The Mechanism of Action of the Neuropeptide Galanin, with Special Reference to Nociception.

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Declaration

The work presented in this thesis has been solely carried out by the PhD candidate at RMIT University, School of Medical Sciences whilst receiving the School of Medical Sciences Associate Lecturer (Cell Biology and Anatomy) Scholarship.

This thesis does not contain any material which has been submitted for the award of any degree or diploma, at any university unless specified otherwise. This thesis does not contain any previously published work or data, from another person.

Jennifer Lawrence
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### List of Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>AGX</td>
<td>-agatoxin GVA</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_i$</td>
<td>intracellular calcium</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca$^{2+}$-induced Ca$^{2+}$ release</td>
</tr>
<tr>
<td>CIPA</td>
<td>congenital insensitivity to pain and anhidrosis</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTX</td>
<td>-conotoxin GVIA</td>
</tr>
<tr>
<td>cyclic AMP</td>
<td>cyclic adenosine 3' 5'-monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>diaglycerol</td>
</tr>
<tr>
<td>DH</td>
<td>dorsal horn</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>DVN</td>
<td>dorsal raphe nucleus</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GAL$_1$</td>
<td>Galanin receptor 1</td>
</tr>
<tr>
<td>GAL$_2$</td>
<td>Galanin receptor 2</td>
</tr>
<tr>
<td>GAL$_3$</td>
<td>Galanin receptor 3</td>
</tr>
<tr>
<td>Gal-OE</td>
<td>Galanin over expressing</td>
</tr>
<tr>
<td>GALP</td>
<td>Galanin-like peptide</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial derived neurotrophic factor</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GIRKS</td>
<td>protein coupled inwardly rectifying K$^+$ channels</td>
</tr>
<tr>
<td>GMAP</td>
<td>galanin messenger associated protein</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>high voltage activated</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol phosphate</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactivity</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
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</table>
LC  locus coeruleus
LPS  lipopolysaccharide
MAC1  macrophage antigen complex
MAPK  map kinase
NA  noradrenergic
NGF  nerve growth factor
NMR  nuclear magnetic resonance spectroscopy
NO  nitric oxide
PRRs  pattern recognition receptors
P2  purinergic receptors
P2X  ionotropic receptors and non-selective cation channels
P2Y  metabotropic GPCR
p38-MAP  mitogen activated kinase pathway
PC12  pheochromocytoma cell line
PDBu  phorbol 12,13-dibutyrate
PKC  protein kinase c
PLC  phospholipase c
PTX  pertussis toxin
PVN  paraventricular nucleus
ROS  reactive oxygen species
RT-PCR  real-time polymerase chain reaction
SD  sprague dawley
SOCE  store operated Ca\(^{2+}\) entry
TLC  thin layer chromatography
TLRs  toll like Receptors
TNF\(\alpha\)  tumour necrosis factor \(\alpha\)
TRPV1  vanilloid receptor 1
VGCC  voltage-gated Ca\(^{2+}\) channels
VIP  vasoactive intestinal peptide
WT  wild type
Summary

Introduction

The neuropeptide galanin has been shown to modulate pain, and can enhance the acute analgesic effects of morphine (1). In addition, endogenous galanin is suggested to modulate chronic pathological pain after nerve injury (2). Galanin binds with high affinity to three G-protein coupled receptors (GPCRs) namely (GAL₁, GAL₂, GAL₃) (3). Receptor expression occurs in key regions for transmission of nociceptive signals including dorsal root ganglion (DRG) neurons, interneurons in the superficial dorsal horn (DH) of the spinal cord (2). Both GAL₁ and GAL₂ are present in small to medium-sized primary sensory neurons of the DRG (4), whereas GAL₂ is present in microglia (5) in the DH. Galanin is up-regulated in DRG neurons after nerve injury, and the cellular actions of galanin via GAL₁ and GAL₂ are linked with the modulation of pain (6). Microglia provide a cellular response to damaged nerves, however prolonged microglial activation can lead to chronic pain states (7). While little is known about the effect of galanin on microglial activation, galanin has been shown to inhibit cytokine release from microglia (5). Therefore the actions of galanin on Ca²⁺ channels and [Ca²⁺], in DRG neurons and microglia were investigated.

Galanin and DRG (in Sensory Neurons)

Dissociated DRG neurons from Sprague-Dawley (SD) rats were used acutely for patch clamp recordings or cultured overnight in medium containing nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) before Ca²⁺ imaging using Fura-2. Calcium transients partially due to voltage-gated Ca²⁺ channels (VGCC) were induced by depolarization with KCl (30 mM). The Ca²⁺ transients in small capsaicin-responsive DRG
neurons were inhibited by galanin (100 nM, 300 nM, 1 μM) and the GAL2 receptor agonist ARM-1896 (100 nM, 300 nM, 1 μM). The extent of the inhibition by galanin and ARM-1896 was similar to that seen with μ opioid receptor agonist DAMGO (1 μM). In addition, the inhibitory effect of galanin on [Ca2+]i was reproducible. In contrast, Ca2+ currents recorded using whole cell patch clamp clearly demonstrated a concentration dependant effect of the GAL2 only at the higher concentrations tested (galanin 1 μM, and ARM-1896 300 nM, 1 μM), which was reproducible. ARM-1896 demonstrated the largest inhibition of Ca2+ currents when compared to galanin and DAMGO, suggesting cellular actions of galanin in DRG neurons are via GAL2. In order to investigate which VGCCs were inhibited by galanin, DRG neurons were pretreated with toxins to block different types of VGCCs. Blockade of L and N-type VGCCs during patch clamp experiments, suggested a role for only N-type VGCCs in the overall inhibition by galanin. During Ca2+ imaging recordings the presence of -conotoxin GVIA (CTX) (100nM) reduced Ca2+ transients. However CTX had no effect on the percentage of neurons that were inhibited by galanin or the amplitude of the inhibition, indicating involvement of channels other than N-type. DRG neurons pretreated with -agatoxin GVA (AGX) (100 nM) to block P/Q-type channels showed a partial reduction in depolarization-induced Ca2+ transients. In contrast to CTX, AGX significantly reduced the percentage of neurons that were responsive to galanin, and in those neurons the amplitude of the inhibition was greatly reduced. These results indicate that galanin’s inhibitory actions on Ca2+ transients in DRG neurons are mainly via inhibition of P/Q-type channels. This suggests that galanin may have the potential to be developed as an alternative to analgesics which act only via N-type VGCCs. VGCC may also contribute to Ca2+ influx due to capsaicin, therefore inhibition of capsaicin responses...
by galanin were investigated. N-type channel blockade with CTX had no effect on capsaicin-induced Ca\(^{2+}\) transients in most neurons. However in a small subset of capsaicin-sensitive DRG neurons, there was a marked inhibition of capsaicin-induced Ca\(^{2+}\) transients, indicating that N-type channels are involved in a small proportion of capsaicin responses. Similarly to CTX, DAMGO which is known to inhibit N-type VGCC, also inhibited capsaicin responses in some neurons, supporting the role of N-type VGCCs as a component of capsaicin responses. In contrast, galanin had no effect on capsaicin responses, suggesting galanin acts via distinct pathways. Galanin’s ability to inhibit Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) was investigated to determine if it was acting at sites other than VGCCs. In the presence of caffeine known to deplete intracellular Ca\(^{2+}\) stores in DRG neurons, the majority of cells tested were not inhibited by galanin. This suggests CICR contributes to the proportion of the response inhibited by galanin, however the mechanism of this effect needs further investigation.

**Galanin and Microglia**

Galanin is up-regulated in response to damaged nerves (2). Similarly, adenosine triphosphate (ATP) released from damaged nerves activates microglia, and leads to the release of inflammatory mediators implicated in generating the symptoms of chronic pain states (8). Both ATP (9), and lipopolysaccharide (LPS) from gram-negative bacteria cell wall activate microglia *in vitro* (10), leading to a cascade of events including an increase in microglial [Ca\(^{2+}\)], an up-regulation of cell surface receptors, and the production of reactive oxygen species (ROS) (11). Galanin acting via GAL\(_2\) has been shown to inhibit LPS-
induced cytokine release, therefore the potential effect of galanin on microglial activation was investigated.

Dissociated microglia from neonatal SD rats were kept in mixed glial culture for 10-14 days before isolation and Ca$^{2+}$ imaging using Fura-2. Pre-treatments with minocycline, a known inhibitor of microglial activation, was used in some cases to maintain microglia in a quiescent state whilst still able to become activated upon a stimulus with LPS or ATP. Results indicated that galanin could modulate both the LPS and ATP responses in microglia, but with substantially different outcomes. Microglia treated with ATP (50 µM, 1 hour), showed a significant increase in [Ca$^{2+}$]$_i$ levels, which has been previously been used as an indicator of microglial activation. This increase was further augmented by the application of galanin (1 µM, 100 nM). These results show that galanin acting via GAL2 can modulate ATP-induced microglial activation suggesting a role for galanin in modulating the early stages of the microglial activation pathway from neural damage. Treatment of microglia with LPS (1 hour) also resulted in a significant increase in [Ca$^{2+}$]$_i$ levels however, upon co-application of galanin, there was no effect on the LPS-response. When examining the effect of galanin on ROS production, galanin in the presence and absence of other treatments did not induce ROS production after 1 hour. Galanin did however reduce basal ROS levels, suggesting galanin may have an inhibitory role on microglial activation. Twenty four hours of microglial exposure to LPS failed to cause nitric oxide (NO) production, but led to an overall decrease in [Ca$^{2+}$]$_i$ levels. Combined exposure to galanin and LPS for 24 hours resulted in an enhancement of the LPS-induced reduction in [Ca$^{2+}$]$_i$ levels. Despite the lowering of [Ca$^{2+}$]$_i$ levels, microglia showed amoeboid morphology, suggesting signs of activation. This suggests that while an increase
in $[\text{Ca}^{2+}]_i$ may be an indication of microglial activation in the short term, this relationship is not so simple in the long term.

**Conclusions and Future Directions**

The current thesis has used $\text{Ca}^{2+}$ imaging to provide novel evidence that galanin, acting via $\text{GAL}_2$, can inhibit depolarisation-induced $\text{Ca}^{2+}$ transients in small nociceptive DRG neurons. In addition, galanin repeatedly inhibits both high voltage activated (HVA) $\text{Ca}^{2+}$ currents and $\text{Ca}^{2+}$ transients, in a concentration-dependent manner. The current thesis provides evidence of galanin acting on DRG neurons to inhibit both N and P/Q-type VGCCs, although the concentration of galanin required were different. This provides a mechanism by which galanin can inhibit transmitter release from DRG neuron axon terminals and differentiates galanin from analgesic agents that inhibit transmitter release only via actions on N-type channels. As galanin has previously been shown to enhance the analgesic effects of morphine (1), galanin receptor agonists have the potential to be used clinically for acute pain relief. In addition, galanin inhibition of P/Q-type VGCC suggests the potential to also be developed for the treatment of chronic pain states, including neuropathic pain resulting from nerve injury. Chronic pain is poorly treated, and one of the few effective treatments, gabapentin, acts via inhibition of P/Q-type channels at the spinal level (12). In DRG neurons $[\text{Ca}^{2+}]_i$ responses to depolarisation are not only comprised of VGCCs, but also CICR and store operated $\text{Ca}^{2+}$ entry (SOCE). While galanin was able to inhibit VGCC, the results also suggested that inhibition of CICR and SOCE may be involved, but further investigation is required.
Evidence from the microglial studies provided evidence that galanin can augment the short-term effects of ATP on microglial activity. Indeed ATP-induced activation of microglia can lead to symptoms of neuropathic pain (8), suggesting a pro-inflammatory role for galanin in the ATP signalling pathway. Therefore, GAL2 receptor antagonists may have the potential to prevent chronic pain states from occurring. While galanin enhances ATP actions in the short-term, this was not the case after short term exposure to LPS. However, galanin did appear to enhance the effects of LPS signalling pathways after longer exposure durations.

This study has provided the basis for future investigation into the effect of galanin on microglial activity. It has also provided information that may inform therapeutic strategies to reduce neuropathic pain states related to prolonged microglial activation. Together with the dual action of galanin on DRG neurons, this suggests galanin receptor mimetics have the potential to be developed as analgesics for both acute and chronic neuropathic pain.
CHAPTER 1:
A REVIEW OF THE LITERATURE

1.1 Neuropeptides: An Overview

The term “neuropeptides”, first referred to by De Wied et al. in the early 1970s, describes biologically active peptides which are released from nervous and visceral tissue (13). Neuropeptides are co-expressed with classical neurotransmitters and can modulate synaptic transmission and the immune system. Neuropeptides combine with and compliment classical neurotransmitters and are capable of pharmacological effects at concentrations in the nanomolar range (14). Most neuropeptides act on subtypes of GPCRs, which are also distributed in nervous and visceral tissue. The key to the versatility of neuropeptides and their associated receptors is in their vast distribution throughout the body. Indeed, neuropeptides can modulate synaptic transmission in neural pathways involved in nociception, depression and anxiety, learning and memory, obesity, stroke, inflammation, and epilepsy (14, 15).

The field of neuropeptide research began upon the discovery of pharmacologically active powder P, or Substance P in 1931. Substance P extracts from horse small intestine and brain, were found to lower blood pressure in rabbits and cats, and to stimulate the smooth muscle of rabbit in organ bath preparations (16). The structure of substance P was soon identified (17, 18), with peptide structural variants and non-peptidergic compounds with antagonist activity soon to follow (19, 20). Substance P was distributed throughout the nervous system including in sensory DRG neurons and the DH. It could modulate neurotransmission, and had a myriad of physiological activities including on gastrointestinal tract motility, nociception and the regulation of mood
(21). Substance P was soon determined to be co-localized in DRG neurons with other neuropeptides involved in the modulation of spinal nociception, including calcitonin-gene related peptide (CGRP) (22, 23). Tatemoto’s laboratory, which greatly contributed to early neuropeptide research, established a new extraction method by focusing their attention on structural characteristics of previously identified neuropeptides (24). The new chemical extraction technique involved isolating peptides from porcine tissue which had an amide group at the C terminal, followed by thin layer chromatography (TLC). Using this technique, the discovery of more neuropeptides soon followed, namely, neuropeptide Y involved in the modulation of vasoconstriction, nociception and the regulation of food intake; and pancreastatin involved in the regulation of insulin secretion (25, 26). With the promise of diverse functions and tissue distribution, the race had begun to discover and harness the versatile biological activity of novel neuropeptides.
1.1.1 Discovery of the Neuropeptide Galanin

Intense neuropeptide research, conducted in the late 1970s to the early 1980s, focussed particularly on purifying novel peptides and determining their receptor associations. During a purification process of other known neuropeptides, an unknown C-terminal was isolated. Thereafter, the remaining sequence was identified and some biological action determined. In this way, the neuropeptide galanin was serendipitously discovered in 1983 by extraction and purification from porcine gastrointestinal tract via TLC and high performance liquid chromatography (HPLC) (27). The same study indicated that galanin dose dependently induced contractions in the porcine and rat fundus strip, ileum, colon and urinary bladder at concentrations above 20 ng/ml and significantly increased blood glucose levels in dogs after fasting. As an alternative to isolating extracts of galanin from porcine intestinal tract, the first studies to clone galanin used cDNA libraries from the rat anterior pituitary tumour and hypothalamus (28, 29). Cloning of galanin cDNA from mouse hypothalamus and human pituitary and neuroblastoma sources soon followed (30, 31).

In humans, cDNA cloning then allowed investigation of the gene coding for galanin. The GAL gene in humans is located on chromosome 11, and codes for the peptide pre-progalanin (32). The human pre-progalanin protein (1-124) amino acids (aa) when cleaved, forms two peptides called galanin messenger associated protein (GMAP) (1-60 aa) and galanin (1-30 aa) (see Fig 1.1A) (33). Unlike human galanin, which consists of 30 amino acids with a non-amidated C-terminal, rat, mouse, and porcine galanin contain 29 amino acids with an amidated C-terminal. The N and C-terminal amino acids glycine and alanine give rise to the peptide’s name (3). Located on chromosome 19, the galanin-like peptide (GALP) gene is also a member of the galanin family.
Unlike galanin, GALP was discovered much later in 1999, by its presence in porcine hypothalamus and gastrointestinal tract, and its action as a ligand for galanin receptors (34). The precursor peptide preproGALP (1-120) is cleaved to form the 60 amino acid GALP.

A few years earlier, structural and biochemical studies were conducted to determine the 3-dimensional structure of galanin using nuclear magnetic resonance spectroscopy (NMR). While several studies have used this method, galanin is a short motile protein which makes identifying the complete structure difficult. In aqueous solution, galanin was found to be relatively unstructured, and remained in a monomer form until receptor interaction (35). However, galanin was found to favour helical formation at 3-11,14,18 and 22-30 aa (36). Chimeric hybrid peptides such as galanin (1-12 aa) attached to the C-terminal of the wasp toxin mastoparan have been used to model the 3-dimentional structural tendencies of galanin (see Fig 1.1B) (37).
Figure 1.1 Galanin Genes and Proteins

(A) Encoded by the GAL gene, galanin is formed upon cleavage of preprogalanin, which separates GMAP from galanin (33). (B) Partial 3D structure of galanin in helix formation (blue and green), when attached to the C terminal of mastoparan (purple) (37).
1.1.2 Galanin Tissue Distribution

The tissue distribution of galanin was first investigated by Tatemoto and colleagues (38), who discovered the neuropeptide two years earlier (27). As galanin was isolated from porcine gastrointestinal (GI) tract, the search began for galanin-immunoreactivity (IR) in gut regions of pig, guinea pig, rat and mouse tissue. Using immunohistochemistry, tissues were treated with antiserum raised in rabbit against porcine galanin to determine the distribution of galanin-IR cells in the enteric nervous system. In the oesophagus, galanin-IR cell bodies and nerve fibres were detected in mice and rats, but galanin was apparently not present in guinea-pig cell bodies, and not tested in porcine tissue. Guinea-pig tissue had a relatively low number of nerve fibres stained for galanin, whereas the remaining species tested demonstrated high numbers of galanin IR nerve fibres. All species showed galanin IR in nerve cell bodies and nerve fibres in the small and large intestine. Unlike other species tested, some mice tissue was treated with colchicine prior to immunostaining, to increase the presence of axonal proteins in cell bodies by blocking axonal transport. If this had been applied to all tissue types, it may have increased the yield of visible galanin-IR, as the study mentioned issues with low levels of fluorescence. As is the case with other neuropeptides, it is more common for galanin antibodies to stain axon terminals more readily than cell bodies. For instance Hokfelt et al. showed immuno-electron microscopy (EM) staining for galanin protein in large vesicles of primary axon terminals of the DH that was more common than in DRG cell bodies (39). In the initial study, primary antibodies were created by using porcine galanin. This may not have been as effective as using rat galanin, but at the time of publication galanin had not yet been isolated from rats (38). Tatemoto et al. (27), initially determined that galanin could modulate blood glucose levels, and as expected galanin-IR was present in nerve fibres that innervate beta islet cells of the pancreas. In addition, galanin reduces
sympathetic nerve activity leading to increased food intake (40, 41), which can be blocked by galanin antagonists (42). As mentioned, galanin-IR was found in sensory DRG neurons, and the sensory terminals and interneurons of the superficial DH of the spinal cord (43, 44), suggesting a potential role in the modulation of pain. Galanin-IR was found in low levels in small DRG neurons at L4 and L5 in normal rats (43). Galanin-IR occurs in both myelinated and unmyelinated dorsal root axons (45, 46), suggesting it may be present in both nociceptive and non-nociceptive neurons (47).

After further investigation, it was soon apparent that galanin was present throughout the CNS. Melander et al. (48) began by demonstrating galanin-IR in the medial septum and basal forebrain. Galanin-IR was also detected in fibres of cholinergic and noradrenergic afferent pathways to the hippocampus (49). This became an early indication that galanin may have a role in the modulation of memory and learning. An early sign of galanin’s potential therapeutic use in neuroinflammatory and neurodegenerative diseases, including Alzheimer’s disease, was the detection of galanin synthesis in glial cells after exposure to colchicine. Galanin peptide-IR was determined via immuno EM and galanin mRNA detected via in situ hybridisation in small glial cells, presumed to be microglia after exposure to colchicine (50), which might suggest an up-regulation in glia as a response to nervous system damage. It was also suggested from the overlap in these two labelling techniques that either could be used to determine galanin expression. More recently, a human study in post mortem brain using riboprobe in situ hybridization determined galanin was present in regions important for sleep regulation, anxiety and depression (51), in noradrenergic (NA) pathways in the locus coeruleus (LC), dorsal raphe nucleus (DVN) and the forebrain.
1.2 Early Evidence for the Existence of Multiple Galanin Receptors

It is now known that galanin is released from vesicles from nerve terminals and modulates communication at synapses throughout the CNS including the hypothalamus, thalamus and spinal cord (6). Galanin is often co-localised with neurotransmitters such as serotonin, acetylcholine and noradrenaline, and neuromodulators such as neuropeptide Y, substance P and vasoactive intestinal peptide (VIP) (23, 52). The function of galanin extends to the modulation of glutamnergic, cholinergic and NA neuronal transmission (49). In particular galanin can modulate NA transmission in the paraventricular nucleus (PVN), hypothalamus (53), and the LC (54), and can inhibit synaptic transmission in the DH of the spinal cord (6). The ability of galanin to modulate the release of various neurotransmitters throughout the CNS and the PNS, leads to the diversity of galanin’s potential for many therapeutic areas (55) (see section 1.4.4).

Soon after the discovery of galanin, pharmacological evidence for the existence of multiple galanin receptors began to take shape (3). Studies on the localisation of the galanin receptors began using radioactively labelled galanin ligands. Binding studies in native systems identified galanin binding sites throughout the rat brain, spinal cord and visceral tissue (56, 57). Manipulation of the amino acid structure of galanin gave rise to various peptide analogues which were then used in binding studies (3). Displacement binding studies using $^{125}$I-galanin in Bowes melanoma cells revealed the order of potency of peptide analogues. This was determined to be galanin (1-30) > galanin (1-16 aa) > D-TRP$^2$ galanin $\geq$ galanin (3-30 aa) (58). In contrast, galanin (3-30 aa) was found to have highest potency in anterior pituitary and hypothalamus of rats (59), and equal potency to galanin (1-30 aa), galanin (1-16 aa), and galanin (1-20 aa) in gastric smooth muscle (57).
The existence of multiple galanin receptors was considered more likely upon the determination of an interaction with multiple intracellular pathways, such as reduction of cyclic adenosine 3’ 5’-monophosphate (cyclic AMP) and inhibiting adenylate cyclase (60), opening K+ channels and closing VGCC via interactions with different G proteins (61, 62). In contrast, a study in gastric smooth muscle cells demonstrated an increase in cyclic AMP concentration (63) suggesting the multiple effects on cyclic AMP can be contributed by the presence of multiple receptors. More evidence of multiple galanin receptors emerged when both an increase and decrease of [Ca^{2+}]_i was found in insulinoma cells (64).

### 1.2.1 Discovery of Galanin Receptor Genes

The first identified galanin receptor referred to as a functional human galanin receptor, was cloned in 1994 (65), and found to consist of a familiar seven trans-membrane structure of recently discovered GPCRs including somatostatin and opioid receptors. Structural differences between galanin receptor subtypes include the number of coding exons, the activation of different types of GPCR, the type of VGCC associated with the receptor, and the number of internal phosphorylation sites (66). The cDNA for the galanin receptor soon known as GAL_1 was isolated from a human Bowes Melanoma cell line expression library using a radioligand binding method (65). The receptor was shown to vary little between human, mouse and rat. GAL_1 was determined to be located on chromosome 18 for mouse, rat and humans, and had 93 percent receptor homology between the three species (67, 68). The gene coding for the GAL_1 contains three exons. The first exon codes for the extracellular N-terminal and the first five trans-
membrane domains. The second codes for the intracellular loop three and the last exon codes for trans-membrane domain seven and the C-terminal (see Fig 1.2A) (68).

Similar to the discovery of GAL1, GAL2 was isolated using the radioligand binding method, although from a rat hypothalamus cDNA expression library in 1997 (69). As a result of an additional 15 amino acids at the C-terminal of human GAL2, there is a lower homology level of 85% between human and rat GAL2. However, in the absence of the additional amino acids, mice and rat species demonstrate a 94% GAL2 homology (70). Upon comparison, there is a 38% identity between rat GAL1 and rat GAL2 (71). The gene coding for GAL2 (387 aa) is localised to chromosomes 17 and 11 in humans and mice respectively (72, 73). Two exons separated by an intron make up the gene, the first intron coding for the N-terminal and the first three trans-membrane domains, and the second exon coding for the intracellular loop and remaining trans-membrane domains to the C-terminus (see Fig 1.2B) (72).

In another study by Smith et al, the last known galanin receptor GAL3 was cloned in two species in 1998. Hypothalamus cDNA libraries, were used to clone both the rat and human GAL3 respectively (66). The gene coding for GAL3 is localised on chromosome 22, and similarly to GAL2 is coded by two exons separated by an intron. When comparing human to rat GAL3, a receptor homology of 89% was demonstrated (73). Rat GAL3 was found to have 35% identity with rat GAL1 and 52% identity with rat GAL2 (see Fig 1.2C) (66).
Figure 1.2 Galanin Receptors

Amino acid sequence and membrane topology for the three galanin receptors GAL₁, GAL₂ and GAL₃ (3) (A) The GAL₁ receptor. Blue circles indicate identical amino acids between GAL₁ and GAL₂, whereas red circles indicate amino acids which are absent in GAL₂. (B) The amino acid sequence for the GAL₂ receptor, where blue circles indicate identical residues to GAL₃, and red circles indicate those residues which are absent in GAL₃. (C) The GAL₃ receptor showing identical residues to GAL₁ in blue, and amino acids absent in GAL₁ shown in red (3).
1.3 Challenges for the Investigation of Galanin Receptors

As mentioned, the three known galanin receptors GAL$_1$, GAL$_2$ and GAL$_3$ belong to the GPCR family, and each play a different role in the modulation of physiological and pathological pathways. This is contributed to by differences in receptor tissue distribution, and differences in their coupling to multiple G proteins and activation of different intracellular events in different tissues (see sections 1.3.1-3). When studying tissue types specifically, interpreting in vitro studies using cell lines and expression systems is often complicated, and may not reflect natural receptor mechanisms as seen in vivo. For instance, a study using a pheochromocytoma cell line (PC12) suggested that galanin activates apoptotic pathways (74), while in contrast an in vivo study of neurons in the hippocampus suggested galanin was neuroprotective and reduced apoptotic cells numbers in response to kainite-induced excitotoxicity & neural damage (75).

Other in vivo studies suggest a neuroprotective role for galanin, when chimeric peptides thought to be antagonists, behaved as partial agonists (76, 77). As such, strategies to fully understand individual receptors are hampered by the lack of investigative tools. Approaches to discover receptor specific agonists and antagonists was to create galanin fragments by removing a proportion of amino acids from the original galanin structure (1-30 aa). The production of chimeric peptides from coupling the N-terminal from galanin with a C-terminal from another neuropeptide, often neuropeptide Y, have produced mixed results (78). As a consequence, there is much controversy in the literature regarding the individual function of the galanin receptors, and caution is advised when interpreting studies using chimeric peptides.

Care must also be taken when evaluating the outcomes of tissue distribution studies. While tissue distribution studies have often used in situ hybridisation (79), alternative studies using galanin
receptor antibodies must be interpreted with caution (80). The use of galanin receptor antibodies designed to target receptors individually were found to label other galanin receptors. As such, controversy surrounds this method, particularly after non specific binding was found for galanin receptors when comparing tissue from wild type (WT) with transgenic receptor knock out (KO) animals (81). Another method used to establish the functional role of galanin was the use of transgenic animals in the investigation of individual receptor function, presents its own unique challenges. Transgenic animals were developed for both galanin and receptor gene KO, but are complicated by developmental changes including the absence of a subset of sensory neurons (see section 1.5.2) (82, 83). It is also possible that other receptors may have higher levels of expression, as a compensatory mechanism.

1.3.1 Distribution and Function of GAL₁

1.3.1.1 Distribution

Using the cloned GAL₁ mRNA, northern analysis indicated GAL₁ distribution in nervous and visceral tissue, in particular in foetal brain and the small intestine (65). Later mRNA in situ hybridization studies demonstrated GAL₁ mRNA distribution in the thalamus, hypothalamus, hippocampus, the LC of the brainstem and the DH of the spinal cord (79, 84, 85). More specifically, GAL₁ mRNA was found to be distributed throughout lamina I and II of the DH, and present in DRG neurons mostly conserved to large DRG somata (4). However, studies using in situ hybridisation have suggested GAL₁ mRNA is also present in smaller DRG neurons and overlaps with GAL₂ expression (86).
1.3.1.2 Receptor Binding and Activation

GAL₁ receptor binding and activation has cellular actions via Gi/o G proteins, as confirmed by pertussis toxin (PTX) sensitivity in the Chinese hamster ovary (CHO) cell line (87). Activation of intracellular pathways via the Gi/o α subunit results in a reduction in the concentration of cyclic AMP, and phosphorylation of cyclic AMP response element binding protein pCREB. The release of the βγ complex results in a downstream increase in map kinase MAPK (65, 87). GAL₁ opens G protein coupled inwardly rectifying K⁺ channels (GIRKS) in GAL₁ when expressed in xenopus oocytes (66). In addition, GAL₁ inhibits VGCC of unknown type, in rat myenteric neurons (88), and inhibits N-type VGCC in mudpuppy neurons (89).

1.3.1.3 Receptor Pharmacology

Galanin shows equal affinity for GAL₁ and GAL₂ and lower levels for GAL₃ (33). The peptide M617 is a GAL₁ agonist, with 25-fold specificity for GAL₁ although also can act via GAL₂ (90, 91). Other peptides M40, C7, M35 and M32, are non-specific receptor antagonists with activity on all galanin receptors. At higher concentrations, they display agonist effects at concentrations higher than 10nM (3, 42). There is however a GAL₁ specific antagonist known as RWJ-57408 which has been used to distinguish GAL₁ effects from non-specific galanin agonists in myenteric neurons (88).
1.3.2 Distribution and Function of GAL2

1.3.2.1 Distribution

Distribution of GAL2 mRNA expression has been examined by both in situ hybridisation and real-time polymerase chain reaction (RT-PCR). It was found in both peripheral and CNS tissue with the highest expression in hippocampus, hypothalamus, cortex, amygdala, spinal cord, DRG, anterior pituitary, lung, and kidney (4, 71, 92). GAL2 was also detected in large intestine, spleen, heart, liver (92), and microglia (5).

1.3.2.2 Receptor Binding and Activation

Of the three galanin receptors, GAL2 has the most complex receptor signalling pathway. Like its sister receptors, GAL2 can have cellular actions via Gi/o G proteins (89). Most commonly, GAL2 acts via Gq/11 G proteins (55), in a PTX-resistant manner in cell lines including CHO cells (87), and in neurons of the hippocampus (93). It is as yet unknown if the more common pathway is used for GAL2 G protein coupling and associated VGCC targets in DRG neurons and microglia. In expression systems it appears GAL2 action on VGCC via Gq/11 is blocked by constitutive activation of Protein Kinase C (PKC) which prevents βγ subunits from binding to N-type channels (94). Activity via Gq/11 increases phospholipase C (PLC) activity leading to inositol phosphate (IP3) hydrolysis and a release of Ca2+ from intracellular stores in expression systems. In addition, release of diacylglycerol (DAG) by GAL2 leads to an increase in PKC activity and an increase of MAPK (69, 71). Whether GAL2 can inhibit VGCC in native cells has not been fully established.
1.3.2.3 Receptor Pharmacology

As mentioned, galanin has equal affinity for both GAL\textsubscript{2} and GAL\textsubscript{1}. While one of the few GAL\textsubscript{2} receptor agonists ARM-1896 (galanin 2-11) predominantly acts via GAL\textsubscript{2}, it has also been shown to have a low affinity for GAL\textsubscript{1} and GAL\textsubscript{3} (88, 95). As mentioned previously, non-specific galanin receptor antagonists M40, C7, M35 and M32, can act via GAL\textsubscript{2} and at higher concentrations, also behave as non specific receptor agonists (3, 42). However a GAL\textsubscript{2} receptor antagonist M871 is available, and has a 30 fold affinity for GAL\textsubscript{2} over GAL\textsubscript{1} (6, 96).

1.3.3 Distribution and Function of GAL\textsubscript{3}

1.3.3.1 Distribution

Despite isolating the cDNA from a rat hypothalamus library, one study suggested there was little if any expression of GAL\textsubscript{3} in the CNS, and was more prominent in the periphery and visceral tissue including the heart, spleen and testis (97). A year later Smith et al. (66), determined that GAL\textsubscript{3} was expressed mainly in the CNS upon using a rat cDNA also isolated from a hypothalamus library. Several studies have confirmed that GAL\textsubscript{3} expression occurs in both CNS and peripheral tissue with the highest expression recorded in the hippocampus, hypothalamus, lung kidney and liver, followed by lower levels detected in the cortex, amygdala, spinal cord, anterior pituitary, large intestine, spleen (66, 92) and lower still in DRG neurons (90).
1.3.3.2 Receptor Binding and Activation

Of the three receptors, the GAL₃ signalling pathway is the least well studied, but GAL₃ actions are also PTX-sensitive, and occur via Gi/o G proteins (87). GAL₃ opens ATP-activated K⁺ channels and opens GIRKS (66).

1.3.3.3 Receptor Pharmacology

GAL₃ has the lowest affinity for the endogenous ligand galanin (88, 95). There are currently no available GAL₃ specific agonists. One of the few GAL₃ specific antagonists available is the small molecule SNAP37889 and SNAP398299, which exhibits anxiolytic and antidepressant behaviour in rats, by reducing galanin inhibition of serotonin transmission in the DRN (98).

1.3.4 Physiological and Therapeutic Roles of Galanin Receptors

It is difficult to fully understand the physiological roles of galanin receptors with limited availability of receptor specific agonists and antagonists. Despite this, the potential use of galanin receptors as therapeutic targets are evident in several physiological pathways that galanin can modulate (55). Galanin inhibits synaptic transmission in the DH of the spinal cord (6), and can modulate glutaminergic transmission (49). Indeed, with galanin up-regulation in response to nerve damage (43, 99, 100), and by cellular actions mediated via GAL₁ and GAL₂ (101), the actions mediated by galanin have been linked to a role in nociception (52, 102). GAL₂ has been linked to the development of a subset of sensory neurons thus GAL₂ is important in neurodevelopment (82), and has been shown to act as a growth factor for neuroendocrine tumours (82, 103).
Building evidence suggests galanin modulates neuronal transmission in the LC and hippocampus, and as such implies GAL$_1$ and GAL$_2$ are potential targets for the treatment of epilepsy (33, 93). An early study demonstrated that galanin injected directly into the hippocampus had anticonvulsant activity (104). In the hypothalamus, galanin can influence appetite and food intake. Injections of galanin directly into the hypothalamic PVN resulted in feeding behaviour in rats via modulating NA transmission (53). Observing the effect of galanin on sleep regulation, a study noted an increase in galanin mRNA in NA neurons of the LC in sleep deprived rats (54). Further investigation in sleep and alertness, galanin was found to modulate growth hormone dependant pathways in the hypothalamus and LC in rats (105), resulting in wakefulness and alertness and also had antidepressant activity by reducing excessive sleeping in depressed patients (106). More recently, direct injection of galanin into the hippocampus in rats caused memory impairment in Morris water maze test (107). As such, there is much interest in the therapeutic use of galanin mimetics in epilepsy, obesity, memory and learning and Alzheimer’s disease.

All three galanin receptors have been considered for their role in the treatment of anxiety and depression (106). However, due to the lack of non-peptide receptor specific pharmacological tools, there has been little progress, with the exception of the small molecule GAL$_3$ specific antagonists SNAP37889 and SNAP398299. After investigation, SNAP37889 and SNAP398299 have been found to have an anxiolytic and antidepressant effects in mice (98). Further progression in the field of galanin therapeutics and better understanding of galanin’s physiological role await the development of better pharmacological tools. It is clear, however,
that galanin plays a major role in how the nervous system reacts to pathophysiological insult such as nervous system injury (43, 99, 100).

1.4 Galanin in Nervous System Injury

As discussed earlier (see section 1.3), the presence of galanin has been established throughout the peripheral and CNS and is up-regulated following neural damage in key areas including the DH of the spinal cord (43, 44), DRG neurons, hypothalamus (53), hippocampus, and LC (54). In addition, galanin is upregulated in the sympathetic nervous system after nerve injury due to the loss of NGF target-derived growth factor (108). Indeed transgenic animal models for galanin and associated receptors indicated that galanin acting via GAL₂ was crucial in the neurodevelopment of a subset of sensory DRG neurons and neurite outgrowth after nerve injury (82, 109). Considering that galanin is both up-regulated after nerve injury (43, 44), and involved in promoting nerve regeneration (82, 109), this suggests that galanin is acting in a neuroprotective manner.

In relation to CNS damage, galanin receptor activation has been linked with both neuroprotection (75), and the progression of pathological processes following nervous tissue damage (55). Galanin is up-regulated in the brains of patients suffering from Alzheimer’s disease, and plays a neuroprotective role acting via GAL₂, against hippocampus neural death associated with amyloid beta plaque formation (110). Also in the hippocampus, galanin acting via GAL₁ and GAL₂ was found to inhibit epileptic seizures, and played a neuroprotective role in hippocampus following neural damage associated with epileptogenesis (33, 93).
Like galanin, microglia are also implicated in the responses to nervous system injury. While galanin is up-regulated in response to nerve injury, microglia respond to release of ATP from damaged neurons among other stimulants. Upon detection of neural tissue damage, a neurodegenerative disease or infection, microglia become activated and migrate to the damaged area (111). However, while the initial inflammatory response to nervous system injury is useful to remove and phagocytose cellular debris, microglia can release pro and anti-inflammatory cytokines, ROS and NO that can contribute to further tissue damage (5, 11). Galanin acting via GAL2 receptors which are expressed in microglia can inhibit cytokine release, suggesting a role for galanin in modulating a myriad of inflammatory pathways as a result of microglial activity (5). While galanin and microglia have separately been shown to play a role after nerve damage, it is unknown if galanin plays a direct role in modulating processes leading to microglial activation. Prolonged activation of microglia can lead to pathologies including chronic pain following nerve injury known as neuropathic pain (7). However, it is unclear what effect the elevated levels of endogenous galanin have on neuropathic pain associated with prolonged microglia activation. Neuropathic pain as a result from nerve injury is a common ailment which is poorly treated. While there are effective analgesics available for acute pain, there are few pharmaceuticals to treat neuropathic pain. Therefore, investigation of the effects of galanin and its receptors following nerve injury may provide new therapeutic targets for the treatment of this debilitating condition (112).

Galanin and its associated receptors are present in nociceptive neurons in multiple areas including the DRG, DH, and can influence nociceptive pathways in both acute and pathological pain, in addition to microglia (see Fig 1.3) (5). Indeed, galanin is up-regulated in response to
nerve injury, in the DRG and the DH (43, 100, 113), and is released from the spinal cord (114). This thesis will therefore focus on the cellular mechanisms involved in galanin’s action in nociceptive neurons and microglia, that are implicated in pathological pain states.

1.5 Neural Basis of Nociception

The nociceptive pathway serves as a method to alert us to present danger that can result in tissue injury. While noxious sensation is unpleasant, it serves as a survival mechanism which becomes apparent when observing humans with a congenital deficiency of the TrkA receptor leading to congenital insensitivity to pain and anhidrosis (CIPA) which is characterized by a lack of nociceptive neurons. As a consequence lacking to respond to noxious stimuli, individuals are at risk of not noticing serious injuries which may result in permanent damage. These individuals also suffer from an inability to sweat, self mutilation and mental retardation (115). Under normal circumstances, nerve fibres transmit noxious information from viscera and the periphery, and include the fast conducting, myelinated A delta fibres and slower conducting unmyelinated C fibres (116). Noxious information is relayed past cell bodies in the DRG and projected to the DH of the spinal cord. From the DH, the ascending nerve pathway carries nociceptive information before information synapse in the thalamus before reaching the somatosensory cortex for interpretation as pain (117). In some cases, pathophysiological pain can result from nervous system injury, leading to inappropriate generation of action potentials transmission in nociceptive neurons which is essentially a false alarm rather than an alert to danger. In the case of nervous system injury, there are regions of the nociceptive pathway where neuromodulators can alter noxious transmission, which can either have an analgesic or algesic effect (2). Galanin
is able to modulate noxious transmission in key areas of this pathway including the DH of the spinal cord and in DRG neurons (100).

Nociceptive DRG express a vast amount of receptors and ion channels, which can mediate and influence the transmission of noxious stimuli. Indeed, DRG neurons express µ opioid and galanin receptors, VGCC and TRPV1 channels (118). While TRPV1 channels are responsible for responding to noxious stimuli, VGCC have a role in synaptic transmission, where galanin and µ opioid receptors can modulate noxious information, and modulate via GPCR subunits acting on VGCCs. It is well known that µ opioids inhibit Ca\textsuperscript{2+} signalling and synaptic transmission via the inhibition of VGCC. Galanin receptors are expressed in the DH, and previous reports indicate galanin can inhibit synaptic transmission in the DH via the actions of GAL\textsubscript{2} (6). Furthermore, galanin has also been shown to increase anti-nociceptive effects of morphine (1). Studies investigating galanin’s cellular actions in sensory pathways are often contradictory. While galanin has also been suggested to inhibit VGCC in other cells (89), it has been shown to augment Ca\textsuperscript{2+} signalling in DRG neurons (86).
As mentioned, this finding in DRG neurons is in complete contrast to evidence of galanin enhancing the analgesic effects of morphine in the spinal cord (1). However, the mechanisms causing a Ca\textsuperscript{2+} increase in response to depolarisation in nociceptive DRG neurons are complicated. Factors suggested to influence Ca\textsuperscript{2+} signalling in nociception is the size of DRG neurons involved (116). More amplification of the Ca\textsuperscript{2+} responses occurs in small DRG neurons, due to the release of Ca\textsuperscript{2+} from intracellular stores suggesting the involvement of CICR and SOCE (119). This suggests amplification of Ca\textsuperscript{2+} signalling at the DRG neuron level may contribute to neurotransmitter release in the DH of the spinal cord. As mentioned, galanin receptors are expressed in the DH, and galanin expression is up-regulated. Indeed, a previous report indicated galanin can inhibit synaptic transmission in the DH via the actions of GAL\textsubscript{2} (6). However, the effect of galanin and its receptors on CICR and SOCE have not been investigated.
**Figure 1.3** The Presence of Galanin Receptors in the Nociceptive Pathway.

Gal1 and Gal2 receptors are present in the nociceptive pathway, both peripherally in the DRG and centrally in the spinal cord, and brain including microglia. Modified from (120)
1.5.1 Galanin Actions in Acute Pain

As discussed, the differences in GAL₁ (92) and GAL₂ (121, 122) receptor distribution in DRG suggests this may relate to different roles modulating sensory information (102, 123). As a consequence of previous reports observing both anti-nociceptive and pro-nociceptive roles for GAL₁ and GAL₂, there is an ongoing debate about the roles of the individual receptors in modulating nociception (55). Galanin and ARM-1896 acting via peripheral GAL₂ has been shown to increase hyperalgesia to hindpaw injection of capsaicin, whereas GAL₁ agonist M617 was found to reduce capsaicin induced flinching (124). This supports the role of GAL₁ being anti-nociceptive and GAL₂ being pro-nociceptive. However, galanin over expressing (Gal-OE) mice have an increased pain tolerance and less sensitivity to noxious heat, when compared to WT animals (125). This suggests the overall effect of endogenously expressed galanin is anti-nociceptive. µ Opioids used for the treatment of acute pain, showed increased analgesia in the presence of galanin (1). Furthermore GAL₂ agonist ARM-1896 has been shown to presynaptically inhibit substance P release from the spinal cord, when applied centrally (44). In addition, actions mediated via GAL₂ have been shown to inhibit presynaptic transmission in primary afferent terminals of the DH (6). These findings support galanin acting via GAL₂ has an anti-nociceptive role. However, the role of GAL₂ in DRG neurons are unclear, and requires investigation.

1.5.2 Galanin Actions in Chronic Pain

With the knowledge of galanin up-regulation after nervous system injury, the current debate in the field is whether galanin has a predominantly pro or anti-nociceptive role in neuropathic pain,
and which galanin receptor(s) are involved. While transgenic animals have been utilised in to address these questions, the results are often difficult to interpret, particularly when galanin and GAL2 KO models lose a subset of DRG neurons during development (82, 83). In receptor KO, it is possible other galanin receptors may have overcompensated and be expressed more than under normal conditions, so that these studies must be interpreted with caution. Previous reports in transgenic animals demonstrated that while GAL-OE models developed neuropathic pain after sciatic nerve injury, the severity and duration of mechanical and heat hypersensitivity was less pronounced compared to WT controls (125). Furthermore, neuron activity was inhibited when galanin was applied to the spinal DH 7-14 days after sciatic nerve injury (126). In contrast, application of the galanin antagonist galantide increased neuron activity in second order nociceptive neurons of the spinal DH. This strongly suggests that endogenous galanin inhibits activity in nociceptive pathways following nerve injuries (126).

However, it is not always possible for nerve regeneration, and a lack of which is associated with the development of chronic pain conditions such as neuropathic pain (8).

Galanin up-regulation has been shown to be as a crucial growth factor required to promote neurite outgrowth and regeneration in the form of growth cones at the site of the damaged axons (109). Therefore it is important to understand the mechanism in which galanin is promoting neuroregeneration and reducing neural degenerative processes, and potential therapeutic use in the future. Studies on transgenic animals lacking galanin (Gal-KO) have shown reduced nerve regeneration and growth cone formation when compared to WT animals (77, 109). Upon application of galanin to the Gal-KO group, nerve regeneration improved, supporting the role of galanin as a neural regenerative factor after nerve injury (77). In support of this, Hokfelt et al.
demonstrated axotomy of the sciatic nerve resulted in elevated levels of galanin peptide-IR, which remained during the process of nerve repair and regeneration (43). Further evidence suggests this was via the cellular actions of GAL2 since GAL2 KO animals nerve regeneration was also impaired and could not be rescued by either galanin or ARM-1896. (82). As galanin is a non-specific receptor agonist, the lack of improvement of nerve regeneration suggests that GAL1 and GAL3 are not involved (82). In addition, recent evidence indicates GAL2-induced cellular mechanisms modulate cytoskeletal mechanisms involved in growth cone formation in both DRG neuron primary culture and PC12 neural cell line (127). Taken together, GAL2 is crucial for not only early neural development, but also neurite outgrowth and regeneration after nervous system injury. Furthermore, with GAL2 been shown to inhibit presynaptic transmission in the DH, it highlights the importance of understanding the cellular actions of GAL2 in DRG neurons which may become a therapeutic target in the future.

1.5.3 Central Mechanisms Influencing Nociception

Chronic pain following nerve injury is not only from peripheral mechanisms, including actions potentials from DRG neurons. ATP released from damaged neurons activates microglia, and as such central mechanisms can react to injury in peripheral sensory axons. In addition to ATP, other neuronal mediators can lead to microglial activation (128, 129). ATP plays an important role in the modulation of pain after nerve injury, which causes the release of ATP and increased expression of purinergic receptors in microglia resulting in symptoms of neuropathic pain (130). Indeed, P2X4,P2Y12 and other purinergic receptor activation has been implicated in the development of neuropathic pain (7, 131). As discussed, galanin is upregulated after nerve injury
and can modulate pain. It is currently unknown if galanin can affect the actions of microglia following nerve injury. Evidence of microglial activation involved in the modulation of pain (11), combined with microglial GAL2 expression and galanin up-regulation following nerve injury (5), suggest this is likely. Under normal physiological conditions, microglia provide CNS immunosurveillance, until detection of stimulants released from damaged neural tissue or infection results in activation (132). ATP released from damaged nervous tissue is detected by some of the purinergic receptors expressed in microglia (see chapter 5). It is known that the metabotropic GPCR P2Y family (133, 134), are involved in migration and chemotaxis (135), whereas the P2X ionotropic receptors and non-selective (K+, Na+, Ca2+) cation channels P2X4 and P2X7 are involved in Ca2+ influx induced activation of p38 mitogen activated kinase pathway (p38-MAP), and apoptosis (136). It is clear there is an up-regulation of many receptors during microglial activation, however the mechanisms involved are not fully understood (137).

LPS, a component of gram-negative bacteria cell walls, is known to activate Toll Like Receptors (TLRs) TLR2 and TLR4, was recently shown to also activate macrophage antigen complex (Mac1) comprised of CD11b/CD18 (10). It is currently known that microglial activation results in the increase in [Ca2+], leading to the production of ROS, nitric oxide (NO), and the release of pro-inflammatory cytokines (5, 11) and anti-inflammatory cytokines (see Fig 1.4) (138). While current knowledge is sparse, a recent report indicated the action of galanin via GAL2 inhibits LPS-induced cytokine release from microglia (5), however it is unclear what stage is galanin inhibiting this pathway. Furthermore, it is unclear whether galanin can modulate the activation of microglia by ATP, which is more relevant to understanding galanin’s actions in chronic pain.
Figure 1.4 Microglial Activation

Under normal conditions, ramified microglia survey the extracellular environment and morphological characteristics including a small soma and long processes. Upon detection of nerve damage (ATP release) or infection (LPS), ameboid microglia undergo morphological changes including a large soma and retraction of processes. As a result, amoeboid microglia lead to an increase intracellular \([\text{Ca}^{2+}]_i\), NO, ROS production cytokine release, migration and chemotaxis. On the other hand, minocycline inhibits \([\text{Ca}^{2+}]_i\), NO, ROS production although the target receptor is unknown. The effect of galanin on the modulation of microglial inflammatory pathways is unknown and need to be investigated. Modified from (139)
Healthy Neuron

Damaged Neuron

Minocycline

↑[Ca^{2+}],
↑ROS
↑TNFα
↑NO

Activated Microglia

Resting Microglia

Infection

(+/-) Galanin (Unclear)

(+/-) Microglia Activation:
LPS: Bacterial Infection
ATP: Neuronal Damage

(-) Microglia Inhibition:
Minocycline

(+/-) Galanin (Unclear)
1.6 Aims and Directions of Experimental Work

The research presented in this thesis will focus on key areas of galanin research into the cellular actions of galanin relevant to acute and chronic pain, which require clarification. Indeed, the action of galanin via GAL₁ and GAL₂ is implicated in the modulation of pain, and further investigation may provide insight into the mechanisms involved. It is known that galanin is up-regulated after nerve injury, and inhibits synaptic transmission via GAL₂ in the DH (6). By using a specific GAL₂ agonist ARM-1896, the current thesis has investigated the actions of GAL₂ activation in the population of small DRG neurons that are mainly nociceptive. While an ongoing issue has been the lack of availability of galanin receptor-specific pharmacological tools, ARM-1896 is the best freely available pharmacological tool to study GAL₂ receptors. As discussed, GAL₂ is present in a subset of DRG neurons, however questions still remain how this receptor acts due to an ability to couple with different G proteins. While galanin has been shown to inhibit N-type VGCC in mudpuppy ganglia, how galanin acts in DRG neurons in terms of target receptors and VGCC and calcium dynamics require more investigation (89). This thesis aims to provide clarification of the role of GAL₂ in DRG neurons, pertaining to GPCR coupling and preferential association with VGCC subtypes. In addition, while TRPV1 channels and galanin receptors are co-expressed in DRG neurons, the existence of any interaction between them is unknown. Considering that galanin modulates, and TRPV1 mediates nociception, the effect of galanin on TRPV1 agonist capsaicin-induced increase in [Ca²⁺]ᵢ is unknown. To address this gap in the literature, the potential effect of galanin on capsaicin responses were also investigated. However, examining the actions of galanin in DRG neurons alone, does not address the effect on all its potential cellular targets relevant to pain, for instance microglia.
Both galanin and microglia are separately implicated in the modulation of pain, yet there is little known about galanin acting via GAL₂ expressed in microglia during pathological responses leading to chronic pain. As mentioned, galanin is upregulated after nerve injury, and nerve damage can result in ATP-induced neuropathic pain conditions via P₂Y₁₂, P₂X₄ amongst other purinergic receptors (7, 131). Early evidence indicates galanin acting via GAL₂ can inhibit microglial cytokine release, which suggests galanin may have a role in modulating microglial inflammatory pathways (5). The process in which microglia become activated in response to ATP release from damaged nervous tissue or detection of LPS in the presence of infection is complicated, and in itself is not fully understood. The current thesis has therefore investigated the effect of galanin on inflammatory responses in microglia.

1.6.1 Overall Aims

1. To investigate the cellular mechanism by which galanin may modulate both peripheral and central nociceptive pathways.
2. To investigate the mechanism by which galanin inhibits VGCCs in nociceptive DRG neurons.
3. To determine the effect of galanin on ATP-induced microglial activation.

1.6.1.1 DRG Hypotheses

DRG hypotheses will be addressed in:

Chapter 3 (see hypotheses 1, 2) and chapter 4 (see hypotheses 3,4).

1. That galanin inhibits Ca²⁺ channels in small nociceptive DRG.
2. That this inhibition is specifically via GAL₂.
3. That galanin inhibits N-type VGCCs.
4. That galanin inhibits increases in $[\text{Ca}^{2+}]_i$, caused by TRPV1 agonist capsaicin.

1.6.1.2 Microglia Hypotheses

Microglia hypotheses will be addressed in chapter five.

1. That galanin inhibits $[\text{Ca}^{2+}]_i$ changes in ATP activated microglia.

2. That galanin inhibits $[\text{Ca}^{2+}]_i$ changes in LPS treated microglia.

3. That galanin inhibits microglial ROS, NO production, and morphological changes associated with activation.
CHAPTER 2:
MATERIALS AND METHODS

2.1 Materials

2.1.1 Drugs and Reagents

Dulbecco’s modified eagles medium (DMEM), nerve growth factor, glial derived nerve growth Factor, DNAse 1, trypsin and nifedepine were obtained from Sigma-Aldridge NSW, Australia. Collagenase type IV, soybean trypsin inhibitor were obtained from Worthington, penicillin (Worthington, Lakewood New Jersey.), whereas DAF-FM, and Fura-2-AM were obtained from Invitrogen, VIC, Australia. Foetal bovine serum (FBS) and bovine serum albumin (BSA) were obtained from Bovogen, Vic Australia. GPCR agonists DAMGO (Ala (2)-MePhe (4)-Gly (5)-enkephalin) and galanin were supplied from Auspep, NSW, Australia and Anaspec NSW, Australia respectively. GAL2 receptor agonists ARM-1896 was a kind gift from Astra Zenica, Canada. -connotoxin GVIA and -agatoxin IVA were supplied from Anaspec and Auspep respectively. Minocycline, PDBu, zymosan, ATP, LPS, and poly-D-lysine were from Sigma, and ionomycin was obtained from Calbiochem.

2.1.2 Chemicals

NaCl, KCl, MgCl2, HEPES, Glucose, CaCl2, TEA-Cl, BaCl2, TEA-OH, Na-OH, CsCl were obtained from Merck, VIC, Australia and Mg-ATP, 0.4 Na-GTP, 10 EGTA, 20 HEPES-CsOH were obtained from Sigma Aldrich, NSW, Australia.
2.2 Methods

2.2.1 Dissociation of Dorsal Root Ganglia

All animal procedures had prior approval by the RMIT Animal Ethics Committee. SD rats (3.5 ± 0.5 weeks) were anaesthetised with ketamine (60 mg/kg) and xylazine (10 mg/kg) and decapitated. DRG neurons were removed from the lumbar region of the spinal cord via spinal laminectomy, and then dissociated as described by White et al. (140), except that Type IV collagenase (Worthington) was used. After enzymatic dissociation, DRG neurons were centrifuged at 1200 revolutions per minute (RPM) for 4 minutes at room temperature. The pellet was resuspended and incubated overnight in DMEM-high glucose (1% penicillin and streptomycin, 10% FBS with NGF 5 ng/ml and GDNF 25 ng/ml) at 37°C, 5% CO₂ on sterile, glass chambers coated with Poly-D-Lysine Hydrobromide (PDL) (World Precision Instruments Inc. Sarasota, Fl.). DRG neurons were used acutely for electrophysiological recordings, (see section 2.2.4) or were incubated overnight at 37°C, 5% CO₂ for intracellular recordings (see section 2.2.5).

2.2.2 Preparation of Mixed Glial Culture

All animal procedures were conducted at the Baker IDI Heart and Diabetes Institute, and tissue collection was kindly permitted by Professor Woodcock. Brains from neonatal SD rats were collected and passed through a series of course (40) mesh using a syringe before being placed in DMEM-high glucose supplemented with 1% penicillin and streptomycin and 10% FBS. The mixed glial tissue was centrifuged at 1500 rpm for 5 minutes, and the pellet was resuspended in sterile PBS where the centrifuge steps were repeated to purify the tissue. The tissue was then enzymatically dissociated in sterile PBS supplemented with trypsin (0.16%) and 0.5 mg DNAse...
(0.01%) for 10 minutes at 37°C with 5% CO₂. The mixed glial tissue was passed through the fine (100) mesh before plating into tissue culture flasks coated with PDL, and supplemented with fresh DMEM-high glucose. The mixed glial culture was monitored regularly and DMEM was replaced as required.

2.2.3 Isolation of Microglia

After 14 days, the mixed glial cultures were used to extract microglia. Flasks containing the mixed glia were placed on a shaker for 120 rpm for 45 minutes at 37°C before collecting the supernatant containing microglia. Microglial culture purity was routinely checked in the laboratory using microglial OX-42 immunocytochemistry and found to be 98% or above (141, 142). The supernatant was plated onto sterile, glass chambers coated with PDL and supplemented overnight with fresh DMEM in the presence and absence of minocycline (10 µM, 100 µM).

2.2.4 Whole Cell Patch Clamp

The whole cell patch clamp technique was used to record Ca²⁺ currents of DRG neurons. DRG neurons were removed from SD rats (see section 2.2.1), except that DRG neurons were plated in DMEM-high glucose at 37°C, 5% CO₂ on sterile, 35 mm culture dishes for at least one hour prior to patch clamp recordings. All neurons were recorded on the day of dissociation. In order to record I[Ca²⁺], using Ba²⁺ as the change carrier, cells were superfused with external solution containing (in mM): 160 TEA-Cl, 10 HEPES, 2 BaCl₂, 10 glucose, adjusted to pH 7.4 with TEA-OH, and osmolarity of 330-340 Osm. Whole cell recordings were made using borosilicate glass patch electrodes (DC resistances 1–3 MΩ) which were previously cleaned with acetone and then
distilled water. Patch electrodes were made on the day of recordings and were fire polished before filling with internal solution contained (in mM): 120 CsCl, 5 Mg-ATP, 0.4 Na-GTP, 10 EGTA, 20 HEPES-CsOH, pH 7.2 and an osmolarity of 310-320 Osm.

Whole cell recordings of DRG neurons were performed using an Olympus IX70 inverted microscope, a faraday cage to reduce electrical interference and vibration isolation using a pressurized air table. A robotic micromanipulator was used to move the electrode, and recordings of DRG Ca\(^{2+}\) currents were captured using Digi Clamp 10 software. A micropipette filled with internal solution was fitted an electrode before being placed into the culture dish also containing an earth electrode, which completed an electrical circuit. The resistance of the electrode filled with the internal solution was measured, and was between (DC resistances 1–3 M\(\Omega\)). Positive air pressure was applied to the electrode to ensure the internal solution was flowing out of the micropipette. A robotic micromanipulator was used to allow an electrode to seal onto a single DRG neuron using a small amount of suction. The Digi Clamp 10 software then depolarized the DRG neuron with voltage steps from -90mV to -10mV. This results in an inward current due to VGCC, which was measured every twenty seconds.

2.2.5 Calcium Imaging

For intracellular calcium recordings, both DRG neurons and microglia (see section 2.2.1 and 2.2.3 respectively) were cultured overnight in preparation for Ca\(^{2+}\) imaging. On the day of experiments, cultured DRG neurons were washed with Krebs-HEPES Buffer (NaCl 14.8 mM, KCl 2.8 mM, MgCl\(_2\) 2 mM, HEPES 10 mM, Glucose10 mM, CaCl\(_2\) 2 mM, pH 7.4) and incubated in 2.5 ml Krebs-HEPES buffer containing Fura-2-AM (2 \(\mu\)M) and BSA (5 mg/ml) for
30 minutes at 37°C with 5% CO₂. Fura-2-AM solution was removed and replaced with 2 ml Krebs HEPES and cells were left in the dark for 30 minutes to allow removal of the ester group, and fluorescent labelling of the neurons.

Ca²⁺ Imaging was performed using an Olympus IX70 fluorescent inverted microscope, a polychrome IV tunable light source (TILL Photonics) and images captured using a cooled CCD camera (Sensicam, PCO) and Axon™ Imaging Workbench software. The Fura-2 was excited at alternating wavelengths of 340 and 380 nm for periods of 100 ms. The fluorescence was measured at 510 nm and the intracellular Ca²⁺ concentration quantified as the ratio of the fluorescence intensity at the two excitation wavelengths. In separate recordings, DRG neurons and microglia were continuously superfused with KREBS HEPES buffer, and drug solutions applied via the inflow lines.

2.2.6 Nitric Oxide Assay
Nitric oxide production in microglia was investigated using light microscopy. Microglia were pre-treated with DMEM or LPS (100 ng/ml, 1 µg/ml) for 24 hours before labelling with the fluorescent dye DAF-FM, which increases in fluorescence intensity in the presence of NO production via iNOS expression. Microglia were labeled with DAF-FM for 30 minutes and cleavage step in KREBS HEPES buffer as described in calcium imaging (see section 2.2.5). Fluorescence intensity at excitation and emission 495 /515 nm wavelengths were examined using NIS viewer microscope software.
2.2.7 Reactive Oxygen Species Assay

Microglia were plated for at least 2 hours in a PDL coated 96 well plate prior to labeling with ROS indicator DCFDA for 30 minutes. After DCFDA removal, 1 hour treatments of galanin (1 pM, 100 nM, 1 µM) in the presence and absence of LPS (100 ng/ml, 1 µg/ml) strains (O26:B6, O111:B4), ATP (3 mM), zymosan (0.05 mg/ml), positive control phorbol 12,13-dibutyrate (PDBu, 100 ng/ml) and negative control KREBS HEPES buffer. ROS production from microglia was quantified using DCFDA (excitation and emission 488/530 nm) and was measured for a total of 60 minutes at 15 minute intervals, using the Flex station Plate Reader and Analysis Software.

2.2.8 Analysis Software

Analysis was performed using Graph Pad Prism, Igor Pro and Microsoft Excel. In addition, analysis using Axon Imaging Workbench and Clamp Fit was performed for Ca²⁺ imaging and patch clamp results respectively. Flex Station software was used to analyse data from microglial ROS production assays.

2.2.9 Data Analysis

All data was analyzed to a significance level of p<0.05. Paired and unpaired t-tests were used when comparing pre and post-treatment or two treatment groups respectively. Otherwise, when multiple comparisons between treatment groups was required, one way ANOVA with Dunnett’s post-hoc test was used. A chi-squared (x²) test with Yates’ continuity correction, was used when comparing proportion of cells inhibited by galanin in the presence and absence of CTX, AGX and caffeine. Please refer to chapter 4, section 2, equation 4.1.
CHAPTER 3:
INVESTIGATION OF G PROTEIN COUPLED RECEPTORS AND THEIR EFFECT ON INTRACELLULAR CALCIUM IN DRG NEURONS IN VITRO

3.1 Introduction

As discussed in chapter 1, galanin is present in both the DRG and superficial DH of the spinal cord that form part of the nociceptive pathway (55). The focus of this chapter was to investigate the mechanism of action of galanin via GPCRs GAL1 and GAL2 in the DRG neurons of the nociceptive pathway. More specifically, the effect of galanin on the modulation of \([\text{Ca}^{2+}]_i\) in DRG neurons was investigated. These neurons have cell bodies located in the PNS, and project to the DH of the spinal cord in the CNS, thus providing a crucial platform to study the likely effects of galanin in the nociceptive pathway.

What is known so far, is that the GAL1 receptor is predominantly expressed in large DRG neurons (92) and interneurons of the DH (85). However, there is controversy in the literature as to whether or not GAL1 and GAL2 receptors expression overlaps amongst different sized DRG neurons (86). GAL2 is expressed in small, mainly nociceptive DRG neurons and afferent terminals of the DH (121, 122). DRG neurons of this small size range (20-27µm) are more likely to be nociceptive and respond to capsaicin due to the presence of TRPV1 channels. Responses to TRPV1 agonists only occurs in nociceptive neurons (143), although not all nociceptors express TRPV1 (47). In fact, there is already evidence of GAL2 and TRPV1 co-expression in DRG neurons (118).

As DRG neurons are heterogeneous by nature, the differences in receptor localisation in subsets of these neurons suggest that each galanin receptor may play a different role in sensory systems and their modulation after nerve injury (102, 123). Indeed, nerve injury resulting in neuropathic
pain leads to an up-regulation of galanin peptide expression (100, 113), and its release in spinal cord (114). Galanin can inhibit nociceptive responses and has also been shown to enhance the analgesic effects of the μ opioid agonist morphine at the spinal level (1, 144). The ability to improve the action of an acute pain treatment used clinically, suggests galanin has a potential role in the development of new analgesic treatments. The known effects of peptide μ opioid DAMGO used in vitro, have been used as a comparison to investigate the effects of galanin agonists in this chapter.

The actions of galanin in the nociceptive pathway via the GPCR’s GAL1 and GAL2, are quite complex. In addition, there is also a lack of specific immunohistochemical tools to study galanin receptors (81). This is complicated further by the limited availability of receptor-specific pharmacological tools, and of those available, most are partial agonists/antagonists depending on the concentration (see chapter 1) (3). One of the few known receptor specific agonists, ARM-1896, has been used in this chapter to investigate the effects of galanin via GAL2 and has been shown to have no effect on GAL1 at the concentrations tested (88, 95). There is also a lack of receptor-specific antagonists. To overcome these issues, transgenic animal models, both galanin and receptor gene KOs have been used in an attempt to investigate the physiological role of galanin, but often the outcomes are complicated by developmental changes (82, 83). As a result, there is intense interest in the role of galanin in the nociceptive pathway, but many gaps remain in the literature. One study using whole cell patch clamp in DRG, suggests galanin increases HVA Ca2+ currents (86). However studies in other neurons have demonstrated that galanin inhibits Ca2+ via G proteins acting directly on Ca2+ ion channels (89, 94). To date, no studies have investigated the effects of galanin on DRG neurons using Ca2+ imaging.
Therefore my intention was to provide insight into the effects of galanin on Ca$^{2+}$ signalling in DRG neurons, using both Ca$^{2+}$ imaging and whole cell patch clamp. The purpose of this chapter was to investigate if galanin inhibits Ca$^{2+}$ channels in DRG, and if this was comparable to a known inhibitor of Ca$^{2+}$ transients, µ opioid peptide agonist DAMGO. In addition, this chapter aimed to determine if effects of galanin were specific to the GAL$_2$ receptor, and if they occurred in nociceptive or non-nociceptive neurons.

3.2 Materials and Methods

Drugs, reagents and chemicals used in the current chapter are described in chapter 2 (see section 2.1). DRG were dissociated as described in chapter 2, (see section 2.2.1), and used acutely for whole cell patch clamp or cultured overnight prior to recordings using Ca$^{2+}$ imaging. All protocols, data analysis and statistics were performed as described in chapter 2, (see section 2.8 and 2.9). Protocols specific to studies of DRG neurons in both techniques are as follows:
3.2.1 Calcium Imaging Recordings of DRG Neurons

Continuously superfused DRG neurons were depolarised with Krebs HEPES containing KCl (30 mM) substituted for NaCl to maintain osmolarity, or capsaicin (1 M) for 30 seconds and the change in [Ca^{2+}] recorded. Galanin receptor agonists and DAMGO were applied for 60 seconds prior to application of KCl or capsaicin and were also included in the solution used to depolarise the neurons. Washout periods of at least 15 minutes were allowed between drug applications, during which the microscope shutter was closed to prevent bleaching of the Fura-2 dye. Responses were measured as the amplitude above the baseline fluorescence ratio level immediately prior to KCl application. DRG recordings were accepted if response amplitudes were >0.25 ratio units. When brief applications of KCl (30 mM, 30 s) were repeated at intervals of 15 minutes under control conditions, the amplitude of the response was consistent for up to 2.5 hours. As DRG neurons are a heterogenous population, it was expected that not all neurons would respond to each treatment. As control responses were very reproducible, it was possible to detect inhibitory responses in individual neurons if that response was large enough. As such, DRG studies are represented as proportion of the population which responded to various treatments. An inhibition was taken to occur if the amplitude of the response in the presence of a drug was reduced by >5% in comparison to the average of both pre and post control responses to KCl (Fig 3.1A). This value was chosen based on the variability of control experiments, in order to provide a conservative measure for the extent of the inhibition taking place.
Figure 3.1 Recording Protocols for DRG Neurons

(A) The Ca$^{2+}$ Imaging Protocol for recording changes in [Ca$^{2+}$]$_i$ in DRG neurons. Traces show changes in [Ca$^{2+}$]$_i$ in a DRG neuron labelled with Fura-2 quantified as the ratio of the two excitation wavelengths 340 and 380nm, and measured at 510 nm. Transient increases in [Ca$^{2+}$]$_i$ occurred when the neuron was depolarised with 30mM KCl, in the presence and absence of treatment. (B) A whole cell patch clamp recording of a DRG neuron, indicating voltage steps (lower panel) from -90 mV to -10 mV and stepped back to -90 mV, via an intermediate step to -40 mV. Voltage steps to depolarize the DRG elicited an inward calcium current (upper panel) and were applied every 20 seconds. Responses were measured as the leak subtracted current at -10 mV immediately prior, during and after treatments were applied via a fast solution changer.
3.2.2 Whole Cell Patch Clamp of DRG Neurons

DRG neurons were superfused with solutions that were designed to isolate Ca\(^{2+}\) currents, as described in chapter 2, (see section 2.2.4) where Ba\(^{2+}\) was used as the charge carrier. Glass patch electrodes (DC resistances 3–5 MΩ) were used to seal onto and break through the cell membrane, so as to obtain whole cell patch clamp recordings. Voltage steps from -90 mV to -10 mV depolarized the DRG resulting in an inward current (Fig 3.1B). One minute applications of galanin and ARM-1896 at various concentrations and DAMGO (1 µM) were applied via close application using a gravity-fed, fast solution changer mounted on a micromanipulator, and Ca\(^{2+}\) currents measurements taken every 20 seconds. When repeat applications of the above treatments were applied, there was a washout period of 5 minutes between treatments. An inhibition was taken to occur if the Ca\(^{2+}\) currents in the presence of a drug was reduced by >2%.

3.3 Results

3.3.1 Effect of Galanin Receptor Activation on Calcium Transients Using Ca\(^{2+}\) Imaging

As discussed in chapter 1, DRG neurons are a heterogeneous population, with respect to galanin receptor expression, which was reflected in their responses to galanin. When galanin (1 µM) was applied to DRG neurons, in the majority of cases the amplitude response to KCl was reduced on average by 18± 2% and this inhibition was reversed upon washout (Fig 3.2). Repeated applications of galanin consistently and reproducibly inhibited responses to KCl (Fig 3.4 B). Overall the second application of galanin produced 15 ± 4% inhibition of calcium transients (n = 4, p > 0.26, paired t-test versus first response; Fig 3.4 B). Inhibition by galanin of Ca\(^{2+}\) transients was consistent at all concentrations tested (100 nM, 300 nM, 1 µM), suggesting the EC\(_{50}\) of the receptor was below the range tested (effect of concentration = n/s p>0.05, one-way ANOVA, Fig
3.2 C). Of the DRG treated with galanin, the proportion of neurons inhibited was 84 percent (16/19), which was comparable to the proportion of neurons inhibited by ARM-1896 (6/12) and DAMGO (6/13), using a chi-squared test (n/s, $x^2$ test, Fig 3.4 C). Of the 84 percent of neurons inhibited by galanin, 70 percent of DRG neurons were activated by capsaicin (1 M), indicating they were nociceptive (Fig. 3.4 D).

KCl-induced Ca$^{2+}$ transients were also inhibited by the GAL$_2$ selective agonist ARM-1896 (1 M) in half of the neurons tested by an average of 16± 2% (Fig 3.3A). Application of ARM-1896 at lower concentrations (100 nM, 300 nM) demonstrated comparable levels of Ca$^{2+}$ transient inhibition suggesting the EC$_{50}$ was below the range tested (Fig 3.3c). As for the galanin results, statistical analysis determined there was no significant effect of concentration over the range tested (one-way ANOVA, p>0.05). Of the 50% (6/12) of DRG neurons inhibited by ARM-1896 (Fig 3.4 C), 83% of neurons were activated by capsaicin (Fig 3.4 D). Thus indicating the majority of GAL$_2$ specific inhibitions by galanin occurred in nociceptive neurons.
3.3.2 Effect of DAMGO on Calcium Transients in Dissociated DRG Neurons

Responses to KCl were recorded before, during and after application of the μ-opioid agonist DAMGO (1 μM, Fig. 3.4 A). KCl response amplitudes were reduced in the presence of DAMGO and the reduction was reversed upon washout. DAMGO reduced the amplitude of KCl-induced Ca\(^{2+}\) transients by an average 18±4% which was comparable to all GPCR agonists tested (one-way ANOVA, p>0.05, Fig3.4 B). Of the DRG neurons treated with DAMGO, 46% were inhibited (Fig 3.4 C). Of the 46% of DRG inhibited by DAMGO, 50% were activated by capsaicin (1 μM), suggesting a proportion of these DAMGO sensitive DRG neurons were nociceptive (Fig 3.4 D). The proportion of neurons responding to capsaicin was comparable across all treatments tested (n/s, \(x^2\) test, Fig 3.4 D).
Figure 3.2 Inhibition of Calcium Transients in DRG Neurons Treated with Galanin.

(A-B) Recordings of \([Ca^{2+}]_i\) from individual DRG neurons using \(Ca^{2+}\) imaging during application of various concentrations of galanin as indicated by horizontal bars. Transient changes in \(Ca^{2+}\) were induced by 30 mM KCl. (A) Galanin (1 μM), inhibited the \(Ca^{2+}\) transients in this neuron by 16 percent compared to control amplitudes. This neuron responded to capsaicin (1 μM) indicating the presence of TRPV1 receptors. (B) In another neuron Galanin (100 nM) inhibited \(Ca^{2+}\) transients by 19% when compared to control. As in (A), this also occurred in a capsaicin-sensitive neuron.

(C) The percentage inhibition of the response amplitude caused by galanin at 100 nM (n= 15), 300 nM (n = 9) and 1μM (n = 16) Bars represent mean ± S.E.M.
Figure 3.3 Inhibition of Calcium Transients in DRG Neurons Treated with Galanin.

(A-B) Intracellular Ca²⁺ recordings from DRG neurons treated with GAL₂ receptor agonist ARM-1896 at various concentrations. Ca²⁺ transients were induced by 30 mM KCl.

(A) ARM-1896 (1 μM) inhibited Ca²⁺ transients by 19% in this capsaicin-responsive neuron.

(B) In another neuron ARM-1896 (300 nM) demonstrated 11% inhibition of Ca²⁺ transients in a capsaicin-sensitive neuron. (C) The percentage inhibition of the response amplitude by ARM-1896 100 nM (n= 4), 300 nM (n = 4) and 1 μM (n = 6) respectively. All concentrations tested demonstrated comparable percent inhibitions of Ca²⁺ transients, (one-way ANOVA). Bars represent mean ± S.E.M.
A

KCl  KCl  KCl  Capsaicin
ARM-1896 1μM

B

KCl  KCl  KCl  Capsaicin
ARM-1896 300 nM

C

<table>
<thead>
<tr>
<th>Concentration</th>
<th>n</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td>100nM</td>
<td>4</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>300nM</td>
<td>4</td>
<td>20 ± 2</td>
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<tr>
<td>1μM</td>
<td>6</td>
<td>20 ± 2</td>
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Figure 3.4 Inhibition of Calcium Transients by GPCR Agonists in Nociceptive DRG Neurons.

(A) DAMGO (1 μM), inhibited the $Ca^{2+}$ transients in this neuron by 12% compared to control. This neuron lacked a response to capsaicin (1 μM) indicating an absence of TRPV1 receptors.

(B) The percentage inhibition of the response amplitude caused by galanin (n = 16), ARM 1896 (n = 6) and DAMGO (n = 6) were all comparable. A second application of galanin (n=4) also produced a similar degree of inhibition. Bars represent mean ± S.E.M. (C) The proportion of DRG neurons inhibited by GPCR agonists (1μM) were comparable ($x^2$ squared test, Yates continuity correction). Galanin inhibited 84% (16/19) of DRG tested, whereas 50% (6/12) of ARM-1896 and 46% (6/13) of DAMGO treated DRG were inhibited. (D) Of the DRG neurons inhibited by GPCR agonists, the proportion which also responded to capsaicin was quantified, and found to be comparable across treatments tested ($x^2$ squared test, Yates continuity correction). Eighty three percent of neurons inhibited by ARM-1896, 70% of galanin and 50% of DAMGO were capsaicin sensitive.
3.3.3 Effect of Galanin Receptor Activation on HVA Ca\(^{2+}\) Currents

When applied to DRG neurons during whole cell patch clamp, galanin (1 µM) inhibited Ca\(^{2+}\) channel currents by an average of 17 percent, which was partially reversed upon washout (Fig 3.5 A). Galanin responses were reproducible, with an average of 17 percent Ca\(^{2+}\) channel current inhibition of 17 percent for both first (17%) and second (17%) applications (p>0.05, t-test, Fig 3.5 B,C). Of the concentrations of galanin tested, an apparently smaller proportion of DRG neurons responded to the lower concentration tested with 5 of 7 DRG neurons at 100 nM versus 12 of 13 DRG neurons treated with galanin 1 µM. Galanin had significantly lower average percentage inhibitions of Ca\(^{2+}\) channel currents at 100 nM (4.5%) in comparison to 1 µM (17%), indicating a concentration dependant effect, (p<0.05, t-test, Fig 3.5 D).

3.3.4 Effect of ARM-1896: GAL2 Receptor Activation on HVA Ca\(^{2+}\) Currents

ARM-1896 (1 µM) inhibited Ca\(^{2+}\) channel currents suggesting the effect was via GAL\(_2\), which was at least partially reversed upon washout (Fig 3.6 A). As seen with galanin responses, ARM-1896 (1 µM) responses were reproducible, with comparable average percent inhibition of Ca\(^{2+}\) channel currents for first and second applications of 39 percent and 32 percent respectively (p<0.05, t-test, Fig 3.6 B,C). Consistent with results for galanin, ARM-1896 demonstrated concentration dependant effects at higher concentrations tested, with only 6 percent inhibition seen at 100 nM (9 of 9 DRG) compared to 38 percent and 39.6 percent at 300 nM (6 of 8 DRG) and 1µM (11 of 12 DRG) respectively. ARM-1896 (100 nM) demonstrated significantly lower average percent inhibition of Ca\(^{2+}\) channel currents when compared to the higher concentrations tested (ARM-1896 100 nM versus 300 nM, p<0.05, and 1µM, p<0.01, one-way ANOVA post-hoc Dunnett’s test, Fig 3.6 D).
Of the galanin sensitive neurons, 12 of 13 DRG neurons were also inhibited by DAMGO (1 μM), as represented in (Fig 3.7 A) as a time course of this response. Similar to repeated galanin (Fig 3.7 B) and ARM-1896 (Fig 3.7 C) responses, DAMGO demonstrated comparable Ca$^{2+}$ channel currents inhibition after first (22 %) and second (20 %) applications (p>0.05, t-test, Fig 3.7 D). The amount of Ca$^{2+}$ channel current inhibition demonstrated by galanin (1 μM) and that seen by DAMGO (1 μM) was comparable (p>0.05, one-way ANOVA). However, ARM-1896 (1 μM) demonstrated significantly higher levels of Ca$^{2+}$ channel current inhibition when compared to both galanin (1 μM) and DAMGO (1 μM) inhibition (p<0.01, one-way ANOVA, post-hoc Dunnett’s test, Fig 3.7 E).
Figure 3.5 Whole Cell Patch Clamp Recordings of Calcium Channel Currents: Effect of Galanin

(A-C) Whole cell patch clamp recordings using solutions to isolate Ca\textsuperscript{2+} channel currents and Ba\textsuperscript{2+} as the charge carrier. Cells were activated using voltage steps from -90 mV to -10 mV, resulting in an inward current. (A) Galanin (1 µM) inhibited Ca\textsuperscript{2+} channel currents in a neuron by 34%. Arrows indicate initial baseline, galanin treatment, and washout. Lower panel indicates the voltage protocol used to generate inward currents (upper panel). (B) Graph showing time course of inhibition by galanin in another neuron. A first application of galanin inhibited Ca\textsuperscript{2+} channel currents by 20%. (C) In the same neuron as (B), a second application of galanin inhibited Ca\textsuperscript{2+} channel currents by 19%, which was reversed upon washout. (D) Percent inhibition of Ca\textsuperscript{2+} channel currents by galanin was concentration dependent (galanin 1 µM versus galanin 100 nM, * = p<0.05, t-test). Bars represent mean ± SEM.
Figure 3.6 Calcium Channel Current Recordings From DRG Neurons: Effect of ARM-1896

(A) Whole cell patch clamp recording of a DRG neuron treated with ARM-1896 (1 µM) showing inhibition of $\text{Ca}^{2+}$ channel currents by 41 percent. Upper panel arrows indicate initial baseline, ARM-1896 treatment, and washout. Lower panel indicates the voltage steps from -90 mV to -10 mV which results in inward current (upper panel). (B) Time course of the change in $\text{Ca}^{2+}$ channel currents indicating the 41% inhibition of calcium transients by ARM-1896 (1 µM). (C) Application of ARM-1896 at 100 nM inhibits $\text{Ca}^{2+}$ transients in another neuron by 7%. (D) Percent inhibition of calcium currents at the range of ARM-1896 concentrations tested. * = $p<0.05$, ** = $p<0.01$ versus ARM-1896 100 nM (one-way ANOVA, pos-hoc Dunnett’s test). Bars represent mean ± SEM.
A

B

C

D

% Inhibition of IBa by ARM-1896

ARM-1896
Figure 3.7 Repeated Inhibition of Calcium Channel Currents by GPCRs: Investigations using Whole Cell Patch Clamp.

(A) Whole cell patch recordings showing inhibition of Ca\(^{2+}\) channel currents by both galanin (1 µM) (20%) and DAMGO (22 %), in the same neuron. (B) Percent inhibition of Ca\(^{2+}\) currents was comparable between 1st and 2nd application of galanin. (C) Percent inhibition of Ca\(^{2+}\) currents by ARM-1896 applied repeatedly was also comparable. (D) Similar to data shown in (Fig3.7 B,C), 1st and 2nd applications of DAMGO resulted in comparable levels of inhibition of Ca\(^{2+}\) currents. (E) Comparison of percent inhibition of Ca\(^{2+}\) currents by various GPCR agonists (1µM) after one application. ** = p<0.01 versus ARM-1896 1 µM (one-way ANOVA, post-hoc Dunnett’s test). Bars represent mean ± SEM.
3.4 Discussion

The results presented in this chapter demonstrate that the neuropeptide galanin inhibits both HVA Ca\(^{2+}\) channel currents and depolarisation-induced Ca\(^{2+}\) transients in nociceptive DRG neurons, as seen in both whole cell patch clamp and Ca\(^{2+}\) imaging. In the current study, repeat applications of galanin, ARM-1896 and DAMGO demonstrated an inhibition of Ca\(^{2+}\)-channel currents that was reproducible. This was also the case for Ca\(^{2+}\) imaging recordings, which showed no evidence of response desensitisation for galanin, ARM-1896 and DAMGO (1 µM). In Ca\(^{2+}\) imaging experiments, the average percentage inhibition of Ca\(^{2+}\) transients was comparable between galanin, ARM-1896 and DAMGO, suggesting galanin’s inhibitory effects are via GAL\(_2\) and that galanin can inhibit nociceptors as potently as a µ-opioid agonist. However in whole cell patch clamp experiments, whilst Ca\(^{2+}\)-channel current inhibition was comparable between galanin and DAMGO, their inhibition was significantly lower than that by ARM-1896. The Ca\(^{2+}\) channel inhibition by both galanin and ARM-1896 was concentration dependent, but this effect appeared to require higher concentrations of GAL\(_2\) agonists, when compared to their inhibition of Ca\(^{2+}\) transients.

3.4.1 Galanin Inhibits HVA Ca\(^{2+}\) Currents and Ca\(^{2+}\) Transients in DRG Neurons via GAL\(_2\) Activation

The size of the inhibition of Ca\(^{2+}\) responses by galanin agonists in DRG neurons was dependant on the recording technique used. Ca\(^{2+}\) imaging results indicated ARM-1896 had comparable inhibition levels to that of galanin. In contrast, the percent inhibition of Ca\(^{2+}\) channel currents by ARM-1896 (1 µM) was significantly greater than the percent inhibition produced by galanin (1 µM). While findings from both techniques indicate that galanin receptor agonists cause
inhibition in DRG neurons via GAL₂, it is unclear as to why the effects of ARM-1896 are more pronounced in whole cell patch recordings. It is clear, however, the two techniques do not measure exactly the same thing. As described in Chapter 2, whole cell patch clamp solutions isolate the Ca²⁺ channel current, allowing direct recording of the Ca²⁺ channel activity. In comparison, Ca²⁺ imaging records the total change in intracellular Ca²⁺ concentration. This comprises both KCl depolarisation-induced opening of VGCCs and in small DRG neurons, can also involve store-operated Ca²⁺ influx and release from stores (119).

3.4.2 Mechanism of Galanin’s Actions in DRG Neurons

As discussed, whole cell patch clamp recordings using a fast application system demonstrated repeatable inhibitions by galanin, ARM-1896 and DAMGO. However, whole cell patch clamp recordings obtained previously by our laboratory provided evidence that galanin responses desensitise (145) (unpublished). Differences between the previous and the current whole cell protocol may account for the different behaviour of the galanin agonist responses seen during applications. The response desensitisation occurring in the earlier whole cell patch recordings may be due to the patch electrode taking up intracellular contents of the neuron, leading to the washout of intracellular components of the receptor signalling pathway. The glass electrodes used for this chapter were of a higher resistance than those used previously. A higher resistance electrode with a smaller opening of the electrode contacting the cell membrane would reduce the rate of intracellular contents leaving the cell and apparently made responses more robust. Indeed similar findings were reported by Merriam et al. using whole cell patch clamp to record galanin responses from parasympathetic neurons (89). Another patch clamp study, this time in DRG neurons, determined that galanin had concentration dependant effects on Ca²⁺ currents in small
DRG neurons (86). In particular, that low galanin concentrations ranging from $10^{-10}$ to $10^{-6}$ M augmented Ca$^{2+}$ channel currents. In contrast, the current experiments showed that galanin (1 µM) inhibited HVA Ca$^{2+}$ channel currents during patch clamp experiments, rather than augmenting them. In addition, galanin (100 nM) inhibited Ca$^{2+}$ transients during our Ca$^{2+}$ imaging recordings. A possible explanation of the differences in results may be related to the method of drug application. The current study used a fast application system for patch clamp experiments, which allowed a fast washout of the drug after treatment. In contrast to the current study, Kerekes et al. (86), used a slow application system which may have led to responses becoming desensitised, and therefore an absence of an inhibition of Ca$^{2+}$ channel currents.

The inhibitory effects of galanin in this study in both whole cell patch clamp and Ca$^{2+}$ imaging were comparable to that of the µ-opioid agonist DAMGO. In addition, all DRG neurons treated with both galanin (1 µM) and µ-opioid peptide agonist DAMGO (1 µM) demonstrated inhibition of Ca$^{2+}$ channel currents when applied in the same neuron. Furthermore, inhibition of Ca$^{2+}$ transients by galanin and ARM-1896, occurred in a similar proportion of nociceptive neurons to that inhibited by DAMGO. These findings compliment previous studies showing that galanin improves morphine’s anti-nociceptive effects (1), and can inhibit nociceptive reflexes and substance P release from primary afferent terminals (144, 146). This suggests galanin agonists may specifically inhibit synaptic transmission from nociceptive nerve endings to DH neurons, and have the potential to be developed as an alternative or an adjunct to morphine to treat acute pain (6).
3.4.3 Galanin’s Effects in the Modulation of Pain

The results presented here suggest an anti-nociceptive role for galanin agonists acting via GAL₂. However galanin has demonstrated both pro and anti-nociceptive roles depending on the concentration tested (146), if the treatment duration is acute or chronic, and the site of application (95). Due to a lack of receptor specific pharmacological tools, determining which receptor mediates which effect, becomes problematic (3). Studies using galanin and GAL₂ receptor knockout models have been used to address this, but as previously mentioned (see chapter 1), often gives rise to complicated findings (82, 83). However, galanin over-expressing mice have shown an increased pain tolerance and decreased sensitization after nerve injury, suggesting an anti-nociceptive effect (125, 147, 148). The ARM-1896 inhibition of Ca²⁺ transients and Ca²⁺ channel currents in the current thesis provide strong evidence that the inhibition seen in DRG neurons is mediated via GAL₂. This is the first study demonstrating GAL₂ mediated inhibition of calcium responses in DRG neurons using Ca²⁺ imaging, and to date there are no reports of GAL₂ inhibition of calcium currents in expression systems (69). The ARM-1896 concentrations presented here do not act on GAL₁ (88, 95), and cannot be binding to GAL₃ as the third receptor subtype is not present in DRG neurons (92). The concentration dependence of the effect of galanin and ARM-1896 on Ca²⁺ transients was consistent with their published EC₅₀ values in the low nM range (95). In contrast, the inhibition of Ca²⁺ channel currents required higher concentrations of both galanin and ARM-1896 (> 300 nM). While this is difficult to explain, it is consistent with some of the reported actions of galanin receptor agonists via GAL₂ in vivo. More specifically, a recent study demonstrated high concentrations of galanin and ARM-1896, acting via peripheral GAL₂ inhibited nerve injury-induced pain states (149). Furthermore, high concentrations of intrathecal galanin inhibited the nociceptive flexor reflex in
GAL₁-KO mice *in vivo*, suggesting the effect was via GAL₂ (150). These findings combined with recent evidence of GAL₂ inhibition of presynaptic transmission of primary afferent terminals of the DH (6), provides further evidence that GAL₂ may be responsible for antinociceptive effects of galanin. From the Ca²⁺ imaging results, the majority of the DRG neurons responding to galanin or ARM-1896, also responded to capsaicin, indicating they were nociceptive. Thus providing further evidence of co-expression of GAL₂ and TRPV1 channels in a subset of DRG neurons (118).

The fact that ARM-1896 and galanin inhibited Ca²⁺ transients at low concentrations whereas higher concentrations were required to inhibit Ca²⁺ channel currents is intriguing. One possible explanation for this is that the inhibitory responses seen with the different techniques actually occur via different cellular mechanisms. It is conceivable that the galanin receptor agonists bind to the same receptor, yet activate different pathways dependant on the concentration tested. Indeed, GAL₂ is capable of signalling via Gi/o and Gq/11 g proteins (55). This phenomenon has recently been shown to occur with other GPCRs, where different agonists can induce signalling in different pathways through the same receptor, termed “biased signalling” (151). In the case of GAL₂, there may be a concentration dependant bias towards a particular pathway. In support of this, most galanin receptor antagonists are reported to have concentration dependant agonist activity (3, 42), which suggests that biased GPCR signalling is possible through galanin receptor activation. Biased GPCR signalling may also explain why ARM-1896 demonstrated a significantly more pronounced inhibition of Ca²⁺ channel currents than that by galanin (1 µM) in whole cell patch clamp experiments, but not in Ca²⁺ imaging experiments. Thus ARM-1896 may be better at activating inhibition at higher concentrations that galanin, even though the two have
roughly the same affinity for binding to the receptor at low concentrations (95). Although quite complicated, this seems to offer the most logical explanation for the current results and would also account for much of the seemingly contradictory data on galanin receptor effects in vivo.

Furthermore, different subtypes of g proteins can associate with different VGCCs, including N and P/Q-type channels (152). This suggests that the GAL2 receptor may be coupling via different G proteins and inhibit different VGCCs depending on the concentrations. The current chapter has established that galanin activation of the GAL2 receptor inhibits VGCC and Ca\(^{2+}\) transients in DRG neurons. However, the mechanisms in which this inhibition occurs and which VGCC type is involved, will be evaluated in chapter 4.
3.4.4 Conclusions

In conclusion, the results presented here demonstrate that galanin can inhibit Ca^{2+} channels in nociceptive sensory neurons. Furthermore, inhibition of Ca^{2+} transients and Ca^{2+} channel currents specifically by ARM-1896, suggests the inhibition of Ca^{2+} channels is mediated primarily via GAL\textsubscript{2}. Taken together with evidence from \textit{in vivo} studies (6), my results support the anti-nociceptive role of GAL\textsubscript{2}, and suggest galanin may play an important role in inhibiting nociceptive transmission. Furthermore, galanin receptor agonists demonstrated comparable inhibition to that of \mu- opioid peptide agonists DAMGO, suggesting galanin agonists may be developed as an alternative analgesic to morphine. This seems likely, considering galanin has already been shown to augment the analgesic effects of morphine. In addition, the responses were concentration dependant and reproducible; which suggest galanin receptor agonists, particularly acting via GAL\textsubscript{2}, may be used to develop alternative analgesics to those used clinically. As galanin is up-regulated after nerve injury and GAL\textsubscript{2} agonists can inhibit injury induced nerve activity, galanin agonists acting via GAL\textsubscript{2} may also be useful in the development of treatments for chronic pain states including neuropathic pain. While high concentrations were required for Gal\textsubscript{2} activation in the current study, this may be partially due to being in peptide form. However it is possible upon application of an agonist in a non-hydrolysable form, to have an enhanced interaction with the receptor and an increased level of inhibition. While not all nerve injuries lead to the development of chronic pain states, galanin agonists acting via GAL\textsubscript{2} may be useful as an alternative analgesic to morphine in acute pain.
CHAPTER 4:
INVESTIGATION OF VOLTAGE GATED CALCIUM CHANNELS AND INTRACELLULAR CALCIUM IN DRG NEURONS IN VITRO

4.1 Introduction
Calcium influx through VGCCs allows this ion to act as an important signalling molecule in synaptic transmission, and in many biological pathways including nociception (153). GPCRs modulate neurotransmitter release at nerve terminals by regulating the actions of VGCCs (154). Galanin can modulate synaptic transmission via inhibition of VGCCs, and has also been shown to have a role in modulating nociception (83). From the current study (see chapter 3), galanin inhibits VGCC currents and KCl-induced Ca\(^{2+}\) transients in DRG neurons. Inhibition by galanin of VGCC is apparently mediated via GAL\(_2\), but the mechanism involved remains unclear, including the type(s) of VGCC inhibited.

Targeting of different subtypes of VGCCs by GPCRs leads to regulation of diverse intracellular processes (155). With the exception of T-type channels, classified as being low-voltage activated (LVA), the remaining subtypes N, L, P/Q and R VGCC are HVA channels (116). HVA Ca\(^{2+}\) channels initiate the release of neurotransmitters at nerve terminals, and the various subtypes have different pharmacological responses to various toxins (156). For example, N-type VGCC actions are blocked in the presence of CTX, a peptide toxin produced by marine cone snails (157). P/Q-type VGCC are CTX-resistant, yet are blocked by AGX, one of several agatoxins produced by Funnel Web spiders (158). The research performed in the current chapter has utilised this pharmacological differentiation of VGCCs to investigate which VGCCs are inhibited by galanin in DRG neurons. DRG neurons express N, L, P/Q and R-subtypes of
VGCCs (116). As mentioned, GPCRs act on VGCCs, with most targeting N-type channels. However there is also some evidence of GPCR receptors targeting P/Q-type channels (152).

I have previously shown that the inhibition of N-type VGCC by galanin was comparable to that seen by the peptide μ opioid receptor agonist DAMGO. μ Opioid receptors are well known GPCRs that inhibit N-type HVA Ca\(^{2+}\) channels, and are a major target for analgesia for the treatment of acute pain (159, 160). Galanin too, has previously been shown to inhibit N-type VGCC for instance in parasympathetic neurons and Bowes melanoma cells (3, 89). Therefore galanin receptors may act via inhibiting N-type VGCCs in DRG neurons, and have the potential to be developed as an alternate target for pain treatment, but the mechanism of action needs to be established.

Galanin has been shown to inhibit N-type VGCC in other cells via the actions of GAL\(_1\) (89), but it is unclear which VGCC is targeted via GAL\(_2\). Although DRG neurons express GAL\(_1\) and GAL\(_2\) receptors (92, 121, 122), my results indicate the inhibition by galanin of VGCC in DRG neurons is predominantly via GAL\(_2\) (see chapter 3). The evidence to date shows that both GAL\(_1\) and GAL\(_2\) can act via Gi/o G proteins (87), and galanin acting via the Gi/o pathway has been shown to cause N-type VGCC inhibition (89, 94, 161, 162). However GAL\(_2\) has more commonly been shown to act via Gq/11 G proteins (55). Gq/11 G proteins may be able to bind and inhibit VGCC Ca\(^{2+}\) influx depending on the phosphorylation state of the channel (94). Studies by Simen et al. determined that GAL\(_2\) action via Gq/11 was blocked when constitutive activation of PKC prevented the βγ subunit from binding to N type VGCC in expression systems (94).
It is also possible that VGCC may not be the sole target of inhibition by galanin in DRG neurons. An intracellular Ca\(^{2+}\) increase occurs in these cells following KCl depolarisation which is initiated by VGCCs but not exclusively due to Ca\(^{2+}\) channels. According to Lu et al. (119), small capsaicin-sensitive neurons have different mechanisms involved in mediating and modulating intracellular Ca\(^{2+}\) changes compared to other DRG subtypes. More amplification of Ca\(^{2+}\) responses occurs in small DRG neurons, suggesting the involvement of SOCE (119). Caffeine, known to deplete intracellular Ca\(^{2+}\) stores, has been shown to modulate KCl-induced Ca\(^{2+}\) transients in both mudpuppy sympathetic neurons and DRG neurons (163), suggesting CICR is important. Although galanin has been shown to inhibit VGCCs, it is as yet unclear whether galanin can also modulate CICR in DRG neurons. It is also unclear if galanin can inhibit Ca\(^{2+}\) increases due to other agonists for instance, capsaicin induced calcium transients. Unlike KCl-induced Ca\(^{2+}\) transients, capsaicin-induced Ca\(^{2+}\) entry occurs through TRPV1 channels. DRG neurons express µ opioid receptors, galanin receptors and TRPV1 channels (118), which may interact during receptor activation. Indeed µ opioids have been shown to inhibit capsaicin-induced Ca\(^{2+}\) currents in DRG neurons (164), however it is unknown if galanin can modulate capsaicin-induced Ca\(^{2+}\) transients. I therefore investigated the mechanism of action of galanin in DRG neurons. Various toxins used to block specific VGCC were used to identify which VGCC contribute to the overall inhibition by galanin. PKC activation blocks galanin inhibition of VGCC via GAL2 in other neurons, so was investigated in this study to evaluate the same phenomenon in DRG neurons. As discussed, Ca\(^{2+}\) signalling in DRG neurons are comprised of not only VGCC but also CICR, SOSE and activation of TRPV1 channels. Capsaicin was applied in the presence of galanin to investigate if
galanin can inhibit capsaicin-induced Ca\textsuperscript{2+} transients. As CICR are a component of the overall Ca\textsuperscript{2+} transients in DRG neurons, galanin was applied in the presence of caffeine to investigate if galanin inhibits CICR from intracellular stores.

4.2 Materials and Methods

DRG were dissociated as described in chapter 2, (see section 2.2.1), and used acutely for whole cell patch clamp recordings or cultured overnight prior to recording using Ca\textsuperscript{2+} imaging. KREBS HEPES buffer was used to superfuse DRG neurons during Ca\textsuperscript{2+} imaging recordings, in addition to providing a means to dilute drugs prior to application. Patch clamp electrodes were filled with internal solution prior to being placed into the recording chamber. HEPES buffered external solution was used to superfuse DRG neurons during whole cell patch clamp recordings, and was also used to dilute drugs before application.

DRG Ca\textsuperscript{2+} imaging recording protocols described in chapter 3 (section 3.2.1) were used with the additional use of AGX (100 nM), nifedipine (10 µM) and CTX (100 nM), applied for fifteen minutes in KREBS HEPES prior to depolarisation with KCl (30 mM) in the presence or absence of galanin (1 M). The contribution of each channel type to the galanin response was estimated using equation 4.1. Similarly PDBu (1 µM) and caffeine (20 mM) were applied for 5 minutes and 15 minutes respectively prior to evoking KCl-induced Ca\textsuperscript{2+} transients in the presence and absence of galanin (1 µM). Capsaicin responses were reproducible every 30 minutes and were recorded in the presence and absence of galanin (1 µM), DAMGO (1 µM) and CTX (100 nM). Responses were measured as the amplitude above the baseline ratio level immediately prior to KCl or capsaicin applications. Statistics were performed as per chapter 2 (see section 2.2.9).
\textbf{Equation 4.1}

$$IhT = (%\text{Inhibition amplitude}) \times (\text{Proportion of cells inhibited})$$

\text{Contribution from each VGCC} = \frac{IhT \text{ control} - IhT \text{ toxin}}{IhT \text{ control}}

\textbf{Definition of Terms:}

Where \(IhT\) is a measure of the total inhibitory effect of galanin on the population of cells under various conditions. The contribution of different VGCC was estimated by applying toxins including AGX (blockade of P/Q-type VGCC) and CTX (blockade of N-type VGCC).

\textbf{4.3 Results}

\textbf{4.3.1 Calcium Imaging Recordings; Effect of VGCC Blockers on Inhibition by Galanin}

Having established that galanin inhibits VGCC in nociceptive DRG neurons, I investigated which types of VGCCs were involved, using Ca\(^{2+}\) imaging. Pre-treatment with CTX (100 nM) to block N-type channels caused a partial reduction in KCl-induced Ca\(^{2+}\) transients in 46 percent of the DRG neurons tested by an average of 20% ± 6 but the majority of neurons were still inhibited by galanin (Figure 4.1 A). In the presence of CTX, galanin further inhibited Ca\(^{2+}\) transients in 15 of 24 DRG neurons (63% of neurons) by an average of 20 ± 4% (Figure 4.1 C, D). Of the 24 DRG neurons tested, 29 percent showed an inhibition of responses by galanin despite showing no evidence of N-type Ca\(^{2+}\) channel contributing to the response (i.e. the initial response to KCl was not attenuated by CTX). Blockade of P/Q-type channels by pre-treatment of neurons with AGX (100 nM) reduced the amplitude of depolarisation-induced Ca\(^{2+}\) transients by 14% ± 2 in
86% of neurons tested (Fig 4.1B). In the presence of AGX, the majority of neurons were not inhibited by galanin (Figure 4.1 C). Galanin inhibited Ca\textsuperscript{2+} transients in only 36% (5 of 14) of DRG studied and in these five neurons the average inhibition was small (8 ± 1%; Fig. 4.1 C, D). Galanin inhibited a significantly smaller proportion of the neurons tested in the presence of AGX (n=14) than in control (n=19), (\(\chi^2\) test, p=0.012, Fig. 4.1D). The L-type Ca\textsuperscript{2+} channel blocker nifedipine (10 M), in the presence and absence of CTX (100 nM), did not significantly reduce Ca\textsuperscript{2+} transient amplitudes (result not shown). In summary, AGX significantly reduced galanin inhibition of Ca\textsuperscript{2+} transients (Fig 4.1C), and the proportion of DRG neurons responding to galanin (Fig 4.1D). Based on both findings presented (Fig 4.1C, D), the galanin response appears to be mediated primarily by the inhibition of P/Q-type channels (Fig 4.1E).

Cadmium chloride was used as a control as it is known to non-specifically block VGCC. Cadmium chloride (1 mM) reversibly blocked 80 percent of the Ca\textsuperscript{2+} transients in (10 of 14) DRG neurons; however some residual Ca\textsuperscript{2+} transients remained (Fig 4.2). In the remaining four cells there was a lack of recovery upon washout. Overall these results confirm that KCl-induced responses are initiated by VGCC.
**Figure 4.1** The Effect of VGCC Blockers on the Inhibition by Galanin of KCl-Induced Ca\(^{2+}\) Transients.

(A) Blockade of N-type VGCC using ω-conotoxin GVIA (CTX, 100 nM), reduced Ca\(^{2+}\) transient amplitude by 13% in this neuron. In the presence of CTX, galanin was still able to inhibit Ca\(^{2+}\) transients by 11.5% compared to galanin alone. (B) Blockade of P/Q-type VGCC using ω-agatoxin IVA (AGX, 100 nM) reduced the Ca\(^{2+}\) transient amplitude by 21% in another neuron. In the presence of AGX, application of galanin produced no further reduction in this neuron, as was the case in the majority of neurons tested. (C) In cells that were inhibited by galanin, KCl-induced Ca\(^{2+}\) transient amplitude was inhibited by 18 ± 2% (n=16). In the presence of CTX, galanin inhibition was comparable to control (n=15). In the presence of AGX, in the few cells showing a galanin inhibition, the amplitude was significantly reduced compared to galanin alone. (*= p<0.05, n=5, 8± 1%). (D) The proportion of neurons tested that were inhibited by galanin in the presence of CTX (n=24) was comparable to control (n=19). Galanin inhibited a significantly smaller proportion of neurons in the presence of AGX than galanin alone. * (n=14) (χ\(^2\) test with Yates continuity correction, p=0.012) (E) This graph shows an estimate of the contribution of each VGCC type to the inhibitory effect by galanin on the DRG neuron population. Inhibition by galanin of VGCC is comprised of 80% by P/Q-type inhibition and 20% N-type VGCC inhibition. Please refer to Equation 4.1 for the calculations used to determine these values. Bars represent mean ± S.E.M.
A

KCl  KCl  KCl  KCl
Galanin

CTX

B

KCl  KCl  KCl  KCl
Galanin

AGX

C

KCl Response Amplitude as % of Control

n=16  n=15  n=5
Gal  Gal + CTX  Gal + AGX

Drug Treatment

D

% of Neurons Inhibited by Galanin

16/19  15/24  5/14
Gal  Gal + CTX  Gal + AGX

Drug Treatment

E

% of Overall Galanin Inhibition

P/Q  N

Ca²⁺ Channel Type
Figure 4.2 Effect of Calcium Channel Blockers on Ca$^{2+}$ Responses to KCl in DRG Neurons

In the presence of CdCl$_2$, KCl-induced Ca$^{2+}$ transients were reduced by 80%, which was reversed upon washout in this neuron. A similar effect was seen in all neurons tested.
4.3.2 Electrophysiological Recordings Examining The Effect of Voltage Gated Calcium Channel Blockers on Galanin Inhibition

Whole cell patch clamp recordings of responses to galanin in the presence of VGCC blockers yielded different results from those demonstrated with Ca\textsuperscript{2+} imaging. Currents resulting from voltage steps from -90 mV to -10 mV were reduced when N-type and L-type VGCCs were blocked in the presence of CTX (100 nM) and nifedipine (10 μM) (Fig 4.3). Blockade of N and L-type channels resulted in a reduction of Ca\textsuperscript{2+} currents in all (5 of 5) neurons tested (Fig 4.3A, B). The reduced Ca\textsuperscript{2+} currents in the presence of both VGCC blockers, was subsequently reduced by galanin (1 μM) in only one neuron (1 of 5), and washout was not obtained (Fig4.3 A, B). The majority (4 of 5 DRG neurons) failed to be inhibited by galanin (Fig 4.3C). The proportion of DRG neurons inhibited by galanin in the presence of VGCC blockers (n=5) was significantly smaller than galanin alone (n=13), (χ\textsuperscript{2} test, p=0.0025), see chapter 3 (Fig 3.2). These results suggest that N-type and/or L-type channels contribute to the overall inhibition by galanin, since galanin failed to reliably inhibit Ca\textsuperscript{2+} currents when N-type VGCC were blocked (Fig 4.1E). N and L blockade of VGCC led to a large reduction in Ca\textsuperscript{2+} currents, leaving only a small amount of current left in the DRG neurons tested, so that the contribution of P/Q-type channels was likely to be minimal.
Figure 4.3 Inhibition of Calcium Channel Currents by Galanin in the Presence of VGCC.

(A) Blockade of VGCC with CTX (100 nM) and nifedipine (10 μM), resulted in a 38 percent reduction in Ca\(^{2+}\) channel currents in this neuron (top panel), evoked by voltage steps (-90 mV stepped to -10 mV) in the lower panel. Subsequent application of galanin (1 μM) reduced Ca\(^{2+}\) channel currents by 40 percent in the same neuron but the reduction did not reverse upon washout. (B) A time course of the change in Ca\(^{2+}\) channel currents over time of the experiment in (A) demonstrating VGCC reduction of Ca\(^{2+}\) channel currents and inhibition of currents by galanin (1 μM). (C) A time course of the change in Ca\(^{2+}\) channel currents over time in another DRG neuron demonstrating a reduction in current in the presence of CTX (100 nM) and nifedipine (10 μM). This neuron shows a lack of inhibition of Ca\(^{2+}\) channel currents in the presence of galanin (1 μM). Only one (1 of 5) neurons were inhibited by galanin in the presence of CTX and nifedipine.
4.3.3 The Effect of Voltage Gated Calcium Channel Blockade on Capsaicin-Induced Calcium Transients

Of the 22 DRG neurons treated with repeated applications of capsaicin (1 μM) using Ca$^{2+}$ imaging, 81% (18 of 22) were capsaicin sensitive (Fig 4.4A). Capsaicin responses were reproducible every thirty minutes. These responses were not affected by desensitization, and showed little or no reduction in amplitude over time. As discussed, capsaicin-sensitive DRG neurons of small diameter have been shown to amplify the response to an influx of Ca$^{2+}$ from VGCC. From previous studies, it is suggested that capsaicin responses are also in part due to depolarisation-induced activation of Ca$^{2+}$ channels (164), and hence may also involve some amplification. The effect of CTX blockade of N-type VGCCs on the TRPV1 mediated capsaicin response was therefore investigated. Of the 18 capsaicin-sensitive DRG neurons, in 77% (14 of 18 neurons) blockade of N-type channels with CTX had no clear effect on capsaicin responses (Fig 4.4A). However in 22 percent of the population tested (4 of 18), CTX induced an average of 66 percent inhibition of the capsaicin response (Fig 4.4B). Overall, CTX was found to have no effect on the total capsaicin responses, when compared to capsaicin responses alone (one-way ANOVA, Fig 4.4C). However in a small subset of the DRG neurons tested, there was a large inhibition of capsaicin-induced Ca$^{2+}$ transients when compared to capsaicin alone, indicating that N-type channels were involved (p<0.01, one-way ANOVA, Fig 4.4C). The distribution of responses was clearly bimodal and is consistent with the heterogeneous nature of DRG neuron population being studied.
Figure 4.4 The Effect of CTX on Capsaicin-Induced Calcium Transients

(A) A Ca\(^{2+}\) imaging recording of repeated capsaicin-induced Ca\(^{2+}\) transients. Application of CTX (100 nM) to block N-type VGCC had no effect on capsaicin (1 µM) responses in this neuron. The same occurred in (14 of 18) neurons tested. (B) In another DRG neuron, capsaicin-induced Ca\(^{2+}\) transients were inhibited in the presence of VGCC blocker CTX. This indicates that CTX has two opposing effects on capsaicin responses in DRG neurons. (C) Represented as a percentage of the capsaicin response (100%), the total population of DRG neurons (solid bar) showed no effect of N-type VGCC blockade. When divided into responders and non-responders, it was clear that some neurons had a large reduction (66%) of capsaicin responses in the presence of CTX (4 of 18). **p<0.01, (one-way ANOVA). Bars represent mean ± SEM.
4.3.4 The Effect of G Protein Coupled Receptors on Capsaicin-Induced Calcium Transients

As discussed previously, small capsaicin sensitive DRG neurons can amplify the increase in Ca\(^{2+}\) caused by Ca\(^{2+}\) influx. The TRPV1 receptors required for capsaicin responses are co-expressed with \(\mu\)-opioid and galanin GPCRs (118). GPCR interaction with capsaicin-induced Ca\(^{2+}\) transients was therefore investigated. Firstly, the effect of galanin receptor activation on repeated capsaicin responses in DRG was evaluated. Of the 16 DRG treated with concurrent treatments of capsaicin (1 μM) and galanin (1 μM), 62% (10 of 16 neurons) were capsaicin-sensitive. Galanin was found to have no effect on the overall capsaicin-induced Ca\(^{2+}\) transients (Fig 4.5A) and was comparable to capsaicin alone (one-way ANOVA). Galanin was therefore compared to the effects of DAMGO (1 μM). Of the 29 DRG neurons sensitive to capsaicin, 52% (15 of 29 neurons) demonstrated a DAMGO-induced inhibition of capsaicin responses by 47% ±7 (Fig 4.5B). In comparison to capsaicin alone, DAMGO significantly reduced capsaicin-induced Ca\(^{2+}\) transients (p<0.01, one-way ANOVA, Fig 4.5 C). In summary, galanin acting via GPCR failed to inhibit capsaicin-induced Ca\(^{2+}\) transients. Whereas, DAMGO caused a substantial reduction in approximately half of the neurons tested (Fig 4.5C).
Figure 4.5 The Effect of GPCR on Capsaicin-Induced Calcium Transients

(A) Repeated applications of capsaicin (1 µM) in the presence of galanin were performed during Ca²⁺ imaging in DRG neurons. Capsaicin-induced Ca²⁺ transients were not affected when evoked in the presence of galanin (1 µM). (B) In the presence of DAMGO (1 µM), capsaicin-induced Ca²⁺ transients were inhibited in a proportion of DRG neurons. (C) Represented as a percentage of the capsaicin response (100%), galanin had no effect on capsaicin responses (n=10). In contrast, when separated into non-responders and responders, the results indicate that DAMGO showed a reduction in capsaicin-induced Ca²⁺ transients (15 of 29). This indicates that the mechanism by which DAMGO inhibits capsaicin responses is not activated by galanin. **=p<0.01 versus capsaicin alone, one-way ANOVA, post-hoc Dunnett’s test. Bars are represented as mean ± SEM.
4.3.5 Manipulation of Intracellular Calcium with Caffeine and the Effect on Galanin Inhibitions and Capsaicin-Induced Calcium Transients

Caffeine was utilised to deplete intracellular Ca$^{2+}$ stores in DRG neurons to determine their role in the response being studied. This was of particular importance to investigate if galanin could inhibit CICR in addition to its demonstrated action on VGCCs. When DRG were treated with caffeine (n=17), there was an increase in baseline Ca$^{2+}$ concentration resulting in an overall reduction of response amplitudes during treatments (Fig 4.6A). When caffeine was exerting an increase in baseline Ca$^{2+}$, galanin was able to inhibit Ca$^{2+}$ transients in only one neuron. The remaining 94%, (16 of 17) failed to demonstrate an inhibition of Ca$^{2+}$ transients by galanin (4.6C, D). The proportion of DRG neurons inhibited by galanin in the presence of caffeine (n=17) was significantly smaller than that of control (n=19), ($\chi^2$ test, p<0.0001). In the presence of caffeine, the majority of neurons did not respond to capsaicin (Fig 4.6A). Of the two capsaicin-sensitive neurons, the amplitude of the responses were small.
4.3.6 Activation of PKC with PDBu and the Effect on Responses to Galanin and Capsaicin.

The study by Simen et al. suggested that GAL₂ action on VGCCs via the Gq/11 pathway is blocked by PKC activation (94). My previous work has determined that the inhibition by galanin in DRG neurons is mediated by GAL₂. Therefore the ability of galanin to inhibit VGCC via GAL₂ in the presence of the PKC activator PDBu was investigated. In the presence of PDBu, KCl-induced Ca²⁺ transients progressively decreased over time. Of the 7 DRG treated with PDBu (1µM), one neuron treated with PDBu (1µM) was inhibited by galanin, which recovered upon washout. In contrast, the majority of neurons showed a reduction of Ca²⁺ in the presence of galanin, although there was no recovery during washout (result not shown). This suggests PDBu may affect the mechanism required for generating Ca²⁺ transients in response to KCl. Under these circumstances it is not possible to test if PKC inhibits galanin actions using Ca²⁺ imaging.
Figure 4.6 The Effect of Calcium Modulators on Galanin and Capsaicin Responses in DRG Neurons

(A) Application of caffeine (20 mM) progressively increased the baseline level of Ca$^{2+}$ in this DRG neuron. As seen in most neurons tested, galanin failed to inhibit Ca$^{2+}$ transients and capsaicin responses were absent in the presence of caffeine (B) The proportion of neurons inhibited by galanin in the presence of caffeine was significantly smaller than the proportion of neurons inhibited by galanin alone ($\chi^2$ test, p<0.0001). (C) The percent of KCl-induced calcium transients (100%) in the presence of galanin alone, and in the presence of caffeine. Bars represent the overall effect (inhibition and no effect). $\chi^2$ test, Proportion inhibited: caffeine vs galanin, (** = p<0.0001), t-test, overall caffeine and galanin vs galanin alone (* = p<0.05). Bars represent mean ± SEM.
A

![Graph showing neuronal inhibition by Galanin and KCl response with Caffeine and Capsaicin](image)

B

| 16/19 | 1/17 |

C

| n=19 | n=17 |

**Graph showing % Control KCl Response**

- Galanin
- Galanin + Caffeine

**Graph showing % of Neurons Inhibited by Galanin**

- Galanin
- Galanin + Caffeine

***Statistical Significance***

- **A**
- **B**
- **C**
4.4 Discussion

Experiments presented in this chapter have indicated that inhibition by galanin of Ca\(^{2+}\) transients and Ca\(^{2+}\) currents in DRG neurons comprises both N and P/Q-type VGCC inhibition. Although analogies can be drawn between galanin and \(\mu\)-opioid inhibition of sensory neurons, there were some clear differences. \(\mu\)-Opioid receptors appear to also act on TRPV1 channel responses, with an inhibition of capsaicin-induced Ca\(^{2+}\) transients evident in some neurons of this study. In contrast, galanin failed to inhibit capsaicin responses, suggesting that galanin does not activate this mechanism. This was complicated by the fact that the majority of capsaicin responses were not affected when N-type VGCC were blocked directly by CTX. My results in caffeine treated neurons suggest that Ca\(^{2+}\) transients in DRG due to SOCE and CICR may also be inhibited by galanin. These results provide an insight into the mechanism of inhibition by galanin in nociceptive DRG neurons, and how the generation of Ca\(^{2+}\) transients in these neurons can affect the overall Ca\(^{2+}\) signalling in nociceptive DRG neurons.

4.4.1 Inhibition by Galanin is via N and P/Q-Type Voltage-Gated Ca\(^{2+}\) Channels

The mechanism of inhibition by galanin of VGCCs was observed using both Ca\(^{2+}\) imaging and whole cell patch clamp recordings. As seen in chapter 3, there were different findings dependant on the recording techniques used. When CTX and nifedipine were applied during whole cell patch recordings, the VGCC blockade left only a small amount of Ca\(^{2+}\) current for potential inhibition by galanin. Indeed with N and L-type channels blocked, the majority of DRG neurons failed to be inhibited by galanin. While it is possible that L-type VGCC contribute to the whole cell current, it is unlikely since Ca\(^{2+}\) imaging recordings indicated that this channel did not contribute to the overall Ca\(^{2+}\) transients in the neurons tested. This indicates therefore that the
inhibition by galanin is partially mediated by N-type VGCC inhibition. In addition, it is common for many G-protein-linked receptors to inhibit HVA IBa mainly via effects on N-type channels in DRG neurons (89, 161, 162). Galanin has been shown to inhibit N-type VGCC in other cells (89, 94). The whole cell patch clamp technique looks directly at VGCC currents in DRG neurons. While it is advantageous to look directly at VGCCs, the technique suffers from consequences of the recording process. For instance, intracellular components of the DRG neuron can be dialysed and taken up into the patch pipette. Other studies using whole cell patch clamp have identified this as being a factor affecting electrophysiological recordings and galanin receptor function (165). As whole cell path clamp does look directly at VGCCs, it cannot record any Ca\textsuperscript{2+} signalling amplification which can occur in DRG neurons from CICR and SOSE (119). Therefore, the Ca\textsuperscript{2+} imaging technique was used to record the Ca\textsuperscript{2+} transients in DRG neurons.

Unlike whole cell patch clamp, Ca\textsuperscript{2+} imaging recordings indicated there were still many DRG neurons inhibited by galanin in the presence of N-type VGCC blockade. Ca\textsuperscript{2+} imaging recordings of DRG Ca\textsuperscript{2+} transients are initiated by VGCC, but may also reflect CICR from intracellular stores and SOCE (119). As mentioned, amplification of Ca\textsuperscript{2+} caused by Ca\textsuperscript{2+} influx (119), may couple differently to intracellular Ca\textsuperscript{2+} amplification machinery depending on different VGCCs involved. This may also explain why CTX and AGX applications individually yielded a smaller reduction in Ca\textsuperscript{2+} transients than observed for whole cell inhibition of IBa\textsuperscript{2+} voltage gated currents patch clamp recordings. In the presence of N-type VGCC blockers, the degree of Ca\textsuperscript{2+} transient reduction was relatively small, with a large percentage of Ca\textsuperscript{2+} increase still available for subsequent inhibition by galanin. In addition, performing Ca\textsuperscript{2+} imaging recordings during blockade of N-type Ca\textsuperscript{2+} channels with CTX did not significantly change the inhibitory effect of
galanin. In contrast, in the presence of AGX blockade of P/Q-type channels, galanin’s activity was significantly reduced according to two different metrics. Firstly in the presence of AGX, galanin inhibition of Ca\(^{2+}\) transients occurred in only a small proportion of DRG neurons. Secondly, of the DRG that were inhibited by galanin in the presence of AGX, the inhibition was small. These findings support another study which report that galanin receptors can inhibit P/Q-type channels in other cells (166). Galanin’s actions via GAL\(_2\) has already been shown to inhibit synaptic transmission in primary afferents of the dorsal horn of the spinal cord (6), and presynaptically inhibit the release of Substance P (44). Indeed, blockade of P/Q-type VGCC has been shown to inhibit substance P release and has anti-nociceptive effects in the superficial DH of the spinal cord (167, 168). Interestingly, analogies can be drawn between the mechanism of action of galanin and gabapentin, one of the few available neuropathic pain treatments available which inhibits P/Q-type VGCC in the spinal cord (12, 169).

4.4.2 Intracellular Mechanism of Inhibition of Ca\(^{2+}\) by Galanin.

Experiments manipulating PKC were designed to provide insight into the mechanism by which galanin acts via GAL\(_2\) in DRG neurons. Simen et al. studied the effect of PKC activation on the mechanism of action of GAL\(_1\) and GAL\(_2\) receptor inhibition. Genetic manipulation of hypothalamic and cell line HEK-293 expressing N-type VGCC indicated PKC constitutive activation prevented GAL\(_2\) mediated inhibition coupled via Gq/11 GPCR (94). While GAL\(_1\) and GAL\(_2\) receptors are both known to be Gi/o coupled GPCR, GAL\(_2\) receptors have more often been shown to couple via Gq/11, which can activate different intracellular pathways. Increasing evidence suggests that GPCRs can act via multiple G proteins. Once activated, Gi/o coupled GPCR generally result in the \(\beta\gamma\) subunits binding to and inhibiting VGCC. Alternatively, Gq/11
coupled GPCR inhibition of N-type VGCC is prevented by PKC activation. Whether this occurs in DRG neurons is unclear. From Ca$^{2+}$ imaging results, the effect of PDBu on galanin-induced inhibition of [Ca$^{2+}$], was unclear. It was evident that there was an overall reduction in Ca$^{2+}$ transients in the presence of PDBu, and whether this was reduced further in the presence of galanin could not be determined. Findings in other cells indicate that GAL2 acts via the Gq/11 pathway, which is blocked by PKC activation (94). It seems the only way to investigate PKC activation specifically in DRG neurons, is via the VGCC subunit manipulations performed by Simen in hypothalamic neurons and HEK293 transfected cells.

As discussed, DRG neurons have other sources of Ca$^{2+}$ including CICR, therefore this was investigated using caffeine. All caffeine treated DRG neurons saw a gradual increase in baseline Ca$^{2+}$ transients, as the Ca$^{2+}$ from stores were released. As such, amplitudes of repeated response in the presence of caffeine became smaller, as the basal Ca$^{2+}$ levels continued to increase over time. Responses to KCl were small as expected, but more consistent than following PDBu. The one inhibition by galanin in the presence of caffeine was clear, although small. The gradual increase in baseline calcium suggests caffeine may have caused depletion of intracellular stores, and possibly SOCE. The manipulation of intracellular Ca$^{2+}$ with caffeine prevented the inhibition by galanin of Ca$^{2+}$ transients in the majority of DRG neurons tested. However there is a possibility that any galanin inhibitions in the presence of caffeine which were smaller than the gradual incline in baseline Ca$^{2+}$ levels, may not have been visible. As caffeine treatment of DRG neurons released Ca$^{2+}$ from intracellular stores in the presence and absence of galanin, caffeine may have affected the inhibition by galanin via another mechanism.
4.4.3 Manipulation of Intracellular Calcium Effects Capsaicin-induced Calcium Transients

As seen with KCl, DRG neurons treated with caffeine also saw a reduction in capsaicin responses. It was apparent that there were a smaller number of capsaicin responsive cells despite evidence of the repeated KCl-induced Ca\(^{2+}\) transients. In the presence of caffeine, capsaicin induced Ca\(^{2+}\) transients were absent or small in amplitude, which was perhaps surprising for a response due to a TRPV1 channel. A previous study has suggested a desensitisation of the capsaicin response in the presence of caffeine in DRG neurons (170). Capsaicin acts by causing an influx of calcium directly via the capsaicin receptor TRPV1, but these results suggest it may also involve the amplification of Ca\(^{2+}\) changes as seen with KCl responses. Whether or not the mechanisms of this amplification are the same, are not clear.

Capsaicin responses have previously been shown to be affected by N-type VGCC blocker CTX in sensory neurons of the respiratory tract (171). The results from the current study indicated that in the majority of DRG neurons with CTX treatment, had no effect on capsaicin responses. However in a small population of neurons tested, capsaicin responses were indeed inhibited when N-type VGCC were blocked. This may be due to the differential distribution of VGCC subtypes in different sizes of DRG neurons, and/or differences in resting membrane potentials (116, 119).
4.4.4 G Protein Coupled Receptors Effect Capsaicin Responses

As discussed, analogies can be drawn between μ-opioids and galanin receptors in which they both inhibit N-type VGCC. Therefore the current study sought to provide insight into the effect of DAMGO and galanin on capsaicin responses in DRG neurons. In the presence of galanin, there was no effect on the capsaicin-induced Ca^{2+} transients. Despite this, in the presence of DAMGO, capsaicin-induced Ca^{2+} transients were significantly inhibited. As discussed above, findings above indicate capsaicin-induced Ca^{2+} transients mediated via TRPV1 required N-type VGCC in only a small subset of DRG neurons. Both galanin and μ-opioid receptors target N-type VGCC, and therefore have the potential to effect the overall capsaicin response in this small subset of DRG neurons. It seems likely that galanin has no effect on capsaicin responses, and yet μ-opioids inhibit capsaicin via inhibition of N-type VGCC in this subset of DRG neurons. In the majority of cells however, N-type channels were not involved. This suggests that DAMGO may be inhibiting capsaicin responses by acting on TRPV1 channels, an action which is not activated by galanin. This study is the first using calcium imaging, to suggest DAMGO inhibits TRPV1 channels in DRG neurons, and supports similar findings in DRG neurons using whole cell patch clamp (164).
4.4.5 Conclusions

Findings from this chapter from manipulation of [Ca\(^{2+}\)]\(_i\) revealed that galanin’s mechanism of inhibition of Ca\(^{2+}\) in DRG neurons is not solely inhibition of VGCC, and may also inhibit CICR or SOCE. While galanin and µ-opioid receptors both inhibit N-type VGCCs (118), only µ-opioids could inhibit capsaicin responses suggesting different cellular mechanisms are involved. It is likely that galanin can act via more than one G protein, although this remains to be investigated. In support of this, results from this chapter have determined that GAL2-mediated inhibition of Ca\(^{2+}\) transients occurred via both N and P/Q-type VGCC in DRG neurons. As discussed, based only on galanin sharing VGCC targets with µ-Opioids (N-type) and gabapentin (P/Q-type), galanin may have potential for the possible development of alternative treatments for both acute and chronic pain.
5.1 Introduction

The nociceptive pathway conveys sensory information detected as noxious stimuli by peripheral nerve fibres to the spinal cord and beyond. While this information is relayed to higher centres of the brain for interpretation as pain, it can be modulated in the DH of the spinal cord, via various mechanisms (44, 55). Galanin is present in sensory neurons and their central axon terminals and in DH neurons, and has been shown to modulate both acute and pathological pain in the nociceptive pathway (see chapter 1). My current investigation in DRG has demonstrated that galanin inhibits N and P/Q-type VGCCs in DRG neurons, and this inhibition is mediated via GAL2, providing a mechanism for the presynaptic inhibition seen in DH. Whilst investigation of galanin actions via GAL2 in DRG neurons is important, there is much recent evidence that microglia in the CNS can also modulate pain. After nerve injury, chemicals released into the extracellular environment activate microglia and can lead to the development of chronic pain conditions including neuropathic pain. As discussed previously, galanin is up-regulated after nerve injury and can also modulate pain. While both galanin and microglia have been shown separately to modulate pain, the effect of galanin on microglial activity is unknown. Indeed, microglia express GAL2, and suggests this is likely. Therefore to fully understand the extent of nociceptive modulation by galanin, this chapter has focussed on the effect of galanin on the function of microglia.
In recent years, there has been intense investigation into the various roles of microglia in the CNS. In their resting state, long processes provide immunosurveillance to the surrounding neural tissue. Microglia undergo activation when these processes detect a variety of stimulants released from damaged neural tissue and during infection (132). Central nerve terminals of damaged sensory neurons interact with microglia which has major implications in neuropathic pain states. It has also been shown that microglia are involved in the development of hyperalgesia as a result of opiate withdrawal (172). In their activated state, microglia show an increase in intracellular Ca\(^{2+}\), resulting in the release of pro and anti inflammatory cytokines, NO and ROS (5, 11).

A multitude of receptors are involved in modulating microglial activation, and many of their roles have not been fully elucidated (137). As such, this study has focussed on the two major pathways for microglial activation. As a result of damage to neural tissue, ATP present at a high intracellular concentration is released into the extracellular environment. Microglia can detect the presence of ATP via the purinergic P2 receptor family which is comprised of two sub-families; P2Y and P2X receptors. Firstly, the metabotropic GPCR sub-family includes P2Y (1,2,4,6,11-14), with several (P2Y1,2,4,6 and 12) expressed in microglia (133, 134). ATP-activated P2Y12 receptors are involved in chemotaxis and migration to damaged tissue (135), and are necessary for the development of neuropathic pain states in animals (7). The second group of receptors are ionotropic receptors and non-selective cation channels permitting the passage of K\(^+\), Na\(^+\) and sometimes Ca\(^{2+}\). Known as P2X receptors, there are seven subtypes and microglia express P2X4 and P2X7. P2X4 receptor activation results in calcium influx which activates the p38 mitogen-activated kinase pathway (p38-MAP), which in turn results in the production and release of mediators known to contribute to neuropathic pain states (see Fig 5.1) (131). P2X4 is activated
by lower ATP levels (low µM range), whereas the P2X7 receptor is activated upon detection of higher levels (low mM range) of ATP. The p38-MAP kinase pathway is activated by P2X7 but with the higher ATP concentrations comes an activation of apoptotic pathways and a release of interleukin 1β and cathepsin S (136).

The effect of galanin on microglial function is relatively unknown, although the GAL₂ receptor is expressed in microglia (11). Galanin has been shown to be up-regulated in microglia after nerve injury, and can inhibit cytokine release (5, 173). Another study suggests that galanin causes an increase in intracellular Ca²⁺ levels in a subset of microglia (174). Although this suggests a role for galanin in modulating microglial function, however how an increase in [Ca²⁺] would prevent cytokine release is unclear. Therefore this chapter investigated the effect of galanin on ATP-induced microglia activation.

Galanin may also have a role in regulating another major activation pathway in microglia. LPS, found on the cellular membranes of gram-negative bacteria has been used by many studies to mimic the effects of a bacterial infection (11). Microglia detect the presence of invading pathogens by possessing pattern recognition receptors (PRRs) such as TLR, where TLR2 and TLR4 play a key role in microglial activation (111). TLR4 recognises LPS and activates signalling pathways which will eventually result in the release of pro-inflammatory cytokines and phagocytosis. LPS was thought to act solely via TLR, however this is complicated by recent evidence of LPS induced microglial activation via the macrophage antigen complex (MAC1) (Fig 5.1). Similarly to TLR, MAC1 is a PRR integrin complex comprised of CD11b/CD18 and is capable of activating microglia in the presence of LPS. In fact in a recent study, using knockout
mice, it has been shown that LPS acting via MAC1 can activate microglia in the absence of the TLR (10). Due to the complexities of LPS binding pathways, the full mechanism of LPS activation of microglia remains to be determined. As a result, galanin’s role in the modulation of the LPS-induced pathway needs investigation. One study has indicated that galanin can inhibit LPS-induced TNFα release from microglia (5). In order to release TNFα, LPS action via TLR4 first induces superoxide production. This superoxide release into the extracellular space acts as a trigger for an autocrine positive feedback mechanism which further activates TLR4, and more superoxide production resulting in TNFα release (129). Because the process for TNFα release is complex, it is unclear where in the pathway galanin is acting. Therefore this chapter has focused on the effect of galanin on LPS-induced microglial activation.

The complex nature of microglial activation is further complicated by studying microglia in vitro. This chapter initially developed a method of preparing mixed glial cultures in a quiescent state, using a tetracyclic antibiotic known to inhibit microglia activation (175). This ensured the results presented observed the full extent of microglia activation in the presence of ATP or LPS, to properly elucidate the effect of galanin on the separate activation pathways.

Subsequent experiments were intended to provide an insight into the potential effect of galanin on the LPS-induced activation seen in nervous system infections, and ATP-mediated states of neuronal tissue damage. The effects of galanin on markers for ATP and LPS-induced microglial activation were observed. Changes in microglial intracellular Ca²⁺ were studied using Ca²⁺ imaging, whereas ROS production and NO release were determined using fluorescent markers at 1 hour and 24 hours post activation respectively.
Figure 5.1 Proposed Activation/Inhibition Pathway of Microglia in CNS

LPS activates microglia by mimicking the presence of bacterial infection. LPS (open square) acts via TLR4 and the lectin binding site of MAC1 (CD11b/CD18) resulting in an increase in Ca$^{2+}$ leading to ROS and cytokine release. ATP activates microglia after release from damaged neurons. ATP (black circle) acts via P2X (+ P2Y not shown) receptors leading to an increase in Ca$^{2+}$ resulting in ROS and cytokine release. The effect of galanin on microglial activation via GAL2 is unclear (176).
5.2 Materials and Methods

Drugs, reagents and chemicals used in the research presented in this chapter are as described in chapter 2. Mixed glial culture and microglial isolation were prepared as described in section 2.2.2, and 2.2.3. Also described in chapter 2, NO production from microglia was measured (section 2.2.6), as was the release of ROS from microglia (section 2.2.7).

5.2.1 Calcium Imaging Recordings of Microglia

Changes in microglial [Ca^{2+}]_i were recorded using Ca^{2+} imaging, as described in chapter 2, section 2.2.5. Protocols specific to Ca^{2+} imaging in microglia are as follows. Initial baseline [Ca^{2+}]_i levels were recorded for 100 seconds before applying treatments for one hour. In the case where galanin was applied in addition to LPS or ATP, the peptide was present for five minutes prior to the 1 hour treatment and remained in the bath thereafter. In order to avoid bleaching the Fura2 dye, microglia remained in darkness during incubation periods. Fura-2 fluorescence ratios of microglia were then recorded after one hour. Statistics were performed as per section 2.2.9. Studies investigating the initial [Ca^{2+}]_i levels of microglia used various concentrations of minocycline (10-100 µM), either applied post isolation or directly to the mixed glial flask at least 24 hours prior to isolation (Fig 5.3). Minocycline was also applied at a lower concentration (10 µM) after microglial isolation with medium topped up either to 2 mls to fill the recording chamber, or to 500 µl to maximize the concentration of residual factors secreted from astrocytes in the mixed glia culture flask (177). Some microglia were pre-treated with LPS (100 ng/ml, 1 ug/ml) and galanin (1 pM,100 nM, 1 µM) for 24 hours. Other microglia were incubated overnight and then treated with 1 hour of either LPS (100 ng/ml, 1 µg/ml) or ATP (50 µM) in the presence and absence of galanin (1 pM,100 nM, 1 µM) (Fig 5.2).
Figure 5.2  Protocol for Treatment and Recording Microglia.

Some microglia were cultured in the presence of minocycline for 24 hours prior to recordings. In most Ca\textsuperscript{2+} imaging experiments, microglia were treated for 1 hour with LPS or ATP in the presence and absence of galanin. Microglia prepared for the ROS assay were treated for 1 hour with ATP, Zymosan, PDBu, or LPS in the presence or absence of galanin. Alternatively, control microglial were compared to microglia treated with LPS for 24 hours to observe any iNOS mediated NO production.
**Microglial Flask Pre-isolation**
- ± 24 hours
- ± Minocycline (100μM)

**Microglia Post-Isolation**
- ± 24 hours
- ± Minocycline (10,100 μM)
- ± LPS (1μg/ml, 100ng/ml)

**Ca²⁺ Imaging (Fura 2)**
- Labelled with Fura-2
- ± ATP (50 μM)
- ± LPS (1μg/ml, 100ng/ml)
- ± Galanin (1pM, 1nM, 100nM, 1μM)

**Flex Station**
- ± 24h LPS

**Confocal Microscopy**
- 24h Medium Alone

**ROS Production Assay (DCFDA)**
- ≤ 1 hour (measurements 15, 30, 45, 60 mins)
- ± PDBu (100ng/ml)
- ± ATP (3mM)
- ± Zymosan (0.05mg/ml)
- ± Galanin (1pM, 100nM, 1μM)
- ± LPS strain C26.86 (1μg/ml, 100ng/ml)
- ± LPS strain C111.B4 (1μg/ml, 100ng/ml)

**Nitric Oxide Production Assay (DAF-FM)**

**Morphology**
5.3 Results

5.3.1 Microglia Culture Optimization

When microglia were studied using Ca\(^{2+}\) imaging, Fura-2 ratios and therefore initial [Ca\(^{2+}\)]\(_i\) were variable but high in some cases indicating a variable level of pre-activation (Fig 5.3A). Time control recordings (no treatment) over one hour were also variable, but many cells showed a marked rise in [Ca\(^{2+}\)]\(_i\) levels (Fig 5.3B). This suggests an unavoidable degree of activation during the isolation process, and activation variability between different batches of microglia. In order to investigate the actions of galanin on microglial activation, the current study aimed first to standardise the level of microglial activation after isolation. Microglia in the presence and absence of the different types of minocycline treatments, were studied. When compared to control, minocycline treated microglia had significantly lower initial [Ca\(^{2+}\)]\(_i\) baselines suggesting a lower activated state (one-way ANOVA, p<0.05, Fig 5.3A), but there were some differences among minocycline treatment regimes. Minocycline (10,100 µM) applied to microglia for 24 hours post-isolation in the presence of both high and low concentrations of conditioned medium (H.C.M), demonstrated a significant reduction in the initial [Ca\(^{2+}\)]\(_i\) baselines. Minocycline (100 µM) applied pre-isolation in the flask was unable to effectively reduce baseline [Ca\(^{2+}\)]\(_i\) levels. In addition, when minocycline (100 µM) was applied pre-isolation, there was an observed lack of longevity of mixed glial cultures leading to loss of proliferation, and a lower yield of microglia as a result. Time control recordings observed the change in [Ca\(^{2+}\)]\(_i\) over one hour (Fig 5.3 B). All microglia demonstrated some change in [Ca\(^{2+}\)]\(_i\), although there were no significant differences between treatment variants, (one-way ANOVA, p>0.05). Despite this, the microglia pre-treated in the flask with minocycline (100 µM) pre-isolation, had a higher level of variability after one hour. The most effective treatment categorized by the lowest variability and lowest mean change
in \([\text{Ca}^{2+}]\), over the one hour in KREBS HEPES was the minocycline 10 \(\mu\text{M}\) treatment post-isolation. This treatment was therefore used for \(\text{Ca}^{2+}\) imaging recordings to assess the effects of 1 hour application of ATP or LPS in the presence and absence of galanin.

### 5.3.2 ATP-Induced Microglial Activation

When ATP (50 \(\mu\text{M}\)) was applied to control microglia (no pre-treatment with minocycline), there was an initial large increase in \([\text{Ca}^{2+}]\), and a large increase in \([\text{Ca}^{2+}]\), levels after one hour, suggesting microglial activation (Fig 5.3C, 5.4A). This change at 1 hour was compared in microglia pre-treated with several minocycline (10,100 \(\mu\text{M}\)) treatments. In each case minocycline was removed upon preparation for \(\text{Ca}^{2+}\) imaging and cells superfused with KREBS HEPES for at least 1 hour prior to recording. All minocycline treatment variations (10 \(\mu\text{M}\), 100 \(\mu\text{M}\)) demonstrated a significant ATP-induced increase in \([\text{Ca}^{2+}]\), levels after one hour when compared to their initial \([\text{Ca}^{2+}]\), levels prior to ATP treatment, (paired t-test, \(p<0.0005\), Fig 5.3, C). This indicated that minocycline treated microglia were able to be activated in the presence of ATP despite apparently beginning in a lower activated ion state. The degree of ATP-induced change in \([\text{Ca}^{2+}]\), levels over one hour for all minocycline treated groups were comparable to control, (one-way ANOVA, \(p>0.05\), 5.3,C). Overall, minocycline (10 \(\mu\text{M}\)) application post-isolation was identified as the most suitable concentration to be applied to microglia prior to \(\text{Ca}^{2+}\)recordings due to significant lowering of initial baseline \([\text{Ca}^{2+}]\), levels and the ability to be activated by ATP during subsequent recordings.
The Effect of Galanin on ATP-induced Microglia Activation

ATP is known to increase microglial $[Ca^{2+}]_i$ levels, leading to chemotaxis and cytokine release (Fig 5.1) (176), and galanin is reported to inhibit cytokine release from microglia (5). Upon application of ATP for 1 hour in the presence of galanin, it was clear that galanin was capable of modulating responses to ATP. In comparison to control, galanin (1 pM, 100 nM, 1 µM) significantly augmented the ATP-induced change in $[Ca^{2+}]_i$ after 1 hour, (one-way ANOVA p<0.01 1 pM, p<0.05 100 nM, 1 µM, Fig 5.4). This suggests that in the presence of ATP, galanin may augment microglia activation. From $Ca^{2+}$ imaging recordings, application of galanin alone showed no evidence of direct microglial activation, based on the lack of change in $[Ca^{2+}]_i$ (Fig 5.4B).
Figure 5.3 Microglia Culture Optimisation: The Effect of Modifying Microglia Activation States

(A) Initial [Ca\(^{2+}\)]\(_i\) levels in microglia as recorded by Ca\(^{2+}\)imaging. Minocycline was able to maintain microglia in a less activated state, as shown by the significantly lower initial [Ca\(^{2+}\)]\(_i\) levels when compared to control. (B) The change in Fura-2 fluorescence ratios for time control experiments following various regimes for pre-treatment with minocycline. There were no significant differences across all treatment groups, over the one hour duration between recordings. (C) In the same experiment as (A), treatment with 1 hour ATP (50 µM) significantly increased the initial baseline [Ca\(^{2+}\)]\(_i\) levels in control and minocycline treated microglia. The degree of change after 1 hour ATP was comparable between control and minocycline groups.

* = p<0.05, *** = p<0.0005 versus control (one-way ANOVA, post-hoc Dunnett’s test), bars represent mean ± SEM.
ATP Activation in the presence of Minocycline

Control

Mino 10µM (H.C.M, Post-isolation)

Mino 10µM (Post-isolation)

Mino 100µM (Flask, Pre-isolation)

Mino 100µM (Post-isolation)

---

**A**

<table>
<thead>
<tr>
<th>n=37</th>
<th>n=38</th>
<th>n=21</th>
<th>n=49</th>
</tr>
</thead>
</table>

**B**

| n=19 | n=21 | n=19 | n=38 | n=16 |

**C**

| n=37 | n=38 | n=21 | n=49 |

---

**Legend**

- Control
- Mino 10µM (H.C.M, Post-isolation)
- Mino 10µM (Post-isolation)
- Mino 100µM (Flask, Pre-isolation)
- Mino 100µM (Post-isolation)
Figure 5.4 The Effect of Galanin on ATP-Induced Microglial Activation

(A) Ca²⁺ imaging recording of a microglial cell treated for 1 hour with ATP (50 µM). ATP application induced an initial spike in \([\text{Ca}^{2+}]_i\), and after 1 hour application resulted in a sustained increase in \([\text{Ca}^{2+}]_i\) levels. (B) Another microglial cell was treated for 1 hour with ATP (50 µM) in the presence of galanin (1 pM). In comparison to (A), ATP-induced \([\text{Ca}^{2+}]_i\) changes were augmented in the presence of galanin. (C) In the presence of galanin at a range of concentrations, ATP-induced \([\text{Ca}^{2+}]_i\) changes were augmented. Duration of drug applications is indicated by horizontal bars. 1hr ATP vs Control *** = p<0.001, Gal 1 pM vs ATP 1hr ## = p<0.01, Gal 100 nM, 1 µM vs ATP 1hr # = p<0.05, Gal 1 nM vs ATP 1hr , one-way ANOVA, post-hoc Dunnett’s test. Bars represented as mean ±SEM.
5.3.3 The Effect of Galanin on Microglial Responses to Short Term LPS Exposure

Similarly to experiments involving activation with ATP, modulation of LPS-induced microglial responses by galanin was also investigated. In contrast to ATP application, LPS did not cause a fast initial increase in \([\text{Ca}^{2+}]_i\). After one hour exposure to LPS (100 ng/ml), there was a significant increase in \([\text{Ca}^{2+}]_i\) levels in comparison to control, (one-way ANOVA \(p<0.001\), Fig 5.5A,C). The addition of the \(\text{Ca}^{2+}\) ionophore ionomycin, resulted in only a slight increase in \([\text{Ca}^{2+}]_i\) levels suggesting a large increase in \(\text{Ca}^{2+}\) permeability had already occurred during LPS treatment (data not shown). LPS-activation of microglia occurs via a variety of targets including TLR receptors and MAC1 reportedly leading to cytokine release and ROS production (see Fig 5.1). The LPS-induced increase in \([\text{Ca}^{2+}]_i\), over one hour was unaffected by co-applications of a series of concentrations of galanin (1 pM, 1 nM, 100 nM, 1 µM, one-way ANOVA \(p>0.05\), Fig 5.5B,C). This suggests that galanin may not modulate the short term effect of LPS activation pathway in microglia.
Figure 5.5  The Effect of Galanin on One Hour Exposure of LPS in Microglia

(A) $Ca^{2+}$ imaging recording of a microglial cell before and after 1 hour treatment with LPS (100 ng/ml). (B) $Ca^{2+}$ imaging recording of a microglial cell treated with LPS (100 ng/ml) for 1 hour in the presence of galanin (1 µM). (C) In the presence of LPS (100 ng/ml) calcium levels were elevated, when compared to control. Galanin had no effect on the LPS-induced increase in intracellular $Ca^{2+}$. 1hr LPS 100ng/ml vs control *** = $p<0.001$, galanin vs LPS 1hr alone, one-way ANOVA, post-hoc Dunnett’s test. Bars represented as mean ± SEM.
5.3.4 The Effect of Galanin on Responses to 24 Hour LPS Exposure in Microglia

In contrast to short term LPS exposure, longer term exposure to LPS produced a decrease in 
$[\text{Ca}^{2+}]_i$ levels. Treating microglia with LPS (100 ng/ml, 1 µg/ml) for 24 hours, resulted in a significant decrease in initial $[\text{Ca}^{2+}]_i$ levels when compared to control, t test, (p<0.001) (Fig 5.6 A). At both concentrations tested, LPS (100 ng/ml, 1 µg/ml) demonstrated a comparable reduction in $[\text{Ca}^{2+}]_i$ levels. Microglia treated for 24 hours with LPS were observed for morphological changes using light microscopy. Despite the observed reduction in $[\text{Ca}^{2+}]_i$, images clearly show characteristics of microglial activation including round, amoeboid appearance with retracted processes in contrast to control microglia with extended processes and a flat ramified appearance (Fig 5.6 B). In contrast, to the lack of effect of galanin on the early response to LPS exposure (see Fig 5.5B,C), galanin was able to modulate the response to 24 hour application of LPS. Upon application of the highest concentrations of galanin (100 nM, 1 µM), there was a significant enhancement of the LPS-induced reduction in $[\text{Ca}^{2+}]_i$ levels, (one-way ANOVA p<0.001, Fig 5.6C). This suggests that LPS modulates microglial Ca$^{2+}$ signalling differently after short term versus long term exposure, and that galanin can enhance the LPS-induced changes in microglia after 24 hours.
5.3.5 The Effect of Twenty Four Hour LPS Exposure on Nitric Oxide Production

As illustrated in Figure 5.1, LPS acts on several receptors leading to cytokine release, ROS production and nitric oxide release. Upon 24 hour application of LPS (100 ng/ml), microglia were loaded with DAF-FM, which increases in fluorescence intensity upon NO production, as described in SECTION 2.2.6. In comparison to control, mean fluorescence intensity was not increased following 24 hour LPS exposure (100 ng/ml) (Fig 5.6D). Indeed, there was a trend for a decrease in NO production in the presence of LPS, (t test p=0.078 versus control).
Figure 5.6 The Effect of Galanin on the Response to 24 Hour LPS Exposure in Microglia

(A) $Ca^{2+}$ imaging recordings revealed LPS (1 µg/ml, 100 ng/ml) decreases microglial $[Ca^{2+}]_{i}$ after 24 hour treatment. This was unlike the activation seen in the presence of 1 hour LPS treatment indicating a biphasic response. (B) Morphological changes from a ramified to a more amoeboid morphology are apparent in 24 hour LPS treated microglia. (C) Upon application of galanin, there was a concentration dependant enhancement of the LPS induced decrease in $[Ca^{2+}]_{i}$ levels. (D) Microglia were stained with DAF-FM which increases in mean fluorescence intensity in the presence of nitric oxide production. Microglia treated with LPS (1 µg/ml) for 24 hours showed no difference in DAF fluorescence to that of control microglia, (t-test LPS 1 µg/ml, 24h vs 24h control, n/s ). *** = $p<0.001$ LPS 24h 1 µg/ml, 100 ng/ml vs Control, (t-test ). ### = $p<0.001$, LPS 100 ng/ml 24h vs Control, LPS and Galanin 100 nM, 1 µM, *** = $p<0.001$ 24h vs LPS 24h alone,(one-way ANOVA post-hoc Dunnett’s test). Bars represented as mean ± SEM.
A

B

C

D

135
5.3.6 The Effect of Galanin on ROS Production in Microglia

Microglia were treated with various microglial activators in the presence and absence of H2 DCFDA, a cell permeable fluorescent dye which increases in mean fluorescent intensity upon the production of ROS, see section 2.2.7. As illustrated in Fig 5.1, microglial activation reportedly leads to NO and cytokine release, and the production of ROS. LPS has been linked to the production of ROS, however effects can vary between different strains. LPS bacterial strain O26:B6 used for all previous LPS treatments in this study, was compared to another strain of LPS O1 11:B4. The mean fluorescence of microglia loaded with DCFDA, increased in intensity due to basal ROS production over one hour, when measured at fifteen minute intervals (Fig 5.7A). The response to microglial activator PDBu, known to stimulate ROS production, was used as an internal standard and data was expressed as percent of the PDBu response. Whilst basal DCFDA oxidation increased steadily over time, PDBu induced microglial ROS production was much greater than basal production over the one hour period. When both LPS strains were applied in the absence of DCFDA, there was no measurable fluorescence, indicating that LPS did not autofluoresce (Fig 5.7A). Fluorescence measurements for both strains of LPS (1 µg/ml, 100 ng/ml) in the presence of the DCFDA, were comparable to DCFDA alone, indicating LPS was unable to cause ROS production above basal levels, (one-way ANOVA p>0.05, Fig 5.7A,B). This prevented further investigation of the effect of galanin on LPS-induced ROS production. Therefore, galanin was applied in the presence and absence of other microglial activators ATP, Zymosan and PDBu (Fig 5.8). As previously, all data is represented as a percentage of the control PDBu response. Zymosan is known to act on the integrin CD11b receptor in the microglia cell membrane, and caused comparable ROS production to that of PDBu treatment. Galanin alone (1 pM, 100 nM, 1 µM) did not produce ROS, and had no effect when combined
with other treatment regimes (one-way ANOVA p>0.05, Fig 5.8 B,C,D). However, galanin did significantly lower basal ROS production levels when compared to DCFDA alone, suggesting galanin has an inhibitory rather than stimulatory effect on basal microglial ROS production (one-way ANOVA, p<0.01, Fig 5.8 A).
Figure 5.7 The Effect of LPS Strain Variants on ROS Production in Microglia

(A) ROS assay showing fluorescence intensity measurements from microglia. Two LPS variants were investigated for ROS production in microglia. Neither strain of LPS (1 µg/ml, 100 ng/ml) altered ROS production with measurements comparable to DCFDA alone over 1 hour. (B) Results of assay are expressed as a percentage of the PDBu response. Both strains and both concentrations tested were, significantly lower than PDBu, known to produce ROS (one way ANOVA, p <0.001). NS=not significant, LPS strains O26:B6 and O1 11:B4 (1 µg/ml, 100 ng/ml) 1h vs DCFDA (one-way ANOVA). Bars represented as mean ± SEM.
Figure 5.8 The Effect of Galanin on ROS Production in Microglia.

(A) DCFDA fluorescence after 1 hour in the presence of galanin, Zymosan, and ATP. All values represented as a percentage of the control PDBu response. Galanin significantly inhibited basal oxidation of the dye while ATP had no effect, despite the large response to zymosan. (B) The addition of galanin at all concentrations had no effect on PDBu-induced ROS production. (C) Zymosan stimulated levels of ROS production comparable to PDBu, but the addition of galanin did not affect the response to zymosan. (D) ATP did not increase ROS production levels above those seen basally, and this did not change when galanin was added. **= p<0.01, *=p<0.05 compared to DCFDA alone.
5.4 Discussion

The current study provides evidence that galanin can modulate the processes underlying microglial activation, including responses to both ATP and LPS. Galanin was able to augment the ATP-induced increase in intracellular Ca\(^{2+}\) levels after one hour. This suggests that galanin may have an effect on the early stages of microglia activation via tissue damage and pathologies. The known inhibitor of microglia activation minocycline (10 µM) (175), significantly lowered basal [Ca\(^{2+}\)]\(_i\) prior to recordings, suggesting lower activation, but following its removal increased [Ca\(^{2+}\)]\(_i\) was seen upon ATP application. This study also showed evidence of galanin acting on signalling pathways activated by LPS in microglia, in particular the later phase of the LPS response which is thought to be activated in the presence of infections. While galanin did not have an effect on the 1 hour LPS-induced increase in [Ca\(^{2+}\)]\(_i\) levels, galanin enhanced the LPS-induced reduction of [Ca\(^{2+}\)]\(_i\) levels after 24 hours. Whilst [Ca\(^{2+}\)]\(_i\) levels were reduced after 24 hours, LPS treated microglia demonstrated amoeboid morphology consistent with activation. These findings combined measurements following 24 hour LPS, showed a trend for a decrease in NO production suggests that LPS has time dependant effects which are biphasic. After an hour treatment two different bacterial LPS strains, were unable to induce ROS production despite large responses to PDBu and zymosan. Galanin showed no evidence of ROS production either in the presence or absence of other treatments and instead appeared to reduce basal ROS levels.

5.4.1 Complexities of Microglial Activation

Morphological changes from ramified microglia to amoeboid phenotype is generally accepted to be associated with an increase release of cytotoxic mediators and inflammatory cytokines. However, the results presented here clearly suggest that this view is too simplistic. In addition,
microglial intracellular $[\text{Ca}^{2+}]_i$, levels are often linked with an increase in the level of activation, however from the current study the relationship between the two is also not simple. When assessing the degree of microglial activation, therefore a multitude of factors which must be taken into consideration including morphology, $[\text{Ca}^{2+}]_i$, levels, ROS production, cytokine release and, for in vitro studies, the degree of any prior-activation. To address prior microglial activation, this study used minocycline to reduce basal $[\text{Ca}^{2+}]_i$ levels. Minocycline prevents microglial NO release and ROS production (175). However, since minocycline was removed before recordings, microglia were still able to be stimulated by ATP, producing a sustained increase in $[\text{Ca}^{2+}]_i$ levels. Microglia prepared for the ROS and NO assays, were not pre-treated with minocycline, which may explain the lack of increase in ROS in ATP-treated microglia. However, large responses to PDBu and Zymosan treated microglia suggest this is unlikely. On the other hand, there was a basal level of ROS production that was reduced in the presence of galanin. A previous study from galanin has been shown to inhibit cytokine release from LPS treated microglia however the mechanism involved is not fully understood and requires further investigation. While there may be differences in microglial behaviour in vitro and in vivo, this is the most optimal method to investigate the in vitro cellular mechanisms involved.
5.4.2 Short Term LPS Exposure: Effect of Galanin on Changes in $[\text{Ca}^{2+}]_i$, Levels and ROS Production

While extensive studies have investigated the effects of LPS on microglia activity, the functional outcomes of activation of the several pathways remain unclear. Only recent evidence has shown that the MAC1 complex is involved in LPS responses, in addition to the previously known TLR4 receptor (10). Studies have shown that LPS causes an increase in functional signs of microglial activation by an increase in $[\text{Ca}^{2+}]_i$ and ROS production (11). My findings indicated that short term exposure to LPS increased $[\text{Ca}^{2+}]_i$ levels, there was an absence of LPS induced ROS production. While an LPS-induced increase in $[\text{Ca}^{2+}]_i$ can be an indication of microglial activation, this can vary from LPS strain variants, and is not always the case (141). However, the two LPS bacterial strains tested in the current study failed to produce ROS, although this may have been due to a lack of cross-linking of receptors. Indeed zymosan used in the current study, can cause cross linking and ROS production (178). Due to the lack LPS-induced ROS production, it was then not possible to establish the effect of galanin on this pathway. Galanin, either alone or in the presence of ATP, zymosan or PDBu did not lead to ROS production. However, galanin significantly lowered basal levels suggesting galanin may have an inhibitory effect on ROS production in the early stages of the LPS pathway.

5.4.3 Long Term LPS Exposure: Effect of Galanin on Changes in $[\text{Ca}^{2+}]_i$, Levels and NO Release

In contrast to the short term effects of LPS, 24 hour LPS exposure reduced microglial $[\text{Ca}^{2+}]_i$, and did not cause NO release. This may be due to the lack of cross linking or receptors, or possibly due to the potential interference of one of the myriad of receptors which are involved in microglial activation. For example, sigma receptor activation has been shown to reduce LPS
induced NO production and rising intracellular Ca\(^{2+}\) levels in chronically (≤24 hours) treated microglia (137). Furthermore, evidence by Hoffmann et al. relates an increase in Ca\(^{2+}\) levels with NO production (11). Taken together, other receptors may have played a role in the current study in influencing the LPS induced reduction in intracellular Ca\(^{2+}\) levels and a lack of an increase in NO production after 24 hours. Hoffmann et al. did show that an increase in [Ca\(^{2+}\)]\(_i\) was necessary, but not sufficient for LPS induced NO production. As discussed (see section 5.4.3) an LPS-induced increase in [Ca\(^{2+}\)]\(_i\) can indicate microglial activation, however this does not always occur (141). In concert with the current study, Beck et al. demonstrated an LPS reduction in [Ca\(^{2+}\)]\(_i\) levels after 24 hours. The reduction of [Ca\(^{2+}\)]\(_i\) was due to a down regulation of Ca\(^{2+}\) release activated Ca\(^{2+}\) channels I\(_{(CRAC)}\) (179). My results suggest that long term activation of LPS receptors could be in fact decreasing microglial activation, or that an increase in [Ca\(^{2+}\)]\(_i\) levels is required for microglial activation in the long term.

From the current study, galanin appears to inhibit long term responses to LPS. The results from the current study suggest that galanin augments the effects of LPS when it is reducing [Ca\(^{2+}\)]\(_i\), and not increasing NO production. It is unclear what effect galanin may have when LPS increases [Ca\(^{2+}\)]\(_i\), and increases NO production, and needs further investigation. By LPS and galanin cooperatively lowering [Ca\(^{2+}\)]\(_i\), this suggests that galanin can modulate long term LPS activation of microglia. Indeed, galanin has already been shown to inhibit LPS induced cytokine release (5), which suggests that galanin has a beneficial role in reducing LPS activation from infections. The evidence so far suggests that galanin appears to be acting exclusively on later steps of the LPS pathway in microglia.
5.4.4 ATP exposure: Effect of Galanin on Changes in \([\text{Ca}^{2+}]_i\) Levels and ROS Production

Similarly to LPS, ATP exposure for 1 hour failed to cause ROS production, which was not changed by the addition of galanin. However, ATP did result in an increase in \([\text{Ca}^{2+}]_i\) levels. A recent study showed that ATP activation of microglia via P2X4 receptors causes a long-term increase in \([\text{Ca}^{2+}]_i\) via the MAP kinase pathway, leading to BDNF production (133). In addition to BDNF release via P2X4 activation, the prolonged release from damaged CNS nervous tissue is implicated in mechanical allodynia seen during neuropathic pain (8). P2X7 is reported to indirectly activate the same p38-MAP kinase pathway, to contribute to microglial-induced inflammation by resulting in release of Interleukin 1beta and cathepsin S (180). Similarly to ATP, galanin has been shown to promote migration of microglia to damaged neural tissue acting via the MAP kinase pathway (174), which suggests this as a mechanism by which galanin may modulate ATP-induced effects. In support of this, whilst galanin alone had no effect on microglial \([\text{Ca}^{2+}]_i\) levels in the current study, galanin enhanced the ATP-induced increase in \([\text{Ca}^{2+}]_i\) levels, suggesting galanin is able to augment functional changes in microglia in the presence of ATP. Indeed, ATP-induced microglial activation after nerve damage leads to the release of inflammatory mediators implicated in symptoms of neuropathic pain (131). This suggests that galanin may have a role by increasing ATP-induced microglia activation, and may be acting in a pro inflammatory manner.
5.4.5 Conclusions

The current chapter has determined that galanin augments ATP-induced increase in $[\text{Ca}^{2+}]_i$. As discussed, prolonged ATP activation of microglia can lead to neuropathic pain symptoms, and suggests that galanin may act in a pro inflammatory mechanism via ATP interaction. It is possible that by enhancing the initial inflammatory stage, this may lead to less chronic inflammation and associated neuropathic pain states. In the current study, galanin was also found to modulate another signaling pathway, namely responses to LPS, by enhancing LPS-induced decrease of $[\text{Ca}^{2+}]_i$ levels after 24 hours. Combined with recent evidence of galanin inhibiting LPS-induced cytokine release, it suggests that galanin may be capable of reducing microglial activation during infections. It is important to note that LPS is involved in infection, and not associated with either analgesia or neuropathic pain development. With this in mind, it is possible that galanin enhancement of LPS-induced reduction in $[\text{Ca}^{2+}]_i$ levels may be used in future studies to further investigate inflammatory pain associated with infection. While there is little known about the effect of galanin on microglial activation, the current study provides evidence of galanin modulating two major pathways, which suggests a diverse role for galanin in modulating microglial activation.
CHAPTER 6:
GENERAL DISCUSSION

6.1 General Conclusions and Future Directions

As discussed in chapter one, the neuropeptide galanin is up-regulated in response to nervous system injury in key areas of the sensory pathway including DRG, DH, and most recently in microglia. The rationale for the current thesis was to investigate areas of the galanin field which required more clarification, including the new area of interest into the role of galanin in microglial activation. Galanin had been shown in previous reports to increase microglial [Ca$^{2+}$]$_i$, and migration (174), and inhibit cytokine release (5), suggesting galanin had a role in modulating microglial activation. The present thesis has investigated the role of galanin on microglial activation in pathways involved in infection and nervous tissue injury. Prolonged activation of microglia can lead to neuropathic pain states (8), which is a common condition, yet poorly treated. One of the only neuropathic pain treatments gabapentin inhibits P/Q VGCC in the DH (12), and galanin acting via GAL$_2$ has been shown to also inhibit synaptic transmission in this region (6). However activation of GAL$_1$ in DRG neurons have been shown to increase [Ca$^{2+}$]$_i$ (86). It is clear that the role of GAL$_2$ in DRG neurons, including the mechanisms and target VGCC required investigation.

As shown in chapter three, this study indicates that galanin acts via GAL$_2$ to repeatedly inhibit HVA Ca$^{2+}$ currents and Ca$^{2+}$ transients, in both whole cell patch clamp and Ca$^{2+}$ imaging. This is the first evidence to date of galanin inhibiting [Ca$^{2+}$]$_i$, acting via GAL$_2$ in small, nociceptive DRG neurons, and addresses a gap in the field of galanin. The currently accepted theory is that galanin
increases [Ca^{2+}], and membrane excitability in DRG neurons (86), which suggests galanin increases Ca^{2+} signalling during synaptic transmission, and a possible cellular explanation of galanin in a pro-nociceptive role. Whilst the current study disagrees with the previous report, it is important to note that the common method used in each study was whole cell patch clamp. As discussed in chapter 3, there were key differences in the methodology used, which may have led to desensitised responses and the apparent differences in the previous study. While this thesis has contributed an important finding, it is clear that further investigations are required to establish more evidence on the GAL₂ mediated effects in DRG neurons. The most appropriate recommendation for future in vitro research would ultimately be the perforated patch clamp technique, which utilises the properties of gramicidin during recordings, which allows membrane pore formation without dialysing the cell contents, and indeed a direct recording of HVA Ca^{2+} currents. Indeed, a previous study in other cells using perforated patch clamp has indicated galanin-induces HVA Ca^{2+} currents inhibition in parasympathetic cardiac neurons (89). While debate in the literature suggests two possible roles for galanin, evidence from Merriam et al. (89), of an inhibition of Ca^{2+} currents in cardiac neurons in addition to Ca^{2+} currents and Ca^{2+} transients in DRG from this thesis, suggests an inhibitory and anti-nociceptive role for galanin. In support of this, further evidence in the DH indicates galanin acting via GAL₂ inhibits synaptic transmission (6), suggesting an inhibitory effect on noxious transmission.

My results support the inhibitory role of galanin, as demonstrated by galanin inhibition of Ca^{2+} transients and Ca^{2+} currents in DRG neurons. Furthermore, galanin demonstrated a comparable amount of inhibition of [Ca^{2+}], in DRG to DAMGO, and exceeded these inhibition levels when acting via GAL₂. Indeed, galanin has already been shown to enhance the analgesic effects of
morphine in an acute pain behavioural study (1). Evidence of galanin’s ability to enhance the effect of morphine in the DH, provides hope of galanin being developed as an alternative analgesic to morphine. While morphine is an effective treatment for acute pain, chronic pain is not effectively treated due to µ opioid receptor desensitisation. This can lead to break through pain, where additional morphine is required to treat intermittent increases in the pain intensity. From the current thesis, responses from galanin receptor agonists are reproducible, which is a desirable quality for the potential development into an analgesic. Whilst being in peptide form is a disadvantage, a human study has successfully established an effective method of administrating hourly doses of galanin intravenously (106), which suggests galanin would be an effective method to intermittently enhance the effects of morphine to combat break through pain.

As discussed, it is well known that µ opioids inhibit HVA N-type Ca^{2+} channel currents specifically via G i/o G proteins (see chapter 4) (159, 160). Likewise, the accepted model of galanin binding is via GAL_{1} and GAL_{2} is also by coupling via Gi/o G proteins (87), which can result in N-type VGCC inhibition (89, 94, 161, 162). In addition to N-type VGCC inhibition, previous reports indicate µ opioids inhibit TRPV1-induced increases in [Ca^{2+}]_{i} (164), however galanin was not found to share this ability in the current study. Despite this, the current thesis determined that GAL_{2} induced inhibition of N-type VGCC by galanin in DRG neurons, was not an exclusive interaction. This study provides novel evidence to address a gap in the literature that GAL_{2} receptor targets not only N-type VGCC, but also P/Q-type VGCC in DRG neurons. This finding compliments evidence that GAL_{2} more commonly acts via Gq/11 G proteins (55), and the emerging evidence which indicates inhibition of P/Q channels in central regions for food intake, cardiovascular and hormonal regulation (166).
From the current thesis, it is clear there is a need for further investigation of galanin-induced GAL2 interactions with P/Q-type channels, rather than the generally accepted view of a specific N-type channel interaction. It would be of particular interest to examine the extent of galanin interaction with P/Q-type channels, and if this could be a possible therapeutic target in the future. As mentioned in chapter one, neuropathic pain is a chronic pain condition which is poorly treated. One of the few treatments known as gabapentin, inhibits P/Q-type VGCC in DRG neurons and the DH (12, 169). As mentioned earlier, galanin may have a potential role at enhancing morphine’s analgesic effect for the treatment of acute pain. With the new findings from this thesis, galanin inhibition of N and P/Q-type channels in DRG neurons has provided new hope of becoming a possible therapeutic agent for the treatment of both acute and chronic pain states such as neuropathic pain.

It is well known that galanin is up-regulated after nervous system injury and modulates neuropathic pain (see chapter 1), however only recently this was discovered to also occur in microglia (11). While little is currently known about the interaction between galanin and microglia, early evidence indicates GAL2 expression in microglia, and upon galanin binding can inhibit cytokine release from activated microglia (5). As shown in chapter five, the current thesis has contributed findings to an area of galanin research which is only in the early stages of investigation. Galanin was found to augment ATP-induced activation of microglia in the current study, suggesting galanin may have a role in enhancing initial microglial activation after nervous system injury. In support of this, a recent report has implicated galanin in the increase of microglial [Ca2+]i and migration in microglia (174). Unlike the augmentation of ATP, galanin had no effect on the early stages of LPS-induced microglial activation, and after 24 hour LPS
exposure galanin enhanced the reduction of microglial [Ca\(^{2+}\)]. This supports the idea that galanin may reduce long term LPS induced microglial activation. Unfortunately, after many studies, the current knowledge of the LPS-pathway in microglia is not complete and as such, can be difficult to draw conclusions on where in this pathway galanin is acting. Until recently, LPS was thought to act via TLR4, however it has now been shown to act via MAC1 (10). What appeared to be a marker for microglial activation was a LPS-induced increase in [Ca\(^{2+}\)], however evidence from the current study and others clearly indicate this is not the case (179). Perhaps the lack of LPS-induced NO production, yet activated morphology combined with a decrease in microglial [Ca\(^{2+}\)], enhanced in the presence of galanin, eludes to complexities of microglial activation which the current field is not yet aware of. It is quite clear that extensive amount of investigation is still required to understand the full extent of the role galanin plays in microglial activation.

Microglial activation is complex and not fully understood, however is known to result in an increase microglial [Ca\(^{2+}\)], pro and anti-inflammatory cytokines, NO release and ROS production (11). However, recommendations for future in vitro investigations would be to use the primary microglial culture regime we developed using minocycline (see chapter five), as this would allow the activation to unfold during treatments rather than previously during culture preparation.

Taking complexities into consideration, the ideal approach would be to focus on the effect of galanin on each individual outcome before looking into in vivo models both wild type and galanin transgenic over expressing and KO models. Indeed, there is some evidence to date that P2X4, P2Y12 and other purinergic receptors are involved in the development of neuropathic states (7, 8), therefore the acute pain and initial stages of microglial activation during this process
could also be investigated in the same studies. While only speculative at this early stage, possible therapeutic applications may involve utilizing galanin augmentation of the initial ATP-induced microglial activation, in particular for newly admitted traumatic brain injury patients. Administered via a spinal tap, the microglia pre-activated by galanin and ATP would reach and enhance migration to the damaged area, promote removal of damaged tissue so as to reduce secondary damage and chronic inflammation. Galanin augmentation of the initial ATP-induced microglial activation could be utilized as a preventative measure to reduce the likelihood of the development of chronic inflammation and neuropathic pain symptoms, which are associated with prolonged purinergic receptor activation. However, it is yet to be seen if this approach would be useful therapeutically. As discussed, galanin could also be administered to enhance the analgesic effect of morphine in the DH during the acute phase of hospitalisation, and again at a later stage if neuropathic pain symptoms develop. However adjuvant therapies of minocycline and galanin may be the best approach for pain therapy to promote analgesic effects, yet prevent galanin from augmenting ATP-induced microglial activation. A second administration of microglia pre-treated with minocycline and galanin may then be used to reduce long term activated microglia and act as a neuroprotective agent. Indeed, long term activation of ATP receptors P2X4 and P2Y12 have been implicated in leading to neuropathic pain (7, 8), and hyperalgesia from morphine treatment (172). However, GAL2 receptor antagonists may be developed in future studies to block the ATP- induced purinergic receptor activity, and reduce the occurrence and severity of these debilitating conditions.

It is hoped that findings from the present study has provided an insight into the potential application of galanin as a new therapeutic alternative in the future, and provides hope for
patients suffering from acute and neuropathic pain. Indeed, with neuropathic pain being so common, there is a clear need for more options for patients to enjoy a better quality of life. I hope the microglia culture technique developed and observations of galanin modulating microglial activation through two separate activation pathways via GAL$_2$, will provide a basis for future studies investigating the role of galanin in microglial activation. The current study has provided sound evidence of GAL$_2$ specific inhibition of both N and P/Q-type channels in DRG neurons, and hope these findings lead to future studies investigating the mechanism in which galanin targets P/Q-type channels via GAL$_2$. The current thesis has contributed strong evidence about the role of galanin in DRG neurons and microglia, and provided a strong platform for future investigations and possibly therapeutic applications.
REFERENCES


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