 7 had no significant effect on 5-HT-induced plasma extravasation in the airways and upper GI tract compared with vehicle pre-treated mice, indicating that only 5-HT$_{2A}$ plays an important role on plasma extravasation.

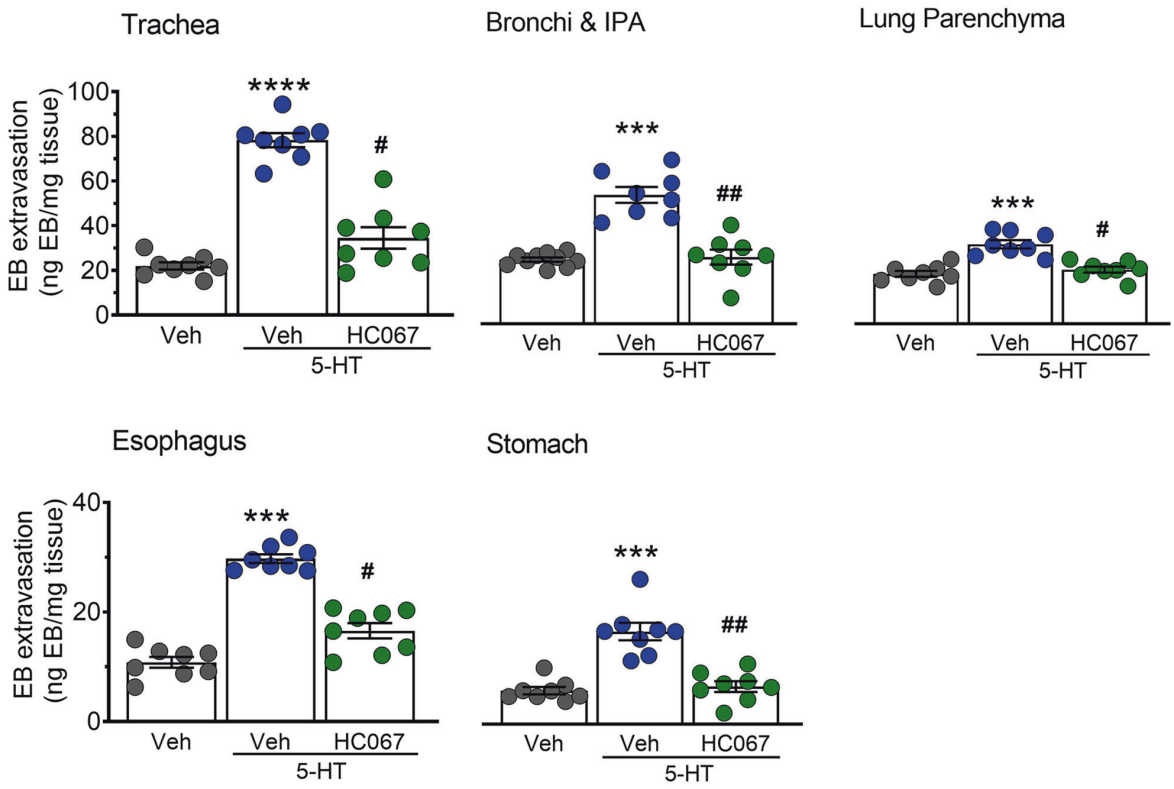

Fig. 3 Selective inhibition of TRPV4 suppresses 5-HT-induced edema. Effects of pre-treatment with the TRPV4 inhibitor HC067047 (10 mg/kg, HC067) on 5HT-induced plasma extravasation. HC067 significantly reduced Evans Blue leakage induced by 5-HT (100 µg/kg, i.v.). Data are expressed as mean ± S.E.M., $n = 8$ mice per group. $^{***}p < 0.001$; $^{****}p < 0.0001$; significantly different compared to vehicle treatment. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; significantly different compared to 5-HT treatment; one-way ANOVA and Dunnett's multiple comparisons test.

TRPV4 mediates 5-HT-induced plasma extravasation in the airways and upper GI tract

We have previously demonstrated that TRPV4 contributes to PAR1- and PAR2-dependent intracellular signaling and to PAR2-induced plasma extravasation [20, 26]. To determine whether TRPV4 plays an equivalent role in 5-HT-induced plasma extravasation, we administered the selective TRPV4 blocker HC067047 (HC067; 10 mg/kg, i.p.) prior to delivery of 5-HT. Inhibition of TRPV4 significantly decreased 5-HT-induced Evans Blue extravasation in the airways and upper GI tract, consistent with a TRPV4-dependent mechanism of action (Fig. 3).

5-HT-induced plasma extravasation requires TRPV4 expression

To confirm that 5-HT-induced plasma extravasation requires TRPV4 expression, we performed equivalent studies in TRPV4$^{-/-}$ mice or matched TRPV4$^{+/+}$ littermates. Previously, we reported that the selective TRPV4 activator GSK1016790A (GSK101) induced a dose-dependent increase in plasma extravasation in wild-type mice [20]. Consistent with our prior report, the administration of GSK101 (100 µg/kg) to wild-type mice induced a significant increase in plasma extravasation in the airways and upper GI tract (Fig. 4). Both GSK101- and 5-HT-induced plasma extravasation were abolished in TRPV4$^{-/-}$ mice (Fig. 4) when compared to TRPV4$^{+/+}$ mice. These data demonstrate the TRPV4-dependence of the 5-HT-evoked extravasation described.

TRPV4 enhanced 5-HT$_{2A}$ calcium signaling in HEK cells

The direct effect of 5-HT receptor signaling on TRPV4 activity was examined in an isolated cell system using HEK cells expressing the serotonin receptors (1A, 1B, 2A, 2B, or 4) alone or with co-expression of TRPV4. Assessment of 5-HT-mediated Ca^{2+} signaling over time (100 µM) demonstrated that HEK cells expressing 5-HT$_{1A,\ 1B,\ 2B}$ did not exhibit increased $[Ca^{2+}]_i$ in response to 5-HT (Fig. 5A).

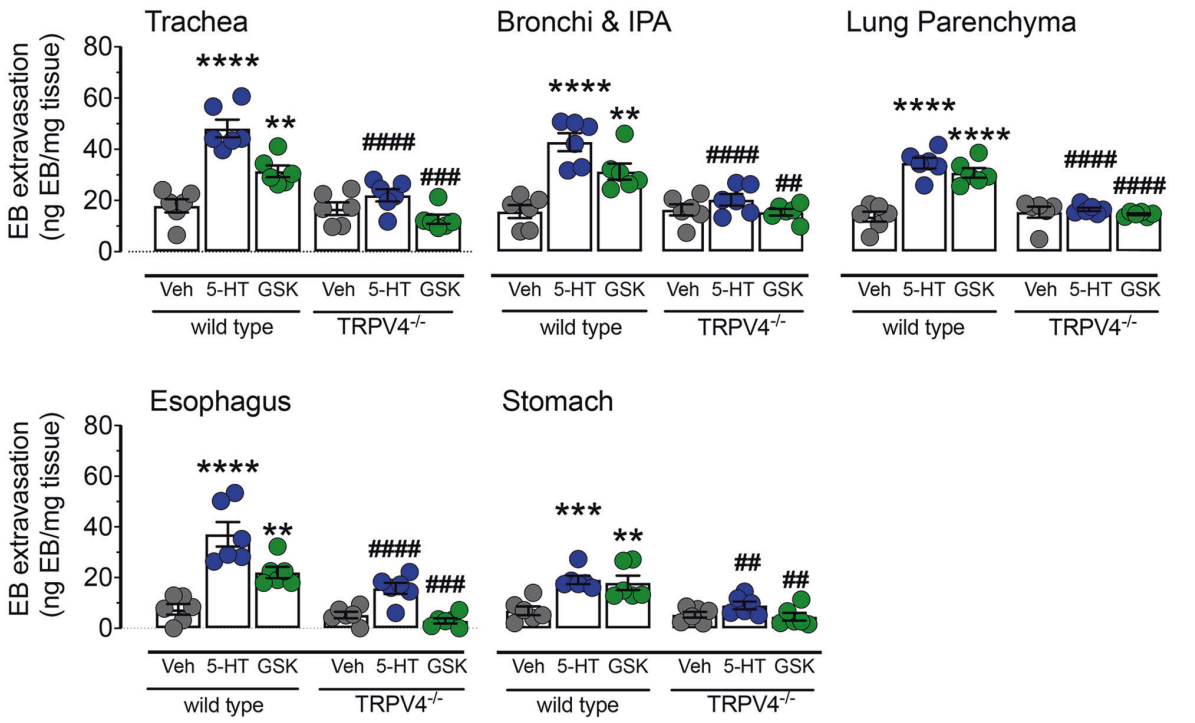

Fig. 4 5-HT and TRPV4-induced edema is absent in TRPV4$^{-/-}$ mice. The TRPV4 agonist GSK1016790A (100 µg/kg, i.v.; GSK) or 5-HT (100 µg/kg, i.v.) caused significant leakage of Evans Blue in the airway and upper GI tract of wild-type mice. Both 5-HT- and TRPV4-induced edema was significantly reduced in TRPV4$^{-/-}$ mice compared to wild-type littermate controls. Data are presented as mean ± S.E.M., $n = 6$ mice per group. * Significantly different compared to vehicle treated wild-type; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. # Significantly different compared to 5-HT- or GSK1016790A-treated wild-type; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$; one-way ANOVA and Dunnett's multiple comparisons test.

Expression of TRPV4 did not influence this response. In contrast, stimulation of HEK-5-HT$_{2A}$ cells resulted in a rapid, transient elevation in $[Ca^{2+}]_i$ that returned to baseline within 40 s, consistent with Gq-coupled signaling. Furthermore, the duration of the Ca^{2+} response was markedly sustained in cells functionally expressing TRPV4 (Fig. 5B). Stimulation of HEK-5HT$_4$ also revealed a Ca^{2+} transient that was only moderately enhanced in cells co-expressing TRPV4 (Fig. 5C).

Based on the robust nature of 5HT$_{2A}$-TRPV4 coupling, we focused on 5-HT$_{2A}$ and used known mediators of GPCR-TRPV4 coupling [24] to define the signaling mechanisms involved. The 5-HT$_{2A}$ dependence of the Ca^{2+} response was initially confirmed using ketanserin (Fig. 5B). Changes in $[Ca^{2+}]_i$ were then quantified over time by assessing the amplitude of the acute phase after 5-HT stimulation (0–20 s) and the magnitude of the sustained plateau phase (20–80 s post-stimulation). 5-HT$_{2A}$ transactivates phospholipase A2 (PLA$_2$) to generate arachidonic acid (AA), an endogenous activator of TRPV4 [50–53]. G$_q$-coupled activation of Protein Kinase C (PKC) can lead to rapid phosphorylation of intracellular regulatory domains of non-selective cation channels to modulate their ionic permeability [24]. To determine if PLA$_2$ and PKC serve as intermediates of 5-HT$_{2A}$-TRPV4 coupling, cells were treated with the PLA$_2$ inhibitor YM 26734 (30 µM) or the PKC inhibitor GF 109203X (GFX; 100 nM). Neither inhibitor affected the initial peak of the 5-HT response (Fig. 5D, E). Both inhibitors significantly suppressed the sustained phase (Fig. 5D, F). In addition, removal of extracellular Ca^{2+} abolished the transient and sustained phase of the 5-HT-evoked $[Ca^{2+}]_i$ response (Fig. 5D). These results suggest that coupling to TRPV4 enhances 5-HT$_{2A}$ receptor signaling predominantly through influx of extracellular Ca^{2+}.

Neuropeptide receptors contribute to TRPV4- and 5-HT-induced edema

Neuropeptides including CGRP and SP are released from sensory terminals that innervate blood vessels. These neuropeptides can influence endothelial barrier function and promote tissue edema through direct actions on microvascular endothelial cells [22, 54]. We assessed the contribution of CGRP and SP receptors to 5-HT- and TRPV4-induced plasma extravasation using selective antagonists of either the CGRP receptor (Olcegepant) or NK$_1$R

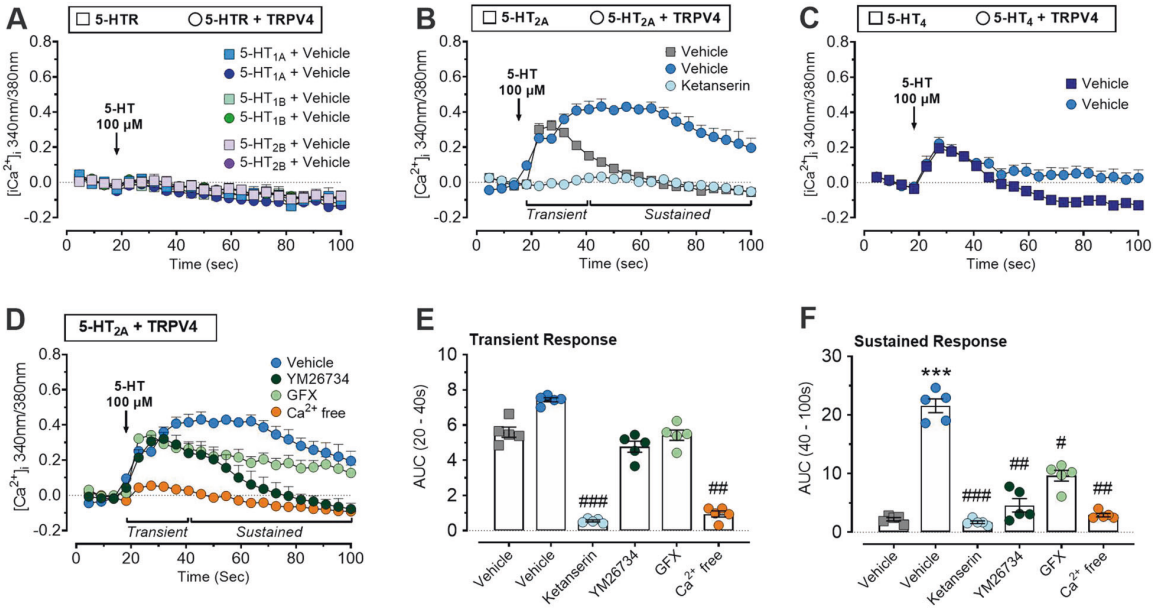

Fig. 5 5-HT induced a sustained increase in $[Ca^{2+}]_i$ in HEK cells co-expressing 5-HT_{2A} receptor and TRPV4. A Time traces showing responses to 5-HT (100 µM) by HEK cells expressing 5-HT_{1A}, 5-HT_{1B} or 5-HT_{2A} alone (circles) or with coexpression of TRPV4 (squares). **B** Time traces showing responses to 5-HT (100 µM) by HEK cells expressing 5-HT_{2A} or coexpressing 5-HT_{2A}/TRPV4. 5-HT-induced $[Ca^{2+}]_i$ was abolished by ketanserin (10 µM). **C** Time traces showing responses to 5-HT (100 µM) by HEK cells expressing 5-HT_4 alone or with coexpression of TRPV4. **D** Effect of the depletion of extracellular Ca^{2+}, PLA_2 inhibitor YM 26734 (30 µM), or PKC inhibitor GF 109203X (GFX; 100 nM) in HEK cells co-expressing 5-HT_{2A}/TRPV4. **E**, **F** Area under the curve analysis from 60 to 100 s post 5-HT (100 µM) addition. Data are expressed as mean ± SEM for n = 5–6 independent replicates. *p < 0.05; **p < 0.01; One-way ANOVA and Dunnett's multiple comparisons test. * Indicates statistical significance compared to HEK cells expressing 5-HTR subtype, # indicates statistical significance compared to HEK co-expressing 5-HTR and TRPV4.

(SR140333). Both antagonists significantly decreased tissue edema in the airways, esophagus and stomach in animals treated with GSK101 (Fig. 6) and 5-HT (Fig. 7), consistent with a neurogenic mechanism of action.

5-HT signaling in vascular endothelial cells is independent of TRPV4

The direct effects of 5-HT-TRPV4 coupling on vascular endothelial cells were examined using HUVEC, which are known to functionally express both targets [20, 55]. Focusing initially on Ca^{2+} mobilization, exposure to 100 nM or 1 µM 5-HT did not increase in $[Ca^{2+}]_i$. This is consistent with signaling through a G_q-independent mechanism (Fig. 8A). In contrast, GSK101 evoked a concentration-dependent elevation of $[Ca^{2+}]_i$ and this was attenuated with prior treatment with HC067 (Fig. 8A), thus confirming functional expression of TRPV4. Pre-treatment with 5-HT (100 nM; 30 min) enhanced GSK101-mediated $[Ca^{2+}]_i$ signaling in HUVECs (Fig. 8A, B). Specifically, 5-HT pre-treatment promoted a modest shift in pEC_{50} from −8.69 M to −9 M and increased E_{max} from 49.52 to 64.58 (Fig. 8A). This demonstrates a significant 5-HT-evoked amplification of TRPV4 signaling. Functional expression of 5-HT receptors was further confirmed by measuring levels of phosphorylated ERK (pERK), which allows for assessment of signaling through convergent pathways downstream of GPCRs. ERK activation was maximal at 2 min post-5-HT addition (100 nM or 1 µM) and decreased gradually over the 30 min assessment period. In contrast, GSK101 did not stimulate pERK in these cells (Fig. 8C). We confirmed that exposure to either SP (100 nM or 1 µM) or CGRP (100 nM or 1 µM) promotes a rapid and robust increase in pERK levels in HUVEC (Fig. 8D). Together, these data indicate that 5-HT receptors can sensitize and augment TRPV4 activity. These observations suggest that enhanced vascular permeability in response to 5-HT is potentially mediated through an indirect mechanism involving the TRPV4-dependent release of the neuropeptides SP and CGRP, possibly from external cellular sources such as primary afferent terminals.

Discussion

TRPV4 activation is important for the pathogenesis of pulmonary edema associated with heart failure or

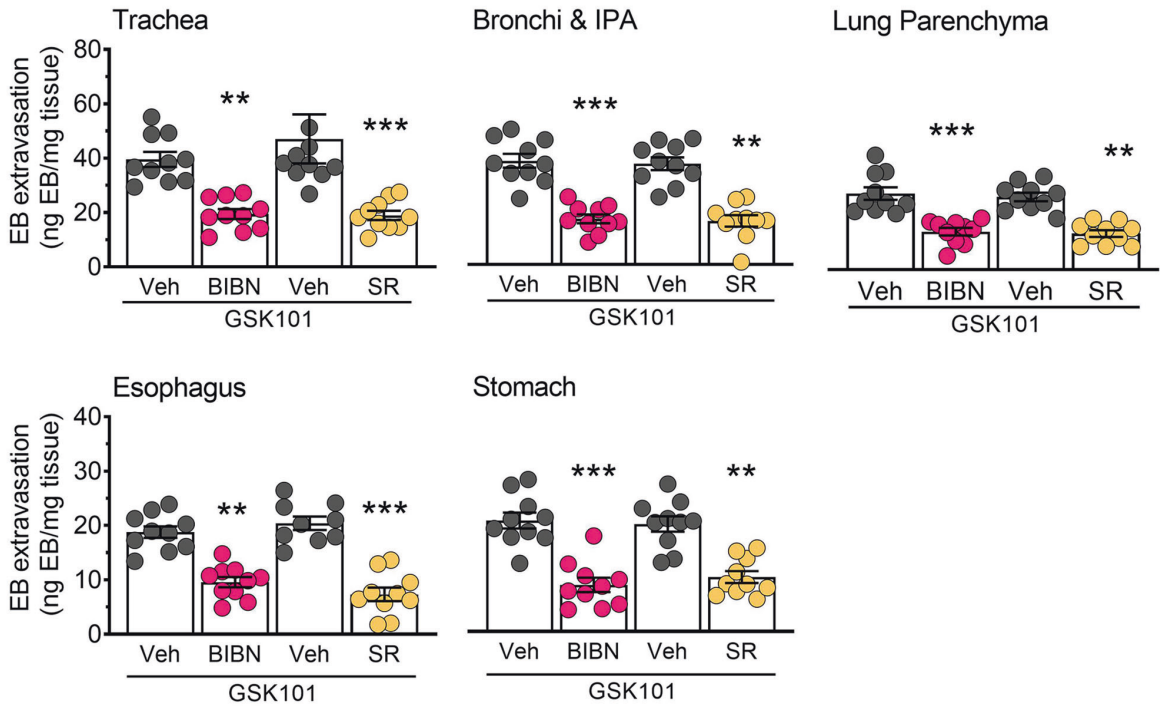

Fig. 6 TRPV4-induced edema is decreased by inhibition of CGRP or NK_1 receptors. Pre-treatment with the CGRP receptor antagonist BIBN4906 (BIBN; 1 mg/kg) or NK_1R antagonist SR140333 (SR; 1 mg/kg) significantly decreased tissue edema induced by GSK1016790A (100 µg/kg) compared to vehicle treatment in the airways and upper GI tract. Data are presented as mean ± S.E.M., $n = 9–10$ mice per group. $^{**}p < 0.01$; $^{***}p < 0.001$; One-way ANOVA and Dunnett's multiple comparisons test, significantly different compared to vehicle treated control (Veh).

chemically-induced acute lung injury [18, 21]. TRPV4 is also a mediator of sepsis-induced endothelial dysfunction and increased vascular permeability [56]. Consistent with this, 5-HT is also a potent vasoactive and signaling mediator and can promote disruption of cell–cell junctions at concentrations not much higher than those normally present under resting conditions [56, 57]. Here, we showed that 5-HT promotes pulmonary and esophageal plasma extravasation through a TRPV4-dependent mechanism. This also involves activation of NK_1R and the CGRP receptor, consistent with a putative neurogenic mechanism involving release of SP and CGRP from nerve fibers innervating the vasculature.

5-HT is mainly produced by enterochromaffin cells of the intestine, and is largely taken up and stored by platelets, or metabolized by the liver [57]. However, the lungs also play an important role in both 5-HT production and removal, and release of 5-HT by platelets may be important in the pathology of certain pulmonary diseases [57–60]. Additionally, 5-HT can be locally synthesized and released from peripheral arteries [61–63]. The 5-HT-TRPV4 signaling pathway may mediate a number of pathologies, including pulmonary hypertension, arterial smooth muscle proliferation, visceral hypersensitivity, and itch [8, 28, 30, 31]. Results of the present study suggest that the 5-HT receptor-TRPV4 axis could be an important pathway in pathologies, such as sepsis, where plasma 5-HT levels are known to be significantly elevated [64].

We have recently demonstrated that the potent and selective TRPV4 agonist, GSK101, caused dose-dependent extravasation in the airways and upper GI tract of mice, which was inhibited by the selective TRPV4 antagonist HC067 [20]. In contrast, GSK101 did not cause plasma extravasation in the bladder, heart, liver or kidney, suggesting that edema is not a general systemic effect of TRPV4 activation [20]. In the present study, we report that 5-HT induces plasma extravasation in the airways, esophagus and the stomach. Plasma extravasation induced by 5-HT was decreased by HC067 or TRPV4 deletion and limited to the tissues in which the TRPV4 activation caused edema, namely the airways and upper gut. These results support a role for TRPV4 in promoting 5-HT-induced plasma extravasation in the airways, esophagus and stomach. Extravasation in response to 5-HT was almost completely blocked by the TRPV4-specific

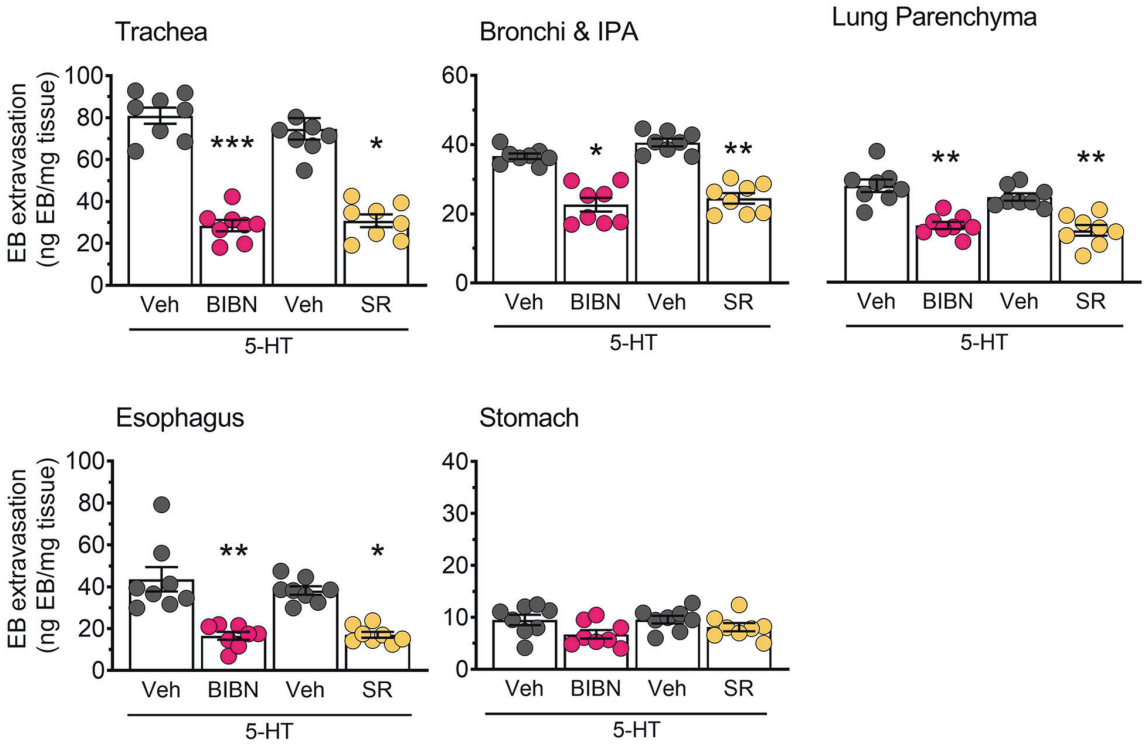

Fig. 7 Inhibition of CGRP or NK_1 receptors blocks 5-HT induced edema. Pre-treatment with the CGRP receptor antagonist BIBN4906 (BIBN; 1 mg/kg) or the NK_1R antagonist SR140333 (SR; 1 mg/kg) decreased plasma extravasation induced by 5-HT (100 µg/kg) in the airways and upper GI tract. Data are presented as mean ± S.E.M., $n = 9–10$ mice per group. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; One-way ANOVA and Dunnett's multiple comparisons test, significantly different compared to vehicle treated control (Veh).

inhibitor HC067. In contrast, the extent of GSK101-induced TRPV4-dependent vascular leak was markedly lower. Although this may be due to differences in the respective signaling pathways involved, it may also reflect the physicochemical properties of the ligands investigated and their relative bioavailability following systemic administration.

The release of neuropeptide transmitters from airway innervating nerves leads to inflammation and to vascular leak. This neurogenic response can also be initiated by exogenous irritants via airway nerves and may contribute to the development of airway pathologies [65–67]. We demonstrated that inhibitors of CGRP and SP receptors reduced TRPV4- and 5-HT-induced plasma extravasation in the airways and esophagus, supporting a mechanistic role for these neuropeptide receptors. It has been reported that CGRP does not cause microvascular leak in the airways and bladder of the guinea pig [68]. In contrast, CGRP has been reported to contribute to edema formation in mice [66] and rats [69], indicating potential species differences. Our results suggest that 5-HT-induced plasma extravasation in the airways and esophagus is mediated by activation of afferent nerves, requires TRPV4, and is likely to involve release of pro-inflammatory peptides (SP and CGRP) (Fig. 9).

Pre-clinically, TRPV4 plays important roles in pathological pulmonary edema and may therefore be a therapeutically useful target. Importantly, chronic treatment with a TRPV4 inhibitor in animal models did not affect osmoregulation or interfere with the activity of diuretics, which are often used to resolve edema in the clinic [18, 70–72]. Recently, a double-blind, placebo-controlled study using a selective TRPV4 antagonist reported that treatment with GSK2798745 resulted in a trend to improve pulmonary gas exchange in symptomatic patients with chronic heart failure [71, 73]. However, the use of inhibitors that directly target TRPV4 in pulmonary injury may be contraindicated by the role that TRPV4 plays in the complex signaling cascade that mediates hypoxic pulmonary vasoconstriction [74]. This mechanism helps to redistribute blood flow from poorly ventilated to more aerated lung areas, and inhibition of this response could be detrimental to patients with lung

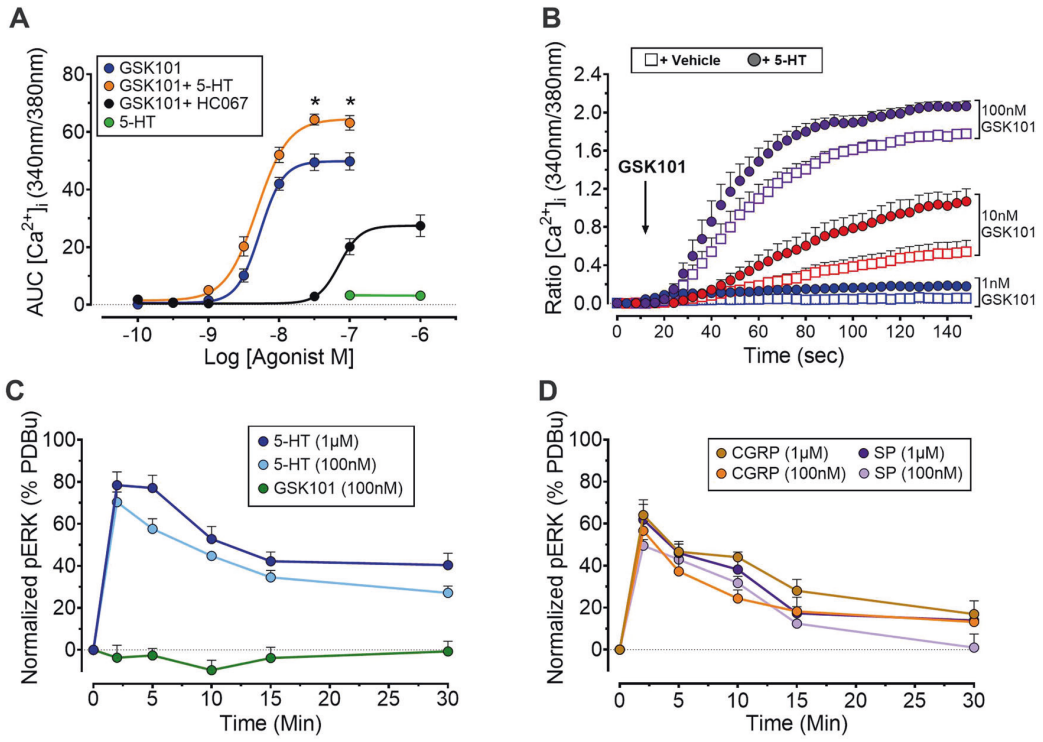

Fig. 8 Functional expression and interaction of TRPV4 and 5-HT receptors in HUVEC. A Pre-treatment with 5-HT augmented the magnitude (E_{max}) of responses to GSK101. GSK101-induced Ca^{2+} signaling was attenuated by the TRPV4 antagonist HC067. No change in $[Ca^{2+}]_i$ was detected following treatment with 5-HT. **B** Time traces demonstrating the effect of pre-treatment with 5-HT (open circle, 100 nM; 30 min) on GSK101-induced elevations in $[Ca^{2+}]_i$ (closed circles). **C**, **D** Elevated ERK phosphorylation (pERK) in response to treatment of HUVEC with 5-HT, GSK101, CGRP or SP. Data are presented as mean ± S.E.M., $n = 6$ technical replicates, pERK data are normalized to the positive control (PDBu, 1 μM).

disease [71, 73]. The benefits of TRPV4 antagonists for reducing pulmonary edema-associated lethality from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have also recently been proposed to outweigh the risks of contraindications such as these. Together, this highlights the need to further understand the relative cellular contribution of TRPV4 activity and also the upstream signaling mediators that lead to TRPV4 activation, to provide potential alternatives to these potent antagonists that directly inhibit TRPV4.

Our pharmacological data indicate that 5-HT_{2A} is the primary receptor subtype involved in promoting 5-HT-evoked plasma extravasation. Evidence supporting the in vivo requirement for TRPV4 activity is provided by the demonstration that 5-HT_{2A} mediated Ca^{2+} signaling is augmented by TRPV4 through a PLA_2- and PKC-dependent mechanism. Although our data support an indirect neurogenic mechanism of action involving enhanced neuropeptide release [23], the specific locations where 5-HT_{2A}-TRPV4 interactions occur (pre- or post-synaptic [75]) could not be definitively determined using available methodology. We cannot exclude a direct effect on endothelial cells as the HUVEC that we examined may not be the most suitable model for the microvasculature involved in 5-HT-dependent vascular leak as they are of a different origin and may not express the precise machinery required. Our results suggest that 5-HT and TRPV4 receptors are also expressed by endothelial cells and may cause protein leak via disruption of the vascular junctions in mice. In addition to expression by peptidergic afferent nerves [76] and vascular endothelial cells [77, 78], 5-HT receptors and TRPV4 are also expressed by immune cells, including macrophages [79–81]. Given the important immunomodulatory role of 5-HT, it is possible that the TRPV4-dependent effects of 5-HT on vascular permeability that we describe are mediated in part through immune cell activation. Future analysis to better define the relative contributions of 5-HT receptors and TRPV4 in endothelial and immune cells and on nerve endings of the different vascular beds would help to clarify the primary location of TRPV4-driven edema and the precise mechanisms involved.

In summary, we have established that TRPV4 mediates 5-HT-induced plasma extravasation in the airways and

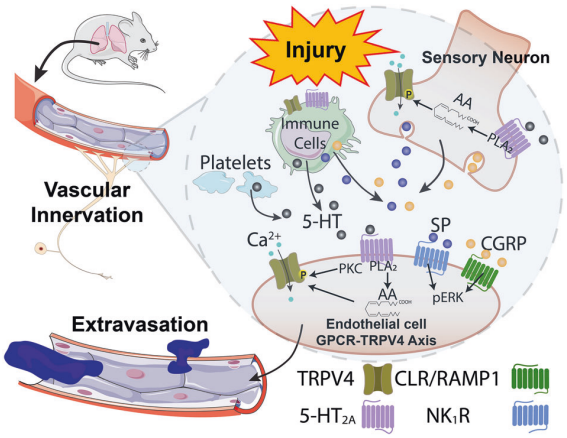

Fig. 9 Postulated neurogenic and direct mechanisms through which 5-HT receptors and TRPV4 may induce tissue edema. 5-HT activates 5-HT_{2A} receptor subtypes on afferent nerve terminals, immune cells, and vascular endothelial cells. Activation of peripheral sensory neurons or local immune cells promotes release of neuropeptides including SP and CGRP, activating NK_1R and CGRP receptors expressed by endothelial cells. Signaling downstream of these receptors leads to retraction of cell-cell junction proteins, leading to increased vascular leak and to tissue edema. Activation of 5-HT_{2A} sensitizes or activates TRPV4 through a PLA_2- and PKC-dependent mechanism, leading to enhanced neuropeptide release.

upper GI tract of mice through interaction with the 5-HT_{2A} receptor subtype. We have provided evidence to support an indirect, potentially neurogenic mechanism of action involving the neuropeptides SP and CGRP.

Data availability

Data presented in this study are available upon request from the corresponding authors.

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Author contributions *Participated in research design*: JSR, MSG, NWB, NAV, DPP, and PM. *Conducted experiments*: MSG, LKD, SP, FB, JSR, JGA, and SA. *Performed data analysis*: JSR, NAV, DPP, MSG, and PM. *Contributed to writing or critical assessment of the paper*: JSR, MSG, ABG, PR, PRG, SEC, NAV, DPP, and PM.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval Studies using mice were approved by the Animal Ethics Committees of RMIT and Monash Institute of Pharmaceutical Sciences.

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Summary

1. The challenges of treating pain

Pain incorporates a complex range of disorders that affects up to 30% of adults at some point during their lifetime and requires a combination of physical therapy and pharmacological approaches for treatment. From a pharmacological perspective, treatment is possible by administering one or more therapeutic agents such as paracetamol/acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase-2 inhibitors (Coxibs) followed by careful use of opioids for elevated pain (e.g., morphine or oxycodone). Unfortunately, each of these drugs has associated side-effects that limit their use. Opioids, for example, remain some of the most effective, efficacious analgesic classes available and continue to be prescribed, especially for extreme acute pain (*e.g.*, post-surgery) or for chronic pain conditions. However, opioids have high abuse potential due to euphoric or addictive properties, as well as receptor desensitisation or tolerance. To overcome tolerance, patients with chronic pain can be subjected to sustained increases in dosing or switching to other more potent opioids to improve analgesia, which often provides only temporary gains in pain relief. However, this approach may increase the risk of tolerance and addiction over time, in addition to increasing the likelihood of debilitating side-effects such as constipation and respiratory depression (Boudreau et al., 2009; Corbett et al., 2006; Scholz and Woolf, 2002; Volkow et al., 2011). Thus, there is a clear need for other analgesics that are both safe and effective. But to do this, we need to better understand other pain targets and investigate how they can modulate pain.

2. Beyond opioids – analgesic Drug discovery in GPCR targets

Alternative GPCR targets have been identified to be to major therapeutic targets that offer effective pain relief without the safety concerns posed by opioids. Cannabinoids, for example, are emerging as effective non-opioid analgesics and primarily function through activation of Gi/o-coupled cannabinoid receptors (CB1, CB2) to downregulate excitatory processes and modulate serotoninergic (5-HT) and noradrenergic pathways. Although widely available and used for millennia, cannabinoids are also associated with behavioural or psychological liabilities that require further investigation. We also are yet to see the outcomes of systematic use in the clinic and the benefit of more recently discovered synthetic ligands for treating pain (Banister et al., 2019; Brennan et al., 2007).

GPCRs are highly dynamic proteins that achieve distinct signalling outcomes by adopting different conformational states (Latorraca et al., 2017). Extracellular ligands that bind cell surface GPCRs promote receptor conformations that activate heterotrimeric G proteins to transduce downstream signalling that engages with βArr. βArr can function as adaptor proteins to mediate distinct signalling processes such as MAPK activity, and also facilitate interactions with clathrin-coated membranes to promote endocytosis (Ferguson et al., 1996). This was historically considered to facilitate termination of signalling, either by directing receptors to degradative lysosomal pathways, or by promoting rapid receptor recycling to reset the activity cycle during the internalisation process, and increase the potential for sustained signalling once the receptor is recovered at the plasma membrane (Ferguson et al., 1996; Shukla et al., 2014).

There are an increasing number of studies interrogating the relationship between GPCR trafficking and signalling suggest a third possibility exists for the cellular need for GPCR endocytosis. Receptors can remain on intracellular membranes such as endosomes for sustained periods of time, to facilitate a unique signalling complex distinct signalling processes in a βArr- or a G protein-dependent manner. This paradigm shift was initially revealed by studying signalling and trafficking relationships on Gs-coupled receptors such as the parathyroid (PTHR), thyroid-stimulating hormone (TSHR) and β2 adrenergic receptors to demonstrate that endosomal-mediated sustained cyclic adenosine monophosphate (cAMP) production could be observed after endocytosis has occurred. This has been extensively reviewed (Calebiro et al., 2010; Tsvetanova et al., 2015; Vilardaga et al., 2014). This was initially studied by combining standard signalling assays with endocytic inhibitors and trafficking-deficient receptors. Recently, the development of sensitive new signalling tools has been advantageous for providing an expanded view of the spatiotemporal nature of signalling. Genetically encoded tools such as conformation-selective nanobodies and FRET or BRET-based biosensors, for example, measure real-time location-specific GPCR activity and downstream signalling cascades respectively, (Halls and Canals, 2018; Irannejad et al., 2017). Given the prevalence and importance of trafficking GPCRs in neurons, the internalisation and location-specific signalling relationships for several GPCRs has been described, including NK_1R, CLR/RAMP1, $mGlu_5$, PAR2 and MOR (Jensen et al., 2017; Jiménez-Vargas et al., 2021; Mantyh et al., 1995; O'Malley et al., 2003; Poole et al., 2015; Ramírez-García et al., 2019a; Stoeber et al., 2018; Yarwood et al., 2017). Three pro-nociceptive GPCRs have been closely studied and shown to be strong candidates for mediating nociceptive signals from an endosomal location. The first receptor reported was NK_1R, followed PAR_2 in dorsal root ganglia and CLR/RAMP1 in trigeminal neurons and in spinal cord (Yarwood et al., 2017). Although

trafficking of NK_1R during pain states has been known for some time (Mantyh et al., 1995). Some of the first insights showing NK_1R-mediated sustained neuronal excitability and pain transmission in spinal neurons was associated with endosomes, was demonstrated with application of endocytosis inhibitors or endosome-biased antagonism (Jensen et al., 2017; Ramírez-García et al., 2019a).

However, endosomes are one of many sites to which GPCRs can be recruited to form unique signalling complexes and promote spatiotemporally distinct signalling. Activation of MOR by lipophilic agonists such as morphine, but not more soluble peptides, for example, can occur on Golgi membranes (Stoeber et al., 2018). Another example, $mGlu_5$ in spinal neurons of neuropathic rats can be recruited to the inner nuclear membrane, increasing nuclear Ca^{2+} and transcriptional factor such as c-Fos and Jun, proteins associated with pain. Although $mGlu_5$ trafficking is known to require dynamin-dependent processes, and the importance for endocytosis or endosome-specific complex formation in the context of pain has not been explored to the best of our knowledge.

3. The challenges of targeting and controlling endosomal signalling

There are several challenges that need to be addressed in order to effectively target and pharmacologically manipulate endosomal signalling. A therapeutic agent of interest needs to be able to remain in solution, traverse the plasma membrane, accumulate within endosomes and be retained in this location for a sufficient period of time to allow efficient blockade of endosomal signalling without disrupting endosomal formation, dynamics and structure. This raises questions about whether the intrinsic properties of analgesic agents that have been enhanced by chemical modification, to increase activity or partitioning into membranes. One strategy to achieve this goal, for example, is the use of lipid-drug conjugated antagonists to promote endosomal delivery and retention (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Mai et al., 2021; Yarwood et al., 2017). These lipidated drugs are comprised of a sterol-based anchor such as cholestanol, attached to an antagonist via a flexible PEG linker, allowing antagonist presentation in an aqueous environment. This strategy was initially investigated for delivery of BACE inhibitors (Rajendran et al., 2008) and subsequently investigated by Jensen *et al.* (2017) and Mai *et al.* (2021) for the NK_1R. These authors used a lipidation approach for the endosomal delivery of the water-soluble NK_1R antagonist Spantide, thereby promoting association into membranes – first in the plasma membrane to inhibit βArr-coupling, with subsequent accumulation in endosomes over time to block signals associated with pain transmission (Jensen et al., 2017; Mai et al., 2021). Additional studies by Yarwood *et al*., and Jimenez-Vargas *et al.,* supported the utilisation of lipidated antagonists for the successful targeting of endosomal of CLR/RAMP1 and

PAR_2 receptors, respectively (Jimenez-Vargas et al., 2018; Yarwood et al., 2017). Disadvantages of this system are that studies have been limited to the use of peptide antagonists, and the slow drug internalisation observed on *in vitro* and *in vivo*, whereby a minimum of 3 h is required for drug quantities to accumulate on endosomal membranes and achieve effective inhibition. Future studies are warranted to optimise the linker type and length, as well as exploring other small molecule drug candidates to advance beyond these peptide-focussed studies.

With the emergence of nanomedicine, this field offers great promise for specialised drug delivery that may increase delivery at sites where it is needed, while avoiding unwanted side-effects. Particularly, studies in the cancer field have benefitted from this approach, where simple, safe nanomedicines (e.g., based on liposomes, or recent lipid nanoparticle vaccine used for mRNA delivery) are increasingly being approved by regulatory agencies such as the FDA and TGA, thus suggesting that we are making safe, effective delivery systems. A new alternative for selective delivery of drugs into endosomes is the use of pH-responsive nanoparticles. Nanoparticles passively internalised within the endosomal network approximately one minute from the formation of early endosomes to 5-15 min until endosome maturation (Ramírez-García et al., 2019b), Pre-Chapter 3) . Our studies showed that pH-responsive nanoparticles remain within endosomes (*i.e.*, no cytoplasmic escape) and then disassembly for cargo release can occur in response to small reductions in pH (<6.5), consistent with the environment nanoparticles encounter within early and late endosomes. Once internalised and exposed to this acidic environment, DIPMA units within the hydrophobic core are proposed to become protonated, leading to charge-charge repulsion and micelle disassembly, which facilitates rapid drug release (Gao et al., 2010; Ramírez-García et al., 2019a). Nanoparticles present advantages compared to lipidated antagonist due to the small size and uncoated structure, which allow a passive uptake, and the ability to bypass the cell surface. Nanoparticles such as these can be modified in a variety of ways, including engineering tuneability pH, as is the focus on this thesis, as well as temperature, oxygen radicals, pressure, ultrasound and light, as reviewed previously (Beiranvand and Sorori, 2019; Hua et al., 2018).

In Pre-chapter 3, we developed a pH-responsive nanoparticle (DIPMA) loaded with a NK_1R antagonist, aprepitant, as a tool to enhance selective delivery of NK_1R antagonists into endosomes. pH-responsive nanoparticles demonstrated to abolish *in vitro* signalling proteins induced by NK_1R (Jensen et al., 2017; Ramírez-García et al., 2019b). While this was a large multi-authored, collaborative international study, I contributed several critical insights that were essential to our understanding of the importance of NK_1R endosomal inhibition and its participation in transmission.

Particularly, I assessed the antinociceptive efficacy of DIPMA in three different models of pain (acute, inflammatory, and neuropathic pain), which showed that a single intrathecal dose of nanoparticles improved the antinociceptive properties compared to free drug. In addition, I examined the effect of nanoparticles on electrophysiological C-reflex parameters in neuropathic rats, demonstrating that endosomal NK_1R inhibition decreases neuronal excitability *in vivo*.

The second part of this PhD thesis (Chapter 4) extended these initial insights from the NK1R to a new receptor, by investigating if and how $mGlu_5$ located on endosomes contributes to signalling and whether $mGlu_5$ endosomal signalling is a suitable therapeutic target for pain. We report that $mGlu_5$ activation leads $G\alpha_{q/11}$ and $G\alpha_s$ protein recruitment in the plasma membrane. This is followed by internalisation into Rab5-positive endosomes, where $mGlu_5$ continue to recruit $G\alpha_{q/11}$ and promote sustained intracellular Ca^{2+} mobilisation and ERK activity using heterologous expression systems. Further studies are required to confirm if these same signalling and trafficking observations are also possible in neurons or microglia where $mGlu_5$ is expressed in the spinal cord. Consistent with the prior studies in NK_1R (pre-Chapter 3), where HEK and cultured neuron signalling data was translated to *in vivo* studies, this led to the hypothesis that endosomal $mGlu_5$ signalling mediates pain transmission and that by selectively targeting these endosomal $mGlu_5$, enhanced analgesia could be achieved. Finally, to demonstrate the importance of signalling from endosomes, we utilised pH-responsive nanoparticles to selectively deliver the $mGlu_5$ negative allosteric modulator VU058 into acidified endosomes. DIPMA micelle employed was proposed to bypass the cell surface and bias drug action toward endosomal $mGlu_5$, to improve the efficacy VU-058-mediated inhibition of intracellular Ca^{2+} mobilisation and ERK activity *in vitro*. In addition, administering pH-responsive nanoparticles intrathecally in mice, as a carrier for endosomal uptake and release of $mGlu_5$ ligands, we achieved superior analgesia in acute and chronic models of pain.

These two studies (pre-chapter 3 and 4) provided an important proof of concept demonstrating that pH-responsive nanoparticles have potential for the reversal of pain not just for NK_1R but also for $mGlu_5$ and addressing current challenges in providing genuine alternatives to current analgesics. The proposed benefit of this approach lies in the repurposing of clinically approved drugs while simultaneously avoiding the broad distribution of drugs due to selective endosomal targeting.

In our initial study (pre-chapter 3), we confirm that pH-responsive nanoparticles are not toxic *in vitro*, and we also confirmed that these nanoparticles do not induce clinical behavioural observation such as shivering, increase of breading, sleeping. However, further histological studies are required to confirm

that pH-responsive nanoparticles does not induces detrimental effect at cellular levels such as spinal cord. Furthermore, in both studies we used highly hydrophobic drugs due to encapsulation limitations. Thus, pH responsive nanoparticles need improvement in the encapsulation process to allow hydrophilic drugs encapsulation. In addition, pH-responsive nanoparticles are highly tuneable, the monomer DIPMA has pKa of 6.1, where this monomer unit can be exchanged and modify the pH responsiveness, where a slower or quicker release can be achieved to inhibit the endosomal network at different levels.

4. TRPV4 ion channels – an important effector protein for GPCR signalling

In contrast to these studies on GPCR trafficking-signalling relationships, the final results chapter of this thesis (chapter 5) investigated another interesting and understudied phenomenon, to further understand how GPCR can achieve their complete signalling repertoire. To do this, we focussed on 5-HT receptor signalling, to explore the role of TRPV4 ion channels as effector proteins in 5-HT-induced plasma extravasation, with particular interaction of 5HT subtype 2A.

TRP channels are established enhancer of vascular permeability, oedema and pain. Particularly, TRPV4 is a recognised as a central mediator of neurogenic inflammation through enhanced release of neuropeptides such as SP and calcitonin gene-related peptide (CGRP) from peptidergic peripheral nerve endings (Ding et al., 2010; Lin et al., 2007; Poole et al., 2013). GPCRs can modify the sensitivity to ligand or mechanical activation, as well as the magnitude and duration of TRPV4 ion channel gating by functional interactions (termed ‘coupling’ or the GPCR-TRP axis). These interactions are reciprocal, where functional coupling of a GPCR to an ion channel such as TRPV4 can lead to augmentation of GPCR signalling. Furthermore, coupling between GPCRs and TRPV4 is proposed to contribute to disease-associated processes, including neurogenic inflammation and pain (Veldhuis et al., 2015).

An example of reciprocal GPCR-TRP coupling is illustrated through functional interactions between protease- activated receptors 1 and 2 (PAR1 and PAR2) and TRPV4 (Peng et al., 2020; Poole et al., 2013; Zhao et al., 2014). PAR activation “sensitise” or reduce the activation threshold through channel phosphorylation and enhance TRPV4 signalling through kinase signalling cascades and activation of phospholipases such as PLA2, to promote the production of endogenous TRPV4 activators, arachidonic acid and the eicosanoic acid, 5′,6′-EET (Grace et al., 2014; Grant et al., 2007; Poole et al., 2013). GPCR-TRP axis studies provide strong mechanistic understanding of the important

contribution in the bidirectional interactions of GPCR and TRP channel in fundamental biological processes such as vascular permeability and oedema.

We reported that activation of either TRPV4 or 5-HT receptors promoted significant plasma extravasation in the airway and upper gastrointestinal tract of mice. Functional interaction studies reveal that TRPV4 enhances the duration of 5-HT-evoked Ca^{2+} signalling through a PLA_2 and PKC-dependent mechanism. In summary, this study demonstrates that TRPV4 contributes to 5-HT_{2A}-induced plasma extravasation in the airways and upper GI tract, with evidence supporting a mechanism of action involving SP and CGRP release.

Here, we provided relevant evidence that GPCRs not just signal within the plasma membrane and intracellular location but also can exert influence on other receptors through downstream pathways, affecting cell or tissue-specific signalling such as oedema. Particularly, 5HT2A couples to Gαq, to activate PLC, PLA2 and PKC. PLA2 promotes the production of endogenous TRPV4 activators, arachidonic acid and 5′,6′-EET, whereas PKC phosphorylated TRPV4 to enhance its activity. While 5HT2A is receiving significant recent attention in drug discovery due to its known role in depression, anxiety, schizophrenia, these studies are also an important reminder of the role serotonin signalling plays in peripheral processes such as the neurogenic inflammatory processes described. While this requires further studies to demonstrate how important 5HT2A signalling is chronic states, this preliminary study at least suggest that the 5HT2A-TRPV4 axis may be important for the acute phase of inflammation.

To summarise, this PhD thesis explored location-specific signalling of GPCRs in endosomes involved in pain, where pH-responsive nanoparticles that selectively delivery drugs into endosomes, decrease pain signalling *in vitro* and enhance the antinociceptive effect of antagonist *in vivo.* In addition, we demonstrated that GPCRs can also interact with other receptor such as TRP channels to enhance or modify their activity, fundamental biological processes such as vascular permeability and oedema. While further studies are required to fully understand GPRCs signalling, it provides valuable new information about the requirement for GPCR recruitment to specific membranes and how it engages with other effector proteins in a spatiotemporal manner to achieve its complete signalling response. This also includes new understanding of how 5HT-mediated signalling utilises ion channels to amplify signalling, modify cell secretion and contractility to promote oedema.

5. References

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Appendix 1

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ARTICLES

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A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain

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Nanoparticle-mediated drug delivery is especially useful for targets within endosomes because of the endosomal transport mechanisms of many nanomedicines within cells. Here, we report the design of a pH-responsive, soft polymeric nanoparticle for the targeting of acidified endosomes to precisely inhibit endosomal signalling events leading to chronic pain. In chronic pain, the substance P (SP) neurokinin 1 receptor (NK_1R) redistributes from the plasma membrane to acidified endosomes, where it signals to maintain pain. Therefore, the NK_1R in endosomes provides an important target for pain relief. The pH-responsive nanoparticles enter cells by clathrin- and dynamin-dependent endocytosis and accumulate in NK_1R-containing endosomes. Following intrathecal injection into rodents, the nanoparticles, containing the FDA-approved NK_1R antagonist aprepitant, inhibit SP-induced activation of spinal neurons and thus prevent pain transmission. Treatment with the nanoparticles leads to complete and persistent relief from nociceptive, inflammatory and neuropathic nociception and offers a much-needed non-opioid treatment option for chronic pain.

Nanoparticle encapsulation improves drug efficacy by enhancing the stability, tolerability, delivery and retention in diseased tissues[1–3]. Interest in using nanoparticles to deliver anticancer drugs is perpetuated by the prospect of targeted delivery to tumour cells, and by the leaky vasculature and poor lymphatic drainage of tumours, which promote nanoparticle accumulation and uptake[4]. Stimulus-responsive nanoparticles can enhance targeted delivery and avoid undesirable exposure, further improving efficacy[5]. Triggers for nanoparticle disassembly and drug release include acidity, protease activity and redox imbalance within tumours. Inflammation and infection also acidify extracellular microenvironments[6–9]. However, few nanoparticle-based chemotherapeutics have been tested in patients, and the rationale has been questioned[10].

Acidification of intracellular compartments, including endosomes and lysosomes, can be exploited for intracellular drug delivery[11]. For applications that require therapeutics to reach cytoplasmic or nuclear targets, the necessity and challenges of endosomal escape limit the usefulness of nanoparticle-mediated endosomal delivery[12]. The identification of drug targets within endosomes provides opportunities for harnessing pH-sensitive materials to chaperone drugs to intracellular targets. The realization that G protein-coupled receptors (GPCRs) can signal from endosomes has created opportunities to improve drug efficacy and repurpose medicines[13].

GPCRs are a large (>800) family of seven transmembrane proteins that control most physiological and pathological processes, and such GPCRs are the target of more than 30% of therapeutic drugs[14]. GPCR signalling is not confined to the plasma membrane, but also occurs within endosomes[15,16]. Location-biased compounds favour interactions with GPCRs in subcellular locations, leading to distinct signals[17,18]. Endosomal signalling of the substance P (SP) neurokinin 1 receptor (NK_1R), calcitonin receptor-like receptor and protease-activated receptor-2 in primary sensory and spinal neurons mediates nociception[19–21]. Inhibitors of endocytosis and lipid-conjugated antagonists that target these receptors in endosomes provide effective anti-nociception[19–21]. Because these compounds are unlikely to be drug candidates, there remains the need to explore endosomal delivery of existing medicines. Endosomal delivery of GPCR ligands could enhance the treatment of many disorders[13].

Herein, we demonstrate that soft polymeric pH-responsive nanoparticles alter the distribution and efficacy of an FDA-approved NK_1R antagonist, aprepitant, which is used to treat emesis but has

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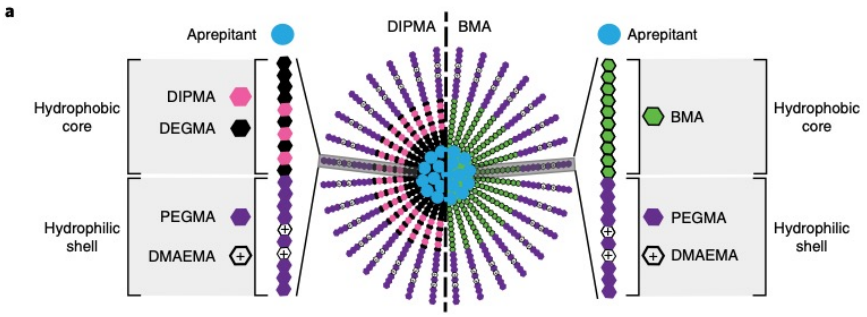


b

Nanoparticle	Diameter (nm)	ζ potential (mV)	Aprepitant loading (%)	CMC (μg ml^{-1})	pH of disassembly
DIPMA-AP	40.4 ± 5.1 (30)	−0.2 ± 1.6 (18)	57.8 ± 11.1 (27)	2.4 ± 0.5 (3)	6.08 ± 0.064 (3)
DIPMA-Ø	37.0 ± 4.2 (18)	−0.5 ± 2.0 (11)	NA	1.9 ± 1.1 (3)	6.08 ± 0.064 (3)
BMA-AP	28.0 ± 2.5 (27)	−1.1 ± 2.8 (16)	61.5 ± 12.5 (10)	1.5 ± 0.8 (3)	NA
BMA-Ø	30.0 ± 4.4 (13)	−0.3 ± 0.3 (11)	NA	1.5 ± 1.1 (3)	NA

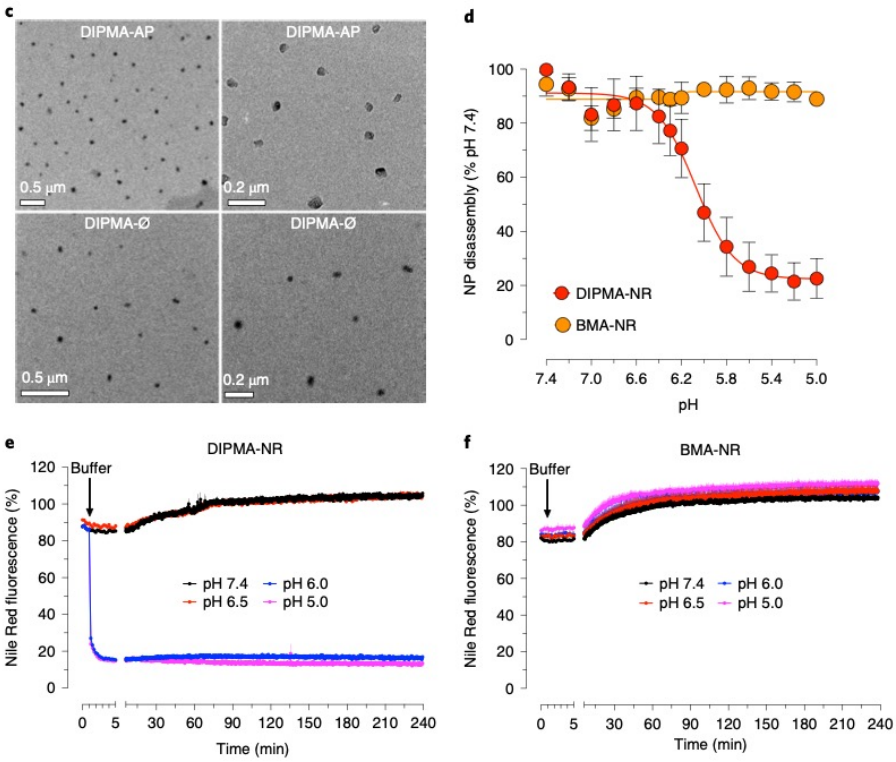


Fig. 1 | Characterization of DIPMA and BMA nanoparticles. a, Structure of pH-responsive (DIPMA) and pH-non-responsive (BMA) nanoparticles. Nanoparticles share the same hydrophilic shell of P(PEGMA-co-DMAEMA) but have different hydrophobic cores: P(DIPMA-co-DEGMA) or BMA. **b**, Properties of DIPMA-Ø, BMA-Ø, DIPMA-AP and BMA-AP (100 nM aprepitant) nanoparticles. Ø, empty; AP, aprepitant. Data are presented as mean ± s.d. Values in parenthesis indicate the number (*n*) of independent experimental replicates. Aprepitant incorporation for nanoparticles containing lower aprepitant concentrations (% initial aprepitant (mean ± s.d.)): DIPMA-AP, 50 nM, 58.4 ± 7.7, *n* = 9 experiments; 25 nM, 62.6 ± 16.3, *n* = 9; BMA-AP, 50 nM, 62.4 ± 11.7, *n* = 9; 25 nM, 65.2 ± 16.2, *n* = 9. CMC, critical micellar concentration. NA, not applicable. **c**, Transmission electron microscopy images of DIPMA-AP (100 nM aprepitant) and DIPMA-Ø nanoparticles. Representative images of *n* = 2 independent experiments are shown. **d**, pH-dependent Nile Red (NR) quenching of DIPMA-NR and BMA-NR nanoparticles in vitro, indicative of nanoparticle disassembly. Data are presented as mean ± s.e.m., *n* = 3 independent experiments, triplicate observations. **e**,**f**, Time course of NR quenching of DIPMA-NR (**e**) and BMA-NR (**f**) nanoparticles in vitro and pH levels of 7.4, 6.5, 6.0 and 5.0. Data are presented as mean ± s.e.m., *n* = 3 independent experiments, triplicate observations.

failed in trials for other indications[22–24]. Nanoparticles delivered aprepitant to endosomes containing activated NK_1R, and induced a more complete and sustained anti-nociception in preclinical models than conventional therapies, including opioids. Nanoparticle delivery minimized the dose of aprepitant required for anti-nociception, which might avoid off-target effects. Thus, nanoparticles have

potential beyond bulk drug delivery for cancer therapy and in fields where, to date, their applicability has been unrecognized. The use of nanoparticles to direct drugs to subcellular compartments from which GPCRs generate disease-relevant signals has broad applicability. The discovery that nanoparticle encapsulation enhances and prolongs analgesia provides opportunities for developing much-needed non-opioid therapies for pain.

Results

Synthesis and pH-dependent disassembly of nanoparticles. Diblock copolymers were synthesized with the same hydrophilic shell of P(PEGMA-co-DMAEMA), but with different hydrophobic cores of P(DIPMA-co-DEGMA) to form pH-responsive nanoparticles or P(BMA) to form non-pH-responsive nanoparticles (DIPMA or BMA nanoparticles, respectively; Fig. 1a and Supplementary Fig. 1a,b). Gel permeation chromatography and ^{1}H-NMR confirmed the molecular weight and composition of the nanoparticles (Supplementary Fig. 1c,d).

Nanoparticles were self-assembled with aprepitant (MK-869[22]), a hydrophobic NK_1R antagonist, forming DIPMA-aprepitant (DIPMA-AP) and BMA-aprepitant (BMA-AP) (Fig. 1b). To generate nanoparticles for delivery of graded concentrations of aprepitant (25, 50 or 100 nM) but a constant concentration of polymer, nanoparticles were self-assembled with graded amounts of aprepitant and a fixed amount of polymer. For most studies, nanoparticles containing 100 nM aprepitant were used, with similar incorporation efficiency (Fig. 1b).

Nanoparticles are dynamic structures that remain assembled when the concentration of polymer exceeds the critical micelle concentration (Supplementary Fig. 2). The critical micellar concentrations of DIPMA-empty (DIPMA-Ø), BMA-Ø, DIPMA-AP and BMA-AP were comparable (Fig. 1b). Nanoparticles were uniformly spherical, with similar diameters and ζ potentials (Fig. 1b).

To examine pH-dependent disassembly, nanoparticles were loaded with Nile Red (NR), which fluoresces only in the hydrophobic core. Fluorescence quenching in aqueous solutions of graded pH was used to evaluate nanoparticle disassembly. DIPMA-NR fluorescence declined with increasing acidity (50% decrease, pH 6.08 ± 0.06; Fig. 1d), consistent with the protonation of the DIPMA tertiary amine ($pK_a = 6.1$), charge repulsion and disassembly (Supplementary Fig. 2). BMA-NR fluorescence was unaffected by acidification (Fig. 1d). DIPMA-NR fluorescence declined to minimum levels within 4 min at pH 6.0 or 5.0 (Fig. 1e) whereas BMA-NR did not decline in acidic buffers (Fig. 1f). There was a small unexplainable increase in DIPMA-NR fluorescence at pH 6.5 or 7.4 and in BMA-NR fluorescence at pH 7.4, 6.5, 6.0 or 5.0.

Uptake and disassembly of nanoparticles in cells. Cellular uptake and trafficking of DIPMA nanoparticles labelled with cyanine 5 (DIPMA-Cy5) were examined by confocal microscopy in HEK-293 cells. NK_1R endosomal trafficking and signalling are similar in HEK-293 cells and spinal neurons[19]. After incubation for 30 or 60 min, DIPMA-Cy5 nanoparticles were localized to early and late endosomes (Fig. 2a, Supplementary Fig. 3a and Supplementary Videos 1 and 2). HEK-293 cells expressing rat (r)NK_1R-GFP were treated with SP to evoke NK_1R endocytosis. At 30 and 60 min after SP, DIPMA-Cy5 nanoparticles co-localized with rNK_1R-GFP in endosomes (Fig. 2b, Supplementary Fig. 3b and Supplementary Video 3). Determination of the Manders overlap coefficient[25] confirmed DIPMA-Cy5 co-localization with rNK_1R-GFP, Rab5a-GFP and Rab7a-GFP (Fig. 2c).

The uptake and disassembly of DIPMA nanoparticles loaded with Coumarin 153 (DIPMA-CO), which fluoresces in an aqueous environment but not in the hydrophobic core, were examined by confocal microscopy and high content imaging. When DIPMA-CO nanoparticles were incubated with HEK-293 cells, there was an increase in intracellular fluorescence from 1 to 10 min that continued for 30 min (Fig. 2e,f). Inhibitors of clathrin (PitStop2)[26], dynamin (Dyngo4a)[27] and endosomal acidification (Bafilomycin A1, which inhibits the vacuolar H^+ATPase; NH_4Cl, a lysosomotropic weak base) attenuated cellular fluorescence (Fig. 2c–f). These results are consistent with clathrin- and dynamin-dependent endocytosis, and pH-dependent disassembly of DIPMA nanoparticles in acidified endosomes. When non-pH-disassembling BMA-Cy5 nanoparticles were incubated with HEK-293 cells, there was a smaller increase in fluorescence from 1 to 10 min (Fig. 2d,e). Although PitStop2 and Dyngo4a suppressed the fluorescence, Bafilomycin A1 and NH_4Cl had no effect (Fig. 2d–f). BMA nanoparticles also enter cells by clathrin- and dynamin-dependent endocytosis, and release cargo by mechanisms that do not require endosomal acidification.

Biodistribution and delivery of nanoparticle cargo. To examine nanoparticle distribution in vivo, DIPMA-Cy5 or BMA-Cy5 nanoparticles were injected intrathecally (L4/L5), which delivers NK_1R antagonists to spinal neurons[19]. Non-invasive imaging revealed that Cy5 fluorescence, which might be incorporated within nanoparticles or disassembled fluorophore, remained within the injection site for up to 24 h (Fig. 3a,b). Confocal imaging showed that DIPMA-Cy5 and BMA-Cy5 nanoparticles accumulated in a perinuclear region in cells throughout laminae I, II and III of the dorsal horn (Fig. 3c and Supplementary Videos 4 and 5). DIPMA-Cy5 nanoparticles were present in neurons, identified by co-localization with the neuronal marker PGP9.5, although detailed analysis of the cellular distribution was not possible due to loss of nanoparticle fluorescence during immunostaining.

To evaluate the usefulness of nanoparticles for drug delivery, free aprepitant, DIPMA-AP or BMA-AP was injected intrathecally to mice, then liquid chromatography–mass spectrometry (LC-MS) was used to quantify aprepitant in the spinal cord. At 1 h after injection, the spinal aprepitant concentration was approximately twofold higher for DIPMA-AP than for BMA-AP and approximately fourfold higher than for free aprepitant (Fig. 3d). At 4 h, spinal aprepitant was similar for DIPMA-AP and BMA-AP, and almost undetectable for free aprepitant. Thus, nanoparticle encapsulation causes retention of aprepitant within the spinal cord.

Effects of nanoparticles on nociception. To examine the hypothesis that incorporation into nanoparticles enhances the anti-nociceptive actions of aprepitant due to delivery to spinal neurons, the efficacy of free or nanoparticle-encapsulated aprepitant was evaluated in preclinical models of nociceptive, inflammatory and neuropathic pain (Fig. 4d). Nanoparticles, free aprepitant or vehicle was injected intrathecally before or after intraplanar injection of algogens or nerve injury. Mechanical nociception was studied in mice by measuring withdrawal responses to stimulation of the plantar surface of the hindpaw with calibrated von Frey filaments (VFFs) and in rats by measuring the pressure that induced withdrawal of the hindpaw (Randall–Selitto test).

Assessment of nociception requires normal motor coordination, which allows paw withdrawal from painful stimuli. The latency to fall from a rotarod was the same in mice after intrathecal injection of vehicle, DIPMA-AP, BMA-AP or DIPMA-Ø (Supplementary Fig. 4). Nanoparticles do not interfere with motor coordination.

Capsaicin. Intraplantar capsaicin activates transient receptor potential-1 on primary sensory neurons to release SP in the dorsal horn, which evokes NK_1R endocytosis in spinal neurons and allodynia[19,28]. In mice pretreated with intrathecal vehicle or DIPMA-Ø, capsaicin decreased the VFF threshold from 0.5 to 4 h, which returned to baseline after 24 h (Fig. 4b,c). Free aprepitant (100 nM) and DIPMA-Ø mixed with free aprepitant (100 nM) caused a modest anti-nociception after 1 h (16 ± 4 and 15 ± 3% inhibition, respectively). BMA-AP

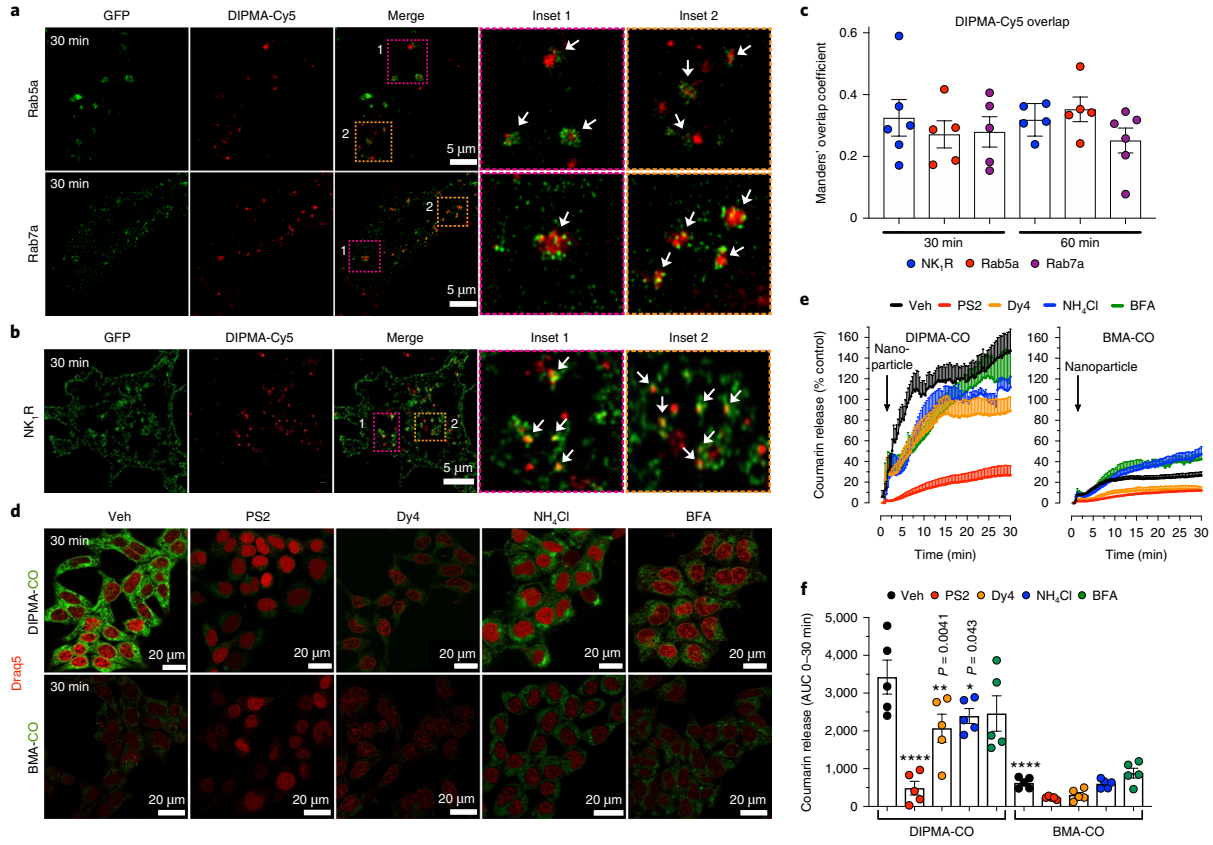


Fig. 2 | Uptake and disassembly of DIPMA and BMA nanoparticles in HEK-293 cells. a, To examine trafficking to endosomes, DIPMA-Cy5 nanoparticles were incubated with HEK-293 cells expressing Rab5a-GFP, which identifies early endosomes, or Rab7a-GFP, which marks late endosomes. Images show localization of DIPMA-Cy5 nanoparticles in Rab5a-GFP-positive early endosomes and Rab7a-GFP-positive late endosomes after incubation with HEK-293 cells for 30 min. Representative images of $n = 5$ independent experiments are shown. **b**, To determine whether nanoparticles traffic to endosomes containing the NK_1R, DIPMA-Cy5 nanoparticles were incubated with HEK-293 cells transfected with rNK_1R-GFP; after 30 min, cells were challenged with 10 nM SP to promote internalization of rNK_1R-GFP. The images show co-localization of DIPMA-Cy5 nanoparticles and NK_1R-GFP in HEK-rNK_1R cells at 30 min after stimulation with SP to induce NK_1R endocytosis. Representative images of $n = 5$ independent experiments are shown. **c**, Manders overlap coefficient to assess the degree of co-localization of DIPMA-Cy5 with NK_1R-GFP, Rab5a-GFP and Rab5a-GFP at 30 min and 60 min. Data are presented as mean ± s.e.m., $n = 5$ independent experiments. **d**–**f**, Uptake of DIPMA-CO and BMA-CO nanoparticles into HEK-293 cells. **d**, Representative images of $n = 5$ independent experiments at 30 min after addition of DIPMA-CO or BMA-CO nanoparticles; Coumarin 153 is green, Draq5 nuclear stain is red. **e**,**f**, Kinetic analysis and quantification of uptake and disassembly of DIPMA-CO and MBA-CO nanoparticles assessed by Coumarin 153 fluorescence. In cells treated with DIPMA-CO, Coumarin 153 rapidly accumulated in the cytosol, indicative of rapid nanoparticle disassembly. PitStop2 (PS2), Dyngo4a (Dy4), Bafilomycin A1 (BFA) and NH_4Cl inhibited the appearance of Coumarin 153 and nanoparticle disassembly. In cells treated with BMA-CO, Coumarin 153 slowly accumulated in the cytosol, indicative of minimal nanoparticle disassembly. PitStop2 and Dyngo4a, but not Bafilomycin A1 and NH_4Cl, inhibited appearance of Coumarin 153 and nanoparticle disassembly. Data are presented as mean ± s.e.m., $n = 5$ (**e**,**f**) independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ compared to DIPMA-CO treated with vehicle. Two-way analysis of variance (ANOVA), Dunnett's post-hoc test.

(100 nM aprepitant) had a similar effect after 0.5–1 h, although the effect was sustained for 2 h. DIPMA-AP (100 nM aprepitant) caused marked anti-nociception at 0.5–1 h (1 h, 34 ± 3% inhibition) that was sustained for 4 h (35 ± 2% inhibition).

Complete Freund's adjuvant. Intraplantar complete Freund's adjuvant (CFA) causes sustained mechanical allodynia and NK_1R endocytosis in spinal neurons[19,29], which allowed examination of the capacity of nanoparticle-encapsulated aprepitant to reverse inflammatory nociception. By 48 h after CFA injection, there was a marked decrease in VFF threshold (Fig. 4d–f). Intrathecal vehicle did not affect mechanical hyperalgesia, which persisted for 24 h. Aprepitant (100 and 300 nM) dose-dependently reversed hyperalgesia for 2–3 h (1.5 h, % inhibition: 100 nM, 30 ± 6; 300 nM, 47 ± 3%). BMA-AP (100 nM aprepitant) was as effective as free aprepitant (300 nM). DIPMA-AP (100 nM aprepitant) produced a larger inhibition of allodynia than the same dose of free aprepitant (1.5 h, % inhibition: 54 ± 4%), and the inhibition was maintained for 6 h, when other treatments were ineffective. Although systemic morphine (3 mg kg^{-1}, intraperitoneal) fully reversed the mechanical allodynia after 0.5 h, the effect waned after 3 h.

Nerve injury. The sural nerve spared (SNS) model produces a mechanical hyperalgesia in rats for >50 days[30,31], which permitted examination of the efficacy of nanoparticle-encapsulated aprepitant to relieve chronic neuropathic nociception in another species.

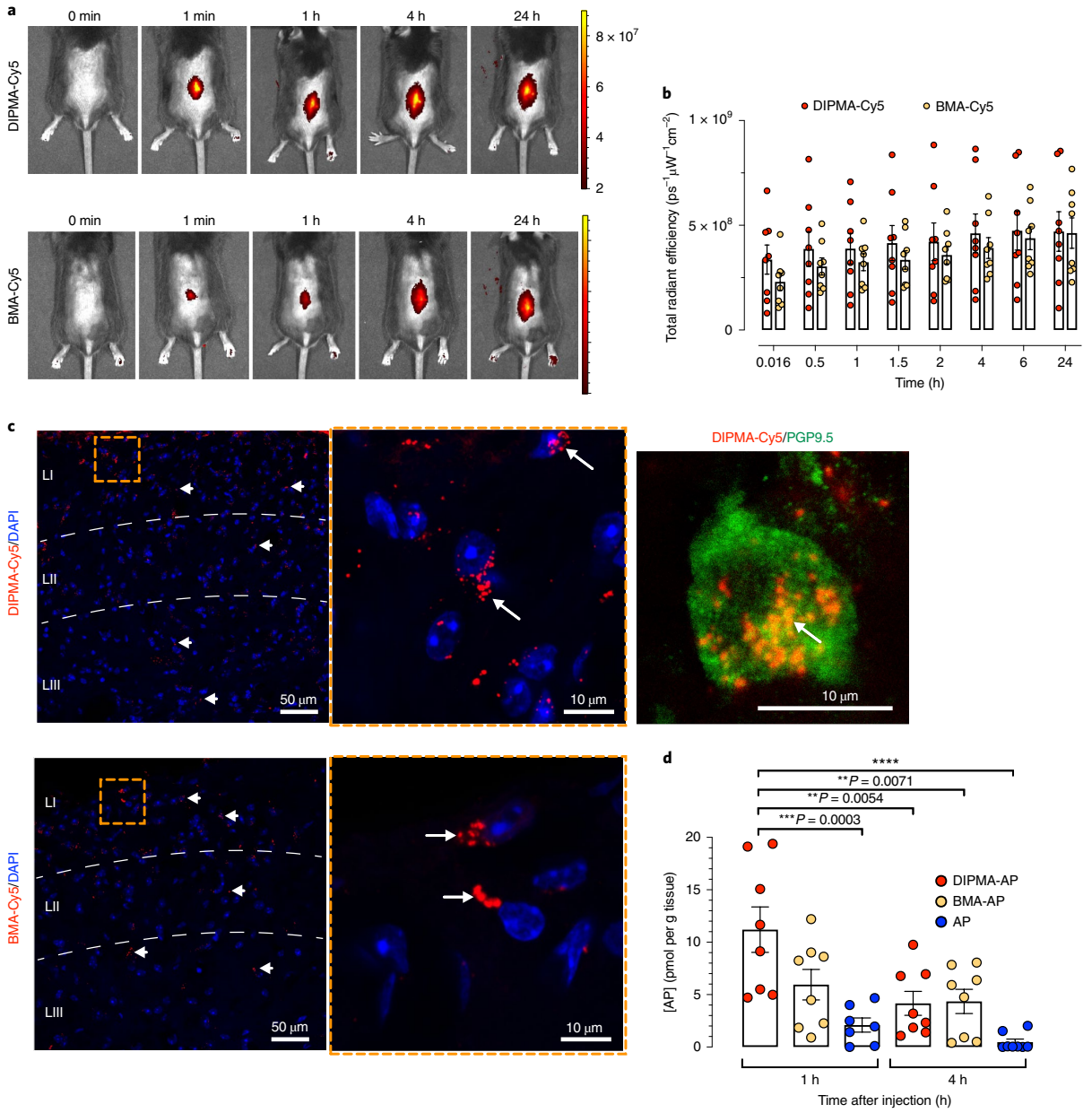


Fig. 3 | Biodistribution and cellular uptake of nanoparticles and aprepitant delivery. a, Distribution of DIPMA-Cy5 and BMA-Cy5 nanoparticles at various times after intrathecal injection of mice. Representative images of experiments on $n = 8$ mice are shown. Scale bar, Cy5 fluorescence intensity measured as radiant efficiency with units $ps^{-1}\mu W^{-1}cm^{-2}$. p, photons. s, seconds. W, watts. **b**, Quantification of the distribution of DIPMA-Cy5 and BMA-Cy5-nanoparticles at various times after intrathecal injection of mice assessed as the radiant efficiency of the images. Data are presented as mean ± s.e.m., $n = 8$ mice. **c**, Localization of DIPMA-Cy5 and BMA-Cy5 nanoparticles in the dorsal horn (laminae, LI–III) 1 h after intrathecal injection. The inset to the right shows accumulation of DIPMA-Cy5 nanoparticles in a perinuclear region of a spinal neuron, as confirmed by labelling with anti-PGP9.5. Representative images from $n = 5$ mice. **d**, Aprepitant concentrations in the spinal cord measured 1 h and 4 h after intrathecal injection of DIPMA-AP, BMA-AP or free aprepitant (100 nM). Data are presented as mean ± s.e.m.; $n = 7$ mice for aprepitant at 1 h and $n = 8$ mice for aprepitant at 4 h (and DIPMA-AP and BMA-AP at 1 h and 4 h). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Two-way ANOVA, Tukey's post-hoc test.

To confirm activation of the SP/NK_1R system, we localized NK_1R immunoreactivity (IR) in spinal neurons at 10 days after sham or SNS surgery by immunofluorescence. In sham rats, NK_1R-IR was confined to the plasma membrane of the soma and neurites of lamina I neurons (Supplementary Fig. 5a and Supplementary Video 6). In SNS rats, NK_1R-IR was detected in endosomes of ipsilateral lamina I neurons but was localized to the plasma membrane of contralateral lamina I neurons (Supplementary Fig. 5a and Supplementary

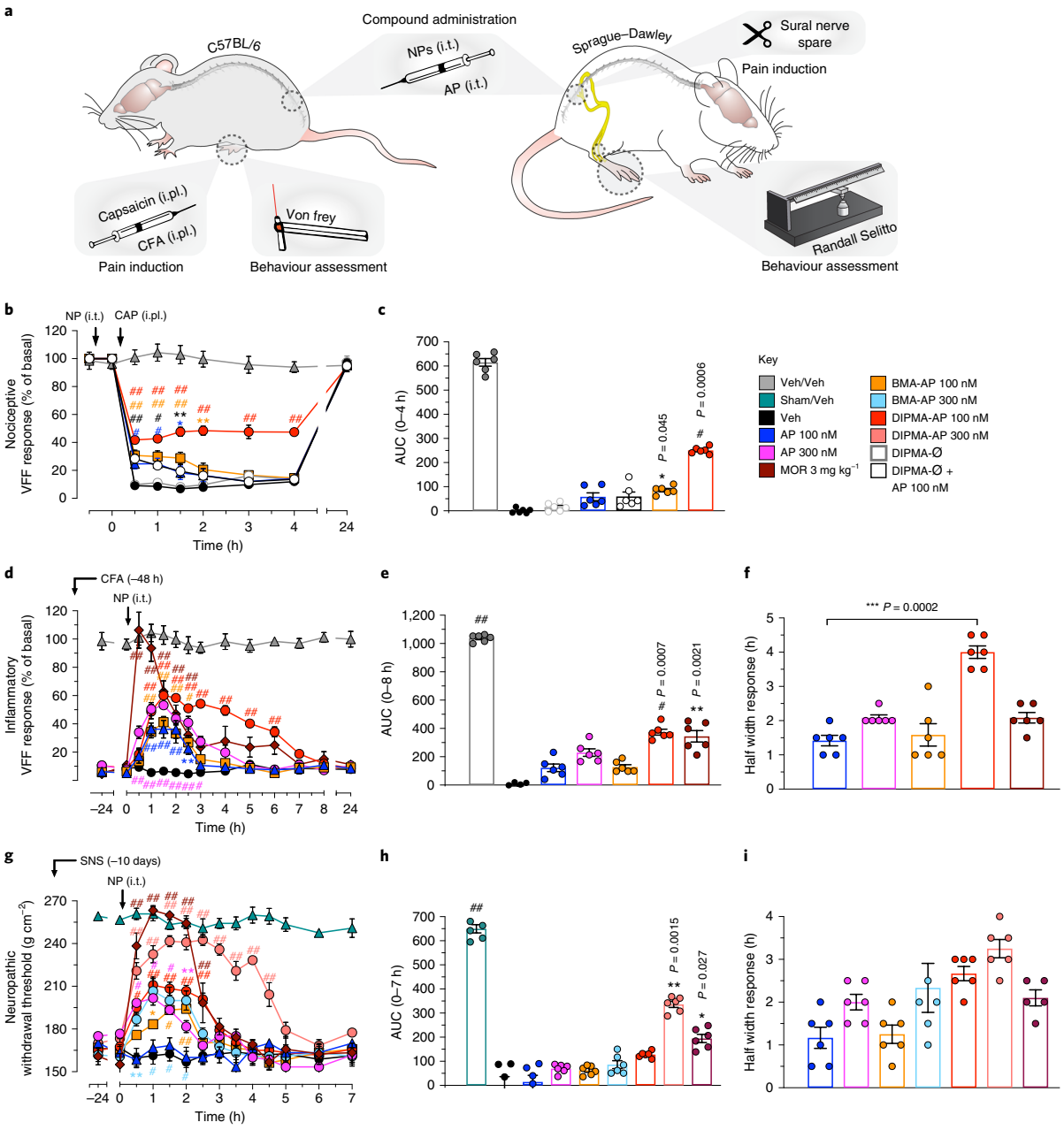


Fig. 4 | Effects of nanoparticles on nociceptive, inflammatory and neuropathic nociception. a, Preclinical models of nociceptive, inflammatory and neuropathic pain. In the capsaicin-evoked model of acute nociceptive pain in mice, AP, nanoparticles (NPs) or vehicle (Veh) (5 µl) was injected intrathecally (i.t.) 30 min before intraplantar (i.pl.) injection of capsaicin (CAP) or Veh. Withdrawal responses were measured to stimulation of the plantar surface of the injected hindpaw with VFF. In the complete Freund's adjuvant (CFA)-evoked model of sustained inflammatory nociceptifon in mice, CFA or Veh was administered by i.pl. injection; after 48 h, AP, NP or Veh was administered by i.t. injection (5 µl). Withdrawal responses were measured to VFF stimulation of the plantar surface of the injected hindpaw. In the sural nerve spared (SNS) model, rats underwent SNS or sham surgery; after 10 days, AP, NP or Veh (10 µl) was injected i.t. Withdrawal responses were assessed using the Randall–Selitto test. **b**,**c**, Capsaicin-induced mechanical allodynia in mice: kinetic VFF response (**b**) and integrated response as area under the curve (AUC) (**c**). **d**–**f**, CFA-evoked mechanical hyperalgesia in mice: VFF response (**d**), AUC (**e**) and half width response (**f**). **g**–**i**, SNS-evoked mechanical hyperalgesia in rats: neuropathic withdrawal threshold response (**g**), AUC (**h**) and half width response (**i**). Data are presented as mean ± s.e.m., $n = 6$ animals for all experiments. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***,\#}P < 0.001$, $^{\#\#}P < 0.0001$ compared to vehicle. Two-way ANOVA, Dunnett's post-hoc test (**b**,**d**,**g**); one-way ANOVA, Dunn's post-hoc test (**c**,**e**,**f**,**h**,**i**).

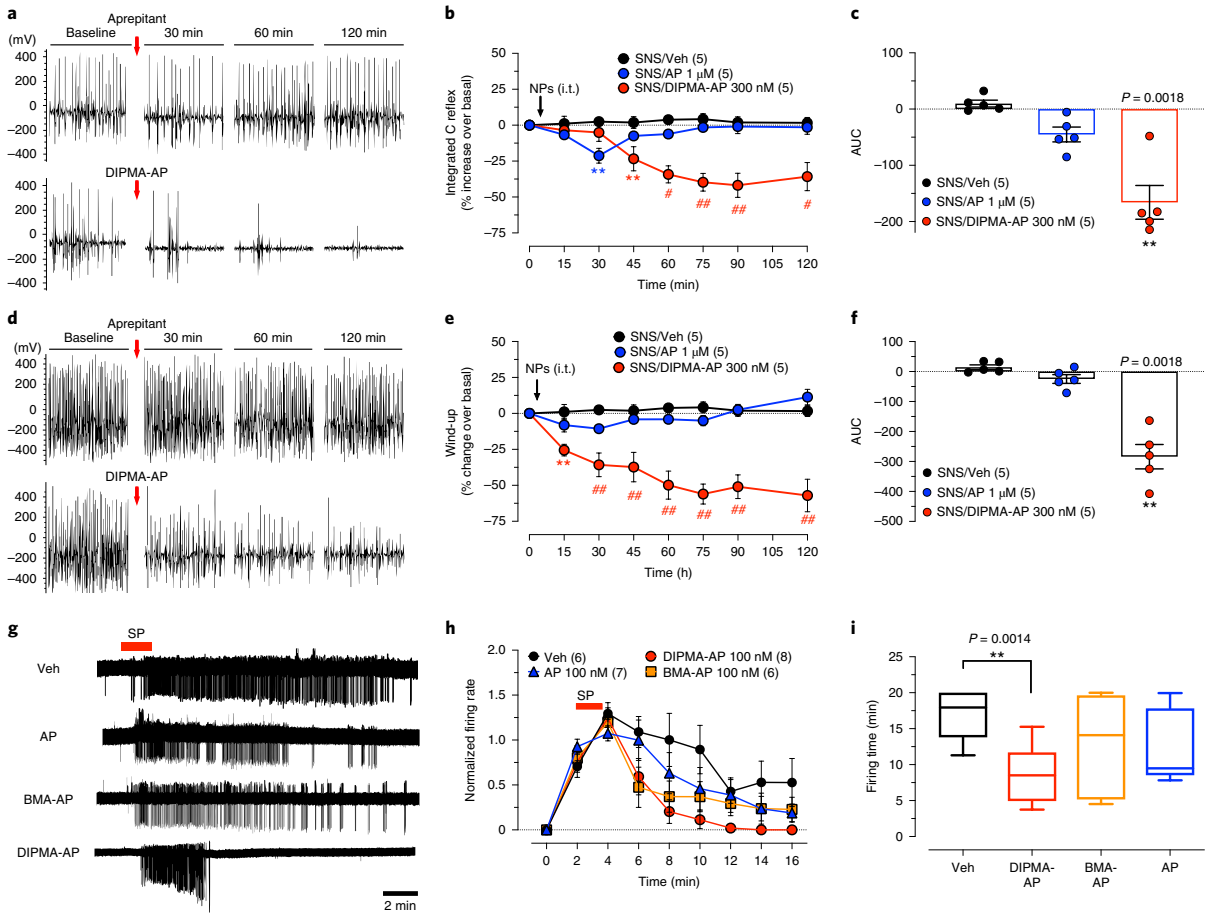


Fig. 5 | Sensitization and activation of nociceptive transmission. a–f, C-fibre reflex and wind-up in SNS rats. C-fibre reflexes (**a–c**) and wind-up (**d–f**) were measured at 10 days after SNS. AP, DIPMA-AP NP or Veh was administered by i.t. injection (10 μl). **a**,**d**, Representative recordings comparing AP and DIPMA-AP. **b**,**e**, Time course of effects. Data are presented as mean ± s.e.m., $n = 5$ rats per group (in parentheses). $^{**}P < 0.005$, $^{\#}P < 0.001$, $^{\#\#}P < 0.0001$ compared to vehicle. Two-way ANOVA, Dunn's post-hoc test. **c**,**f**, Integrated responses (AUC, $n = 5$ rats). $^{**}P < 0.005$, vehicle compared to DIPMA-AP, one-way ANOVA, Dunn's post-hoc test. **g–i**, Cell-attached patch-clamp recordings of SP-induced excitation of lamina I neurons in slices of rat spinal cord. Tissues were preincubated with AP, NP or Veh, and then superfused with SP (1 μM, 2 min). Action potential firing was measured: representative traces (**g**); normalized firing rate (**h**); firing time (**i**). Data are presented as mean ± s.e.m., $n = 6$ for rats for Veh, $n = 7$ rats for AP, $n = 8$ rats for DIPMA-AP and $n = 6$ rats for BMA-AP. $^{**}P = 0.005$, vehicle compared to DIPMA-AP. Unpaired *t*-test (two-sided).

Video 7). Quantification confirmed NK_1R endocytosis. These results suggest activation of the SP/NK_1R system, and are consistent with NK_1R upregulation in the dorsal horn during neuropathic pain[32].

At 10 days, SNS reduced the pressure that induced withdrawal of the hindpaw when compared to sham-operated rats, indicating mechanical hyperalgesia (Fig. 4g–i). Intrathecal vehicle did not affect mechanical hyperalgesia, which persisted for 7 h. Although low doses of aprepitant (100 nM) did not modify the withdrawal threshold, higher doses (300 nM) inhibited withdrawal thresholds after 0.5 h to a maximum of 40 ± 2% inhibition after 1 h, with return to baseline after 2.5 h. Aprepitant (1 μM) almost fully reversed hyperalgesia after 1 h (75 ± 4% inhibition), although hyperalgesia returned to baseline after 3 h (Supplementary Fig. 4). BMA-AP (100 and 300 nM aprepitant) inhibited hyperalgesia to a similar degree as free aprepitant (300 nM). DIPMA-AP (100 and 300 nM aprepitant) strongly reversed hyperalgesia, with almost complete inhibition after 1.5 h (300 nM, 80 ± 4% inhibition) and maintenance for 4.5 h, when none of the other treatments were effective. DIPMA-AP (500 nM) provided complete relief from hyperalgesia for 4.5 h (Supplementary Fig. 6). Although morphine fully reversed hyperalgesia for 2 h, the effect was absent after 2.5 h.

The enhanced effects of DIPMA-AP could be related to delivery and retention of aprepitant in endosomes of spinal neurons containing activated NK_1R, and the continued release of aprepitant as nanoparticles encounter increasingly acidified endosomal compartments. The anti-nociceptive actions of BMA-AP might be due to non-pH-responsive aprepitant release by unknown mechanisms.

Effects of nanoparticles on neuronal activity. Nociceptor C-fibres transmit painful stimuli centrally by releasing SP, calcitonin gene related peptide and glutamate in the dorsal horn[33]. Central sensitization (that is, amplified nociceptive transmission, decreased nociceptive threshold) is a hallmark of chronic pain. To examine sensitization, we measured the threshold current required to activate C-fibre reflexes, and assessed wind-up, a frequency-dependent

increase in the excitability of spinal cord neurons induced by electrical stimulation of C-fibres[31]. The threshold current required for activation of the C-fibre-mediated reflexes in the ipsilateral biceps femoris muscle was reduced in SNS rats compared to sham controls (SNS, 3.2 ± 2.8 mA; sham, 10.3 ± 1.2 mA, $P < 0.05$), consistent with sensitization. Repeated 0.1 Hz electrical stimuli caused a constant and stable C-reflex activity over time, while repeated 1.0 Hz stimuli evoked a progressive increase in C-reflex frequency or wind-up (Fig. 5a–f). In SNS rats, intrathecal aprepitant (1 μM) decreased the C-reflex only at 30 min, but did not affect wind-up. In contrast, DIPMA-AP (300 nM aprepitant) decreased C-reflex within 45 min and wind-up activity within 15 min, and inhibited responses for the duration of observations (120 min).

The effectiveness of DIPMA-AP to suppress nociception could be due to antagonism of sustained SP-induced excitation of spinal neurons, which requires NK_1R signalling from endosomes[19]. To examine this possibility, we made cell-attached patch-clamp recordings from lamina I neurons in slices of rat spinal cord. In vehicle-treated slices, SP (1 μM, 2 min) caused a rapid onset in action potential firing that persisted for 16 min after washout (Fig. 5g–i). Aprepitant (100 nM) or BMA-AP (100 nM aprepitant) had minimal effect on the onset, rate or duration of SP-induced firing. DIPMA-AP (100 nM) did not affect the initial onset of SP-evoked firing, but inhibited the rate of discharge after washout and the duration of excitation. When delivered in pH-responsive nanoparticles, aprepitant antagonizes endosomal NK_1R signals that drive sustained excitation of spinal neurons.

Effects of nanoparticles on endosomal signalling. Endosomal NK_1R signalling in HEK-293 cells activates nuclear extracellular signal-regulated kinase (ERK), which mediates SP-induced excitation of spinal neurons[19]. Painful stimuli (capsaicin) evoke phosphorylation of ERK (pERK) in spinal neurons, which requires NK_1R endocytosis[19]. We examined whether nanoparticle-encapsulated aprepitant prevents capsaicin-evoked ERK activation in spinal neurons in vivo. Capsaicin induced a 3.9-fold increase in the number of pERK-IR expressing neurons in laminae I, II and III of the ipsilateral but not contralateral dorsal horn (Fig. 6a,b). Free aprepitant caused a 43% reduction, BMA-AP a 63% reduction and DIPMA-AP an 81% reduction in pERK-IR neurons. The more complete inhibitory action of DIPMA-AP on ERK signalling concurs with its enhanced anti-nociceptive actions.

ERK signalling in vitro was studied in primary cultures of mouse striatal neurons. SP increased $[Ca^{2+}]_i$ in striatal neurons; pretreatment with aprepitant abolished responses, which are NK_1R-dependent (Supplementary Fig. 7a,b). After neurons were incubated with DIPMA-Cy5 nanoparticles and SP (100 nM) for 30 min, nanoparticles were detected in close proximity to endosomes containing NK_1R-IR (Fig. 6c). SP (100 nM) and phorbol 12,13-dibutyrate (positive control, 10 μM) stimulated nuclear pERK in striatal neurons (Fig. 6d and Supplementary Fig. 7c). DIPMA-AP reduced responses to basal levels, whereas free aprepitant was ineffective.

Förster resonance energy transfer (FRET) biosensors allow analysis of signalling in living cells with high spatiotemporal fidelity[34]. To examine activation of nuclear ERK, HEK-293 cells expressing human (h) NK_1R were transfected with NucEKAR (nuclear ERK biosensor). SP (100 pM–1 μM) activated nuclear ERK (half-maximal effective concentration, EC_{50}, of 5.9 nM) (Supplementary Fig. 8a,b). Aprepitant inhibited the response to 5 nM SP (~EC_{50}), but only at high concentrations (0.1, 1 and 10 μM; half-maximal inhibitory concentration, IC_{50}, of 45 nM) (Supplementary Fig. 8c,d). To determine the requirement for NK_1R endosomal signalling, we transfected HEK-hNK_1R cells with wild-type dynamin or dominant negative dynamin K44E, which inhibits NK_1R endocytosis[19]. Compared to cells expressing wild-type dynamin, dynamin K44E attenuated ERK responses to all concentrations of SP, abolished the response to 10 nM SP, and reduced the potency of SP by approximately twofold and the efficacy by ~30% (Supplementary Fig. 8e–g).

DIPMA-Ø or BMA-Ø nanoparticles (10, 20 and 30 μg ml^{-1}, 30 min) did not activate nuclear ERK in HEK-293 cells (Supplementary Fig. 8h). DIPMA-Ø nanoparticles had no effect on SP (5 nM) stimulated activation of nuclear ERK in HEK-hNK_1R cells, although 30 μg ml^{-1} BMA had a small inhibitory effect (Supplementary Fig. 8i). DIPMA-Ø or BMA-Ø nanoparticles (1–100 μg ml^{-1}, 24 or 48 h) did not affect the viability of HEK-293 cells (Supplementary Fig. 8j).

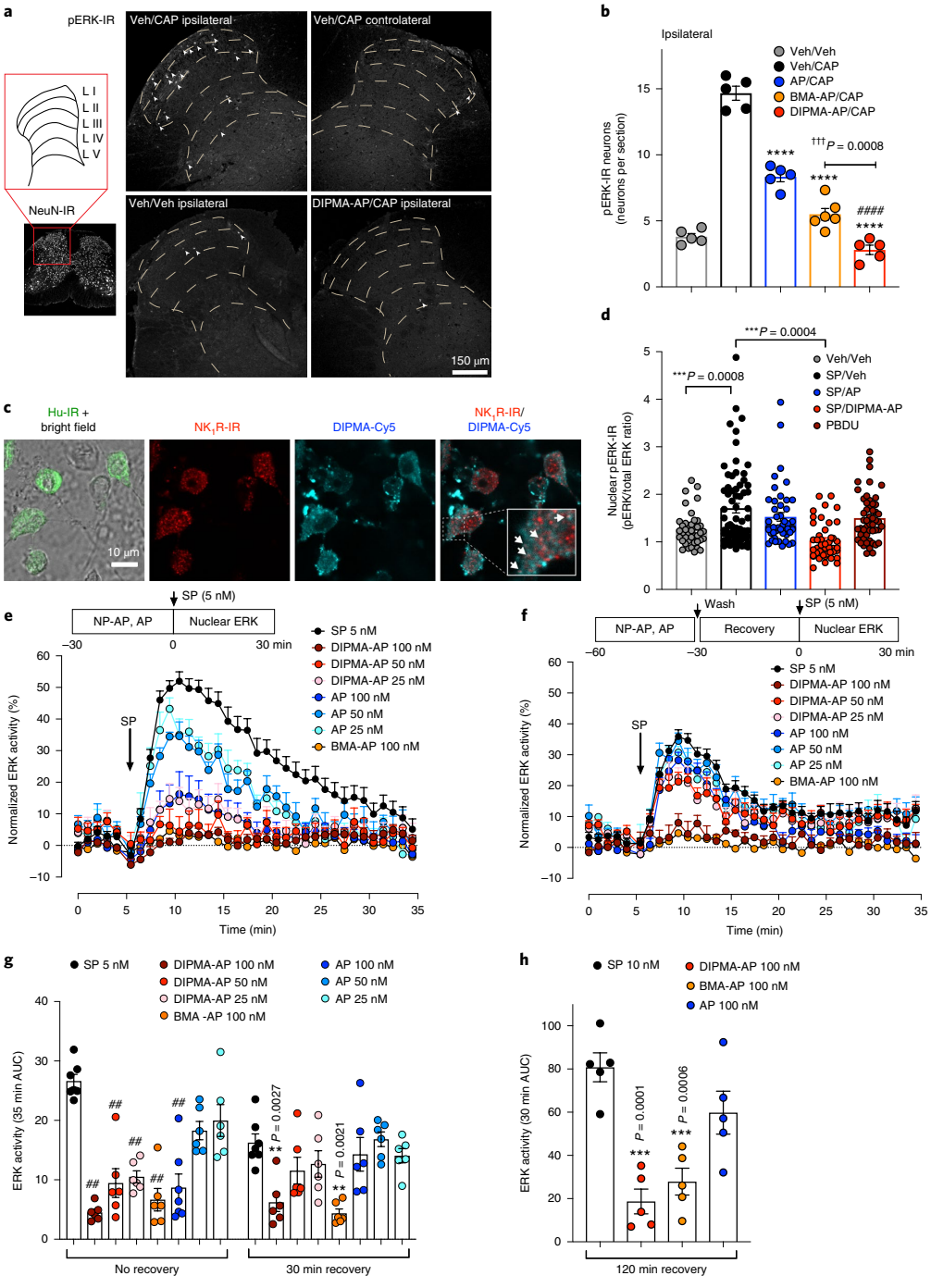
To compare the capacity of free aprepitant and nanoparticle-encapsulated aprepitant to antagonize the NK_1R in endosomes, we measured SP-induced activation of nuclear ERK in HEK-hNK_1R cells. Cells were preincubated with vehicle, free aprepitant or DIPMA-AP (25, 50 and 100 nM aprepitant) for 30 min, and were challenged with SP (5 nM). In vehicle-treated cells, SP stimulated a rapid and sustained activation of nuclear ERK (Fig. 6e,g). At all concentrations, DIPMA-AP more completely inhibited this response than free aprepitant. To compare sustained antagonism of endosomal NK_1R, cells were preincubated with vehicle, aprepitant or DIPMA-AP (100 nM) for 30 min, washed, recovered in medium without antagonist for 30 or 120 min, and then challenged with SP. Free aprepitant was now inactive, whereas DIPMA-AP (100 nM) abolished SP-induced activation of nuclear ERK (Fig. 6f–h). Although BMA-AP was less efficacious than DIPMA-AP in assays of nociception and ERK activity in spi-

Fig. 6 | Antagonism of NK_1R signalling in endosomes. a,**b**, Localization of pERK in the spinal cord: representative images (a) and the number of pERK-IR neurons per section (**b**). AP, BMA-AP, DIPMA-AP or Veh was injected i.t. into mice. After 30 min, CAP or Veh was administered by i.pl. injection. After 4 h, the spinal cord was collected for localization of pERK-IR or NeuN-IR (pan-neuronal marker). Data are presented as mean ± s.e.m., $n = 5$ mice for Veh/Veh, Veh/CAP, AP/CAP, DIPMA-AP/CAP groups or $n = 6$ mice for BMA-AP/CAP groups. $^{****}P < 0.0001$ compared to Veh/CAP; $^{\#\#\#\#}P < 0.0001$ compared to AP/CAP; $^{\dagger\dagger\dagger}P < 0.001$ compared to BMA-AP/CAP. One-way ANOVA, Bonferroni post-hoc test. **c**, Uptake of DIPMA-Cy5 nanoparticles in proximity to NK_1R-IR endosomes in cultured striatal neurons stimulated with 100 nM SP for 30 min. Representative images from $n = 4$ independent experiments are shown. **d**, Nuclear ERK signalling in primary cultures of mouse striatal neurons. Neurons were preincubated with Veh, free AP or DIPMA-AP (100 nM, 30 min), washed and recovered for 30 min. Neurons were challenged with SP (100 nM) or phorbol 12,13-dibutyrate (positive control, 10 μM) for 30 min. Nuclear pERK-IR and total ERK-IR were detected by immunofluorescence and confocal imaging, and expressed as the ratio of phospho-ERK1/2 (Thr202/Tyr204) to total ERK. Data are presented as mean ± s.e.m., $n = 41$ neurons for Veh/Veh, 68 neurons for SP/Veh, 46 neurons for SP/AP, 43 neurons for SP/DIPMA-AP, 52 neurons for SP/BMA-AP and 51 neurons for phorbol 12, 13-dibutyrate (PBDU, positive control), from four experiments (Veh, SP/AP, SP/DIPMA, SP/BMA) or six experiments (SP/Veh, PBDU) tested with independent nanoparticle preparations. $^{***}P < 0.001$. One-way ANOVA, Tukey's post-hoc test. **e**–**h**, Effects of free AP, DIPMA-AP and BMA-AP on SP-induced activation of nuclear ERK in HEK-hNK_1R cells. Cells were preincubated with Veh, AP or DIPMA-AP for 30 min; they were either challenged with SP (no recovery, **e**), or were washed, recovered in antagonist-free medium for 30 or 120 min and then challenged with SP (30 min recovery, **f**). **g**,**h**, AUC of ERK assays. Results are expressed as normalized values by the maximum nuclear ERK response to 1 μM phorbol 12,13-dibutyrate. Data are presented as mean ± s.e.m., $n = 7$ independent experiments for SP (no recovery and 30 min recovery) and AP (100 nM, no recovery), $n = 5$ independent experiments for 120 min recovery and $n = 6$ independent experiments for all other data points; observations are in triplicate. $^{**}P < 0.005$, $^{***}P < 0.001$, $^{\#\#}P < 0.0001$ compared to vehicle. One-way ANOVA, Tukey's post-hoc test.

nal neurons in vivo, BMA-AP inhibited SP-induced activation of nuclear ERK in HEK-hNK$_1$R cells to a similar degree as DIPMA-AP (Fig. 6e–h). Intracellular disassembly of BMA-AP nanoparticles by unknown mechanisms might release sufficient quantities of aprepitant to effectively antagonize the NK$_1$R in endosomes. Non-selective effects of BMA on SP-stimulated nuclear ERK (Supplementary Fig. 8i) could also contribute.

Discussion

The rationale for the current study is that painful stimuli evoke NK$_1$R endocytosis in spinal neurons[24,28], where NK$_1$R endosomal signals mediate excitation and nociception[19]. Clathrin and dynamin inhibitors and lipid-conjugated antagonists that target the NK$_1$R in endosomes inhibit nociception[19]. Considerable effort will be required to advance these compounds to the clinic. Dynamin



and clathrin inhibitors disrupt trafficking of many receptors and channels that control nociception. Lipid-conjugated antagonists can lose potency. Because lipidated antagonists incorporate into plasma and endosomal membranes, they cannot exclusively target endosomal signalling. pH-responsive nanoparticles deliver aprepitant to endosomes, without loss of potency. Nanoparticle encapsulation enhanced the anti-nociceptive actions of aprepitant in preclinical models of pain. These findings are consistent with the improved capacity of nanoparticle-encapsulated aprepitant to inhibit SP-induced excitation of spinal neurons and to cause a sustained inhibition of endosomal signalling. Nanoparticle uptake and sustained release of aprepitant in acidic endosomes containing the activated NK_1R could account for these enhanced and persistent anti-nociceptive effects.

Further studies are necessary before nanoparticle-encapsulated analgesics can be advanced to clinical trials. They include toxicology, pharmacokinetic and pharmacodynamic studies in disease-relevant preclinical models. Therapeutic efficacy could be improved by combining into the same nanoparticles antagonists of different GPCRs that co-mediate pain transmission (for example, NK_1R, calcitonin receptor-like receptor)[19,21]. By incorporating targeting groups into the nanoparticle shell, it might be possible to deliver drugs selectively to pain-transmitting neurons. Limitations of our study include the following: analysis of nociception rather than the perception of pain, which requires human studies; study of evoked rather than spontaneous nociception; examination of nanoparticle actions in cell lines or primary striatal neurons, rather than the spinal neurons that are the target of nanoparticle-encapsulated aprepitant.

Nanoparticle encapsulation could improve the therapeutic efficacy of antagonists and agonists of many GPCRs that signal from endosomes[13,15,35]. Although GPCRs are the target of most clinically approved drugs, many drugs fail during development for unknown reasons. Nanoparticle encapsulation could advance the development of drugs to treat multiple diseases by altering their intracellular distribution to fine-tune signalling processes of pathophysiological importance.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41565-019-0568-x.

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Methods

Materials. Reagents were purchased from Sigma-Aldrich unless otherwise specified.

Diblock copolymers and nanoparticles. Polymer synthesis and characterization and nanoparticle assembly, pH-dependent disassembly in vitro and characterization are described in the Supplementary Methods.

Cell lines. The human (h) NK_1R long isoform open reading frame with a CD8 signal sequence and N-terminal FLAG-tag was cloned into pcDNA5 FRT/TO between KpnI and NotI restriction sites using Gibson assembly (NEB). A stable cell line expressing hNK_1R (HEK-hNK_1R) was produced by co-transfecting Flpn HEK-293 cells with hNK_1R vector and pOG44 (0.5 µg and 4 µg, respectively), using polyethylenimine (PEI, Polysciences) at a 1:6 DNA:PEI ratio. Cells (~0.7×10^6) were seeded into T-25 tissue culture flasks (Perkin Elmer) in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (50 U ml^{-1}) and streptomycin (50 U ml^{-1}) (DMEM/pen/strep) and incubated for 24 h (37 °C, 5% CO_2). Culture medium was changed to fresh DMEM/pen/strep prior to transfection and incubated for 24 h (37 °C, 5% CO_2). The medium was changed to DMEM supplemented with 10% (vol/vol) fetal bovine serum (FBS) and hygromycin B (200 µg ml^{-1}, Thermo Fisher Scientific) for stable cell line selection. Cell lines were tested and confirmed free of mycoplasma.

Nanoparticle trafficking in HEK-293 cells. HEK-293 cells were plated on poly-D-lysine coated chambers (ibidi, Germany) in DMEM supplemented with 10% (vol/vol) FBS (DMEM/FBS). After 24 h, cells were transfected with 300 ng of rat (r) NK_1R-GFP per chamber and cultured for 48 h. To identify endosomal compartments, HEK-293 cells were infected with Rab5a-GFP (resident in early endosomes) or Rab7a-GFP (late endosomes) CellLight BacMam2.0 (Thermo Fisher Scientific) for 16 h. To examine localization of nanoparticles, cells were incubated in Leibovitz´s L-15 medium with DIPMA-Cy5 nanoparticles (20 µg ml^{-1}, 30 min, 37 °C) or vehicle, followed by addition of SP (10 nM). Cells were imaged at 30 and 60 min post-SP addition using a Leica SP8 confocal microscope equipped with HCX PL APO ×40 (NA 1.30) and HCX PL APO ×63 (NA 1.40) oil objectives. Images were analysed using Fiji[36] and deconvolved with Huygens Professional version 18.04 (Scientific Volume Imaging, http://svi.nl), using the CMLE algorithm with a signal-to-noise ratio of 10 and 100 iterations. Co-localization was evaluated by determination of the Manders overlap coefficient[25].

Uptake and disassembly of nanoparticles in HEK-293 cells. Nanoparticles were self-assembled with 0.5 mg of Coumarin 153 per mg of DIPMA or BMA polymer (DIPMA-CO, BMA-CO). HEK-293 cells were preincubated for 30 min with vehicle (Hank's balanced salt solution, HBSS), dynamin inhibitor (Dyngo4a, 30 µM)[27], clathrin inhibitor (PitStop2, 30 µM)[26], vacuolar H^+ATPase inhibitor (Bafilomycin A1, 1 µM) or NH_4Cl (20 mM), which acts as a lysosomotropic weak base. Nuclei were stained using Draq5. Images were obtained with a Leica SP8 confocal microscope using an HCX PL APO ×63 (NA 2.0) oil objective. Images were taken every 10 s for 30 min, where the first five readings correspond to baseline images before the addition of DIPMA-CO nanoparticles (20 µg ml^{-1}). All images were analysed using Fiji[36]. Kinetic data were normalized to the fluorescence of free Coumarin 153 (5 µg ml^{-1}) at 30 min.

Animals. Male C57BL/6 mice (6–10 weeks) and pregnant Asmu:Swiss mice were sourced from the Monash Animal Research Platform. Male Sprague–Dawley rats (225–250 g) were obtained from the Faculty of Medicine of the University of Chile. Animals were housed in groups of four, maintained at a temperature of 22 ± 4 °C in a humidity-controlled environment with a 12 h light/dark cycle. Food and water were available ad libitum. For behavioural tests, investigators were blinded to the treatment groups and animals were randomly assigned to treatments and studied during the light cycle. Animals were euthanized by anaesthetic overdose and thoracotomy. Studies on animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and adhered to the ethical guidelines of the International Association for the Study of Pain[37]. Studies were approved by the Animal Ethics Committee of Monash Institute of Pharmaceutical Sciences, Monash University and the Bioethics Committee of the University of Santiago of Chile.

Drug administration. *Mice.* The following drugs were administered by intrathecal injection (5 µl) into the intervertebral space (L4/L5) of conscious mice: aprepitant (100 and 300 nM), nanoparticles delivering an equivalent dose of aprepitant (DIPMA-AP, BMA-AP, 10 µg ml^{-1} 100 nM aprepitant, 30 µg ml^{-1} 300 nM aprepitant), controls (10 µg ml^{-1} of DIPMA-Ø and a mixture of 10 µg ml^{-1} of DIPMA-Ø and aprepitant 100 nM) or vehicle (artificial cerebrospinal fluid, aCSF). Treatments were administered 30 min before rotarod experiments and the induction of acute nociceptive pain or 48 h after the establishment of inflammatory nociception. For biodistribution studies, nanoparticles (50 µg ml^{-1}) were administered intrathecally immediately after obtaining control images. For localization of nanoparticles in the spinal cord, nanoparticles (50 µg ml^{-1}) were administered intrathecally 30 min after the induction of acute nociception with capsaicin (see below). Morphine (3 mg kg^{-1} intraperitoneal) was administered 48 h after induction of inflammatory nociception.

Rats. Drugs were administered by intrathecal injection (10 µl) into the intervertebral space (L4/L5) of conscious rats: aprepitant (100 nM, 300 nM, 1 µM), nanoparticles loaded with aprepitant (DIPMA-AP, BMA-AP, 10 µg ml^{-1} 100 nM aprepitant, 30 µg ml^{-1} 300 nM aprepitant, 50 µg ml^{-1} 500 nM aprepitant), DIPMA-Ø nanoparticles (10, 30 and 50 µg ml^{-1}) or vehicle (aCSF). Treatments were administered 10 days after sural nerve transection or sham surgery. For electrophysiological studies, drugs were administered by intrathecal injection under anaesthesia (isoflurane 1.2–1.5%): aprepitant (1 µM) or nanoparticles (30 µg ml^{-1} 300 nM aprepitant). Morphine (3 mg kg^{-1}, intraperitoneal) was administered 10 days after sural nerve transection.

Biodistribution of nanoparticles in the spinal cord. Mice were sedated (2% isoflurane) and placed in an in vivo imaging system (IVIS spectrum Lumina II, Perkin Elmer). Posterior images were obtained using the Perkin Elmer Living Image software v4.3.1. After collection of a baseline image, nanoparticles (50 µg ml^{-1}) were administered intrathecally (5 µl). Images were collected at 0.5, 1, 1.5, 2, 4, 8 and 24 h post DIPMA-Cy5 or BMA-Cy5 administration.

Uptake of nanoparticles in the spinal cord. Cy5-labelled nanoparticles were administered to mice (intrathecal). After 30 min, capsaicin (5 µg) was administered by subcutaneous intraplantar injection (10 µl) into the left hindpaw under sedation (2% isoflurane). This approach was used to mimic the therapeutic situation where nanoparticle-encapsulated drugs might be used to treat pain. At 1 h after nanoparticle administration, mice were transcardially perfused with 50 ml of PBS followed by 50 ml of ice-cold 4% paraformaldehyde (PFA). The spinal cord was removed, immersion fixed in 4% PFA (2 h, 4 °C) and cryoprotected in PBS containing 30% sucrose (24 h, 4 °C). The spinal cord (L3–L6) was embedded in tissue freezing medium (TFM, General Data), and 30 µm serial coronal sections were cut and mounted on Colorfrost Plus microscope slides (Fisher Scientific). Sections were washed twice in PBS, counter-stained with DAPI (5 µg ml^{-1}, 5 min) and cover-slipped with ProLong Glass mounting medium (Thermo Fisher Scientific). Some sections were processed to detect neurons. Sections were blocked in PBS containing 0.2% Triton X-100 and 10% normal horse serum (NHS; 30 min, room temperature). Sections were incubated with rabbit anti-PGP9.5 (1:500, Abcam ab27053) in PBS containing 0.2% Triton X-100 and 3% NHS (60 min, room temperature). Sections were washed four times in PBS and incubated with donkey anti-rabbit Alexa488 (1:1,000, Thermo Fisher Scientific; 30 min, room temperature). Sections were imaged on a Leica SP8 confocal microscope with HC PLAPO ×40 or ×63 oil objectives.

Determination of aprepitant concentration in the spinal cord. Aprepitant (100 nM) or nanoparticles delivering an equivalent dose of aprepitant (10 µg ml^{-1} 100 nM aprepitant) was administered by intrathecal injection to conscious mice. Mice were killed 1 h and 4 h post-treatment. The spinal cord (L2–L6) was removed for determination of the tissue concentration of aprepitant by LC-MS, as described in the Supplementary Methods.

Acute and inflammatory nociception in mice. *Nociceptive pain.* Capsaicin (5 µg) or vehicle (0.9% NaCl) was administered by intraplantar injection (10 µl) into the left hindpaw of sedated mice (2% isoflurane) 30 min after intrathecal injection of drugs[19].

Inflammatory pain. CFA (0.5 mg ml^{-1}) or vehicle (0.9% NaCl) was administered by intraplantar injection (10 µl) into the left hindpaw of sedated mice (2% isoflurane)[19,29]. Drugs were administered by intrathecal injection 48 h after CFA.

Mechanical allodynia. Mechanical nociception was assessed by measuring withdrawal thresholds to stimulation of the plantar surfaces of the ipsilateral and contralateral hindpaws with calibrated VFFs[19]. Before experiments, mice were acclimatized to the experimental apparatus and environment for 2 h on two successive days. VFF withdrawal thresholds were measured in triplicate to establish a baseline for each mouse. For the capsaicin model, VFF withdrawal thresholds were measured at 30 min intervals for the first 2 h after drug administration, then at 60 min intervals for the next 2 h, and finally after 24 h. For the CFA model, VFF withdrawal thresholds were measured every 30 min for the first 3 h after drug administration, then at 60 min intervals for the next 5 h, and finally after 24 h. Results were normalized to the baseline withdrawal thresholds of each mouse. Results are expressed as a percentage of baseline, as AUC and as the half width response (the duration of the effect of each treatment calculated as the time to attain 50% of the maximal analgesic response).

pERK localization in mouse spinal cord. Vehicle (control), free aprepitant and nanoparticles (all 100 nM aprepitant) were administered by intrathecal injection to mice as described above. After 30 min, mice were sedated (2% isoflurane) and vehicle (0.9% NaCl) or capsaicin (5 µg) was administered by intraplantar injection (10 µl) into the left hindpaw. After 4 h, sections of spinal cord (L3–L6) were prepared as described above. Fixation, staining and imaging of sections are described in detail in the Supplementary Methods.

Rotarod test. Motor coordination was assessed in mice by a rotarod test as described in the Supplementary Methods.

Neuropathic nociception in rats. *Neuropathic pain.* Neuropathic nociception was induced in rats using a variation of the SNS injury model, which induces rapid onset and sustained mechanical and thermal hyperalgesia[30]. Under anaesthesia (2% isoflurane), the three terminal distal branches of the sciatic nerve (tibial, common peroneal and sural nerves) were identified and the sural nerve was transected[31]. For controls (sham), rats underwent a similar surgery but without transection of the sural nerve. After surgery, ketoprofen (3 mg kg^{-1}) and enrofloxacin (5 mg kg^{-1}) were administered subcutaneously for 2 days.

Mechanical hyperalgesia. Mechanical hyperalgesia was assessed in rats by measuring hindpaw withdrawal pressure thresholds using an algesimeter (Ugo Basile) with a cutoff value of 570 g to prevent injury[38,39]. Mechanical hyperalgesia was evaluated before (basal) and 5, 9 and 10 days after surgery. After evaluation at day 10, drugs were administered by intrathecal injection, and withdrawal thresholds were recorded every 30 min for 7 h. Results are expressed as the paw withdrawal pressure threshold (g cm^{-2}), AUC and half-width response.

Electrophysiological assessment of nociception in rats. Nociceptive synaptic transmission was evaluated by measurement of electromyographic activity associated with the hind limb-flexion nociceptive reflex evoked by electrical activation of C-fibres of the sural nerve (C-reflex) as described previously[40] and in detail in the Supplementary Methods.

Cell-attached patch-clamp recordings of rat spinal neurons. Parasagittal slices (340 µm) were prepared from rat lumbar spinal cord as described in refs. [19,41]. Slices were transferred to a recording chamber and superfused with aCSF (2 ml min^{-1}, 36 °C). Dodt-contrast optics were used to identify large (capacitance ≥20 pF), putative NK_1R-positive neurons in lamina I based on their position, size and fusiform shape with dendrites that were restricted to lamina I. Spontaneous currents were recorded from NK_1R-positive lamina I neurons in a cell-attached configuration in voltage clamp. Slices were preincubated in DIPMA-AP (10 µg ml^{-1} 100 nM aprepitant), BMA-AP (10 µg ml^{-1} 100 nM aprepitant) or aprepitant (100 nM) for 120 min, washed and incubated in antagonist-free aCSF for a further 30–60 min before recording. Slices were challenged with SP (1 µM, 2 min) and the firing rate for each cell was normalized to the response between the 2 and 4 min time points, which was not significantly different between groups. The firing time was determined as the duration of the response to the last action potential.

NK_1R localization in rat spinal cord. At 10 days after sham or SNS surgery, rats were anaesthetized and transcardially perfused with 250 ml PBS followed by 250 ml 4% PFA. The spinal cord was removed, immersion fixed in 4% PFA (2 h, 4 °C) and cryoprotected in 30% sucrose in 0.1 M PBS (24 h, 4 °C). The spinal cord (L3–L6) was embedded in TFM (General Data) and 30 µm serial coronal sections were cut into 48-well plates containing PBS. Fixation, immunostaining, imaging and image analysis of spinal cord sections are described in the Supplementary Methods.

Nanoparticle uptake and SP signalling in striatal neurons. *Neuronal isolation and culture.* Primary striatal neurons were dissociated from E15-16 Asmu:Swiss mouse embryos as described in ref. [42]. Neurons (200,000 per well) were plated on poly-D-lysine-coated eight-well chamber slides (ibidi) in Neurobasal medium supplemented with B-27, 2 mM L-glutamine and penicillin/streptomycin.

Nanoparticle uptake and NK_1R localization. At 5 days after isolation, neurons were equilibrated in HEPES-buffered saline (10 mM HEPES, 0.5% BSA, 10 mM D-glucose, 2.2 mM $CaCl_2.H_2O$, $MgCl_2.6H_2O$, 2.6 mM KCl, 150 mM NaCl, pH 7.4) for 30 min and then incubated with 50 µg ml^{-1} DIPMA-Cy5 and 100 nM SP for 30 min. Neurons were fixed in 2% PFA and 1% sucrose in PBS (room temperature, 20 min) and blocked in PBS containing 0.3% Triton X-100 and 5% NHS for 24 h at 4 °C. Neurons were stained as described above for rat spinal cord slices, using rabbit anti-NK_1R and mouse anti-Hu (HuC/HuD Monoclonal Antibody 16A11, Thermo Fisher Scientific; 24 h, 4 °C), washed in 4× PBS, and incubated with donkey anti-rabbit Alexa594 and donkey anti-mouse Alexa488 (1:500, Thermo Fisher Scientific; 24 h, 4 °C). Neurons were counter-stained with DAPI. Images were obtained on a Leica SP8 confocal microscope with HCX PL APO ×63 (NA 1.40) oil objective.

Ca^{2+} imaging. At 5 days after isolation, neurons were incubated with Fura-2 AM ester (2 µM, 45 min, 37 °C, Thermo Fisher Scientific) in HEPES-buffered saline containing 4 mM probenecid and 0.05% pluronic F127. Neurons were recovered in fresh HEPES-buffered saline for 30 min before imaging on a Leica DMI-6000B microscope with HC PLAN APO 0.4 NA ×10 objective at 37 °C. Images were collected at 1.5 s intervals (excitation, 340 nm/380 nm; emission, 530 nm). To assess the functional expression of NK_1R, neurons were preincubated with 300 nM aprepitant or vehicle (DMSO), and challenged with 100 nM SP and followed by 5 mM KCl.

ERK activity. At 8 days after isolation, neurons were equilibrated for 30 min in HEPES-buffered saline and then preincubated with DIPMA-AP (10 µg ml^{-1} 100 nM aprepitant), aprepitant (100 nM) or vehicle (PBS) for 30 min. Neurons were washed, recovered for 30 min and challenged with SP (100 nM) or the positive control, phorbol 12,13-dibutyrate (10 µM), for 30 min. Neurons were fixed in 4% PFA (20 min at 4 °C) and blocked (0.3% Triton X-100 and 5% NHS; 24 h at 4 °C). Neurons were incubated with rabbit anti-phospho-p44/42 MAPK (ERK1/2 phospho-Thr202/Tyr204, 1:100, #4370, Cell Signalling Technology) and mouse anti-p44/42 MAPK (1:100, #4696, Cell Signalling Technology) (24 h at 4 °C). Neurons were washed four times in PBS and incubated with donkey anti-rabbit Alexa488 and donkey anti-mouse Alexa647 (1:500, Thermo Fisher Scientific; 2 h at room temperature). The nucleus was counter-stained with DAPI. Neurons were imaged using a Leica SP8 confocal microscope with an HCX PL APO ×63 (NA 1.40) oil objective. Nuclei of neurons were selected as regions of interest and the ratio of phospho-ERK to total ERK was calculated using mean fluorescence intensity values. The mean ERK ratio for all neurons within a single well was determined and the means of four experiments were compared for statistical analyses.

FRET assays of endosomal NK_1R signalling in HEK-293 cells. HEK-hNK_1R cells (~2×10^6) were seeded into a 90 mm Petri dish (Corning) in DMEM/FBS/Hygro and incubated for 24 h (37 °C, 5% CO_2). Before transfection, the medium was changed to fresh DMEM/FBS/Hygro and the nuclear ERK (nucEKAR) plasmid was transfected (2.5 µg DNA per dish) using PEI at a 1:6 ratio[19]. After 24 h, cells were plated in a poly-L-lysine-coated black 96-well CulturPlate (Perkin Elmer) and incubated for a further 24 h (37 °C, 5% CO_2). On the day of the assay, cells were serum-starved for 6–8 h and then equilibrated in HBSS, supplemented with HEPES at 37 °C in a CO_2-free incubator. FRET was assessed using a PHERAstar FS (BMG LABTECH) with optic module FI 430 530 480 and measurements were made every 1 min. Baseline was measured for 5 min followed by stimulation with SP, vehicle (HBSS) or phorbol 12,13-dibutyrate (1 µM), and further measurements for 30 min. For the SP concentration response curve, half logarithmic dilutions of SP were added (1 µM to 100 pM) and EC_{50} was determined using the AUC after SP addition (30 min reading). For the aprepitant concentration response curve, logarithmic dilutions of aprepitant (10 µM to 1 pM) were added 30 min before baseline measurements, followed by the addition of 5 nM of SP. The IC_{50} was determined for aprepitant as described. To assess the effect of nanoparticles on nuclear ERK signalling, DIPMA-Ø, DIPMA-AP, BMA-Ø or BMA-AP (30, 20 and 10 µg ml^{-1}) was added 30 min before baseline measurements, followed by the addition of SP 5 nM or vehicle. Data were expressed as vehicle corrected values, normalized by the maximum response to the positive control.

Cell viability assays. Studies of the effects of nanoparticles on the viability of HEK-293 cells are described in the Supplementary Methods.

Statistical analysis. Data were analysed using GraphPad Prism 8 (GraphPad Software). Data are presented as mean ± s.e.m., unless noted otherwise. A two-tailed Student's *t*-test was used for two comparisons and exact *P* values are shown in the figures. For multiple comparisons, results were compared using one- or two-way ANOVA followed by post-hoc multiple comparison tests, as described in the figure legends. Exact adjusted *P* values are shown for bar graphs in figures when $P \geq 0.0001$. $P < 0.05$ was considered significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analysed during this study are available in this Article and its Supplementary Information or from the corresponding authors upon request.

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Author contributions

P.D.R.-G. prepared and characterized nanoparticles, examined nanoparticle uptake and disassembly, studied SP signalling in model cells and wrote the manuscript. J.S.R. studied the biodistribution and anti-nociceptive and in vivo electrophysiological actions of nanoparticles. P.S. studied the biodistribution and anti-nociceptive actions of nanoparticles. W.I. conceived and designed electrophysiological studies on spinal neurons. M.S. studied the excitation of spinal neurons, and N.T. prepared and characterized nanoparticles. L.C. conceived and designed neuropathic nociception and in vivo electrophysiological studies. T.P. conceived and designed neuropathic nociception. C.J.N. provided expertise in the analysis of confocal images and S.Y.K. obtained transmission electron microscopy images. L.M.L. characterized the critical micellar concentration and pH-disassembly of nanoparticles. C.L. studied SP signalling in model cells and D.P.P. studied nanoparticle uptake. T.M.L. studied anti-nociceptive actions of nanoparticles, G.D.S. prepared striatal neurons, and Q.N.M. prepared and characterized nanoparticles. D.D.J. examined NK1R endocytosis, nanoparticle uptake into spinal neurons, and SP signalling in model cells and striatal neurons. R.L. examined NK_1R endocytosis and nanoparticle uptake into spinal neurons. N.S.N. studied NK_1R endocytosis in rats. B.L.S. designed experiments to examine NK_1R endocytosis in rats. J.F.Q. designed nanoparticles and wrote the manuscript. M.R.W. designed nanoparticles. N.A.V. conceived experiments, studied SP signalling in neurons, interpreted the results and wrote the manuscript. T.P.D. conceived the experiments and designed the nanoparticles. N.W.B. conceived and designed the experiments, interpreted the results and wrote the manuscript.

Competing interests

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Additional information

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Appendix 2

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SUPPLEMENTARY INFORMATION

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In the format provided by the authors and unedited.

A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain

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Supplementary Information

A pH-Responsive Nanoparticle Targets the Neurokinin 1 Receptor in Endosomes to Prevent Chronic Pain

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1. Supplementary Methods

1.1. Synthesis of diblock copolymers. Prior each synthesis, monomers were deinhibited using basic aluminium oxide. All polymers were synthesized via reversible addition fragmentation chain (RAFT) polymerization[1]. All reactions were carried out in toluene at 70°C and 400 RPM unless stated otherwise. Resulting polymers were purified by dialysis (molecular weight cut off, MWCO 3500, Membrane Filtration Products, USA) against acetone for 96 h. Residual solvent was evaporated and the final products were dried for 24 h in a vacuum oven at 37°C and 1000 mbar.

P(PEGMA-co-DMAEMA) hydrophilic block copolymer. The macromolecular chain transfer agent (macro-CTA), P(PEGMA-co-DMAEMA), was synthesized using 2-cyanoprop-2-yl dithiobenzoate (CPBD, 0.0736 g, $3.34 \cdot 10^{-4}$ mol) as a RAFT agent and azobisisobutyronitrile (AIBN, 0.0054 g, $3.34 \cdot 10^{-5}$ mol) as the initiator in a ratio of 1:0.1. The monomers poly(ethylene glycol) monomethyl ether methacrylate (PEGMA, 6 g, 0.02 mol) and 2-[*N,N*-(dimethylamino)ethyl] methacrylate (DMAEMA, 0.314 g, 0.001 mol) were added at a ratio of 10:1 and the mixture was left to react for 21 h.

P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) diblock copolymer. The chain extension reaction was initiated by AIBN (0.0017 g, $1.033 \cdot 10^{-5}$ mol), using P(PEGMA-co-DMAEMA, 0.89 g, $6.89 \cdot 10^{-5}$ mol) and the monomers 2-[*N,N*-(diisopropylamino)ethyl] methacrylate (DIPMA, 1.47 g, $6.892 \cdot 10^{-3}$ mol) and di(ethylene glycol) methyl ether methacrylate (DEGMA, 0.1427 g, $7.58 \cdot 10^{-4}$) at a ratio of 0.15:1:100:11. The mixture was left to react for 17.5 h.

P(PEGMA-co-DMAEMA)-b-P(BMA) diblock copolymer. Butyl methacrylate (BMA, 0.582 g, 0.0041 mol) was polymerized from the hydrophilic P(PEGMA-co-DMAEMA) block by a chain extension reaction in the presence of AIBN (0.0008 g, $5.124 \cdot 10^{-6}$ mol) at a ratio of 120:1:0.2. The solution was left to react for 15 h.

P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA-co-Cy5) and P(PEGMA-co-DMAEMA)-b-P(BMA-co-Cy5) diblock copolymer. The chain extension of P(PEGMA-co-DMAEMA, 0.5 g, *$3.85 \cdot 10^{-5}$ mol*) was done by adding DIPMA (*0.82 g, $4.6 \cdot 10^{-3}$ mol)* and 4,4-dimethyl-2-vinyl-2-oxazolin-5-one (VDM, 0.027 g, *$1.92 \cdot 10^{-4}$ mol)* in the presence of AIBN (*0.95 mg, $5.77 \cdot 10^{-6}$ mol)* at a ratio of *1:100:11:5:0.15.* The mixture was left to react for *18 h.* For the BMA diblock copolymer only *BMA (0.66 g, 3.87·10-3 mol) was added* and the ratios of macro-CTA:BMA:VDM:AIBN were *1:120:5:0.15 and t*he mixture was left to react for *19* h. Cy5 coupling was achieved by mixing 250 µL of the reaction with Cyanine 5 amine (Cy5, 0.008 g, $1.20 \cdot 10^{-5}$ moles) and reacting at room temperature, 400 RPM for 72 h under dark conditions.

1.2. Analysis of diblock copolymers

Gel permeation chromatography. The molecular weights of polymers were determined by gel permeation chromatography using a Shimadzu (Kyoto, Japan) liquid chromatography system (Shimadzu, Japan) equipped with a (RID-10A) differential refractive index detector and SPD-20A ultraviolet-visible detector (λ = 633 nm). Samples were fractionated using 5.0 µm bead-size guard column (50 × 7.8 mm) and three Shodex KF-805L columns (300 × 8 mm, 10 µm bead-size, 5000 Å pore size) in series at 40°C. The eluent used was *N,N*-dimethylacetamide (DMAC, HPLC grade, with 0.03% w/v LiBr) with a flow rate of 1 mL/min. A molecular weight calibration curve was produced using polystyrene standards with narrow molecular weights distribution ranging from

(50 mm by 2.1 mm, 2.7 µm particle size) equipped with a Phenomenex SecurityGuard precolumn fitted with a Synergi Polar cartridge. Aprepitant loading was quantified against aprepitant standards (0.016 to 20 µM). The mobile phase consisted of 0.05% formic acid in water and acetonitrile and compounds were eluted under gradient conditions. Mass spectrometry was conducted in positive electrospray ionization conditions and elution of compounds monitored with multiple-reaction monitoring.

Determination of critical micelle concentration. The critical micellar concentration was determined by the pyrene I_1/I_3 ratio[2]. A pyrene stock solution (50 µM) was prepared in THF and 5 µL of pyrene stock were added to 995 µL of graded concentrations of nanoparticles (400 to 0.5 µg/mL), obtained by diluting nanoparticle stock solutions in PBS. The mixture was stirred for 3 h at room temperature and the fluorescence spectrum of pyrene was recorded from 360 to 410 nm using an excitation wavelength of 336 nm in a RF5301PC Espectrofluorophotometer (Shimadzu, Japan). The emission intensities measured at 373 nm (I_1) and 384 nm (I_3) were used to calculate the pyrene I_1/I_3 ratio.

Transmission electron microscopy. The morphology of nanoparticles was determined by transmission electron microscopy imaging using a Tecnai F20 transmission electron microscope at an accelerating voltage of 200 kV at ambient temperature. An aliquot (5 µL) of 0.1 wt% nanoparticle solution (diluted with Milli-Q water) was deposited on a Formvar coated copper grid (GSCu100F-50, Proscitech, Australia) and was allowed to dry overnight in air and at room temperature.

1.5. Nanoparticle disassembly. Nile Red (NR) is a solvatochromic dye that fluoresces only in non-polar solvents, allowing determination of the pH of disassembly for the nanoparticles. Specifically, the pH of disassembly is identified by observing the loss of fluorescence of NR due to release of NR from the core of nanoparticles. Nanoparticles were self-assembled using 0.1 mg of NR per mg of polymer and dialyzed as previously described. pH-responsive nanoparticles loaded with Nile red (DIPMA-NR) and non-pH responsive nanoparticles loaded with NR (BMA-NR) were prepared at a concentration of 200 µg/mL. For pH-dependent disassembly studies, nanoparticles were suspended in a mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4 buffer solutions with a pH range from 7.6 to 5.0. pH-dependent disassembly was assessed by measuring NR fluorescence (excitation/emission 552/636nm) using a FlexStation 3 (Molecular Devices, USA). The time course of nanoparticle disassembly was examined by measuring NR fluorescence at pH 7.4, 6.5, 6.0 and 5.0 over a 12 h period using a CLARIOstar (BMG LABTECH, Germany).

1.6. pERK localization in mouse spinal cord. Spinal cord sections were preincubated in 10% normal donkey serum (NDS) in 0.1 M PBS (1 h, room temperature), followed by rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:200; #4370; Cell Signalling Technology, Danvers, MA) and guinea-pig anti-NeuN (1:1000, Millipore, #abN90) in PBS containing 0.3% Triton X-100 and 5% NDS (overnight, 4˚C). Sections were washed 4x in 0.1 M PBS containing 0.3% Triton X-100 and incubated with donkey anti-rabbit Alexa 488 and donkey anti-guinea-pig Alexa 568 (1:1000, Thermo Fisher Scientific) (45 min, room temperature). Sections were washed 5x in PBS, counter-stained with DAPI (10 µg/ml, 5 min) and cover-slipped with ProLong Glass mounting medium (Thermo Fisher Scientific). Sections were imaged on Leica SP8 confocal microscope with HC PLAPO 40X for counting pERK-immunoreactive (IR) neurons or a 20X objective to collect representative images. For each experimental group, 6 sections of ipsilateral and contralateral dorsal horn were imaged. Only pERK-positive neurons co-labelled with NeuN were counted. To avoid re-counting the same neurons, the analysed sections were 100 µm apart. The total number of neurons was averaged for each group.

1.7. Rotarod test. A rotarod test was used to assess whether the intrathecal injection of nanoparticles would affect normal motor function and thereby impede studies of nociception that

require examination of paw withdrawal from a painful stimulus. Prior to experiments, mice were acclimatized and trained on the rotarod apparatus for three consecutive runs on two successive days. On the day of the experiment, three baseline readings were recorded and a cut-off threshold of 120 second was pre-set. Nanoparticles (DIPMA-AP, BMA-AP, DIPMA-Ø, 10 µg/mL) or vehicle (aCSF) was injected intrathecally as described above. Subsequently, the latency of mice to fall (seconds) were recorded at 30, 60, 90, 120, 180 and 240 min post-injection[3].

1.8. Electrophysiological assessment of nociception in rats. Rats were maintained under anaesthesia (1.2-1.5% isoflurane in oxygen using a diaphragm rodent facemask) and placed on a regulated thermal pad (37 ± 0.5°C). EMG activity was measured using a pair of platinum stimulation electrodes inserted subcutaneously into the lateral part of the third and fourth toes, and recording electrodes inserted through the skin into the ipsilateral biceps femoris muscle[4]. The C-reflex corresponds to the integration of the reflex response into a 150-450 ms time window post-stimulus. Wind-up is a potentiation of the C-reflex response when the stimulating frequency is increased to 1 Hz. The wind-up score corresponds to the slope of the first seven consecutive C-reflex recordings obtained at 1 Hz stimulation. After recording to obtain a stable C-reflex response (~30 min), the threshold for C-reflex was estimated and the rats remained stimulated at 2X the threshold intensity for the duration of the experiment. The C-reflex was evaluated by the mean of 15 consecutive stimuli at 0.1 Hz while the next 7 stimuli at 1 Hz were used to evaluate wind-up. Recordings were made 10 days after surgery before (basal) and 30, 60, 90 and 120 min after intrathecal drug administration. The integrated C-reflex responses were expressed as a percentage of basal response.

1.9. NK_1R localization in rat spinal cord. Free floating sections were blocked in PBS containing 0.3% Triton x-100 and 10% NDS (1 h, room temperature). Sections were incubated with rabbit anti-NK_1R (1:1000, #94168) and guinea-pig anti-NeuN (1:1000, Millipore, abn90) in PBS containing 0.3% Triton X-100 and 3% NDS (overnight, 4°C). Sections were washed 4x in PBS and incubated with donkey anti-rabbit Alexa488 and donkey anti-guinea-pig Alexa568 (1:1000, Thermo Fisher Scientific) (2 h, room temperature). Sections were washed 5x in PBS, counter stained with DAPI (10 µg/ml, 5 min), and mounted onto ColorFrost Plus slides (VWR) with ProLong Glass mounting medium (Thermo Fisher Scientific). Sections were imaged on Leica SP8 confocal microscope with a HC PL APO 63x oil objective (NA 1.4). Z stacks of NK_1R-positive neurons in lamina I of the dorsal horn were collected with a digital zoom of 5. To quantify NK_1R endocytosis in lamina I neurons, the border of the cytoplasm of the soma was delineated by NeuN fluorescence. NK_1R immunoreactivity within 5 pixels (0.5 µm) of the border was defined as plasma membrane receptor. The ratio of plasma membrane to cytosolic NK_1R-IR fluorescence was determined in >6 lamina I neurons per condition.

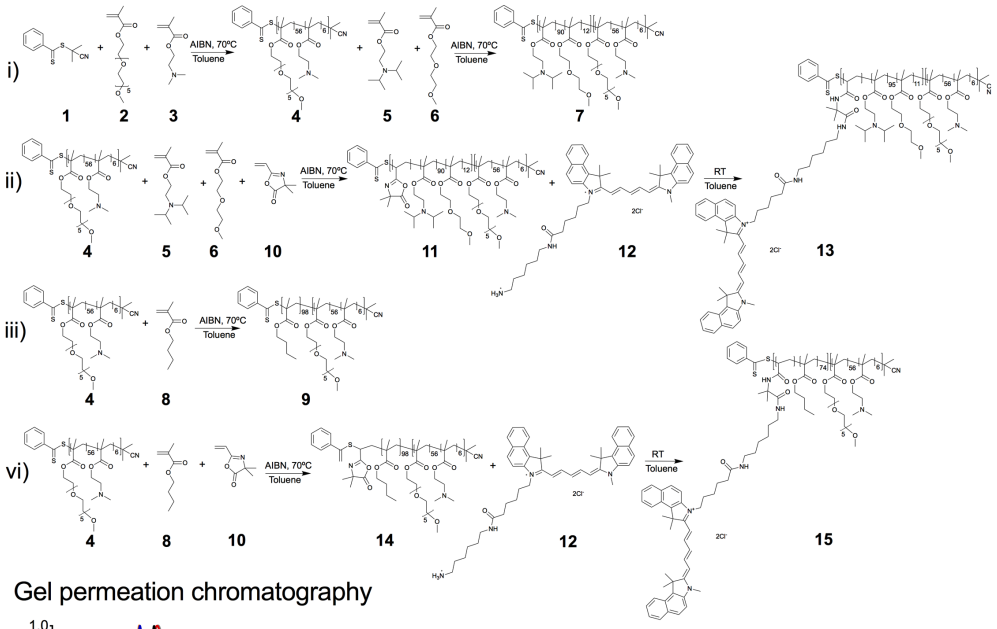
1.10. Cell viability assays. HEK-hNK_1R cells were incubated with empty nanoparticles (1-100 µg/mL) for 24 and 48 h. Medium was replaced by phenol red-free DMEM, followed by incubation for 2 h (37°C, 5% CO_2) with 10% (v/v) alamarBlue reagent (Thermo Fisher Scientific, USA). Fluorescence of the reduced active compound, resofurin, was measured (510/610nm exc/em) using a ClarioStar (BMG LABTECH, Germany).

Supplementary Figures

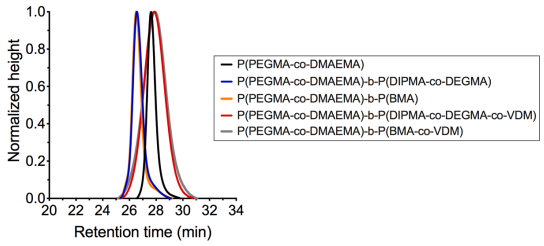
A

Polymer	Conversion (%)	GPC Mn (g/mol)	GPC PDI	^{1}H-NMR Mn (g/mol)	Composition PEGMA	DMAEMA	DIPMA	DEGMA	BMA
P(PEGMA-co-DMAEMA)	54	12,377	1.20	17,600	56	6	-	-	-
P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA)	92	17,581	1.36	39,444	56	6	91	12	-
P(PEGMA-co-DMAEMA)-b-P-(BMA)	82	18,225	1.37	31,654	56	6	-	-	98
P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA-co-VDM)	95	19,380	1.22	39,881	56	6	95	11	-
P(PEGMA-co-DMAEMA)-b-P-(BMA-co-VDM)	62	18,576	1.29	28,220	56	6	-	-	74

B Raft polymerization of diblock copolymers



C Gel permeation chromatography



D Proton nuclear magnetic resonance

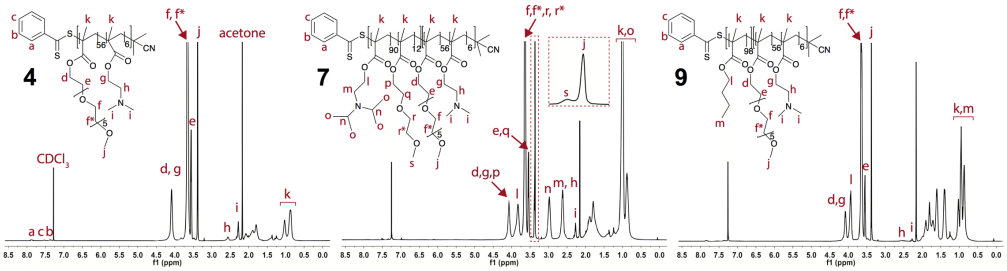


Figure S1. Synthesis and characterization of P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-

DEGMA) and P(PEGMA-co-DMAEMA)-b-P(BMA-co-DEGMA) diblock copolymers. A. Characterization of the hydrophilic block copolymers and the diblock copolymers. **B. i)** Sequential RAFT polymerization indicating synthesis of the hydrophilic block using (**1**) CPDB, (**2**) PEGMA and (**3**) DMAEMA to form (**4**) p(PEGMA-c-DMAEMA); synthesis of P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) by chain extension reaction, where addition of the pH-responsive monomer (**5**) DIPMA and the charge screening monomer (**6**) DEGMA to (**4**) the hydrophilic block forms (**7**) the diblock P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA); **ii)** Addition of (**5**) DIPMA, (**6**) DEGMA and (**10**) VDM to (**4**) the hydrophilic block to form the intermediate (**11**), followed by the addition of (**12**) Cy5 to form the final Cy5 conjugated polymer (**13**) P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA-co-Cy5). **iii)** Addition of (**8**) BMA to (**4**) the hydrophilic block forms (**9**) P(PEGMA-co-DMAEMA)-b-P(BMA). **iv)** Addition of (**8**) BMA and (**10**) VDM to (**4**) the hydrophilic block to form the intermediate (**14**), followed by the addition of (**12**) Cy5 to form the final Cy5 conjugated polymer (**15**) P(PEGMA-co-DMAEMA)-b-P(BMA-co-Cy5). **C.** Gel permeation chromatography traces showing a shift from P(PEGMA-co-DMAEMA) to higher molecular weight (*i.e.*, shorter retention time) after chain extension to form P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) and P(PEGMA-co-DMAEMA)-b-P(BMA-co-DEGMA). **D.** ^{1}H-NMR spectra of the resulting polymers indicating the successful incorporation of the monomers. ^{1}H-NMR was used to estimate molecular weight since gel permeation chromatography was calibrated using polystyrene standards. **C** and **D** are from a single independent experiment because the polymer was made once and used throughout the project.

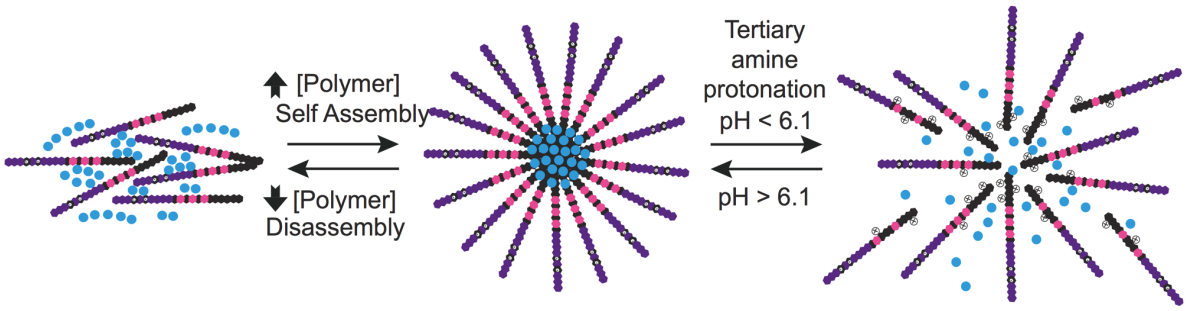


Figure S2. Mechanism of concentration-dependent self-assembly and pH-dependent disassembly of DIPMA nanoparticles. Increasing concentrations of polymer result in nanoparticle self-assembly in an aqueous solution. DIPMA nanoparticles possess a tertiary amine on the DIPMA units located in the core. At pH<6.1, protonation results in a change from neutral to positive charge on DIPMA that induce like-like charge repulsion, which destabilizes the nanoparticle core with the subsequent disassembly and release of cargo.

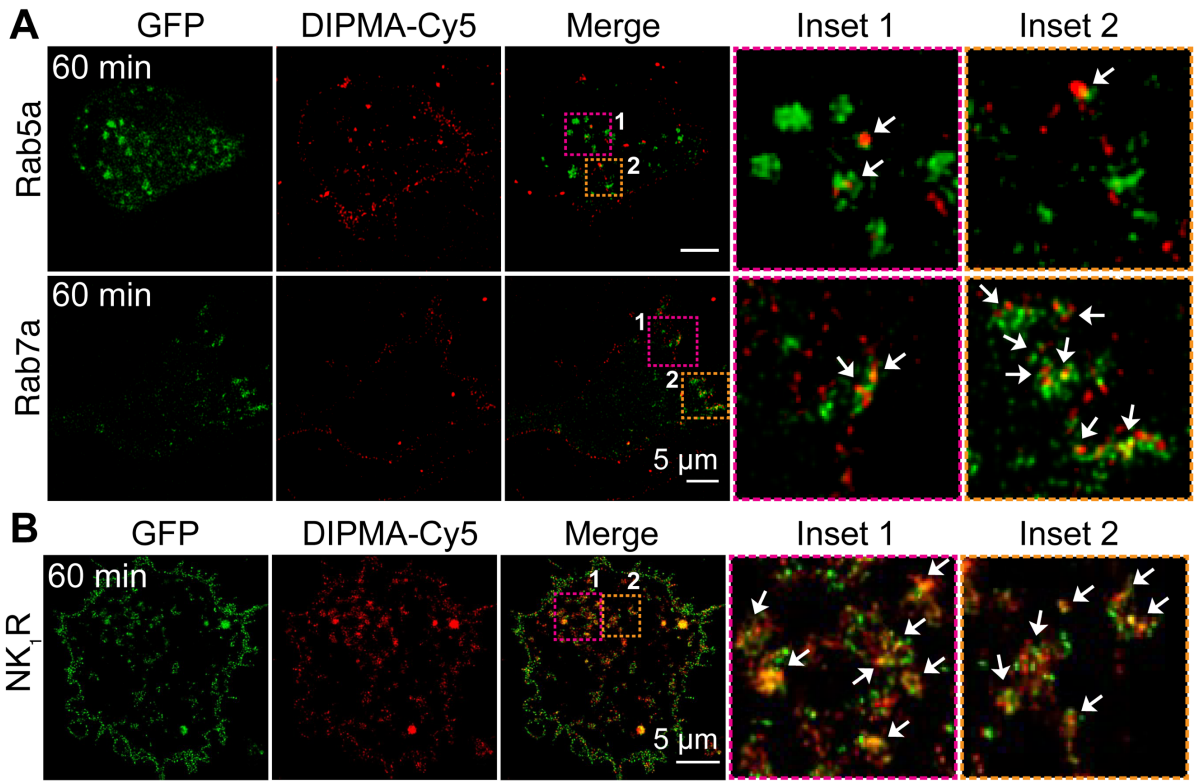


Figure S3. Uptake of DIPMA-Cy5 nanoparticles in HEK-293 cells. A. Localization of DIPMA-Cy5 nanoparticles in Rab5a-GFP early endosomes and Rab7a-GFP late endosomes after incubation with HEK-293 cells for 60 min. Representative results, *n* = 5 independent experiments. **B.** Colocalization of DIPMA-Cy5 nanoparticles and NK_1R-GFP in HEK-rNK_1R cells at 60 min after stimulation with SP to induce NK_1R endocytosis. Representative results, *n* = 5 independent experiments.

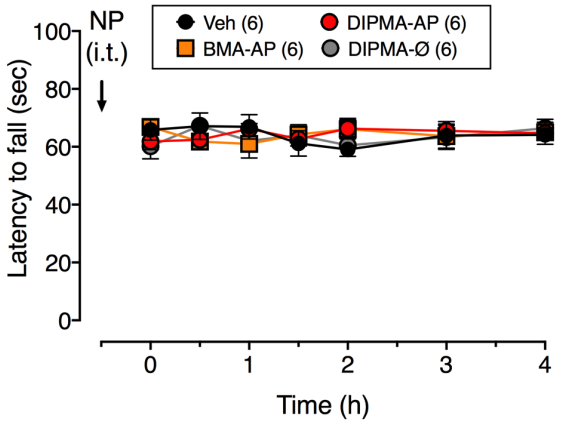


Figure S4. Effects of nanoparticles on rotarod latency. Effects of intrathecal (i.t.) injection of vehicle (Veh), DIPMA-Ø, BMA-AP or DIPMA-AP nanoparticles (NP) on latency to fall in mice, assessed using rotarod. Data are presented as mean ± SEM from *n* = 6 mice (numbers in parentheses) for each treatment group.

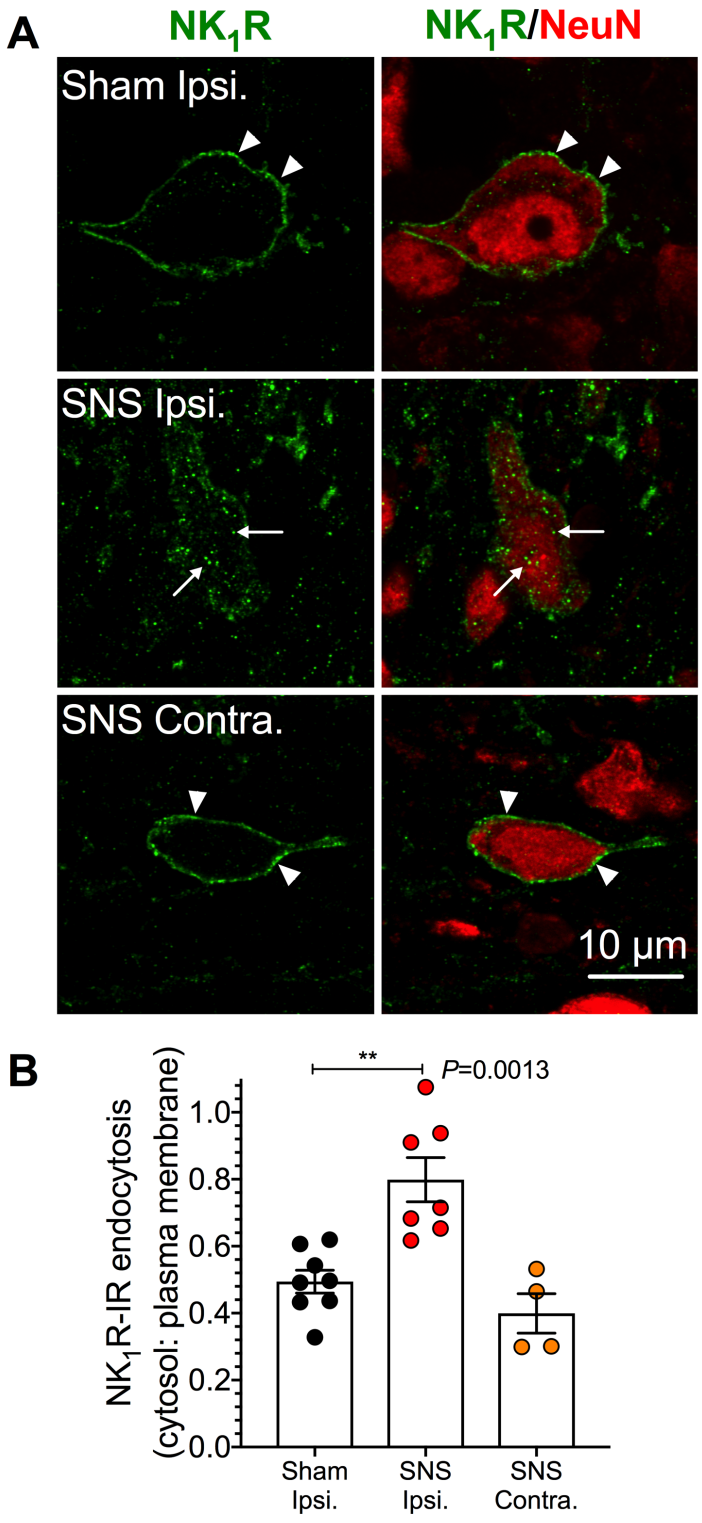


Figure S5. NK_1R endocytosis in chronic neuropathic nociception. To confirm activation of the SP/NK_1R system during chronic neuropathic nociception, the NK_1R was localized in the dorsal

horn of rats 10 days after sural nerve spared (SNS) or sham surgery by immunofluorescence. **A.** Localization of NK_1R-IR and NeuN-IR to the ipsilateral (Ipsi.) or contralateral (Contra.) lamina I dorsal horn of sham or SNS rats. Arrow heads denote plasma membrane. Arrows denote endosomes. **B.** Quantification of NK_1R endocytosis, assessed as the cytosol:plasma membrane pixel intensity for NK_1R-IR neurons. Data are presented as mean ± SEM from sham ipsilateral (*n* = 8 rats), SNS ipsilateral (*n* = 7 rats) and SNS contralateral (*n* = 4 rats) groups. 1-way ANOVA, non-parametric Tukey post-hoc test. $^{**}P<0.01$.

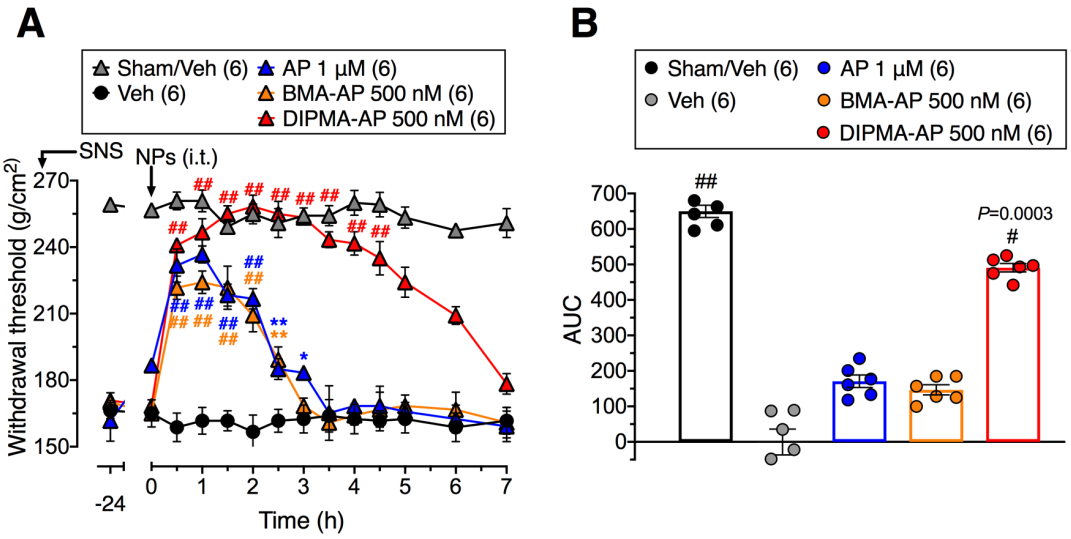


Figure S6. Effects of nanoparticles on neuropathic nociception. The sural nerve spared (SNS) model of chronic neuropathic pain was studied in rats. Vehicle (Veh), aprepitant (AP) or nanoparticle (NP) was administered by intrathecal injection 10 days after SNS or sham surgery. Paw withdrawal responses were assessed using the Randall-Selitto test. **A.** Time course of response. **B.** Area under curve (AUC) from 0-7 h. Data are presented as mean ± SEM from n = 6 rats (numbers in parentheses) for each treatment group. *P<0.05, **P<0.005, $^{\#}P$<0.001, $^{\#\#}P$<0.0001 compared to SNS vehicle. 1-way ANOVA, nonparametric Dunn's post-hoc test.

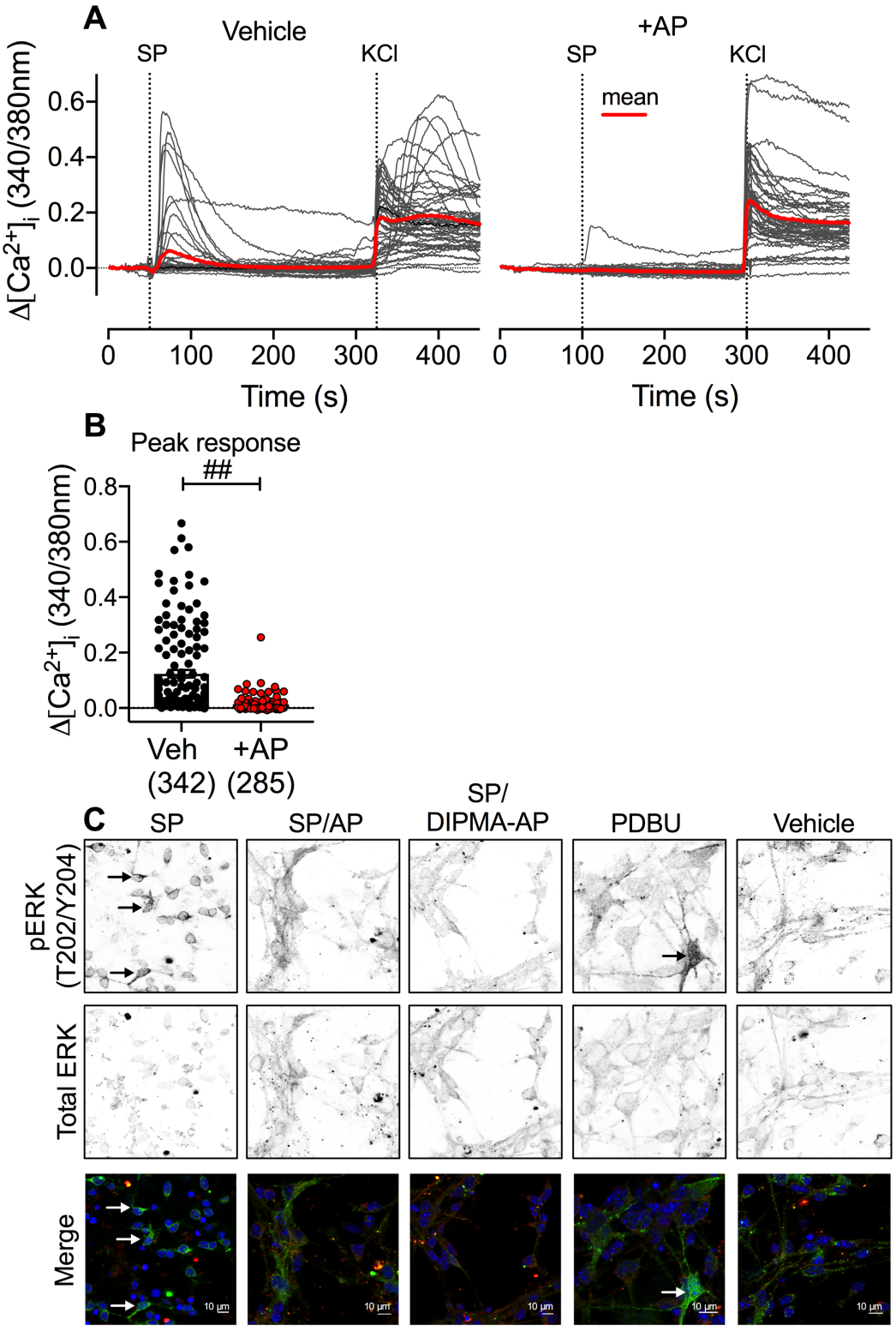


Fig. S7. SP signaling in mouse striatal neurons. SP (100 nM)-induced Ca^{2+} signaling in striatal

neurons pretreated with vehicle (Veh) or aprepitant (AP). **A.** Representative traces of $[Ca^{2}+]_{i}$. Grey lines show responses of individual neurons. Red lines show mean responses. **B.** Peak $[Ca^{2}+]_{i}$ responses. Data are expressed as mean ± SEM from *n* = 342 neurons for SP plus vehicle or *n* = 285 neurons for SP plus aprepitant (numbers in parentheses) from *n* = 3 independent experiments for both treatment groups. ##*P*<0.0001, unpaired *t*-test (2-tailed). **C.** Representative images of phospho-ERK and total ERK immunostaining in cultured striatal neurons from *n* = 3 independent experiments. Neurons were treated with SP, SP plus aprepitant (AP), SP plus DIPMA-AP, phorbol 12,13-dibutyrate (PDBU) or vehicle.

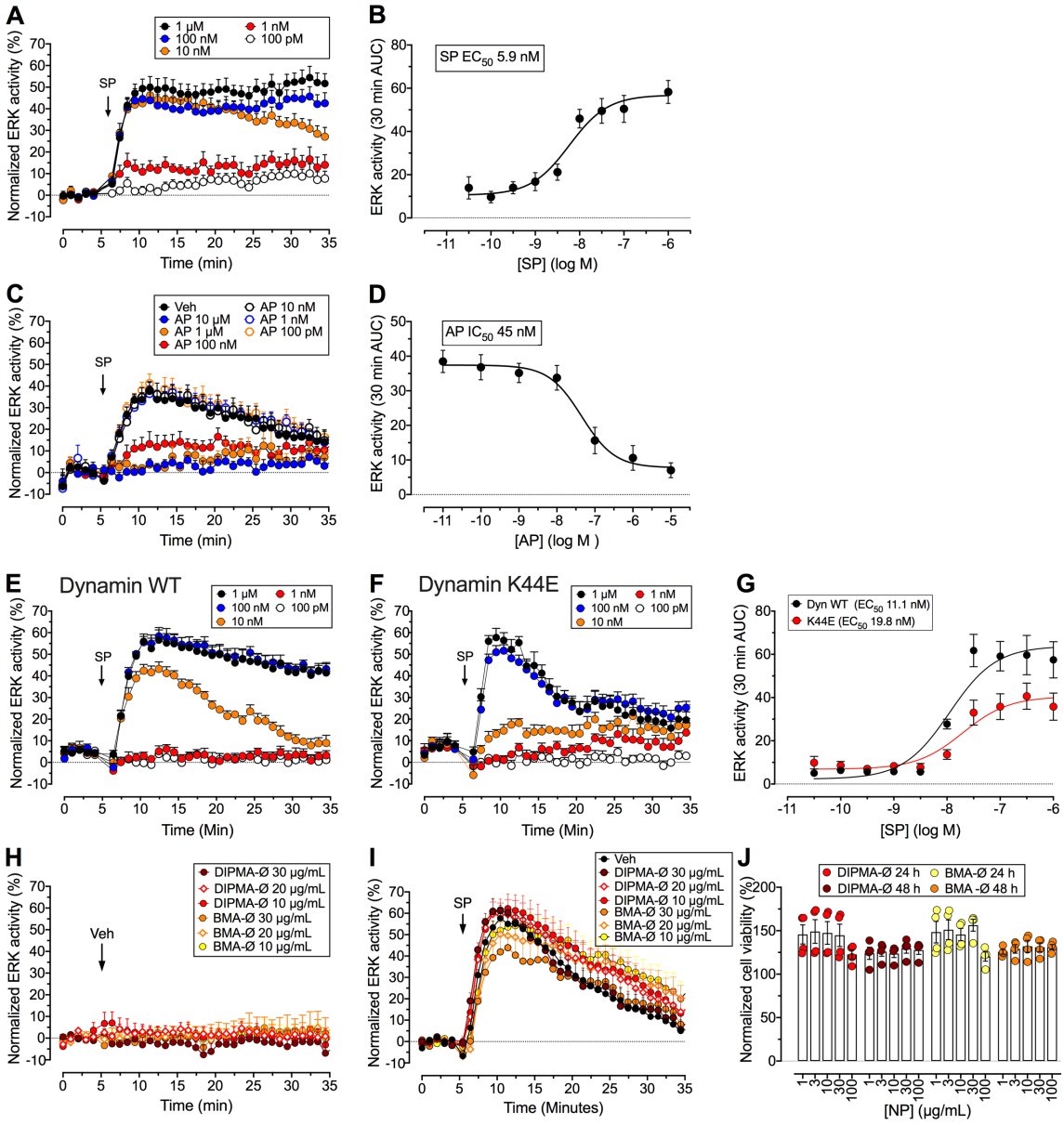


Figure S8. Nuclear ERK signalling and toxicity assays in HEK-293 cells. A-I. SP activation of nuclear ERK in HEK-hNK$_1$R cells. To examine activation of nuclear ERK, HEK-293 cells expressing hNK$_1$R were transfected with NucEKAR (FRET biosensor for nuclear ERK). Data are expressed as mean ± SEM, with triplicate observations made in each experiment. **A.** Effects of graded concentrations of SP on nuclear ERK activity; *n* = 8 independent experiments. **B.** SP concentration-response curves; *n* = 8 independent experiments. **C.** Effects of graded concentrations of aprepitant (AP) on nuclear ERK response to SP (5 nM); *n* = 7 independent experiments. **D.** Aprepitant concentration-response curves; *n* = 7 independent experiments. **E-G.** SP-induced activation of nuclear ERK in HEK-hNK$_1$R cells expressing dynamin wildtype (Dyn WT, **E**) or dynamin K44E (Dyn K44E, **F**); *n* = 6 independent experiments. **G.** SP concentration-response curves; *n* = 6 independent experiments. **H, I.** Effects of DIPMA-Ø and BMA-Ø on basal nuclear ERK activity in HEK-293 cells (**H**) and on SP-stimulated nuclear ERK activity in HEK-

hNK$_1$R cells (**I**) over 30 min; *n* = 7 independent experiments. **J.** Effects DIPMA-Ø and BMA-Ø on viability of HEK-293 cells over 24 h and 48 h. Viability was examined using alamarBlue, which assess the capability of viable cells to reduce rezasurin to resofurin; triplicate observations, *n* = 4 independent experiments.

Supplementary Videos

Video S1. Localization of DIPMA-Cy5 nanoparticles and Rab5a-GFP in HEK-293 cells. The video shows trafficking of DIPMA-Cy5 nanoparticles (red) to Rab5a-GFP early endosomes (green). Cells were incubated with DIPMA-Cy5 nanoparticles for 30 min.

Video S2. Localization of DIPMA-Cy5 nanoparticles and Rab7a-GFP in HEK-293 cells. The video shows trafficking of DIPMA-Cy5 nanoparticles (red) to Rab7a-GFP late endosomes (green). Cells were incubated with DIPMA-Cy5 nanoparticles for 30 min.

Video S3. Localization of DIPMA-Cy5 nanoparticles and NK_1R-GFP in HEK-293 cells. The video shows trafficking of DIPMA-Cy5 nanoparticles (red) and NK_1R-GFP (green). Cells were incubated with DIPMA-Cy5 nanoparticles for 90 min and with SP for 60 min to induce NK_1R endocytosis.

Video S4. Localization of DIPMA-Cy5 nanoparticles in the mouse dorsal horn. The video is a 3D projection of DIPMA-Cy5 nanoparticles in the perinuclear region of lamina I cells in the dorsal horn of the mouse spinal cord. The image was taken at 1 h after intrathecal injection of nanoparticles. Nuclei are stained with DAPI.

Video S5. Localization of BMA-Cy5 nanoparticles in the mouse dorsal horn. The video is a 3D projection of BMA-Cy5 nanoparticles in the perinuclear region of lamina I cells in the dorsal horn of the mouse spinal cord. The image was taken at 1 h after intrathecal injection of nanoparticles. Nuclei are stained with DAPI.

Video S6. Localization of NK_1R-IR in the rat dorsal horn after sham surgery. The video is a 3D projection showing the subcellular localization of NK_1R-IR (green) of lamina I spinal neuron (NeuN, red). The image was taken at 10 days after sham surgery.

Video S7. Localization of NK_1R-IR in the rat dorsal horn after SNS surgery. The video is a 3D projection showing the subcellular localization of NK_1R-IR (green) of lamina I spinal neuron (NeuN, red). The images were taken at 10 days after SNS surgery.

Supplementary References

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