The mechanisms of GABAergic signalling in the peripheral pain pathway

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Abstract

Peripheral pain pathway plays a crucial role in how pain is perceived and felt. The dorsal root ganglia (DRG) which house the primary sensory neurons have become the focus of many emerging pain studies due to its potential as a functional structure in controlling pain transmission, and not only for producing proteins and providing nutrients essential for neuron survival. The major inhibitory neurotransmitter in the nervous system, GABA has been shown to play a significant role in this regard.

Within the present study, the mechanism of GABA release within DRG neurons was investigated by studying the expression of vesicular GABA transporter (VGAT) in the DRG neurons. VGAT was highly expressed in the DRG neuron somata. The VGAT-positive neurons also expressed markers of subpopulations of DRG neurons, including those involved in nociception. The availability of VGAT luminal (VGAT-C) and cytoplasmic (VGAT-N) domains were utilised to investigate the mechanism of GABA release in a live DRG neuron culture. This mechanism involves the recycling process of vesicles following their exocytosis. Imaging of the internalization of VGAT-C domain during vesicle recycling indicates GABA is released via exocytosis and has both, tonic and activity-dependent components. Using the *in vivo* electrophysiological recordings, neuronal firing in the spinal nerve and dorsal branches of the peripheral nerve (before and after the DRG, respectively), was investigated. These data revealed existence of a ‘filter’ in the DRG that decreased the frequency of the neuronal firing passing through the DRG. This filtering effect was overcome by bicuculline, a $GABA_A$ receptor antagonist indicating the role of $GABA_A$ receptor in peripheral pain pathway. This role of $GABA_A$ receptor was also supported by the decrease in $GABA_A$ receptor activation in the presence of bicuculline in DRG neurons co-cultured with HEK293 cells.

In sum, in the DRG, GABA is liberated into the interneuronal space via Ca^{2+}-dependent vesicular exocytosis, which in turn acts on $GABA_A$ receptors. This GABAergic signalling is responsible for filtering the action potentials from the periphery to the central terminals in the spinal cord. These findings identify and further characterize peripheral ‘gate’ within the somatosensory system.

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Abbreviations

$[Cl^-]_i$	Intracellular Cl- concentration
5HT3Rs	Serotonin type 3 receptors
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaCCs	calcium-activated chloride channels
cAMP	cyclic adenosine monophosphate
cdk5	Cyclin-dependent kinase 5
CDS	Ca2+-dependent secretion
CFTR	Cystic fibrosis transmembrane conductance regulator
CGRP	Calcitonin gene-related peptide
CHO	Chinese hamster ovary
CIP	Congenital insensitivity to pain
CiVDS	Ca2+-independent but voltage-dependent-secretion
CNS	Central nervous system
CRPS	Complex regional pain syndrome
CRPS1	Complex regional pain syndrome, type 1
CWP	Chronic widespread pain
DAB	3,3′-Diaminobenzidine
DBI	Diazepam binding inhibitor
DMEM	Dulbecco’s Modified Eagle Medium
DREADD	Designer receptor exclusively activating designed drugs
DRG	Dorsal root ganglia
EC	Extracellular
ECD	Extracellular domain
E_{Cl}	Cl^- reversal potential
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
ER	Endoplasmic reticulum

FBS	Fetal bovine serum
FDA	Food and Drug Administration
FNG	Fluoronanogold
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAT-1	GABA transporter-1
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GlyRs	Glycine receptors
GPCRs	G-Protein coupled receptors
GS	Glutathione synthase
HEK cells	Human Embryonic Kidney 293 cells
HEK indicator cells	HEK cells with a I^--sensitive EYFP mutant (H148Q/I152L) but not the α1, β2, and γ2 $GABA_A$ receptor subunits
HEK_{GABAA} indicator cells	HEK cells transfected with EYFP H148Q/I152L and the α1, β2, and γ2 $GABA_A$ receptor subunits
HIV	Human immunodeficiency virus
IB4	Isolectin B4
IFs	Intermediate filaments
IHC	Immunohistochemical
IPSPs	Inhibitory postsynaptic potentials
ISH	In situ hybridisation
KCC2	Potassium-chloride cotransporter
LTMRs	Low-threshold mechanoreceptors
Mrgprd	Mas-related G-coupled protein receptors
nAChRs	Nicotinic acetylcholine receptors
NF	Neurofibrils
NF200	Neurofilament 200
NGF	Nerve growth factor
NKCC1	Sodium-potassium-chloride cotransporter
NT3	Neurotrophin 3
PAD	Primary afferent depolarisation
PAM	Allosteric modulator
PBS	Phosphate buffer saline

PFA	Paraformaldehyde
PNS	Peripheral nervous system
PTPσ	Protein tyrosine phosphatase sigma
RNA-Seq	RNA sequencing
RT-PCR	Reverse-transcriptase polymerase chain reaction
RVM	Rostral ventromedial medulla
SCS	Spinal cord stimulation
SGC	Satellite glia cells
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors
SV2	Synaptic vesicle antibody
SVZ	Subventricular zone
TIRF	Total internal reflection fluorescence
TMD	Temporomandibular disorder
Trk	Tyrosine kinase
TRPV1	Transient receptor potential subfamily V1
VGAT	Vesicular GABA transporter
VGATgp	VGAT raised in guinea pig
VGATrab	VGAT antibody raised in rabbit
VGCCs	Voltage-gated calcium channels
VPL	Ventroposterolateral
VRAC	Volume-regulated anion channel
YFP	Yellow fluorescent protein

Publication

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Chapter 1 Introduction

1.1 Pain- why is it so important?

Humans are blessed with the ability to enjoy and 'sense' the beauty of the world via five sensory systems; ears (hearing), olfactory organ (smell), eyes (vision), tongue (taste) and the skin (somatosensation). Of these five, somatosensation is multimodal and able to detect different sensations: proprioception, pain, temperature and touch via their own receptors namely, proprioceptors, nociceptors, thermoreceptors and mechanoceptors respectively. Touch can further be subdivided into detection of curvature, hardness, shape, texture, pruriception (itch detection) and pleasurable touch (Johnson et al., 2000, McGlone and Reilly, 2010, Woolf and Ma, 2007). As alluded to above, these sensations can be gratifying but obversely, one sensation, in particular, can cause various degrees of displeasure and discomfort to the body; this is the very familiar sensation known as pain.

The International Association for the Study of Pain has defined pain as 'an unpleasant emotional and sensory experience that is related to actual or potential tissue damage or described in terms of such damage' (IASP). For most individuals, pain is a subjective experience with each individual especially of different ethnicity and race, perceiving pain differently when given the same noxious stimuli (Kim et al., 2017a, Larsson et al., 2017, Rahavard et al., 2017). The resulting experience is influenced by cognitive and contextual factors (Lee and Tracey, 2013); for some, pain can be felt without an identifiable nociceptive input and conversely activation of nociceptors may not cause pain at all (Lee and Tracey, 2010). Regardless of the aetiology, pain is indeed associated with many pathological conditions that has become one of the common reasons for people to seek treatment. Approximately 28 million adults (approximately a third of the population) are affected by chronic pain (see below) in

the UK alone (Fayaz et al., 2016). This places both an economic and social burden on the country as it affects the quality of life of those suffering.

However, looking past the emotional and behavioural aspects of pain that we are all accustomed to, this sensation plays a crucial role in allowing us to function properly and more importantly, ensuring the survival of the body (Karos et al., 2017, Leknes et al., 2006). When one thinks of pain, an unpleasant sensation is almost always pictured but biologically, pain is indeed a very important defence mechanism that protects the body from injury. Of critical importance is that pain aversiveness facilitates learning that could affect decision making preventing potential future injury (Wiech and Tracey, 2013). Examples of such mechanisms include moving a hand away from a flame without physically touching it and hitting on a fractured bone which results in pain– the former protects the body from pain whereas the latter reminds the body to stop further injury and therefore allow healing to occur. Interestingly, some defects occurring along the nociceptive pathway may render the sufferer painless to any noxious stimuli, which may seem to be the holy grail of pain management. However, as mentioned, the protective nature of pain means that inability to feel pain exposes such individuals to more harm. It has been reported that spontaneous and unconscious behaviours such as biting lips and/or the tongue, as well as injuries such as bone fractures may occur unnoticed in people insensitive to pain (Nahorski et al., 2015). The genetic basis of patients suffering from insensitivity to pain usually inherit this disability and suffer from birth; this condition has been termed congenital insensitivity to pain (CIP) Ultimately, the lack of awareness to pain can lead to accumulation of injuries which in many cases can unfortunately affect the life expectancy of these people.

CIP may occur due to mutations involving voltage-gated sodium channels (Cox et al., 2006) or through congenital absence or progressive degeneration of sensory and autonomic neurons (Rotthier et al., 2012). To date, there are a few conditions reported that have been associated with CIP in which several gene mutations were

present. In 2006, an article published in Nature reported a group of six Pakistani children (aged 6 to 14 years) who suffered from a complete insensitivity to pain and due to this, these children endured physical injuries such as burns and fractures. Genetic investigations revealed all six Pakistani children had a nonsense mutation in *SCN9A* which encodes the α subunit of the voltage-gated sodium channel $Na_v1.7$, making them unable to sense thermal and mechanical pain due to inability to transmit signals through axons (Cox et al., 2006). A less common type of mutation, which was found in 4 families worldwide, is the gain of function mutation in the $Na_v1.9$ channel which results in nerve dysfunction and loss of pain perception (Leipold et al., 2013, Phatarakijnirund et al., 2016, Woods et al., 2015). In 2008, another type of CIP was reported in Italy, affecting six individuals across three generations (a mother, her two daughters and their children, two boys and one girl aged 24, 21 and 16 respectively) (Habib et al., 2018). Further investigations by a group of scientists discovered that this family had a defect in the *ZFHX2* gene, a nociceptor-expressed transcriptional regulator (Habib et al., 2018).

However, not all characteristics of pain are in favour of the body. In certain conditions, pain ceases to be protective and occurring only when required. Instead, it becomes persistent and constantly present without any need hence transforming into a nuisance to the body– this is known as chronic pain (Treede et al., 2019). In pathological conditions such as in an individual with a spine injury, the nerve may get affected and the pain becomes chronic in nature tormenting (instead of protecting) the individual.

1.1.1.1 Nociception

The perception of pain from noxious stimuli requires an intact communication between peripheral and central nervous system (CNS). Noxious stimuli trigger generation of action potentials that are propagated via the primary afferent fibres to the spinal cord and finally reach the brain where pain is perceived. Within the spinal cord, these action potentials also activate excitatory interneurons which in turn excite the α and γ motor neurons, leading to subsequent motor responses such as contraction of flexor muscles of the stimulated limb. The ability of the peripheral fibres to detect noxious stimuli and integrate the signals at the level of the spinal cord and the brain is essential for the organism's wellbeing.

Pain and nociception are two distinct terms which are often confused. The distinction was first clarified by Charles Sherrington, a neurophysiologist, in 1906 (Sherrington, 1906), who stated that 'nociceptors' (neurons) were the neural apparatus for noxious signal-sensing. The IASP defines nociception as the neural process encoding the noxious stimuli while pain is the physical and emotional experience resulting from such process (IASP).

Acute pain serves a biological purpose where it warns the body of any disease or injury and is time-limited– once the injury has recovered, the pain subsides as it is no longer required. The peripheral nerve terminals are responsible for sensing the initial noxious stimulus and transmitting this to higher centres for processing. The molecular process of sensing the initial stimulus through receptors and proteins is known as nociception, hence neurons involved in nociception are known as nociceptors. Nociceptors detect painful sensation from the skin, muscle and viscera and are first-order neurons with cell bodies located in the dorsal root ganglia (DRG). Nociception for the orofacial region occurs via the trigeminal nerve whose cell bodies are housed in the trigeminal ganglia.

The process of nociception involves detection of stimuli by the peripheral nerve endings, which convey information through axons and neuronal cell bodies before synapsing at the first-order neurons located in the dorsal horn of the spinal cord. The first-order neurons, also known as primary sensory neurons, are distinguished from each other by their conduction velocity, size or the somatosensory sensation they carry (Dubin and Patapoutian, 2010)- into Aα, Aβ, Aδ and C fibres (**Table 1**).

Table 1 Classification of Sensory Fibres in Peripheral Nerves. (Kandel et al., 2012).

Fibre type	Fibre group	Fibre diameter (μm)	Conduction velocity (m/s)	Receptor type	modality
Myelinated	Aα (Large diameter)	12 - 20	72 - 120	Proprioceptors	Touch
	Aβ (Medium diameter)	6 - 12	36 - 72	- Meissner corpuscle - Merkel disc receptor - Pacinian corpuscle - Ruffini ending - Hair-tylotrich - Field	- Stroking, flutter - Pressure, texture - Vibration - Skin stretch - Stroking, flutter - Skin stretch
	Aδ (Small diameter)	1 - 6	4 - 36	Thermal - Cool receptors - Heat nociceptors	- Skin cooling (<25˚C/77˚F) - Hot temperature >45˚C/113˚F
				Nociceptors - Mechanical - Thermal mechanical (heat)	- Sharp, pricking pain - Burning pain
Unmyelinated	C	0.2 – 1.5	0.4 – 2.0	Thermal - Warm receptors - Cold receptors	- Skin warming >35˚C/95˚F - Cold temperature <5˚C/41˚F
				Nociceptors - Thermal mechanical (cold) - Polymodal	- Freezing pain - Slow, burning pain

Nociceptors are defined as having a high threshold for activation. Thus, they are activated by noxious but not innocuous stimuli. Most literature reports two major classes of nociceptors; the medium diameter myelinated (Aδ) afferent fibres and the small-diameter unmyelinated C afferent fibres (Meyer et al., 2006). Aδ fibres mediate acute, localised fast pain, while C fibres are responsible for the poorly localised, slow pain. C-fibres are also involved in the detection of innocuous, low temperatures (Meyer et al., 2006, Samour et al., 2015).

The Aα/β fibres are considered as low-threshold mechanoreceptors. These fibres have often been overlooked as playing a role in nociception however, a few studies have shown evidence of their involvement. Studies in different species (guinea pig, rat and mouse) have reported that 18% – 65% of A-fibre nociceptors indeed conduct action potentials in Aβ conduction velocity range (Djouhri and Lawson, 2004). The non-nociceptive Aβ fibres respond to innocuous mechanical stimuli such as light touch. In a chronic constriction injury rat model, most of the Aβ fibres did not conduct action potentials upon stimulation, however, these fibres showed the highest percentage of spontaneous discharge as compared to Aδ and C fibres (Kajander and Bennett, 1992). Consistent with this, Truini and colleagues studied neuropathic pain in carpal tunnel syndrome and found that paroxysmal pain was related to high-frequency bursts from the demyelinated Aβ fibres. However, whether the high-frequency bursts are sufficient to provoke pain or require the ephaptic transmission to the neighbouring C-fibres is yet to be investigated (Truini et al., 2009).

The different fibres of the first-order neurons; Aα, Aβ, Aδ and C fibres enter the spinal cord and terminate on second-order neurons and local interneurons at different layers of the dorsal horn. These layers, termed laminae are organized in ten laminae according to their cytoarchitectonic characteristics within the gray matter of the spinal cord (Rexed, 1952). Nociception carried by the C and Aδ fibres predominantly arborize and terminate in laminae I and II with some fibres arborizing deeper into lamina V. **Figure 1.1** (Todd, 2010).

From these laminae, the second-order neurons project to medulla, mesencephalon, and thalamus via the spinothalamic tract before synapsing on the third-order neurons within the somatosensory and anterior cingulate cortices where the sensory-discriminative and affective-cognitive aspects of pain is perceived (Millan, 1999).

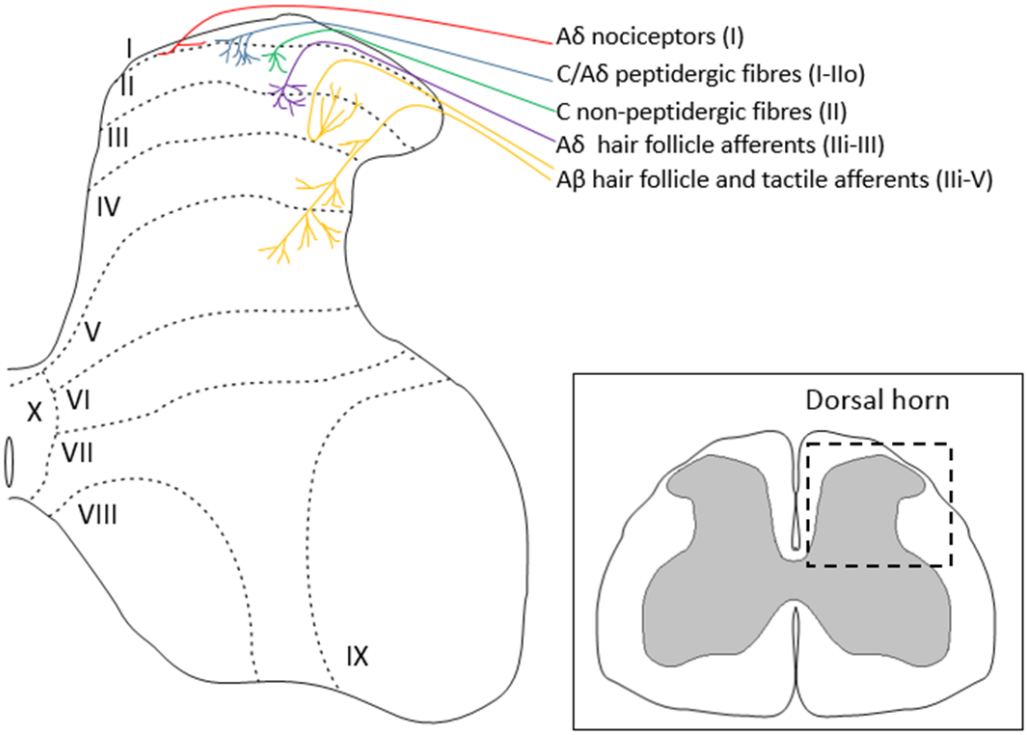


Figure 1.1 The terminations of A and C fibres in different laminae in the rexed dorsal horn of the spinal cord. Aδ nociceptors end mainly in lamina I, peptidergic primary afferents (which also include some Aδ nociceptors) arborize mainly in lamina I and lamina IIo, with some fibres penetrating more deeply, whereas most non-peptidergic C fibres form a band that occupies the central part of lamina II. Aδ hair follicle afferents arborize on either side of the border between lamina II and lamina III, whereas Aβ tactile and hair afferents end mainly in lamina III–V [Adapted from (Todd, 2010)].

1.1.2 The Gate Control Theory of Pain

During the Middle Ages, pain was perceived as a punishment from gods or demons for sins committed, however, this perception was later challenged. In the 17th century, René Descartes' tried to embody the transmission of pain by producing illustrations reflecting how pain occurred (Illustration of the pain pathway in René Descartes' Traite de l'homme (Treatise of Man) 1664 (**Figure 1.2**). Descartes stated that pain was a sensation that came from the body itself following exposure to noxious external stimuli such as fire that caused burning of the skin. The information of the burnt skin was conveyed from the site of injury to the brain where the individual experiencing it would respond to the sensation by removing the burnt part of the body from the flame body. This theory gave rise to specificity theory which maintained that the free nerve endings were pain receptors before being refuted and superseded by pattern theory in the late 19th century. Unlike specificity theory which agreed with Descartes (that free nerve endings were pain receptors), pattern theory proposed that stimulus intensity and central summation were the critical determinants of pain (Perl, 2007). Despite being able to explain many of the clinical phenomena in pain, pattern theory was lack of substantial experimental verifications and failed to comprise a satisfactory general theory of pain which lead to the emergence of Gate Control Theory of Pain (Melzack and Wall, 1965). Using the Gate Control Theory of Pain, Melzack and Wall suggested a clearer pathway of how pain transmission works (Melzack and Wall, 1965). According to this theory, the brain receives information about an injury by way of a gate control system which is influenced by: i) an injury signal that is transmitted via the small C and Aδ fibres, ii) cells that are excited by the injury signal which also receive facilitation or inhibitory inputs from other peripheral nerve fibres which carry innocuous information, and iii) descending control systems that project from the brain and modulate the excitability of cells which transmit the information about the injury. This gate control system involves the interaction of these components within the dorsal horn of the spinal cord; it controls the input from the

peripheral fibres (that carry injury signal) onto the projecting T cells that travel via the ascending pathway to the brain where pain is perceived (Melzack and Wall, 1965).

Figure 1.2 Descartes' concept of the pain pathway. He writes: If for example fire (A) comes near the foot (B), the minute the particles of this fire, which as you know move with great velocity, have the power to set in motion the spot of the skin of the foot which they touch, and by this means pulling upon the delicate thread CC, which is attached to the spot of the skin, they open up at the same instant the pore, i.e., against which the delicate thread end, just as by pulling at one end of a rope one makes to strike at the same instant a bell which hangs at the other end (Descartes, 1901).

The main postulate of the Gate Control Theory of Pain can be exemplified by what happens when one experiences a mosquito bite: it feels itchy when a person is first bitten, however after rubbing the affected area, the urge to itch recedes- even if this is temporary. The mosquito bite serves to sensitize the itch receptor carried by C and Aδ fibres. Upon one rubbing the affected skin in response to the mosquito bite, stimulation of Aβ fibres which are non-nociceptive fibres involved in detecting mechanical sensation such as touch, overwhelms the firing coming from the C fibres, hence 'closing' the gate to resist transmission from the C fibres to the spinal cord and CNS. Ultimately, this renders our perception of the skin as less itchy. More specifically, Gate Control Theory postulates that the stimulation of non-nociceptive fibres gates the nociceptive signals from being transmitted to the brain at the level of the spinal cord resulting in less pain perception.

Pain is indeed a complex condition which intrigues scientists to explore further avenues to gain a better understanding of the mechanisms behind the sensation. Fascinatingly, recent work from our group has proposed a new addition to the Gate Control Theory (Du et al., 2017) whereby in addition to 'classical' spinal gate, an earlier gate exists at the level of peripheral ganglia (see below).

1.1.3 Acute and chronic pain

Depending on its mechanism and duration, pain can be categorised into acute or chronic. Acute pain is a short, self-limited condition that involves sensitisation of nociceptors followed by the perception of pain. Acute pain occurs following tissue injury, surgical procedures or a brief disease process. These conditions produce either thermal, chemical or mechanical stimuli that sensitise the nociceptors. These stimuli are converted into electrical potentials by means of ion channels and G protein-coupled receptors. These potentials are then transduced to the CNS where pain is integrated and modulated. Thus these three components, the stimulus, transduction and central integration and modulation are the important components in the nociceptive pathways.

On the other hand, chronic pain is defined as pain which lasts longer than three months (Treede et al., 2019). Chronic pain is caused by an underlying pathology or pain which healing goes beyond the normal duration. Different types of chronic pain have been described which include conditions such as chronic widespread pain (CWP), fibromyalgia, complex regional pain syndrome, type 1 (CRPS1), temporomandibular disorder (TMD), irritable bowel syndrome (IBS) and most back pain and neck pain conditions (Nicholas et al., 2019). Unlike heart disease or lung cancer, chronic pain is not the most common cause of death but individuals suffering from chronic pain will have limited ability to perform daily activities and depression that may sometimes lead to suicidal thought (Rizvi et al., 2017). Chronic pain affects the sufferers physically and emotionally and inevitably incur a financial burden not only to the individuals but employers, healthcare systems and society in general (Breivik et al., 2006, Breivik et al., 2013).

The aetiology and pathophysiology of chronic pain are sometimes obscure and not well understood, however, three contributing components exist and interplay with each other; biological, psychological and social factors (Fillingim et al., 2014, Gatchel et al., 2007). Biologically, chronic pain is viewed as direct results of disease

processes, while psychological problem such as depression can be a risk factor for chronic pain and vice versa (Fishbain et al., 1997, Lepine and Briley, 2004). The psychosocial factors such as fear-avoidance behaviour, social support and physical environment contribute to the limitation of activities and pain-related disability (Zale et al., 2013, WHO, 2015).

As psychological presentations in patients with chronic pain can be related to the underlying pain mechanisms, a group of researchers in Brazil conducted a clinical study among female human immunodeficiency virus (Chivet et al., 2014) patients suffering from chronic pain. This study identified patients with chronic pain with two different mechanisms, nociceptive and neuropathic, by using Leeds Assessment for Neuropathic Symptoms and Signs (LANSS) (Bennett, 2001). Their results showed that females with chronic neuropathic pain suffered more compared to females living with chronic nociceptive pain. Female HIV patients suffering from chronic neuropathic pain presented with catastrophizing and higher depression scores, more sleep disturbances and less resilience. Meanwhile, females living with chronic nociceptive pain lead a life similar to pain-free females and only differed in having a higher level of anxiety (de Souza et al., 2018). These findings, albeit the first on this subject, may give an insight for better management of HIV patients with chronic pain.

1.1.3.1 Nociceptor sensitisation

Pain alerts the body of the presence of a harm that has the potential to cause injuries to the body. The ability of the nociceptors to undergo sensitisation allows an increase in the pain signal occurring (Woolf and Ma, 2007). There are two main manifestations of sensitisation - hyperalgesia (peripheral sensitisation) and allodynia (central sensitisation) (Woolf, 2011). After injury, nociceptors are exposed to the 'inflammatory soup' consisting of the various inflammatory mediators which are able to exert their effects on the array of receptors present to produce an enhancement of the pain response. This is known as 'hyperalgesia', where there is an exaggerated pain sensation towards standard pain signals (Loeser and Treede, 2008). Meanwhile, allodynia is a mechanism of central sensitisation where innocuous touch stimuli elicit pain responses when normally this would not be the case (Loeser and Treede, 2008). This mechanism requires rearrangement of neuronal connections in the dorsal horn, which are part of the mechanoreceptive Aβ-fibers, into the nociceptive pathway (Woolf, 2011). Differential nerve fibre blocks by compression suggested that allodynia is mediated by low-threshold Aβ fibres (Koltzenburg et al., 1994). Some studies suggested a phenotypic change of Aβ fibres contributes to the spontaneous firing during peripheral nerve injury (Jensen and Finnerup, 2014). The *de novo* expression of neuropeptides such as calcitonin gene-related peptide (CGRP), substance P and brain-derived neurotrophic factor (BDNF) particularly at the level of DRG may influence the neuropathy-induced hypersensitivity. Both of these mechanisms are also hallmarks of chronic pain syndromes; conditions such as fibromyalgia manifests hypersensitivity and allodynia (Henriksson, 2003).

1.1.4 Gender bias in pain study

To gain a better understanding of both the physiology and pathology of pain and potential strategies of pain management, scientists across the globe have conducted many studies involving both sexes in humans and animals. As males and females may show differences in physiological responses towards any physical trauma or insult to the body, scientists are rightly concerned about the consequence of conducting such studies on a single sex. A review by Mogil and Chanda reported 79% of animal studies published in *Pain* involved male subjects only compared to 8% involving female subjects only and only 4% focussing on the differences between both sexes (Mogil and Chanda, 2005). Most research focuses on males as hormonal changes during the oestrous cycle in females may affect nociceptive responses during experiments. Despite this, Mogil and Chanda showed that variability and pain responses between female and male subjects were not significantly different. Thus, they suggested that despite the concerns regarding hormonal changes that may occur in females, they should still be included in studies of pain (Mogil and Chanda, 2005). Another recent review by Bartley and Fillingim on sex differences in pain further suggested that more factors may contribute to variation in pain responses between the two sexes (Bartley and Fillingim, 2013). Their review included both epidemiological and clinical studies which demonstrated that women are subject to an increased risk of chronic pain and may experience a more severe clinical pain compared to men. Females also exhibit more pain sensitivity, enhanced pain facilitation and reduced pain inhibition in experimentally-induced pain studies. Sex-related differences in responses to pharmacological and non-pharmacological pain treatments also exist although they seem to depend on specific pain treatment and management. Surprisingly, gender biases appear to exist and are influenced by the characteristics of both patient and provider. These variations in pain responses in both males and females are attributed to multiple biological mechanisms such as gonadal hormones, endogenous opioid function and genetic factors and

psychosocial factors such as pain coping, catastrophizing and gender roles. Apart from hormonal changes, immunocompetent cells are also important in the pathophysiology of pain. Pain in males is related to microglial activation during injury (Sorge et al., 2015) whilst females are more dependent on the activation of the adaptive immune system, most likely T lymphocytes. However, despite the increasing reports on sex-related differences towards responses to pain, more robust evidence is required before sex-specific tailoring of treatment can be implemented.

1.2 Dorsal root ganglia

1.2.1 Anatomy, physiology and clinical significance

The DRG is a ganglionic organ harbouring a collection of primary sensory neurons that lie between the spinal nerve and the dorsal root. Anatomically, DRG are located on the pedicle of the vertebra body, at the upper third of the intervertebral foramina. The primary sensory neurons are pseudounipolar fibres with one branch extending from the T-junction to the peripheral nerve ending and another branch extends towards the spinal cord **Figure 1.3**. While the estimated number of neurons in the brain is 86 billion (Azevedo et al., 2009), one DRG on average contains approximately 6,000 neuronal somata (Arvidsson et al., 1986). Unlike the brain which permits only small molecules to get through due to the presence of blood-brain-barrier (BBB), DRG are an intensely vascularised structure with high permeability between blood and nervous tissues (Jimenez-Andrade et al., 2008, Hirakawa et al., 2004). Compared to spinal nerves, DRG have a significantly increased permeability to molecules as large as albumin (Olsson, 1968), especially at the cell body-rich area (Godel et al., 2016).

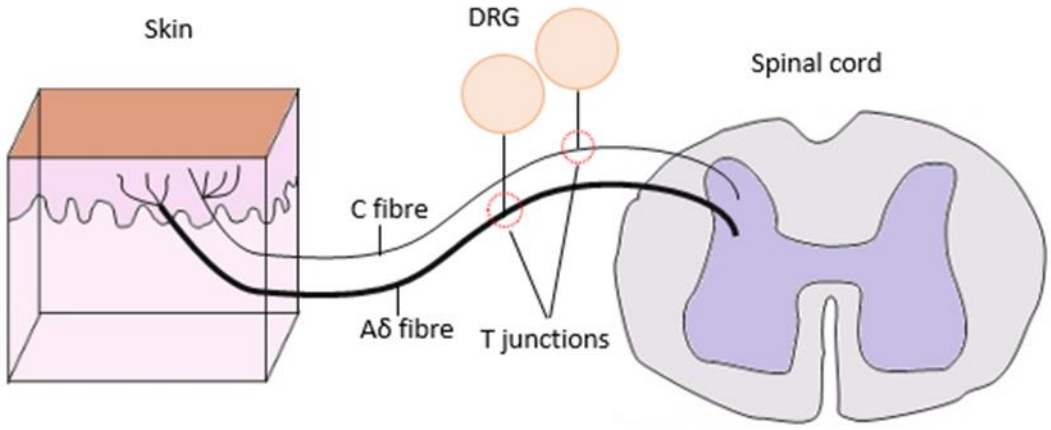


Figure 1.3 Schematic of dorsal root ganglia neuron with its axon bifurcating peripherally to the skin and centrally to the dorsal horn of spinal cord.

DRG neurons convey various somatosensory information from the receptive fields of the skin, visceral organs and joints, to the CNS through the spinal cord. These different sensations are carried by the different nerve fibres running close to each other, enclosed by the insubstantial pia mater. Apart from this very crucial function, DRG have been shown to play an important role in axonal degeneration and the synthesis of proteins and organelles which are transported via its peripheral and central axons [reviewed in (Nascimento et al., 2018)].

Disruption to DRG neurons due to mechanical injury or inflammation may render them more sensitive to nociceptive signalling and thereby increase neuronal excitability and generation of ectopic discharges (Chen et al., 2019, Zhang et al., 2018, Chang et al., 2017). Evidence is present to suggest that various channels are upregulated after nerve injury; Nav1.7 channels have been shown to be upregulated in DRG neurons of rats in Paclitaxel-induced neuropathy and more relevantly in humans with neuropathic pain (Li et al., 2016).

As mentioned above, due to the lack of blood-DRG barrier, DRG have become a viable option for the application of local anaesthesia and drugs of larger molecular size. However this could also be a disadvantage; the permeability unselectively allows the passage of the low and high molecular weight of toxic substances (Cho, 1977, Le and McLeod, 1977, London and Albers, 2007, Viaene et al., 1999, Cavanagh and Barnes, 1973).

Recent years have witnessed more studies on drug delivery via DRG (Berta et al., 2017, Krames, 2015, Sapunar et al., 2012). Gene therapy-based approaches use a different method in delivering the viruses of interest to DRG. In animal studies involving rodents, microneurosurgical injection (Glatzel et al., 2000) and subcutaneous inoculation (Liu et al., 2008) of the selected viral vectors have been shown to be successful in delivering target genes into DRGs. This novel approach of drug delivery may have the potential for future clinical use in the treatment of chronic

pain which could be a potential therapeutic method in drug delivery (Beutler, 2010, Glorioso and Fink, 2009, Goins et al., 2012).

Despite the success of studies on direct injection of drugs onto DRG, access to DRG neurons remains a challenge. DRG are an isolated organ and access is difficult due to overlying structures such as bone. In animal studies, three different approaches have been used for drug delivery to DRG; percutaneous injection, intraganglionic injection after soft tissue removal with advancement of the needle into intervertebral foramen and intraganglionic injection after laminar bone removal and exposure of caudal pole of the DRG (Puljak et al., 2009). Amongst these three methods, partial laminar bone removal (laminectomy) and exposure of caudal pole of the DRG was shown to be the most successful despite the trauma that was caused by the removal of bone– **Figure 1.4.** The accuracy of the injection into the DRG has been validated with dye injections (Du et al., 2017, Puljak et al., 2009).

Direct local administration of drugs into nerves is not a new method. This method of drug delivery has a long history tracing back to the early 19th century. In 1845, Sir Frances Rynd had injected morphine solution via a cannula to relieve intractable neuralgia which became the first ever documented regional nerve block (Rynd, 1845). Targeting drug delivery via injection into nerves has since evolved significantly. Peripheral nerve blocks (Ilfeld, 2011), perineural approaches (Sundara Rajan et al., 2017, Thor et al., 2017, Gharaei and Whizar-Lugo, 2015), sympathetic block (Alexander and Dulebohn, 2017, Cheng et al., 2012, Menon and Swanepoel, 2010), epidural and intrathecal pathways (Kedlaya et al., 2002) have been discovered and developed over time. As ectopic nerve firing generated from the soma of the DRG neurons is believed to be a major contributor to neuropathic pain (Devor, 2009, Han et al., 2000, Koplovitch and Devor, 2018, Nassar et al., 2006), it is imperative to overcome the ectopic nerve firing that comes from the DRG. Thus, a local approach of drug delivery direct into DRG would be a very useful mode of drug delivery and

effective in minimising the unwanted systemic effect of the centrally- or non-locally administered drug.

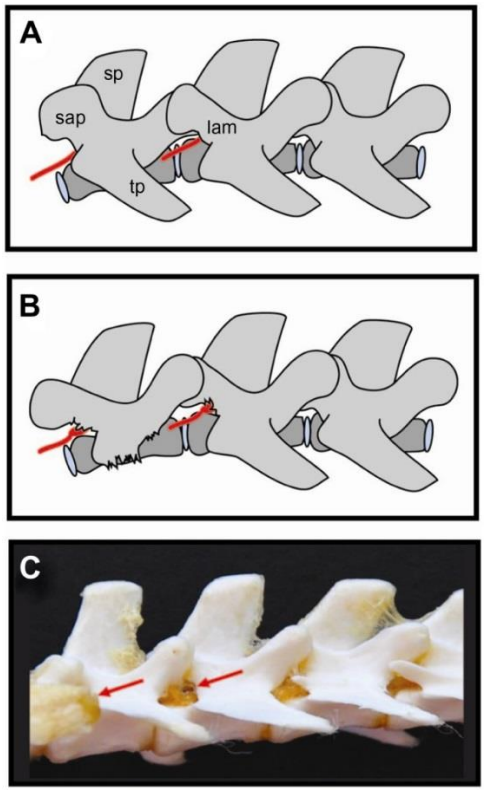


Figure 1.4 Schematic drawings (A and B) and photo (C) of a rat spine depicting the position of dorsal root ganglia and surgical approach used for DRG injection. Reliably precise injection can be performed using minimal laminectomy, while blind needle-insertion approach requires a lot of skill and experience. The dorsal root ganglion is covered by laminar bone. (B) Removal of laminar bone superior to the foramen and the L4 accessory process reveals the distal dorsal root ganglion, recognised by its broader diameter. Abbreviations: red arrows, dorsal root ganglion (L5 and L6); sap, superior articular processes; sp, spinous processes; tp, transverse processes; lam, laminar bone. (Sapunar et al., 2012).

The majority of targets would involve using therapeutic blocks of pro-nociceptive receptors to reduce pain however, inhibitory channels and receptors also exist that function to reduce the firing (Waxman and Zamponi, 2014). One of the examples of these are the chloride ion channels that will be discussed in the next section.

DRG neurogenic modulation was approved almost a decade ago in Europe and Australia. In 2016, the Food and Drug Administration (FDA) in the USA has also approved this new therapeutic advance in treating neuropathic pain (Deer and Pope, 2016). This relatively new therapy is similar to spinal cord stimulation (SCS). Both therapies involve a lead inserted into the epidural space of the spine, but instead of leaving the lead in the spinal cord, it is steered to lie along DRG. The lead is attached to a device that produces electrical impulses to reduce the excessive neuronal firing in neuropathic pain. This therapy has been proven to be effective in the treatment of complex regional pain syndrome (CRPS), phantom pain and postsurgical pain and is superior to SCS (Harrison et al., 2018, Deer et al., 2013, Rowland et al., 2016).

The different techniques for pain management discussed above involve disruption of the electrical signalling from the receptive nerve endings to the higher centres. The electrical signalling is controlled partly by different types of ion channels. In this thesis, I explored the role of one of the ion channels activated by gamma-aminobutyric acid (GABA)– the $GABA_A$ receptor, in the GABAergic signalling in the DRG. Thus the following section will discuss the $GABA_A$ receptor as a chloride ion channel in the mammalian GABAergic system.

1.3 Chloride channels

Cl- channels are a functionally and structurally diverse group of anion-selective channels involved in wide variety of processes including the regulation of the excitability of neurones, skeletal, cardiac and smooth muscle, cell volume regulation, trans-epithelial salt transport, the acidification of internal and extracellular compartments, the cell cycle and apoptosis (Nilius and Droogmans, 2003). Based on their regulation, mammalian chloride ion channels are classified into several subtypes including cystic fibrosis transmembrane conductance regulator (CFTR), cyclic adenosine monophosphate (cAMP)-dependent phosphorylation-activated chloride channels; calcium-activated chloride channels (CaCCs); voltage-gated chloride channels (VGCCs); ligand-gated chloride channels (GABA and glycine); and volume-regulated chloride channels (Verkman and Galietta, 2009). There is no official recommendation exists regarding the classification of chloride channels although IUPHAR classifies chloride channels as CIC1 through CIC7 with two additional groups, CIC-Ka and CIC-Kb (Alexander et al., 2017). However, this thesis will focus on ligand-gated GABA channels which will be discussed in more detail in the coming sections.

1.4 GABA as inhibitory neurotransmitters

1.4.1 GABA neurotransmitter

GABA was first discovered in plants in 1883 as a by-product during plant metabolism. In plants, GABA accumulation occurs as a result of stress such as hypoxia, heat and cold, drought, mechanical wounding and infection (Shelp et al., 2012). Bown and Shelp reported GABA involvement in defending plants from insect herbivory and drought by maintaining root growth via regulation of a malate-transporting plasma membrane channel and inducing stromal closure, respectively (Bown and Shelp, 2016).

In 1950 Awapara and colleagues discovered that GABA was also present in the mammalian CNS when they successfully isolated, identified, and estimated the level of GABA in the different parts of human brain tissue and a few other animal species (Awapara et al., 1950). However, it was not until several years later that the discovery of the GABA as an inhibitory neurotransmitter was made by Florey and Bazemore whom initially referred GABA as factor I (I for inhibitory action on neuronal activity) (Bazemore et al., 1957). A few years later, the same group discovered that factor 'I', extracted from mammalian brain contained GABA, which they suggested to be a type of neurotransmitter (Florey and McLennan, 1959).

Almost a decade later, the exact action of GABA was confirmed by Krnjevic and Schwartz who studied the effect of GABA on cat cerebral cortical neurones and found unequivocal evidence for GABA as an inhibitory neurotransmitter (Krnjević and Schwartz, 1967). Their findings showed that GABA application had similar actions to a physiological neurotransmitter in cortical neurons on membrane potential and resistance and produced inhibitory postsynaptic potentials (IPSPs). Krnjevic and Schwartz also showed that similar to the reversal of IPSP demonstrated by physiological transmitter upon increase in the membrane potential or changes in local ion concentration, GABA was also able to reverse IPSP in these two conditions (Krnjević and Schwartz, 1967).

GABA concentrations have been reported in the literature, in the brain and spinal cord; extracellular [GABA] in the brain has been reported to be 0.1 – 0.8 μM (Cavelier et al., 2005, Hagberg et al., 1985, Lerma et al., 1986), whilst cytosolic [GABA] ranges from 1 to 6 mM (Otsuka et al., 1971) and 0.7 to 1.3 mM in the brain and spinal cord, respectively (Van der Heyden et al., 1979).

1.4.1.1 GABA Synthesis

A neuron which synthesises and releases GABA is termed a GABAergic neuron. In the mammalian system, GABA synthesis takes place in neurons via decarboxylation of glutamate by the enzyme glutamic acid decarboxylase (GAD). Two isoforms of GAD have been identified, which were named according to its different molecular weights; GAD65 and GAD67 (Soghomonian and Martin, 1998). GABA is then transported into vesicles, and upon generation of an action potential at the nerve terminal, influx of Ca^{2+} into the presynaptic terminals causing the release of GABA into the synaptic cleft. Interestingly, GABA can also be released non-vesicularly via the reversal of GABA transporter-1 (GAT1) (Wu et al., 2007). Using Chinese hamster ovary (CHO) cells, Wu and colleagues showed that the action of GAT1 could be reversed in situations where there is strong depolarisation or altered homeostasis, and that this could also occur in physiological conditions (Wu et al., 2007).

Figure 1.5 summarises the synthesis, packaging, release, transport, and metabolism of GABA (Roth and Draguhn, 2012). The released GABA can be recycled via three pathways; 1) reuptake by GABA transporter back into the cell, 2) via neuronal glutamate pathway, where extracellular glutamate is transported into GABAergic neuron by neuronal glutamate transporters, or 3) via glutamate-glutamine pathways which involve supporting glial cells (see chapter 3) and neurons. In glutamate-glutamine pathways, glutamate is converted to glutamine by glutamine synthetase and is then transported out of astrocytes, which are specialised glial cells in the spinal cord and brain, and into GABAergic neurons. In these neurons, glutamine is converted to glutamate and ultimately to GABA by glutaminase and GAD, respectively.

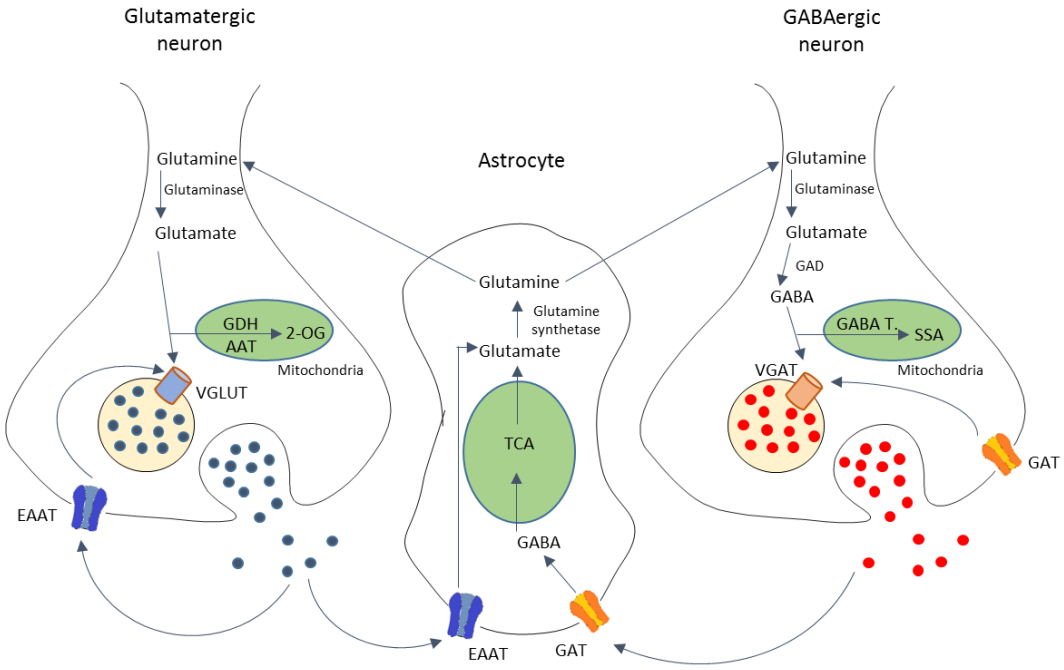


Figure 1.5 Summary on the neurotransmitter glutamate and GABA synthesis, packaging, release, transport, and metabolism in astrocyte, glutamatergic and GABAergic neurons. In GABAergic neuron, glutamine is converted to glutamate then to GABA by glutaminase and GAD respectively. Subsequently GABA is transported into the vesicle by VGAT before it is released into the synaptic cleft. Upon release, GABA is taken up into neurons and glia by GAT where they can be recycled or metabolised via several enzymatic processes. **Abbreviations**: 2-OG, 2-oxoglutarate; AAT, aspartate aminotransferase; Aralar, aspartate-glutamate carrier; Asp, aspartate; EAAT, excitatory amino acid transporter; GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; GAT, GABA transporter; GDH, glutamate dehydrogenase; SSA, succinic semialdehyde; TCA, tricarboxylic acid cycle; VGAT, vesicular GABA transporter; vGlut, vesicular glutamate transporter [adapted from (Rowley et al., 2012)].

1.4.1.2 Functions of GABA

GABA has piqued the interest of many researchers decades ago with regards to its involvement in pain transmission. Such involvement has been reported as early as in 1981 by Krogsgaard in his perspective on the design of GABA agonists, antagonists and uptake inhibitors, with relation to their respective therapeutic uses particularly in pain and other conditions such as neurological and psychiatric disorders, anxiety and epilepsy (Krogsgaard-Larsen, 1981). Enhancement of GABAergic neuronal signalling has been the therapeutic target for treatment of anxiety, panic disorders, epilepsy, sleep disturbances, muscle spasms and for improving learning and memory (Krogsgaard-Larsen et al., 2004, Rowlett et al., 2005, Rudolph and Knoflach, 2011, Rudolph and Möhler, 2006). In the spinal cord, GABA plays an important role as an inhibitory neurotransmitter released by both local interneurons and inhibitory descending fibres. Studies on the presence of GABA in DRG has been reported as early as in the 1970s (Minchin and Iversen, 1974). However, the role this inhibitory neurotransmitter may play in sensory signalling is only beginning to emerge.

1.4.2 Types of GABA receptors

The presence of the GABAergic system including GABA receptors are not unique only to the human nervous system. Other than neurons, GABA receptors are also found in glia (Bernareggi et al., 2011), in the immune system (T cells, glia and dendritic cells) (Barragan et al., 2015) and human pancreatic beta cells (Korol et al., 2018). In the immune system, GABA upregulates cell motility and chemotactic responses during infection and inflammation (Barragan et al., 2015) while in human pancreatic cells GABA stimulates insulin release controlling blood glucose levels (Korol et al., 2018).

There are two main types of GABA receptors present in the body- $GABA_A$, which are ligand-gated ion channels and $GABA_B$ which are G-Protein coupled receptors (GPCRs).

1.4.2.1 $GABA_A$ receptor

$GABA_A$ receptors belong to the Cys-loop superfamily types of ion channels, which includes cation-selective nicotinic acetylcholine receptors (nAChRs), serotonin type 3 receptors ($5HT_3Rs$) and anion-selective glycine receptors (GlyRs) (Grenningloh et al., 1987). $GABA_A$ receptors are heteropentameric ionotropic receptors that form a ligand-gated Cl^- channel; 19 protein subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π and any ρ1-3) are known to exist and any five of these different subunits can form the functional Cl^- channel (Olsen and Sieghart, 2008). In the brain, the $\alpha_1\beta_2\gamma_2$ subunit combination is the most common heteromer that forms the $GABA_A$ receptor (Sieghart and Sperk, 2002). $GABA_A$ receptors have three different domains: an extracellular domain (ECD), a transmembrane domain, and an intracellular domain (Karlin and Akabas, 1995).

$GABA_A$ receptor function is modulated by agents that interact with sites on the receptor subunits other than the GABA ligand-binding domain (Möhler et al., 1997). Benzodiazepine, barbiturates, ethanol, and anaesthetics are examples of exogenous positive allosteric modulators (Olsen, 2018, Sieghart, 1995). **Figure 1.6** depicts heteropentameric structure of $GABA_A$ receptor subunits with ligands binding to its transmembrane and ECD domain. GABA neurotransmitter/ligand binds with high affinity to its binding site which lies on the ECD between α_1 and β_2 subunits (Cromer et al., 2002, Ernst et al., 2005). The benzodiazepine binding pocket that lies between α_1 and γ_2 can be bound by three different types of ligand; positive allosteric modulators (PAMs) (Haefely, 1989), negative allosteric modulators (NAMs) (Braestrup et al., 1982), and antagonists (Hunkeler et al., 1981) There is indeed major heterogeneity of benzodiazepine sensitivity to $GABA_A$ receptors, but considering the

γ_2 subunit is most abundant in all $GABA_A$ subtypes, it reflects benzodiazepine sensitivity to the majority of $GABA_A$ subtypes (Lüddens et al., 1995).

Anaesthetics, such as barbiturates and propofol, bind to $GABA_A$ receptors at α and β interface at the transmembrane domain and not within ECD. Ethanol also binds to $GABA_A$ receptors at α and β interface within the ECD and this binding has been shown to mediate the anxiolytic, mood-enhancing alcohol effects (Kumar et al., 2004, Olsen et al., 2007, Wallner et al., 2006).

The endogenous modulators of $GABA_A$ receptors are neurosteroids (Belelli and Lambert, 2005, Sigel et al., 2011) and a more recently identified endogenous $GABA_A$ receptor modulator, endocannabinoid 2-arachidonoyl glycerol (2-AG) (Sigel et al., 2011). Neurosteroids allosterically bind to $GABA_A$ receptors enhancing GABA-evoked responses (Belelli et al., 2002). Meanwhile, 2-AG potentiates GABA responses by binding selectively to β_2 and δ subunits of $GABA_A$ receptors (Sigel et al., 2011). Interestingly, elevated 2-AG showed super-additive effect when co-administered with exogenous 3α, 21-dihydroxy-5α-pregnant-20-one (THDOC) suggesting 2-AG can modulate the actions of neurosteroid at $GABA_A$ receptors (Sigel et al., 2011). A recent study on mutant mice revealed that an endogenous benzodiazepine-site ligand, diazepam binding inhibitor (DBI) demonstrated actions that mimic benzodiazepine actions as a PAM on $GABA_A$ receptors (Christian et al., 2013).

Orthosteric modulators are compounds that interact directly with the receptor recognition site. $GABA_A$ orthosteric modulators include muscimol and bicuculline, $GABA_A$ receptor agonist and antagonist, respectively.

1.4.2.2 $GABA_B$ receptor

Earlier during its discovery, GABA receptors were found to be sensitive to bicuculline which later, as mentioned above, was identified as an orthosteric antagonist of $GABA_A$. However later in 1980, it was also found that another type of GABA receptor, which differed from the bicuculline-sensitive receptor, also existed (Bowery and Hudson, 1979). This receptor was not sensitive to bicuculline but rather to baclofen (Bowery et al., 1979). Following the discovery of this second type of GABA receptor, Hill and Bowery suggested that these two different GABA receptors be referred to as $GABA_A$– bicuculline-sensitive receptor, and $GABA_B$– baclofen-sensitive receptor (Hill and Bowery, 1981). Unlike the $GABA_A$ receptor, $GABA_B$ receptor is a metabotropic receptor. It is a heterodimeric receptor composed of B1 and B2 subunits (Kaupmann et al., 1998). The discovery of these two distinct subunits of $GABA_B$ receptor made it the first, identified GPCR that exists as a heteromer. Through the activation of Gi/o types of G protein, $GABA_B$ receptors regulate the activity of K^+ and Ca^{2+} channels which reduces neuronal activity via G proteins and secondary messenger systems causing an action similar to that of $GABA_A$ receptor activation (Bettler et al., 2004, Kornau, 2006, Ulrich and Bettler, 2007).

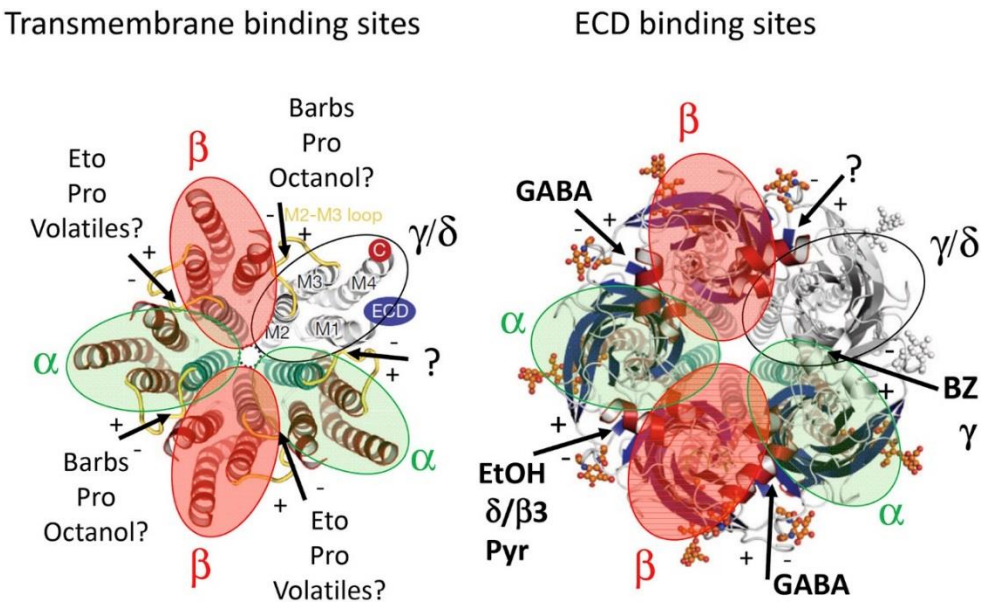


Figure 1.6 $GABA_A$ receptor ligand sites at subunit interfaces identified by mutagenesis and/or affinity labelling. The left panel shows the transmembrane binding sites, while the right panel shows the ECD binding sites. The protein structures are taken from the X-ray crystallography-derived structure of the recombinant mammalian homomeric β3 $GABA_A$ receptor (Miller and Aricescu, 2014), on which is displayed a homologous native $GABA_A$ receptor comprised an α-β-α-β-γ/δ heteropentamer (actual subunits arbitrary, no specific sequence implied although they are all homologous to β3). The protein is viewed looking from the extracellular face, perpendicular to the cell membrane/synapse. Thus the ECD pentamer on right would actually be positioned directly on top of the TMD pentamer at left. Both portions indicate locations of ligand binding sites found in the β3 homomer structure. The two α subunits are indicated by the green (or dark grey) shaded oval, the two β subunits by the pink (or light grey) shaded ovals, and the one γ/δ subunit indicated by the clear (white) oval. An example C-terminus is indicated by a small red circled“C” at the bottom of the TMD of the γ/δ subunit; the M1,2,3,4 domains are also labelled in this example subunit, and the N-terminus of the TMD of each subunit would attach to its ECD at the position indicated by the small blue (or black) oval “ECD”. Ligand binding sites for the compounds listed (in shorthand) are indicated by arrows. The ligands named are BZ (benzodiazepines) and GABA, EtOH,and Pyr (pyrazoloquinolines), in the ECD. In the TMD, Eto (etomidate), Pro(propofol), octanol, volatiles (volatile anaesthetics), and barbs (barbiturates) binding sites are located (Olsen, 2018).

Bornmann and Feigespan reported another GABA receptor which is pharmacologically distinct from $GABA_A$ and $GABA_B$ receptors being insensitive to bicuculline ($GABA_A$ antagonist) and baclofen ($GABA_B$ agonist). This GABA receptor, referred to as $GABA_C$ receptor incorporates another subunit, ρ, and is inhibited by picrotoxin (Bormann and Feigenspan, 1995). $GABA_A$ and GABAc receptors share a few similar characteristics in structure and function. The difference to the ionotropic GABA receptor properties introduced by ρ subunit is comparable to the degree of difference between $GABA_A$ receptors of different subunit composition, thus, the Nomenclature Committee of IUPHAR has designated that the GABAc as part of the $GABA_A$ receptor family and discouraged the use of the term $GABA_C$ receptor (Olsen and Sieghart, 2008).

1.4.3 Synaptic and extrasynaptic GABA receptors

There is a differential GABA receptor subunit distribution dominating different areas of the brain (Pirker et al., 2000). Depending on the actual location in neurons, GABA receptors can be classified as either synaptic or extrasynaptic.

$GABA_A$ receptor subunit composition is very important in determining the type of inhibition generated upon GABA binding. The composition determines the localization of the receptors and, in turn, the type of effects these confer, specifically, presynaptic, transient postsynaptic or tonic GABAergic inhibition is distinguished (Farrant and Nusser, 2005). GABA neurotransmitters that are released via phasic mechanism activate the synaptic GABA receptors (Carver and Reddy, 2013, Farrant and Nusser, 2005, McKernan and Whiting, 1996, Mody, 2001). The most common subunit composition for this activity is $\alpha_1\beta_2\gamma_2$ (Sieghart and Sperk, 2002). Meanwhile, extrasynaptic GABA receptors are activated by tonic GABA release (Glykys and Mody, 2007a). Tonic GABA release which happens at a slower rate is responsible for maintaining the ambient GABA concentration at rest (Farrant and Nusser, 2005).

$GABA_A$ receptors that populate extrasynaptic regions are mostly made up by δ and specific alpha subunits: α_4 and α_6 (Belelli et al., 2009, Mortensen et al., 2012).

1.4.4 GABA in central pain

The dorsal horn of the spinal cord is an essential CNS sensory processing region connecting the periphery to the brain. Studies have shown that pain transmission mediated by myelinated Aδ and unmyelinated C fibres can be inhibited by innocuous stimulation (such as rubbing) that activates Aβ fibres– a phenomenon that has been explained earlier, in the section 1.1.2. According to the Gate Control Theory, the nociceptive 'gate' in the substantia gelatinosa (lamina II) of the spinal cord is controlled by the balance between the inputs from the nociceptive and non-nociceptive primary fibres. The balance between these two inputs determines whether nociceptive transmission will occur. It also determines the strength of transmission, via nociceptive-specific projections to higher centres**– Figure 1.7**. In simple words, an increased innocuous input will push the balance into the favour of less pain sensation. To a lesser extent, this gating theory also emphasizes the influence of descending efferent fibres originating from the brain on these two sensory inputs.

As mentioned above, the descending fibres also contribute to the Gate Control Theory of Pain. Despite the claim that these fibres control nociceptive signalling by both presynaptic and postsynaptic inhibitions (Millan, 2002, Zeilhofer et al., 2012), few other studies suggest that the descending pathways have control only via the postsynaptic inhibition of the nociceptive pathway (Aicher et al., 2012, Antal et al., 1996, Light and Kavookjian, 1985). Presynaptic inhibition involves the following: i) activation of local GABAergic interneurons by the input from either myelinated A fibres or from the descending inhibitory pathways (see below); ii) release of GABA from interneurons; iii) binding of GABA to the $GABA_A$ receptors on primary afferent terminals causing depolarisation, termed primary afferent depolarisation (PAD)

(Carlton et al., 1999, Cattaert and El Manira, 1999, Kennedy et al., 1974) and iv) depolarization-induced block of conductance due to sodium channel inactivation.

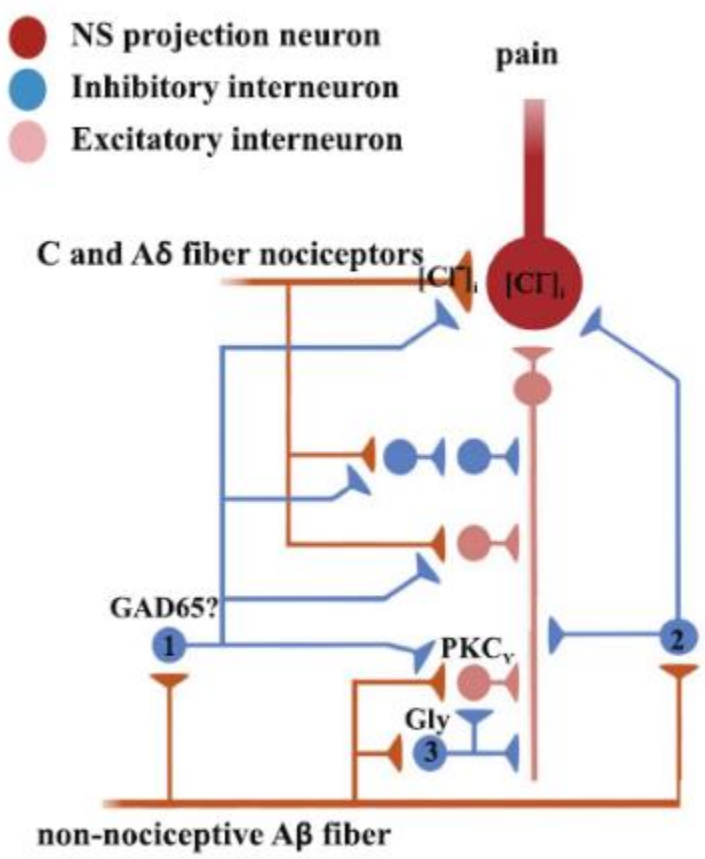


Figure 1.7 A schematic diagram illustrating presynaptic and postsynaptic inhibitions in primary afferent inhibitory and excitatory interneurons in the spinal cord dorsal horn. Excitation of neuron 1 by non-nociceptive Aβ releases GABA which binds to the $GABA_A$ receptor on the C and Aδ fibre nociceptors, leads to PAD and inhibition of glutamate release onto nociceptive specific (NS) projection neuron (presynaptic inhibition). GABA release from neuron 2 directly inhibits NS projection neuron reducing its excitation (postsynaptic inhibition). Non-nociceptive Aβ also stimulates PKCγ + cell and the excitatory neuron which in turn is inhibited by the neuron 3, the glycinergic inhibitory interneuron upon stimulation by the same type of non-nociceptive fibres. During nerve injury, there are various mechanisms that interrupt the linkages of neuron 2 and neuron 3 which enable the innocuous input to access the nociceptive pathway leading to activation of NS projection neuron and consequently tactile allodynia. While in C and Aδ fibre nociceptors, the $[Cl^-]_i$ is increased, accompanied by reduced $GABA_A$ receptor conductance causing a loss of presynaptic inhibition and heat hyperalgesia. Inputs from non-nociceptive Aβ fibres will stimulate the GABAergic interneuron 1 to generate action potentials in the nociceptive afferents. The excitation from the action potential could produce DRR and evoke touch-induced pain and neurogenic inflammation. Abbreviations: PAD, primary afferent depolarization; PKCγ, protein kinase C; DRR, dorsal root reflex (Guo and Hu, 2014).

As mentioned above, the postsynaptic inhibition occurs via the descending pathways. These pathways are primarily modulated by the fibres originating within the periaqueductal grey (Miranda et al., 2002) situated in the midbrain which project to the rostral ventromedial medulla (RVM) before descending to the spinal dorsal horn. Indeed, descending pathways play a complex and crucial role in nociceptive signalling with the involvement of the multiplicity of neurotransmitters and modulators such as GABA (Millan, 2002), serotonin (Zhao et al., 2007), noradrenalin (Pertovaara, 2006), opioid peptides (Watkins and Mayer, 1982) and endocannabinoids (Hohmann et al., 2005). GABAergic postsynaptic inhibition occurs when GABAergic neurons projecting from the RVM act on $GABA_A$ receptors and suppress the nociceptive transmission projecting from the spinal cord as shown in the studies by (Aicher et al., 2012) and (Kato et al., 2006). Kato and colleagues characterised the descending pathways from the RVM by electrically stimulating this area and subsequently recording the IPSCs of the substantia gelatinosa neurons via *in vivo* patch-clamp technique. Their results revealed that the electrical stimulation of the RVM evoked >50% of monosynaptic GABAergic and glycinergic responses and that the action potentials elicited from noxious stimuli applied onto the skin were blocked by the facilitation of the inhibitory inputs on substantia gelatinosa neurons (Kato et al., 2006). These findings were supported by Aicher and colleagues; their anatomical and neurochemical tracing of projections from the RVM to the lumbar spinal cord showed that more than two-thirds of RVM projections are GABAergic which was deduced by measuring the expression of GAD 67 (Aicher et al., 2012).

Knabl and colleagues demonstrated that α_2 and α_3 subunits of $GABA_A$ receptors were abundantly expressed in mouse dorsal horn neurons (Knabl et al., 2008), especially in layer II of both the inner and outer lamina (Paul et al., 2012). In a model of inflammatory pain tested with diazepam, mice lacking α_2-$GABA_A$ receptor showed reduced potentiation of dorsal root potentials with impaired anti-hyperalgesia effects upon thermal and mechanical stimulations (Witschi et al., 2011). Studies in a variety

of pain models have shown that point mutations in the α_1-subunit of the $GABA_A$ receptor, which is responsible for the sedative action of benzodiazepines, did not reduce its analgesic actions (Knabl et al., 2008, Knabl et al., 2009). Analgesia was completely retained in α_1 point-mutated mice, whereas wild type mice developed sedation and analgesia with almost the same dose dependence (Knabl et al., 2009). However, further studies showed that the doses of benzodiazepines that were sufficient to produce hypnosis and anxiety (Guerrini et al., 2011, Rudolph and Knoflach, 2011) might not be adequate to produce analgesic effects (Zeilhofer et al., 2009). Studies on mice with altered α_2- and α_3-subunits containing $GABA_A$ receptors showed a significant reduction in analgesia effects of diazepam (Löw et al., 2000, Zheng et al., 2003) which suggested that these subunits contribute to the analgesic effects. Similar findings were also seen in mutant mice lacking the $GABA_A$ α_2-subunit in peripheral nociceptors which failed to show analgesia to spinally-administered diazepam (Witschi et al., 2011).

1.4.5 GABA in peripheral pain

In the 1970s, emerging data on the presence of GABA neurotransmitter in DRG have been reported. Calcium-dependent GABA release from glial cells following potassium-induced depolarisation was observed in one of the earliest studies by Minchin and Iverson (Minchin and Iversen, 1974). This GABA release was inhibited with raised extracellular Mg^{2+} and the presence of chelating agent ethylenediaminetetraacetic acid (EDTA). Within the same decade, Feltz and Rasminky performed intracellular recordings on adult rat DRG neurons; application of GABA led to depolarisation of the cells and increase in amplitude of the after-potential hyperpolarisation. With these findings, they proposed that GABA could mediate the presynaptic inhibition on the primary afferent terminals (Feltz and Rasminsky, 1974).

After the discovery of GABA in DRG in the 1970s, more studies have been carried out to explore the potential effects GABA may have on DRG. As discussed above, the study by Feltz and Rasminky in 1974 led to the emergence of the theory that GABA may be involved in controlling nociceptive inputs at the level of the DRG. Although their results demonstrated a lack of definite evidence for direct involvement of GABA receptors (i.e. GABA receptor antagonist was not used to confirm the involvement of GABA receptor), they postulated that the receptors on spinal ganglion neurones and those involved in the presynaptic inhibition were identical (Feltz and Rasminsky, 1974). Indeed this was the case; the involvement of GABA receptors was reaffirmed by other studies that followed suit. These studies were carried out focusing on different mechanisms of GABAergic transmission in DRG looking at the involvement of ion channels (Gallagher et al., 1983, Nishio and Narahashi, 1990) and transporters (Price et al., 2006), as well as monitoring mRNA expression levels (Fukuoka et al., 1998, Maddox et al., 2004, Obata et al., 2003) of such proteins. A study by Hanack and colleagues suggested that GABA was endogenously present at the peripheral nerve endings and was able to inhibit TRPV1 sensitisation in cultured DRG neurons of affinity-tagged TRPV1 transgenic mice (Hanack et al., 2015). More recently, Du and colleagues reported functional GABAergic signalling within DRG which may play an important role in peripheral nociception which will be discussed in more detail later (Du et al., 2017).

Despite many recent data suggesting that post-synaptic inhibition may play an important role in the modulation of nociception, earlier studies support the mechanism of pre-synaptic inhibition as the more powerful pathway on inhibition of the somatosensory input into the CNS (Eccles, 1964). However, the precise contribution of these two modes of inhibition (presynaptic vs postsynaptic) still remains elusive due to difficulties to manipulate the pre- and postsynaptic inhibitions in experimental models (Guo and Hu, 2014). The underlying physiological mechanism responsible for presynaptic inhibition is the depolarisation of primary

afferent fibres made by axo-axonal contact, which is mediated by GABAergic interneurons (Carlton, 2014). The release of GABA from one interneuron and its binding to $GABA_A$ receptors on primary afferent terminal leads to $GABA_A$ receptor activation and due to the greater Cl^- concentration present in these neurons (see below), Cl^- efflux from the primary afferent terminal. These events result in PAD as shown in **Figure 1.8** (Szallasi and Sheta, 2012).

Different mechanisms have been proposed to explain GABA-mediated PAD in presynaptic inhibition including: 1) the inactivation of voltage-gated sodium channels on primary afferent neurons, hence interrupting propagation of the action potential; 2) attenuation of the amplitude of propagated action potential due to shunting effect; and 3) inactivation of voltage-gated calcium channels thus reducing Ca^{2+} influx and consequently excitatory transmitter release (Price et al., 2009, Zeilhofer et al., 2012, Rudomin and Schmidt, 1999).

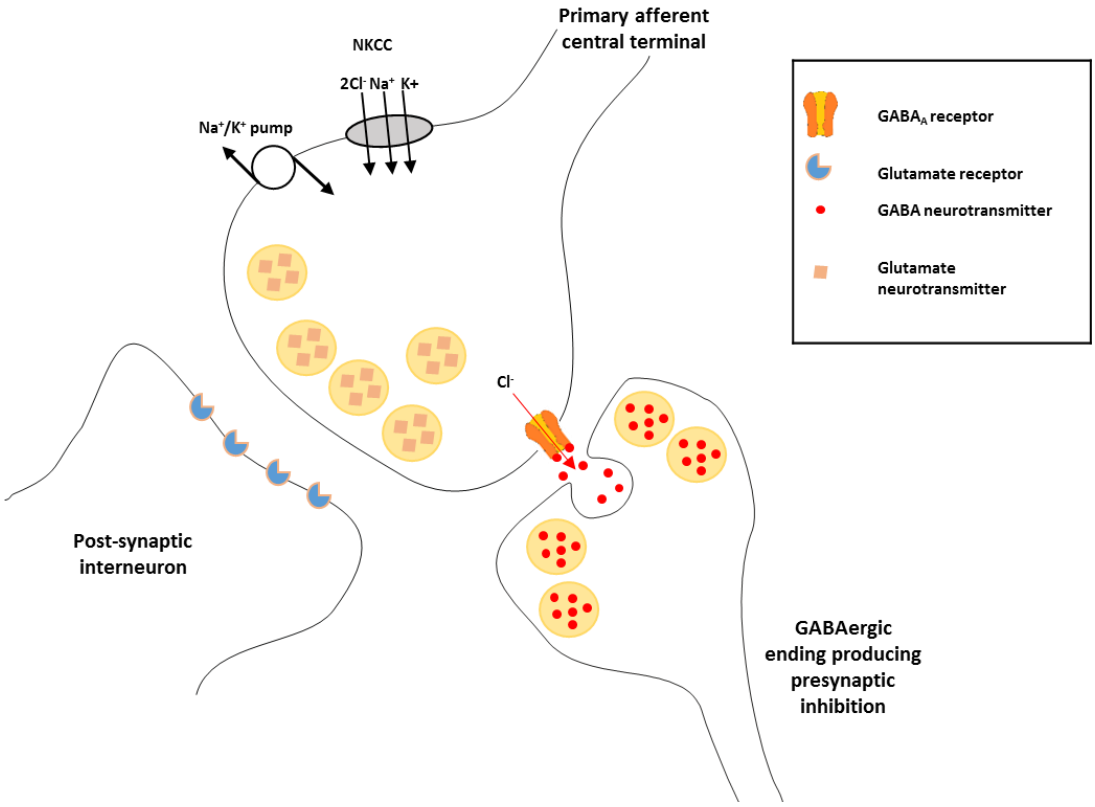


Figure 1.8 Primary afferent depolarisation in central terminals of sensory fibres. Cl^- concentration in primary afferent fibres is controlled by NKCC in the membrane of the afferent. GABA release and activation of $GABA_A$ receptors leads to Cl^- efflux resulting in depolarisation of primary afferent terminal [Based on a concept reported in (Carlton, 2014)].

1.4.5.1 Biophysics of Cl^-

The Cl^- reversal potential (E_{Cl}) plays an important role in determining the effect of GABA on cell excitability. Indeed, $GABA_A$ and glycine receptors are permeable to Cl^- and other common anions, such as HCO_3^- (Kaila, 1994, Staley and Proctor, 1999). Depending on the E_{Cl}, Cl^- movement can cause hyperpolarisation, depolarisation or shunting effect (Price et al., 2009). The direction of Cl^- movement is determined by the electrochemical gradient for each ion which constitutes the driving force. Driving force, in this case, is the difference between membrane potential and anion reversal potential. The reversal potential in turn, is determined by the anions' intra- and extracellular concentrations which is calculated using the Nernst equation.

Shunting effect is a feature of $GABA_A$ receptor activation. Shunting occurs when a neuron's E_{Cl} is near to its resting potential and in this state, the electrochemical gradient of Cl^- is quite small. Thus, the activation and opening of GABA channels will cause small depolarisation and shunting effect due to a very low driving force (Price et al., 2009).

The activity of GABA depends on the electrochemical Cl^- gradient present in cells; this is set and maintained by Cl^- cotransporters. Two cotransporters regulate Cl^- concentrations in cells; the sodium-potassium-chloride cotransporter (NKCC1) and potassium-chloride cotransporter (KCC2) (Delpire, 2000, Payne et al., 2003). NKCC1 moves Cl^- into the cell together with Na^+ and K^+ while KCC2 moves Cl^- out of the cell together with K^+ (transmembrane gradients of both ions are tightly controlled by the Na^+/K^+ ATPase) (Price et al., 2009). In rodent brain, neuronal NKCC1 protein was shown to be present at birth and its expression increased during P14 – 28 however, decreased towards adulthood (He et al., 2014, Zhang et al., 2006). Meanwhile, KCC2 expression peaked at P14 and persists in the adult brain. Therefore, immature cortical neurons accumulate high intracellular Cl^- concentrations, $[Cl^-]_i$ and the primary synaptic action of GABA in these neurons is depolarising. However, due to developmental changes, activation of $GABA_A$ receptors in healthy mature principle

cortical neurons leads to hyperpolarisation (Glickfeld et al., 2009). Thus, changes in the levels of NKCC1:KCC2 contribute to the changes in intracellular Cl^- concentration (Frederikse and Kasinathan, 2015) and is the mechanism which leads to shifting from neuronal depolarisation in immature cortical neurons to hyperpolarisation in the adult mammalian brain. **Figure 1.9** depicts the schematic diagram of the developmental alterations of $[Cl^-]_i$ levels and the polarity of the actions of GABA and the actions of chloride transporters.

In most of the mature CNS neurons, the intracellular $[Cl^-]_i$ is ~5 mM whereas the extracellular $[Cl^-]$ is ~150 mM, thus the Nernst potential for Cl- (E_{Cl}) is lower than that of the resting membrane potential (resting membrane potential is ~-70 mV). In this condition, activation of a Cl^- channel will move Cl^- ions into the cell leading to hyperpolarisation of the cell membrane causing inhibitory effects. When the concentration of Cl^- inside the cell is greater (i.e. due to mechanisms of Cl^- accumulation- see below), the E_{Cl} is also greater than the resting membrane potential and in this scenario, Cl- moves out of the cells, thereby causing depolarisation (Delpire, 2000). A shift in E_{Cl} by as minimal as 10 mV to a less negative value can cause a shift in GABA-induced currents from inhibitory to excitatory (Prescott et al., 2006).

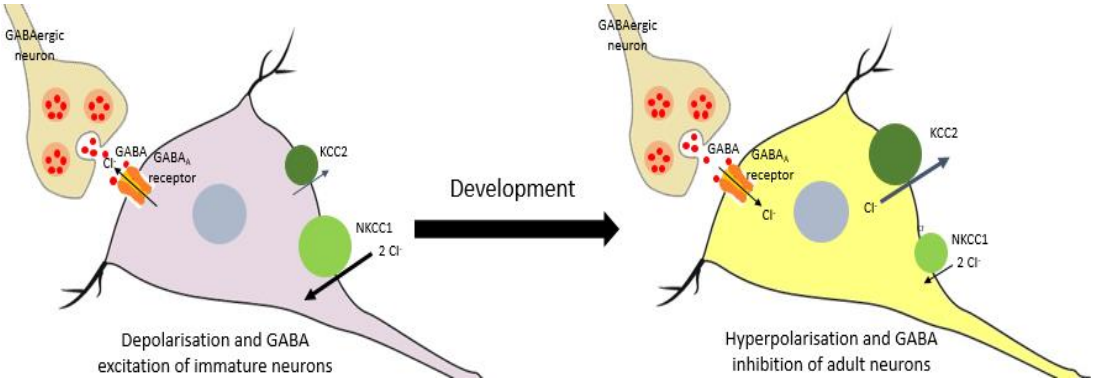


Figure 1.9 Schematic diagram of the developmental alterations of $[Cl^-]_i$ levels and the polarity of the actions of GABA and the actions of chloride co-transporters. GABA depolarises and excites immature neurons and inhibits adult ones. The chloride exporter KCC2 is poorly active initially whereas the NKCC1 is highly active in immature neurons leading to different chloride gradients and actions of GABA [Based on a concept reported in (Ben-Ari, 2014)].

1.4.5.2 Cl^- signalling in pain

Interestingly however, the hyperpolarisation shift mentioned above does not occur in the primary sensory neuron. Instead, activation of $GABA_A$ receptors in these neurons results in depolarisation due to the persistence in NKCC1 expression which causes an accumulation of Cl^-; a higher intracellular [Cl^-] is present (Price et al., 2006). Studies have tried to measure the $[Cl^-]_i$ in DRG neurons and have suggested values in the range of ~40 mM (Rocha-Gonzalez et al., 2008). This is considerably higher than that of 'normal' CNS neurons. During inflammation, NKCC1 is further upregulated and accumulates up to three times more Cl^- compared to control conditions (Funk et al., 2008).

This begs the question; could this be involved in chronic pain conditions? A study on neuropathic pain-induced rodents showed that the increase in NKCC1 was responsible for the increased accumulation of Cl^- and the ensuing depolarising shift in the $GABA_A$ receptor-mediated current (E_{GABA}) in both large (putative mechanoreceptors) and small (putative nociceptors) neurons (Chen et al., 2014). Interestingly, this depolarising shift returned to control levels 21 days after injury albeit neuropathic pain symptoms persisted, thus this was only considered a transient shift (Chen et al., 2014). This transient shift may result in enhanced PAD, sufficient to evoke an action potential, suggesting that a transient reduction in $GABA_A$ receptor-mediated presynaptic inhibition or even a switch to excitation (Chen et al., 2014). Consistently, a study by Modol and colleagues also showed a significant increase in pNKCC1 (phosphorylated NKCC1) along with down-regulation of KCC2 in the main relay centers [dorsal horn of spinal cord, posterolateral nucleus (VPL) of the thalamus and somatosensory cortex]. The increased level of pNKCC1 was observed 3 - 16 days after injury, after which it returned to control levels, fascinatingly coinciding with regeneration of axons (Modol et al., 2014). Enhanced $[Cl\text{-}]_i$ due to pNKCC1 activity may contribute to the regenerative growth of axotomized nerve as reported by (Pieraut et al., 2007) and (Pieraut et al., 2011) which may be the reason the level of

NKCC1 return to control level. In humans, fluctuation in the expression of these cotransporters leads to derangement of Cl^- regulation responsible for the development of GABA disinhibition implicated in chronic pain and other neurological disorders (Kahle et al., 2008, Yousuf et al., 2017).

1.4.5.3 Other Cl^- channels in pain

The $GABA_B$ receptor also plays a role in inflammatory pain signalling (Brewer and Baccei, 2018, Patel et al., 2001, Potes et al., 2006). $GABA_B$ receptor has been shown to form a complex with the TRPV1 receptor to counteract inflammatory pain (Hanack et al., 2015). Fascinatingly, this complex isolated from rat DRG was able to block the pathological aspect of pain whilst leaving the acute TRPV1 pain signals intact (Hanack et al., 2015) Interestingly, it was observed that the effect of $GABA_B$ on TRPV1 relied on the close juxtaposition of $GABA_B$ and TRPV1 and was independent on the more common and established G protein signalling (Hanack et al., 2015).

Another Cl^- channel, the Ca^{2+} activated Cl^- channel, anoctamin 1 (ANO1) also known as TMEM16A, produces excitation of DRG neurons. ANO1 has been shown to be activated by an increase in intracellular Ca^{2+} released from internal Ca^{2+} stores and not through the influx of Ca^{2+} through the voltage-gated Ca^{2+} channels (Jin et al., 2013). Activation of inflammatory mediator-activated Gq-coupled protein receptors (such as bradykinin-activated B2 receptors) leads to IP3 production and subsequent activation of endoplasmic reticulum (ER) -resident IP3 receptor (IP3R), which has been shown to physically interact with ANO1 itself (Jin et al., 2013). The hypothesis regarding this close proximity between ANO1 and its Ca^{2+} source is that the local high concentration of Ca^{2+} reached at the mouth of the channel (which can reach 50 µM) (Bauer, 2001, Bootman et al., 2001, Neher, 1998) is able to activate the poorly-Ca^{2+} sensitive ANO1 channel and induce depolarisation in primary afferent neurons (Jin et al., 2013).

1.5 GABA in the current treatment for chronic pain

Treatment for chronic pain is a significant clinical challenge and requires a multidisciplinary approach. Current strategies include pharmacological treatments, physiotherapy, medication, surgery and counselling. One of the most common medications prescribed for chronic pain are opioids which have caused contentious controversies with regard to its adverse effects for patients, including overdose, dependence and subsequent withdrawal, addiction, and negative impacts on functioning (Vowles et al., 2015). A better medication option with more localised administration (and hence the localised effect) indeed would lead to better pain management.

Increasing the inhibitory signalling in the nociceptive pathway would make sense in terms of targeting for pain relief. Several organic compounds have been found to have activities related to GABA. One of the earliest compounds reported to show structural similarity to GABA was muscimol, found in *Amanita muscaria* and the related mushroom (Eugster and Takemoto, 1967) which is later proven to have the same action as GABA and classified as an ionotropic $GABA_A$ receptor agonist (Johnston, 2014). More compounds were discovered in subsequent years which were related to GABA including THIP, a GABA agonist (Krogsgaard-Larsen et al., 1977), bicuculline, a GABA antagonist (Curtis et al., 1970), and baclofen, a $GABA_B$ receptor agonist (Bowery et al., 1980). These compounds are used until the present day in the study of GABA pharmacology and its potential as a therapeutic agent in central nervous system disorders as well as in the pain management at the spinal cord level.

Recent results from our lab revealed a hitherto unknown GABAergic mechanism within the DRG. We found that delivery of GABA or GABA mimetics to DRG *in vivo* was able to attenuate pain in both chronic neuropathic and chronic inflammatory rat models. Similar results were obtained when endogenous GABA levels in DRG were elevated by focal DRG application of GABA reuptake inhibitor. These actions of

GABA were blocked by bicuculline suggesting the involvement of $GABA_A$ receptors in this signalling pathway. Stimulating GABAergic DRG neurons via optogenetic and designer receptor exclusively activating designed drugs (DREADD) approaches also showed similar results. These and other experiments (Du et al., 2017) suggest that the peripheral nociceptive stimulation triggers activity-dependent somatic release of GABA from DRG neurons and activation of somatic/perisomatic $GABA_A$ receptors. This, in turn, depolarise the stem axons and the T-junctions, resulting in block of throughput conduction due to sodium channel inactivation and shunting, similar to PAD. This action potential filtering reduces excitatory input into the spinal cord, playing the role as another ‘gate’ in the peripheral nociceptive transmission **Figure 1.10**.

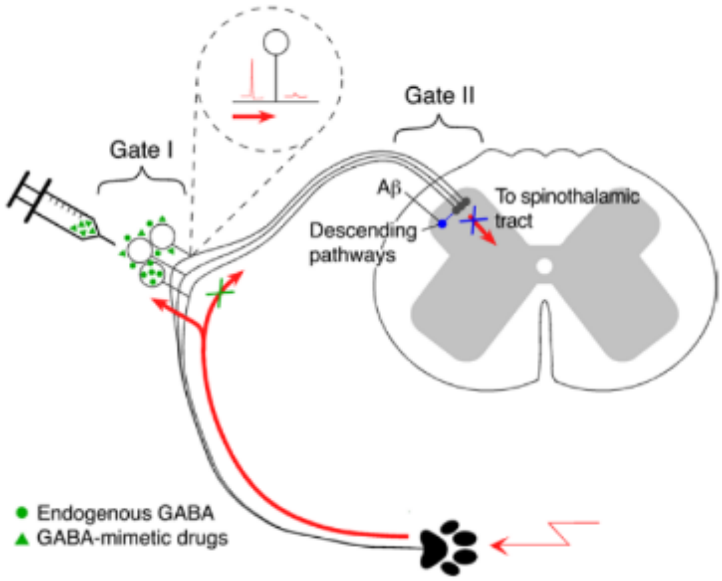


Figure 1.10 Schematic for peripheral somatosensory integration in DRG (Du et al., 2017).

Hypothesis

In this thesis, I hypothesised that DRGs are populated by GABAergic neurons. These neurons release GABA via both tonic and phasic mechanisms and that the released GABA filters the nociceptive signals from the periphery to the spinal cord at the T-junction of the DRG.

Aims

To answer the hypothesis, I investigated the expression of vesicular GABA transporter (VGAT) and its mechanisms of release in the DRG. Specifically, the aims of this study were:

1. To determine the VGAT expression and distribution in DRG neurons

2. To characterize the subpopulations of GABAergic DRG neurons

3. To investigate the mechanisms of GABA release via:

   i. investigating the exposure of luminal domain of VGAT

   ii. all-optical Cl^- channel activity assay

   iii. *in vivo* electrophysiological recording on spinal nerves and dorsal roots

Chapter 2 Materials and methods

2.1 Immunohistochemical staining of Dorsal Root Ganglion

2.1.1 DRG isolation

Ethical implication

Animal handling and humane schedule 1 euthanasia were performed in accordance with the regulations of Animal (Scientific Procedures) Act 1986, under the personal license no. IF8F7648C and project license no. PPL 70/7928.

DRG used in this experiment were isolated from male adult wistar rats weighing 250 – 300 gm obtained from the Central Biological Services (CBS) unit, Faculty of Biological Sciences, University of Leeds. Male rats were used as the hormonal changes during menstrual cycle in females can influence the results of the study. Despite the arguments on the use of male over females rats (Chapter 1, section 1.1.4), a meta-analysis of neuroscience studies showed that data from female rats are not more variable than that of male rats (Becker et al., 2016). The authors reported no sex differences were evident when data from males were compared to either females at random or at specific oestrous cycle, supporting the use for male rats only in this study.

A total number of four rats were used to study the expression of VGAT in DRG and its co-localisation with neuronal markers TRPV1, IB4, NF200, trkC and SV2 as well as the glial marker S100B. Rats were housed in pairs with *ad libitum* access to water and chow in a 12:12 light-dark cycle. Out of four rats sacrificed, one was not included for immunohistochemical (IHC) staining due to poor quality of gelatin used to embed the DRG (gelatin became brittle and difficult to cut). Briefly, rats were anaesthetised with isofluorane (inhalation) before they were sacrificed via decapitation. Following decapitation, the cervical, thoracic and lumbar segments of vertebral column were dissected and longitudinally divided into two halves along the median lines on both dorsal and ventral sides. DRGs were taken out from the inner side of each half of the

dissected vertebrae and transferred into Dulbecco's Modified Eagle Medium (DMEM) and Glutamax (Invitrogen) containing 10% fetal bovine serum (FBS) and 50 U/ml of penicillin and 50 U/ml of streptomycin, immediately.

2.1.2 VGAT immunoreactivity

Immunohistochemical investigation was performed using an antibody against N-terminal epitope of VGAT antibody raised in rabbit (VGATrab) (#131 002; Synaptic Systems). The distribution of VGAT protein expression in the DRG was determined by analysing the total number of neurons per rat (total number of rats, N=3) in sections processed for IHC staining. Following sacrifice, DRG were immediately isolated and washed three times with phosphate buffer saline (PBS). Immersion fixation method was used to fix the DRG for one hour using 4% paraformaldehyde (PFA). This fixation method also known as immersion fixation method, was used over transcardiac perfusion as small tissue such as DRG can be adequately fixed with this method; a simpler method with equal quality of images compared to transcardiac perfusion (Kasukurthi et al., 2009) and is less time-consuming. Fixed tissues were washed three times for 10 min each with PBS. Tissues were embedded in 10% gelatin and cut at 35 µm thickness using microtome (Leica VT1000S Leica Biosciences, UK). The thickness of the sections cut is usually determined by the technique used to store the tissue. DRG tissues in this experiment were embedded in gelatin after PFA fixation, thus thicker section was required to prevent section breakage during cutting. Also, free-floating technique was used for antibody staining, thus in case of thinner tissue section, the tissue may fall off and break during mounting on slides. Most importantly, the thickness chosen was able to detect the expression of the proteins investigated in this study, as shown in the Chapter 3.

Following DRG tissue sectioning, IHC staining protocol for VGAT antibody commenced using the free-floating technique. The slices were permeabilised and blocked with blocking buffer for one - two hours with 0.05% Tween 20, 0.25% Triton

X-100 and 5% normal donkey serum diluted in PBS. Blocking buffer was removed prior to addition of primary antibody. The anti-VGAT antibody was diluted in antibody dilution buffer [PBS and 5% bovine serum albumin (BSA)] using three different dilutions (1:500, 1:1000, and 1:2000); slices were then incubated overnight at 4˚C. Following day, slices were washed three times for 5 min each with PBS and incubated for 2 hours with fluorescent dye-conjugated secondary antibody, Donkey anti-rabbit Alexa Fluor 555 (Invitrogen, Eugene, Oregon, USA) (1:1000 in antibody dilution buffer). Slices were then washed three times with PBS before mounting on glass microscope slides. DAPI was used to stain the nuclei. The imaging was done using inverted confocal microscope LSM700 (Zeiss). Following optimisation, we found that DRG stained in anti-VGAT antibody with 1:2000 dilution gave the best image with the least background noise under confocal imaging. Thus, further experiments were done using this optimised method for IHC.

Optimisation of VGAT antibody raised in guinea pig (VGATgp) (131 004, Synaptic Systems) were done adopting the method used to optimise VGATrab. The dilutions used for optimisation were 1:200, 1:500 and 1:1000. Imaging done under inverted confocal microscope LSM700 suggested that dilution of 1:1000 VGATgp gave the best image with the least background noise. Further experiments were conducted using this dilution.

2.2 Double immunofluorescence staining of VGAT with NF200, trkC, TRPV1, IB4, SV2 and S100B

Using the same protocol, we also performed double IHC staining of VGAT antibody with the following markers: synaptic vesicle antibody (SV2), isolectin B4 (IB4) conjugates, transient receptor potential subfamily V1 (TRPV1), neurofilament 200 (NF200), tyrosine kinase C (trkC) and S100B. Details of all antibodies used in this study are listed in **Table 2**. Analysis of co-localisation of VGAT with each neurochemical marker was done by summing up the total number of neurons per rat expressing VGAT and any one of the markers studied (total number of rats, N=3). Means per rat were calculated and reported as mean ±SEM.

2.2.1 Assessment of positively labelled DRG cells

Antibody labelling on DRG sections observed was compared with that observed from experiment-matched negative controls (omission of primary antibodies). Neurons were considered as positively labelled by a given antibody if the mean fluorescence intensity was greater by two-fold than that of the background level. However, as some antibodies were relatively weakly stained, the determination of positively labelled neurons was also facilitated by visual assessment together with the intensity value of the background level of two-to threefold.

2.2.2 Image analysis

DRG sections were imaged using Confocal Microscopes LSM700 and LSM880 Airyscan (Carl Zeiss, AG, Germany). For neuron quantification, at least two sections were obtained from each of three male adult rats used in this study. Measurements were done by drawing region of interest (ROI) of all visible neurons on the Zen 2 software (blue edition). To quantify the neurons according to their sizes, only neurons with visible nuclei were included. This measurement provides information on the fluorescent intensity of each fluorophore used to label the studied antibodies and the cross-sectional area. Values of fluorescence intensity were used to distinguish the positive and negatively labelled cell bodies while the cross-sectional

area was used to calculate the cell body diameter. Neuronal cross-sectional areas were determined by drawing the clearly defined neuronal cell bodies. According to their size, neurons were classified as small (<32 µm), medium (32 – 40 µm) and large (>40 µm) (Ramachandra et al., 2013). VGAT was co-labelled with either of these five neuronal markers, NF200, trkC, IB4, TRPV1 and SV2. VGAT was also co-labelled with a glial marker, S100B. To determine the co-localisation of VGAT with each of the neuronal markers, the total number of neurons expressing VGAT and the total number of neurons expressing each of the neuronal markers were quantified. The total number of neurons expressing both VGAT and the neuronal markers were also quantified. The percentage of co-localisation was then determined using these values. Data for quantification were obtained from at least two sections of three independent rats.

2.3 Electron microscopy

2.3.1 3,3 diaminobenzidine (DAB) staining

3,3′-Diaminobenzidine (DAB) staining protocol was performed before commencing electron microscopy (EM) protocol. DRG tissue embedded in 10% gelatin was sectioned at 35 µm using microtome (Leica VT1000S, Leica Biosciences, UK). Sections were then permeabilised with 50% alcohol for 30 min and washed with PBS. Blocking buffer with 10% normal donkey serum was then added followed by 3% peroxidase solution for 30 min. Sections were washed 3 times for 5 min with PBS followed by addition of VGATrab (1:2000). Sections were incubated overnight at 4˚C. The following day, sections were washed 3 times with PBS before addition of biotinylated secondary antibody (1:250) and incubated for four hours at room temperature. Avidin peroxidase was added at a concentration of 1:1500 followed by DAB (Vector Lab #SK-4100) staining.

DAB solution was prepared by adding in two drops (84 µl) of DAB buffer, four drops (100 µl) of DAB and two drops (80 µl) of hydrogen peroxide (H_2O_2) in 5 ml of distilled water.

2.3.2 Fluoronanogold (FNG) staining

DRG sections were permeabilised with 50% ethanol followed by blocking with 10% donkey serum, each for 30 min. Tissues were incubated with VGATrab (1:2000) overnight at 4˚C. The following day, DRG tissues were washed with PBS 3x5 min before incubated in secondary biotinylated antibody (anti-rabbit) (1:250) for four hours. DRG sections were washed with PBS 3x5 min, followed by overnight incubation in streptavidin fluoronanogold (anti-rabbit) (1:100). Next day, sections underwent three PBS washes and one gold buffer wash, each for 5 min. Tissues were post-fixed with 2% glutaraldehyde in 0.1M PB, washed with PBS 2x5 min. A final wash was done with distilled water to remove all traces of PBS. During the

washes, sections were transferred to clean wells of a filter plate (Acroprep™ 96-well filter plates, 350 µl).

Silver enhancement

HQ silver enhancement kit (#2012 Nanoprobe) was used to enhance the gold immunolabelling. The reagents were supplied in dropping bottles for easier dispensing of small amount of reagent volumes. In brief, one drop of initiator (solution A) was mixed with one drop of moderator (solution B). Following that, DRG sections were added into the mixed solution followed by addition of activator (solution C). The reaction usually took 4 – 10 min to be completed (appeared dark). The reaction was stopped with distilled water before transferred to 0.1M phosphate buffer ready for osmium fixation.

2.3.3 Tissue preparation for transmission electron microscopy

DRG tissue sections were washed 5 times (3 min each in 0.1M phosphate buffer. A solution containing 3% potassium ferrocyanide in 0.1M PB with 4 mM calcium chloride was combined with an equal volume of 4% aqueous osmium tetroxide (EMS). The tissues were then incubated in this solution for one hour on ice. The tissues were further washed 5 times (3 min each) before placing in the 0.22 µm Millipore filtered thiocarbohydrazide (TCH) solution for 20 min at room temperature. Tissues were rinsed 5 times again and placed in 2% osmium tetroxide in double distilled water for 30 min. Following the second exposure to osmium tetroxide, the tissues were washed again and placed in 1% uranyl acetate and left in the refrigerator overnight at 4˚C. The next day, *en bloc* Walton's lead aspartate staining was performed. Prior to staining, 0.066 g of lead aspartate was dissolved in 10 ml 0.03M aspartic acid solution and pH was adjusted to 5.5 with 1N potassium hydroxide (KOH). This solution was placed in a 60˚C oven for 30 min. Then, the tissues were washed and dehydrated for 5 min each, using ice-cold solutions of freshly prepared 20%, 50%, 70%, 90%, and 100% ethanol. Next, the tissues were placed on ice-cold

acetone and left at room temperature for 10 min. Tissues were then placed in 25%, 50%, and 75% durcupan:acetone sequentially for two hours each. Tissues were placed in 100% durcupan overnight, followed by fresh 100% durcupan for two hours. Tissue sections were then mounted on a glass slide with an aclar coverslip and placed in a 60˚C oven for 48 hours. Tissue sections were then cut off from the coverslip and mounted onto a block before they were cut using ultramicrotome (Leica Ultracut UCT, Germany). Mounted tissues underwent semithin (500 nm) and ultrathin (70 nm) cutting and collected onto a nickel grid for imaging with the transmission electron microscope (Tecnai T12 G2).

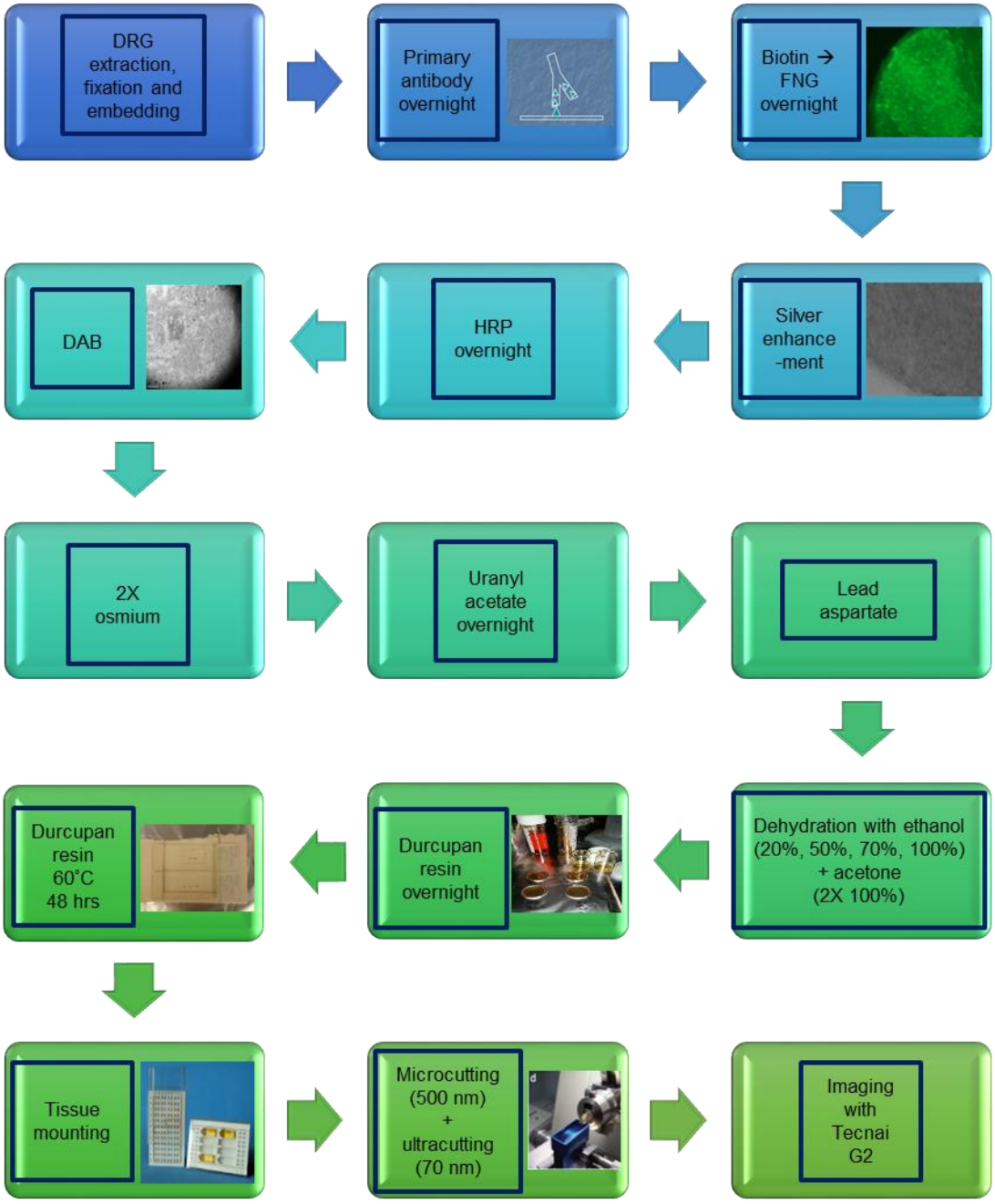


Figure 2.1 The flowchart diagram of antibody labelling for electron microscopy.

Table 2 List of antibodies used in this study

Primary antibody					Secondary antibody		
Antibody	Target protein	Dilution	Dilution determination	Company	Antibody	Dilution	Company
NF160/200 (mouse)	Myelinated neurons	1:2000	Established protocol	Sigma-Aldrich	Alexa-Flour 488 conjugated IgG Donkey anti-mouse	1:1000	Life Technologies the USA
SV2 (mouse)	Synaptic vesicles	1:250	(Hüsken et al., 2014)	Developmental Studies Hybridoma Bank (DSHB)	Alexa-Flour 488 conjugated IgG Donkey anti-mouse	1:1000	
Isolectin IB_4 dye conjugate 488 (IB4) *Griffonia simplicifolia*	Non-peptidergic small neurons	1:50	(Bangaru et al., 2013)	Invitrogen	-		-
Anti-VR1 antibody ab10296 (rabbit)	TRPV1 receptor	1:1000	(Boisvert et al., 2015)	Abcam	Alexa-Flour 488 conjugated IgG Donkey anti-rabbit	1:1000	Invitrogen, Eugene, Oregon, USA
TrkC AF1404 (goat)	Tyrosine kinase receptor marker, marker for large neurons	1:100, 1:200, 1:1000	optimization	R&D Systems	Alexa-Flour 488 conjugated IgG Donkey anti-goat	1:1000	Invitrogen, Eugene, Oregon, USA
S100B (rabbit) (ab52642, EP1576Y)	Glial cell marker	1:1000		Abcam	Alexa-Flour 488 conjugated IgG Donkey anti-rabbit		
CD63 (rabbit)	Exosomal marker	1:200	optimisation	Genetex	Alexa-Flour 488 conjugated IgG Donkey anti-rabbit	1:1000	Invitrogen, Eugene, Oregon, USA
					Alexa Flour™488 streptavidin, 10 nm colloidal gold conjugate	1:100	Thermo-fisher Scientific
Anti-VGAT 131002 (rabbit)		1:2000	optimisation	Synaptic Systems	Alexa-Flour 555 conjugated IgG Donkey anti-rabbit	1:1000	Invitrogen, Eugene, Oregon, USA
					Biotin-SP-conjugate Donkey anti-rabbit 711-065-152	1;250	Jackson Immuno-Research, Pennsylvania, USA
Anti-VGAT 131004 (guinea pig)		1:1000	optimisation	Synaptic Systems	Alexa-Flour 555 conjugated IgG Goat anti-guinea pig	1:1000	Invitrogen, Eugene, Oregon, USA
VGAT polyclonal AB-N44 (VGAT-C) (rabbit)	Vesicular GABA transporter, marker for VGAT vesicular domain	1:200	Optimisation, (Martens et al., 2008)	Atsbio, Advanced Targetting System	Alexa-Flour 555 conjugated IgG Donkey anti-rabbit	1:1000	Invitrogen, Eugene, Oregon, USA

2.4 Live VGAT antibody uptake by DRG neurons in culture

2.4.1 DRG preparation

DRG were isolated from preweaner rats according to the protocol used for immunohistochemical labelling of fixed DRG tissue sections (section 2.1.1). In this experiment, five preweaner wistar rats (6 – 9 days old) were obtained from the Central Biological Services (CBS) unit, Faculty of Biological Sciences, University of Leeds. Out of five rats sacrificed, two were not utilised for IHC staining as the cultured DRG neurons were washed off during the initial steps of the experiment. The collected ganglia were enzymatically digested for 15 min with dissociation solution containing prewarmed HBSS, 10 mg/ml dispase and 1 mg/ml collagenase type 1A (1mg/ml) (Sigma). The dissociation solution-containing DRG was incubated for approximately 13 min at 37˚C to allow dissociation of cells. These dissociated cells were then gently triturated (5 times) to help the dissociation process before being replaced into the incubator for a further 2 min. DRG homogenates were then centrifuged at 800 rpm for 5 min, triturated briefly using 1 ml-Gilson pipette and recentrifuged. The pellet was then suspended in the culture media containing DMEM 1% penicillin-streptomycin and 10% FBS. The homogenates were plated on a coverslip coated with poly-D-lysine placed in a 48-well plate at 85 µl per slip. These homogenates were then incubated at 37˚C for 4 hours to allow cell attachment before adding in another 500 µl fresh media into each DRG homogenates-containing well. DRG cultures were incubated for 48 hours followed by 2 times wash using phosphate buffer saline (PBS) free from the culture media.

2.4.2 Depolarisation of neurons with high KCl (100 mM)

DRG neurons were incubated in either of the following solutions: (i) standard extracellular solution (EC), (in mM): 144 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 5.6 D-glucose, 0.7 NaH_2PO_4, 0.9 $MgCl_2$ and 10 HEPES; (ii) the 'high K^+' (high KCl) EC solution (standard EC solution but with NaCl concentration reduced to 49.8 mM and KCl concentration

increased to 100 mM); (iii) Ca^{2+}-free standard EC; (iv) Ca^{2+}-free high K^+ EC. Antibodies against either the luminal (C-terminal) VGAT epitope ('VGAT-C') or the cytosolic (N-terminal) VGAT epitope ('VGAT-N') were used. Details on the protocol for this VGAT antibody uptake experiments are schematically presented in **Figure 4.3.** This protocol was adopted from Martens *et. al* 2008 with slight changes; following optimisation, I found that incubating DRG neurons with 100 mM KCl concentration for 15 min at 25˚C produced better DRG neurons depolarisation compared to 55 mM KCl for 5 min used to depolarise primary hippocampal neurons of adult wistar rat reported in Martens and colleagues (Martens et al., 2008). This difference could be contributed by the different temperature setting; Martens and colleagues incubated their cells at 37˚C while in my experiment, DRG neurons were incubated at ambient temperature ~25˚C. Indeed, higher temperature has been shown to increase neuron activity in hippocampal neurons (Shibasaki et al., 2007). Thus 100 mM KCl with 15 mins incubation time were used in this experiment protocol. In brief, high KCl EC solution was used to depolarise DRG neurons leading to Ca^{2+} influx and ultimately release of neurotransmitter from synaptic vesicle via exocytosis. N-terminus of VGAT, inserted in the neurotransmitter vesicle membrane, faces the cytoplasm while the C terminus resides in the synaptic vesicle lumen (Martens et al., 2008). During exocytosis and subsequent recycling of a synaptic vesicle, luminal VGAT epitopes are temporarily exposed to the extracellular milieu. During such an exposure, antibodies that recognize these epitopes can bind to these and become trapped and subsequently internalized by endocytosis (Martens et al., 2008). On the other hand, antibodies against the N-terminus of VGAT should not be entrapped in this way as VGAT's N-termini remain cytosolic at all times throughout the exocytosis. The VGAT-C antibodies were labelled with secondary antibody alexaflour donkey anti-rabbit (DAR) 488 and VGAT-N antibodies were labelled with donkey anti-rabbit (DAR) 555 following DRG neurons' fixation using 4% paraformaldehyde (PFA). The DRG neurons (on coverslips) were mounted on cover slides with DAPI to stain the nucleus.

For immunochemical labelling of VGAT-C terminal (#AB-N44, Advance Targeting System), 1:200 dilution was used as described by (Martens et al., 2008). **Figure 2.2** shows the workflow of the aforementioned experiment which included the time for incubation for each of the buffer, PFA and antibodies used.

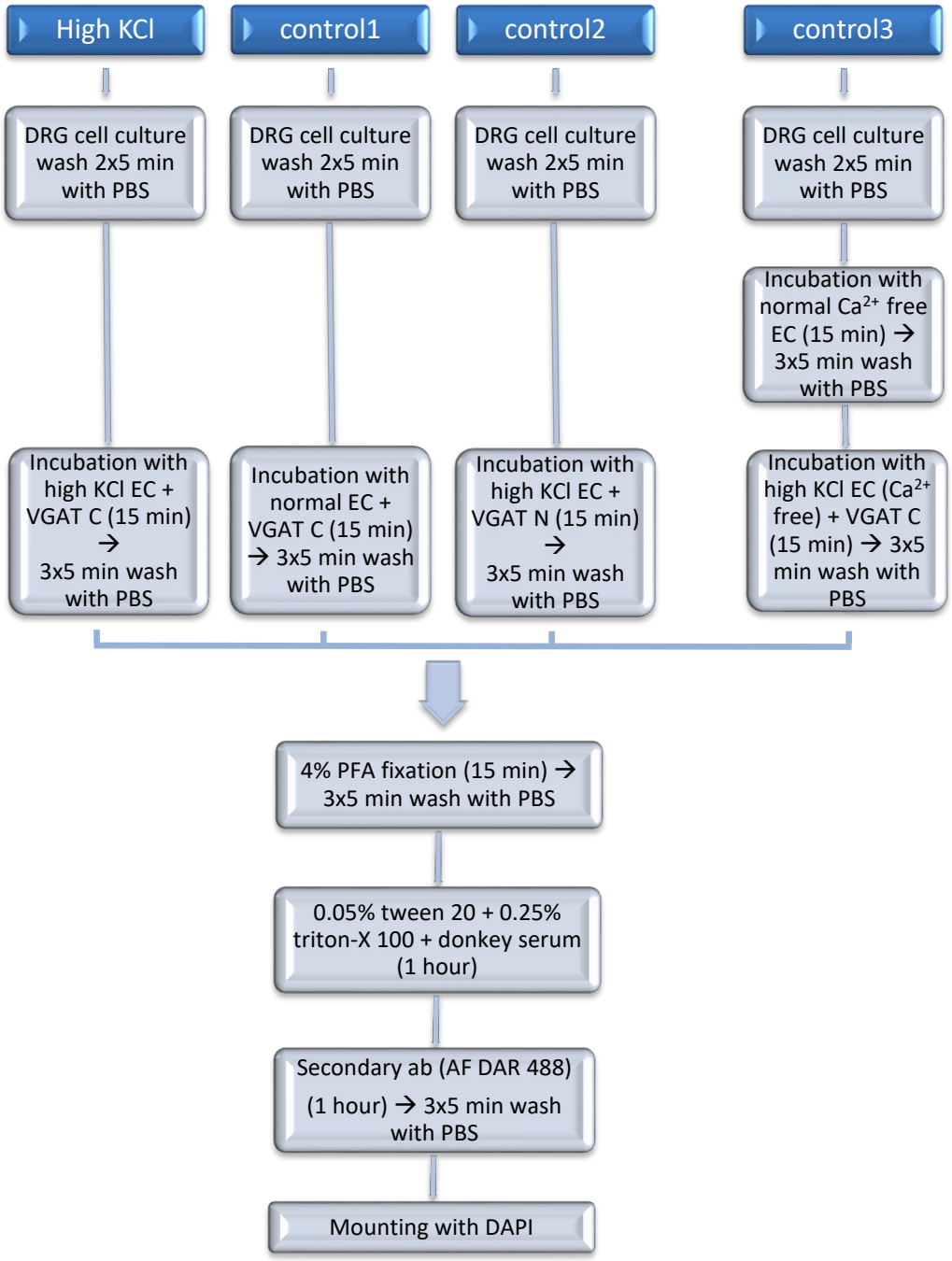


Figure 2.2 Workflow for VGAT-C antibody uptake in DRG neuron culture. [Modified from (Martens et al., 2008)].

2.4.3 Image analysis

Data were obtained from DRG neuron cultures (total number of cultures, N=3). Analysis was performed by quantifying the total number of VGAT-C-positive neurons from three coverslips per rat. Neurons were identified by their morphology (spherical structure) and size (>15 µm). Neurons were considered as positive for VGAT-C if the whole neuron displayed green fluorescence. Imaging was done using Confocal Microscope LSM880 Airyscan (Carl Zeiss, AG, Germany).

2.5 $GABA_A$ receptor activation by GABA released from DRG neurons

2.5.1 Transfection of HEK293 cells with $GABA_A$ receptor subunits (α_1, β_2 and γ_2) and EYFP

HEK293 cells were transiently transfected with cDNA encoding human α_1, β_2 and γ_2 subunits of $GABA_A$ receptors (a kind gift from Prof David Weiss, Department of Physiology, University of Texas Health Science Center, San Antonio, Texas, USA). These HEK293 cells were also co-transfected with halide-sensitive EYFP mutant (H148Q/I152L; EYFP-QL) a fluorescence protein used to study the influx of iodide via $GABA_A$ receptors. The individual plasmids of $GABA_A$ receptor subunits together with EYFP were added into a transfection mixture with the following ratio:

α_1 subunit, β_2 subunit, γ_2 subunit and EYFP-QL 1:1:1:2

For a total volume of transfection mixture solution 25 µl, FuGENE® HD Transfection Reagent 1.7 µl (Promega, USA) and HyPure™ Cell Culture Graded Water (GE Healthcare Life Science, Utah) 18.3 µl. HEK293 cells media was replaced with 500 ml fresh DMEM followed by addition of 25 µl of transfection mixture. Transfection mixture was left at room temperature for 15 – 20 min before 25 µl were added into each of the two wells of HEK293 cells (80 – 90% confluency), cultured in a 24-well plate. Transfected HEK293 cells were then incubated at 37˚C with 5% CO_2 for 24 hours before being co-cultured with DRG neurons.

2.5.2 Co-culture of DRG with HEK293 cells transfected with $GABA_A$ receptor subunits (α_1, β_2 and γ_2) and EYFP H148Q/I152L

Extraction and isolation of DRG neurons were performed as described in section 2.4.1. In this experiment, a total number of six preweaner wistar rats (6 – 9 days old) were obtained from the Central Biological Services (CBS) unit, Faculty of Biological Sciences, University of Leeds. Out of six rats sacrificed, three rats were used for optimisation of transfection of HEK cells with $GABA_A$ receptor subunits (α_1, β_2 and γ_2) and EYFP H148Q/I152L while the other three were used for HEK cells-DRG neurons co-culture experiment. After the second centrifugation, 80 µl of DRG suspension were suspended onto a 10 mm coverslips coated with poly-D-lysine and laminin placed in a 24-well plate 15 min prior to the addition of 500 µl of culture media into each DRG neurons-containing well. In the meantime, transfected HEK293 cells were prepared for co-culture. After a 24-hour incubation period, old culture media were removed and new media was added in. The cells were scraped and together with the culture media, they were centrifuged for 5 min at 800 rpm at 25˚C (room temperature). New media (1.5 ml) was added to the HEK293 cell pellet, cells were resuspended by trituration and added into each DRG neuron-containing well, 250 µl for each well. The HEK293 cells-DRG neuron co-cultures were incubated for 24 hours at 37˚C with 5% CO_2 for 24 hours before imaging could be performed. **Figure 2.3** summarises the workflow for HEK293 cells culture and HEK293 cell-DRG neuron co-culture.

2.5.3 Iodide imaging

For iodide imaging, HEK293 cells-DRG neuron co-cultures were grown onto 10 mm glass coverslips as described above. Cells were perfused with standard extracellular solution. An imaging system comprising a Nikon TE-2000 E microscope with a CCD camera was used for iodide imaging. Cells were located using brightfield and epi-fluorescence (488 nm excitation) before being imaged for iodide quenching: the fluorescence of EYFP H148Q/I152L is quenched by iodide and most anion

channels are permeable to this halide anion, hence, EYFP H148Q/I152L fluorescence quenching by I^- has been used to monitor activation of anion channels, including $GABA_A$ channels (Galietta et al., 2001, Jin et al., 2013, Johansson et al., 2013) HEK293 cells-DRG neuron co-culture was imaged using a 20x objective using whole 10 mm coverslip. For each coverslip, the laser intensity and exposure were optimised to achieve the highest possible fluorescence while avoiding both photobleaching and saturation (Laser auto exposure 400 – 700 ms; aperture 35 µM).

HEK293 cells-DRG neuron co-cultures were perfused with a different solutions using the protocols detailed in **Table 3**. Fluorescence quenching of the HEK293 cells during the sequential application of these solutions was recorded using a Nikon Swept Field confocal microscope equipped with a 488 nm argon laser and images were taken using EM-CCD camera. Data were analysed using NIS Elements 3.2 software (Nikon).

2.5.4 Data analysis

Following iodide imaging, the EYFP H148Q/I152L fluorescence intensity data were transferred to Microsoft Excel for analysis. Data were normalised to t=0 (F/F_0) for each cell. Cells with unstable baseline or oscillations during recording were excluded from analysis. EYFP quenching was determined at the point when the EYFP fluorescence intensity was consistently decreasing from t=0 (quenching started) until the value reached plateau at the end 200-second protocol for each cell. The amount of EYFP quenching for each cell was calculated by subtracting the fluorescence intensity value at the point where quenching started with the value at which the quenching plateaued. Data were analysed by calculating the mean EYFP H148Q/I152L fluorescence intensity of HEK293 cells per transfection (one transfection represents one biological replicate) and per rat and transfection for HEK cell-DRG neuron co-cultures.

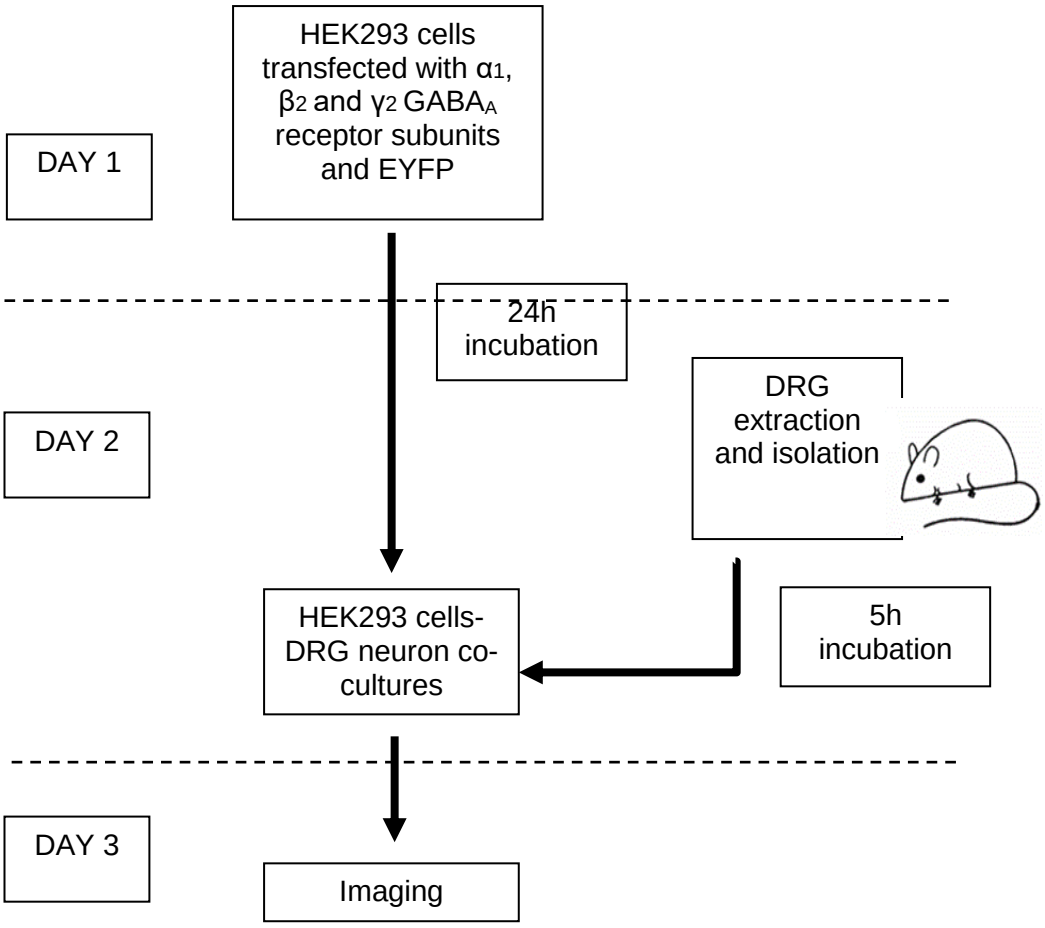


Figure 2.3 Workflow for the study of somatic GABA release by DRG neurons. On day 1, HEK293 cells were transfected with α_1, β_2 and γ_2 $GABA_A$ receptor subunits and EYFP and incubated for ~24 hours. On day 2, rat was sacrificed and DRG were isolated followed by DRG incubation for ~5 hours. At the end of 5 hour-DRG incubation, HEK293 cells were co-cultured with DRG neurons and further incubated for 24 hours before imaging of the HEK293 cells was performed on Day 3.

Table 3 Application protocols for iodide imaging. Muscimol was perfused to HEK cells and HEK_{GABAA} indicator cells single culture as negative and positive control, respectively; KCl was perfused to HEK_{GABAA}-DRG co-culture to induce DRG neuron depolarisation; HEK_{GABAA}-DRG co-culture was perfused only with NaI to confirm the presence of ambient (tonic) GABA; and bicuculline was perfused to HEK_{GABAA}-DRG co-culture to determine the involvement of $GABA_A$ receptor in EYFP quenching of HEK_{GABAA} indicator cells.
HEK cells: Human Embryonic Kidney 293 cells; HEK_{GABAA} indicator cells: HEK cells transfected with EYFP H148Q/I152L and α_1, β_2, and γ_2 $GABA_A$ receptor subunits; HEK_{GABAA}-DRG co-culture: HEK_{GABAA} indicator cells co-cultured with DRG neurons.

Compound	Application protocol
Muscimol (agonist)	Standard EC (50 sec) → 5 mM NaI (30 sec) → 5 mM NaI + 10 µM muscimol
50 mM KCl	Standard EC (50 sec) → 5 mM NaI (30 sec) → 5 mM NaI + 50 mM KCl
Ambient GABA (agonist)	Standard EC (30 sec) → 5 mM NaI (3-5 min)
Bicuculline (antagonist)	Standard EC (30 sec) → 50 µM bicuculline in standard EC solution (60 sec) → 50 µM bicuculline in 5 mM NaI solution

2.6 In vivo electrophysiological recording of L5 dorsal root and its corresponding spinal nerve

This experiment was conducted at the Hebei Medical University, China. Here, animal handling and humane schedule 1 euthanasia were performed in accordance with the Animal Care and Ethical Committee of Hebei Medical University under the project license no. SYXK(Ji)2018-005. A total number of 15 wistar rats were used in this experiment, of which only 13 contributed to the data. Two rats did not survive the surgery thus recordings could not be obtained from them. Four rats were housed per cage with sawdust flooring and ad libitum water and chow access at 23 - 25˚C.

**With the permission from the Head of Department of Pharmacology, Hebei Medical University, Professor Xiaona Du, I performed four surgeries and recordings (out of the total 13) under the supervision of a trained PhD student from Hebei Medical University, while the remaining were performed by the student himself. Data analysis and interpretation for all 13 recordings were performed by me.*

2.6.1 DRG and spinal nerve exposure

All surgical procedures were performed under deep anaesthesia using intraperitoneal injection of sodium pentobarbital (60-80 mg/kg). The depth of the anaesthesia was confirmed and monitored with toe pinch while the body temperature was maintained using a plate placed under the rats. At the end of the experiment, rats were euthanized via cervical dislocation.

To get access to the dorsal root and spinal nerve of L5, a longitudinal incision was made in the middle dorsal part of the skin. Two incisions (on the right and left side of the spinous processes) parallel to the spinous processes were made from T4 - L6. The spinous processes of the T4 - L6 were removed using small scissors. The L5 and L6 vertebrae were identified by locating the midpoint of the line linking the right and left iliac crest of the pelvic bone. The two transverse processes of the L5 were removed to locate the corresponding L5 DRG that lies underneath these transverse processes. Once the L5 DRG was located, the corresponding L5 spinal nerve was

traced down and exposed by removing the overlying bone and muscles. The thin membrane covering the spinal nerve was also removed to get a direct contact between the spinal nerve and the recording electrode. The L5 spinal nerve was isolated from the surrounding structures and lifted by using a thin glass capillary. The dorsal root was identified by locating the L5 DRG and tracing the nerve fibre proximal to it. This fibre lies amongst the dorsal roots of DRGs from other levels, and thus it was isolated using another thin glass capillary. Each of these two nerve branches (the L5 spinal nerve and dorsal root) was suspended on a stainless-steel electrode attached to an amplifier. Paraffin ointment was applied onto the exposed soft tissues to prevent dehydration. The recording of the L5 dorsal root and spinal nerve allowed simultaneous registration of activity in the nerve at two points – before and after the DRG, while the ganglion was accessible for drug application. Recording of the nerve activities was done using BL-420F biological data acquisition and analysis system (Chengdu Techman Software Co., Ltd. China), an instrument integrated with amplifier and analogue to digital conversion. The recording was filtered using low-pass filter which was set at 500 Hz. The activity of both spinal nerve and dorsal root was recorded at rest (no stimulus), upon subcutaneous injection of capsaicin (10 µM, 50 µl) on the plantar surface of the rat's hind paw (to induce acute inflammation) (Du et al., 2017), and upon local application (direct drug application onto L5 DRG) of GABA (200 µM, 3 µl) and bicuculline (200 µM, 3 µl) consecutively. Data were analysed by calculating the mean neuronal firing frequency from recordings of all the six rats (GABA and capsaicin) and seven rats (bicuculline).

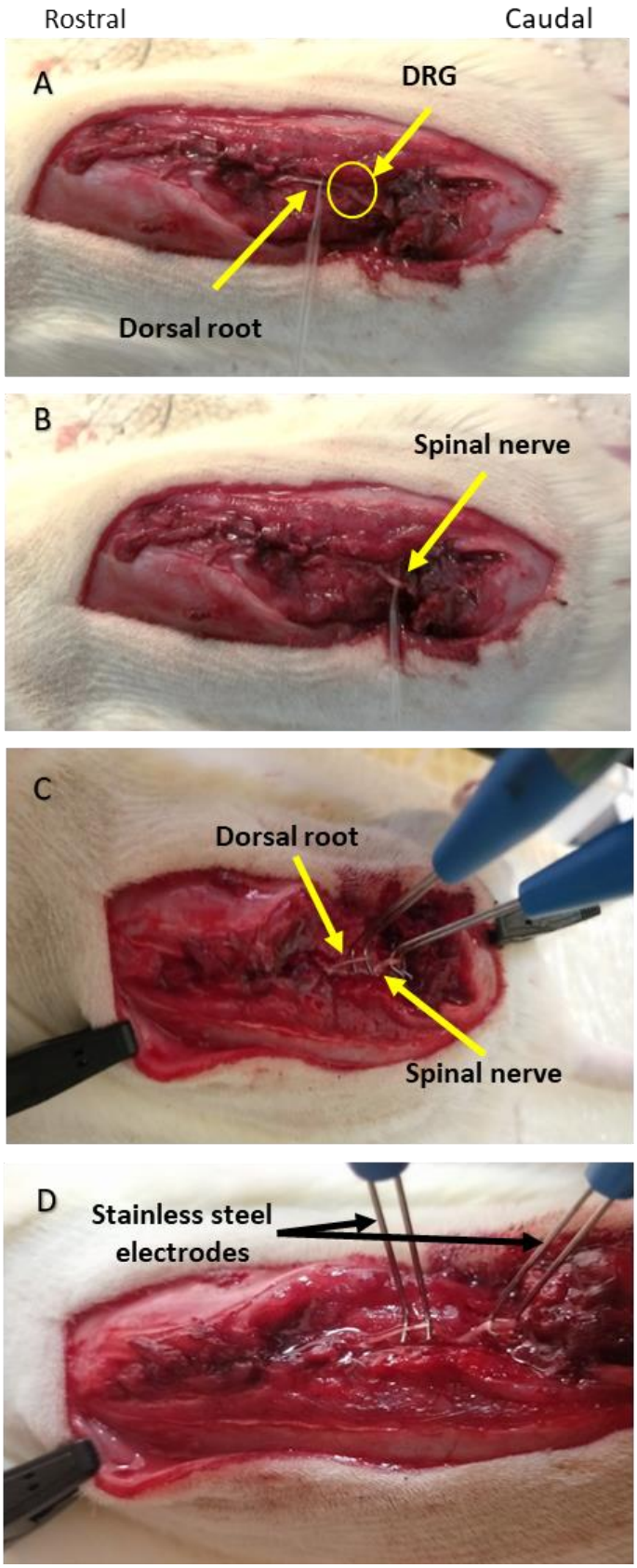


Figure 2.4 Surgical exposure of L5 DRG, dorsal root and spinal nerve. Following identification and exposure of both dorsal root and spinal nerve **(A)** and **(B)**, dorsal root and spinal nerve are suspended onto stainless steel electrodes for recording of their neuronal firings **(C)** and **(D)**.

2.7 Sample size estimation

Sample size for each experiment was estimated using resource equation approach (Arifin and Zahiruddin, 2017). Although power analysis approach is recommended for sample size calculation (Festing and Altman, 2002), in some experiments with no reference to standard deviation and effect size, resource equation approach can be an alternative. Using the resource equation approach, the sample size estimation was 3, 4, 6, and 6 rats for VGAT expression and co-localisation with neuronal markers, VGAT-C uptake, *in vivo* electrophysiological recording and HEK cells-DRG neurons culture, respectively. However, due to time limitation and cost restriction, only 3 animals were used for each experiment except for the *in vivo* recording (where 6 – 7 rats were used). However, to increase the possibility of getting the real effect of the experiment despite the small sample size, technical repetitions were done for each animal used, which yielded high number of cells per rat. For all figures presented in this thesis, the number of rat used and the total number of neurons analysed to generate the data are represented, where appropriate, by “N” and “n”, respectively.

2.8 Statistical analysis

Data for VGAT expression and co-localisation with neuronal markers were analysed using IBM SPSS Statistics software 21 (IBM, UK). Data are presented as frequencies and percentage for the quantification of the total DRG neuronal cell bodies, localisation of VGAT, and co-localisation of VGAT with NF200, IB4, TRPV1, and SV2. Association of VGAT positive neurons with the different sizes of neurons (small, medium, and large or sizes at 5 µm interval) were analysed using Fisher’s exact test with Bonferroni correction. The P value was set at 0.05. The potential significant differences in VGAT-C uptake between different groups (control, high-K^+, high-K^+/Ca^{2+}-free, and high-K^+/N term ab) were analysed using one way ANOVA with Bonferroni correction. P value was also at 0.05.

For experiment on iodide imaging, the time taken to reduce EYFP H148Q/I152L fluorescence in EYFP H148Q/I152L, $GABA_A$ receptors-transfected HEK293 cells by sodium iodide (NaI) and NaI+bicuculline was evaluated by generating the time constant (T) from individual response-curves recorded using Origin 2018 software. The mean T for both NaI and NaI+bicuculline response curves for 100 seconds each were determined and tested for significance using Mann-Whitney U test ($P<0.05$). The significant difference of the EYFP fluorescence intensity quenching in three different groups- indicator cells (HEK_{GABAA} indicator cells: HEK cells transfected with EYFP H148Q/I152L and α_1, β_2, and γ_2 $GABA_A$ receptor subunits), indicator cell+DRG and indicator cell+DRG+BIC was tested using Kruskal wallis ANOVA ($P<0.05$). Data on the *in vivo* electrophysiological recordings of L5 spinal nerve and dorsal root were analysed using one-way ANOVA followed by Bonferroni correction for analysis of firing frequency before and after application of GABA, and Wilcoxon signed rank test for analysis of firing frequency before and after application of bicuculline. Statistical significance was accepted at $P<0.05$.

Chapter 3 Quantification and characterisation of expression of vesicular GABA transporter (VGAT) dorsal root ganglion neuron somata

3.1 Introduction

DRG house somata of the diverse subpopulations of sensory neuron. These afferent neuronal cells differ from each other in a number of ways, including functional diversity (different sensory modalities), as well as different morphological and biophysical parameters such as diameter, myelination, conduction velocity, presence of certain neurochemical markers etc. (see Chapter 1: Introduction).

Since this study focuses on pain, nociceptive neurons will be of particular importance in this and subsequent experimental chapters. Nociceptive C-fibres express receptors which allow the fibre to detect more than one type of sensation and are hence known as polymodal C fibres. However, despite the ability of these polymodal receptors to detect a wide range of thermal, chemical and mechanical stimuli, some of them have preference towards a specific stimulus. For example, Wooten and colleagues have reported three different stimulus-specific polymodal receptors in primates: the capsaicin-sensitive, histamine-sensitive and β-alanine-sensitive receptors (Wooten et al., 2014).

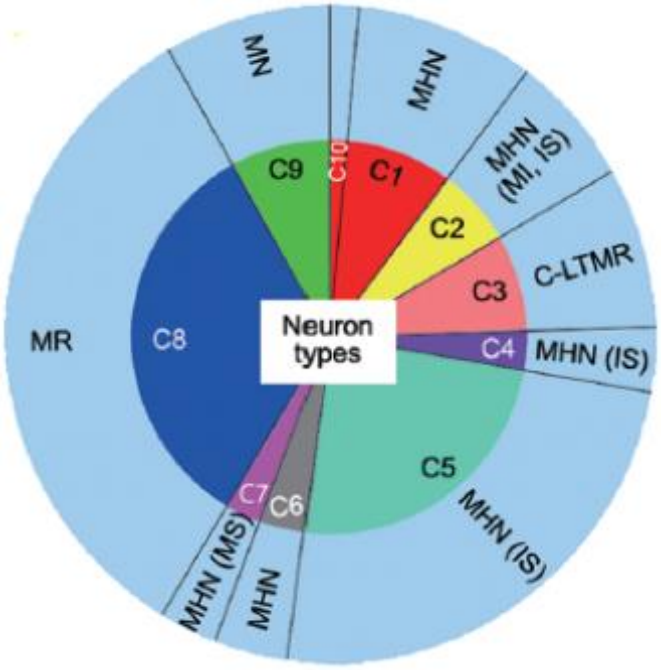
Recent studies from our lab (Du et al., 2017) and from others (Hanack et al., 2015) suggested that some DRG neurons, particularly those among the nociceptive range, are able to produce and release GABA. Yet, in order to be able to release a neurotransmitter, DRG neuron somata must express appropriate machinery. In neurons, GABA is mainly released via activity-dependent exocytosis (Gao and van den Pol, 2000, Missler et al., 2003) and in order to be able to pack GABA into the releasable vesicles, a vesicular GABA transporter (VGAT) is necessary (Chaudhry et al., 1998, Martens et al., 2008, McIntire et al., 1997). In this chapter I investigated the expression of VGAT in dorsal root ganglion neuron somata. I hypothesised that VGAT was expressed in DRG neurons of all sizes–small, medium and large neurons.

3.1.1 Methods for neuronal characterisation

Traditionally, DRG neurons are characterised by size, conduction velocity, degree of myelination, somatosensation modality and the presence of specific neurochemical markers (see below). These parameters are very useful to identify and understand the physiology of a neuron. To determine the type of neurons, more than one method of classifications are usually performed to enable a defined and confirmative neuron types. For example, results from our previous study integrated results from the morphological analysis (size determination), somatosensory profiling (behavioural experiments) and expression of neurochemical markers to understand the role of GABA in the peripheral nociceptive pathway (Du et al., 2017). For this reason, these traditional methods are still widely used nowadays for neuronal characterisation. Today, thanks to the evolving knowledge in science, particularly genetics, more specific methods of DRG neuron classification had been developed, including single-cell RNA sequencing (RNA-Seq). This method involves deep sequencing methods which provide a transcriptome analysis of a cell. It gives high coverage and higher resolution compared with other sequencing methods such as Sanger sequencing and microarray-based method (Kukurba and Montgomery, 2015).

RNA-Seq was recently used by two groups to classify mouse DRG neurons (Li et al., 2016, Usoskin et al., 2015, Zheng et al., 2019). Using this method, Usoskin and colleagues revealed eleven types of sensory neurons: three distinct low-threshold mechanoreceptive neurons, two proprioceptive, and six principle types of neurons. The later include thermosensitive, itch sensitive, type C-low threshold mechanosensitive and nociceptive neurons with distinguished molecular properties (Usoskin et al., 2015). This work also provided markers for new and functionally distinct neuron subtypes. However, as this study performed low-coverage single-cell RNA-Seq, it resulted in transcriptional variations due to a limited number of genes detected in each neuron (Usoskin et al., 2015). By using higher-coverage RNA-Seq,

Li and colleagues were able to classify mouse DRG neurons more specifically by linking transcriptome-based neuron typing with sensory phenotype using in vivo recordings. This allowed them to classify mouse DRG neurons into ten types and 14 subordinates with distinct transcriptional patterns, molecular markers and functional annotations providing a new catalogue for somatosensory receptors (Li et al., 2016). **Figure 3.1** and **Table 4** summarise the types of DRG neurons investigated in these two studies.



MHN: mechanoheat receptor

MI: mechanically insensitive

MS: mechanically sensitive

IS: itch-sensitive

C-LTMR: c-fibre low-threshold mechanoreceptor

MN: mechanical nociceptor

MR: mechanoreceptor

Figure 3.1 Ten types of primary sensory neurons.A schematic showing the proposed framework of DRG neuron types and their proportions based on both transcriptomic analysis and ISH, as well as functional annotations suggested by electrophysiological analysis results and published data. C8 and C10 are predicted to be mechanoreceptors or/and proprioceptors (Li et al., 2016).

Table 4 Sub-classifications of primary sensory neurons(Usoskin et al., 2015).

Neuron subclassification	Type of neurons	Degree of myelination
NF1	LTMRs	Myelinated
NF2		
NF3		
NF4	Proprioceptors	
NF5		
NP1	Nonpeptidergic	Unmyelinated
NP2		
NP3		
PEP1	Peptidergic	
PEP2		Myelinated
TH	C-LTMRs	Unmyelinated

3.1.2 Neurochemical markers for DRG neuron characterisation

Neurochemical markers have been used to identify DRG somatosensory modalities and determine the functional connotation of the fibres studied. For example, distinct markers are used to label large, medium and small neurons, for example, NF200 labels myelinated Aβ and Aδ fibres while IB4 labels a subset of small neurons respectively (Dirajlal et al., 2003, Perry et al., 1991, Stucky and Lewin, 1999). This size-labelling, in turn, helps to determine the likely somatosensation of each of the neurons labelled by these markers as, for example, the small neurons are highly likely to be nociceptive C fibres (Woolf and Ma, 2007) while the large, myelinated neurons are more abundantly represented by low threshold mechanoreceptors-A fibres (Djouhri and Lawson, 2004). Results obtained from other methods such as electrophysiology and behavioural studies can also be correlated with immunohistochemical labelling to enhance conclusiveness (Zheng et al., 2019). In this study, I labelled DRG neurons using a number of sensory modality-specific markers in order to identify the type of neurons expressing GABA-related protein.

3.1.3 Markers for large neurons

The presence of neurofibrils (NFs) in neuron cytoplasm were first discovered by Ramon Cajal, Golgi and others (López-Muñoz et al., 2006). In 1950, via electron microscopy, Schmidt and Geren observed these neurofibrils as 10 nm filaments hence the designated term ‘neurofilament’ that is used today (Schmitt and Geren, 1950). NFs are a member of intermediate filaments (IFs) with diameter ~10 nm, which lies between the diameter of actin filaments (5 nm) and myosin filaments (15 nm) found in muscle cells. Under the IFs classification, NFs are classified as class IV which also include the neurofilament light (NF-L), neurofilament middle (NF-M), neurofilament heavy (NF-H) and α-internexin (Yuan et al., 2017). Other IFs are acidic keratins (class I), basic keratins (class II), vimentin, desmin, peripherin, glial fibrillary acidic protein (GFAP) (class III), nuclear lamina (class V), and nestin (class VI). NFs are very important in radial growth and structural stability of myelinated neurons. For

this reason, NF antibodies have been used as markers for myelinated neurons which are the medium- and large-size neurons in the DRG. The molecular size for NF-L, NF-M and NF-H are 68, 160 and 200 kDa respectively (Yuan et al., 2017). To date, antibodies against NF-M (NF160) and NF-H (NF200) or a combination of NF-M and H (NF160/200) have been used most widely as markers of myelinated neurons in both CNS and peripheral nervous system (PNS). As an example, in the dental primary afferent neurons, NF200 was shown to be highly co-expressed with Piezo2, a recently discovered mechanosensitive ion channel (Won et al., 2017). This result further confirmed the role of Piezo2 in transducing mechanosensitive nociception, in this particular study.

Another set of markers widely used in somatosensory physiology belongs to the family of tropomycin-related tyrosine kinase (trk) receptors, which are a family of growth factor receptors. These are present in different tissues in the body including the CNS and PNS. Trk receptors have been shown to influence neuronal differentiation, neurite outgrowth and synaptic plasticity (Huang and Reichardt, 2001, McAllister et al., 1999, Park and Poo, 2013). To date, three subtypes of trks have been identified, trkA, trkB and trkC. These Trk receptors bind to different ligands (neutrophins) and activate different pathways related to neuronal growth. TrkA receptors bind nerve growth factor (Fayaz et al., 2016), trkB binds BDNF and neurotrophin 4 (NT4), while trkC binds neurotrophin 3 (NT3) (Dechant, 2001, Kaplan et al., 1991, Klein et al., 1991, Nikoletopoulou et al., 2010). TrkA-NGF signalling plays an important role both during the prenatal period (Indo et al., 1996, Miranda et al., 2002) and in adulthood (Lewin et al., 1993, Petty et al., 1994). Individuals born with a mutation in the trkA gene suffer from a form of CIP together with anhidrosis, both caused by defective trkA activity. A mutation in NGF also causes CIP due to the inability of nerve growth factor (Fayaz et al., 2016) to perform its physiological action at the receptors (Hirose et al., 2016).

In adulthood, NGF is one of the proteins released during inflammation. The binding of NGF to trkA, located on the cell membrane of the sensory neurons, phosphorylates other proteins involved in the Rap1/Erk1/2, p38MAPK and PI3K pathways (Delcroix et al., 2003). Ultimately, increased expression of these proteins, in turn, induces peripheral and central sensitisation leading to hyperalgesia and allodynia, respectively (Sousa-Valente et al., 2018). Additionally, a loss-of-function mutation in trkA was shown to abolish heat-hyperalgesia in African dark mole rat (Omerbasic et al., 2016).

Meanwhile, trkB-BDNF signalling was shown to be involved in heat-hyperalgesia inflammatory pain mediated by cyclin-dependent kinase 5 (cdk5) (Zhang et al., 2014). Immunoprecipitation and kinase assay revealed that Cdk5 mediates carrageenan-induced heat-hyperalgesia by phosphorylating TRPV1 receptors in DRG of mice and rats (Pareek et al., 2007, Pareek et al., 2006, Xing et al., 2012). Unlike trkA and trkB, trkC along with its ligand, NT3, are involved more in proprioception than nociception (Patel et al., 2003, Ramer et al., 2002). However, trkC and NT3 have also been shown to modulate aspects of neuropathic pain (Tender et al., 2011). In a nerve injury rat model, intrathecal NT3 injection has been shown to attenuate TRPV1 expression in sensory neurons resulting in inhibition of thermal hyperalgesia (Wilson-Gerwing et al., 2005). NT3 and trkC were found to be expressed in medium- and large-diameter DRG neurons (McMahon et al., 1994) but interestingly, their expression levels were upregulated in small-diameter neurons during neuropathic pain (sciatic nerve injury) rat model (Tender et al., 2011). More importantly, the increase in NT3 and trkC expression decreased in a neuropathic rat model treated with a TRPV1 inhibitor, Resiniferatoxin (RTX), hence supporting the role of trkC and NT3 in modulating neuropathic pain (Tender et al., 2011). Recently, an interaction between trkC and pre-synaptic type-IIa receptor-type protein tyrosine phosphatase sigma (PTPσ) has been reported (Naito et al., 2017). PTPσ is another ligand that binds at different extracellular domains of trkC and this interaction induced excitatory synapses to fire,

this was also enhanced by NT3 (Naito et al., 2017). This finding reveals a novel role of trkC as a synapse organising protein (Naito et al., 2017).

3.1.4 Markers for nociceptive neurons

Most nociceptors are small diameter unmyelinated C fibre neurons (Woolf and Ma, 2007). The C fibre neurons can be further divided into peptidergic and non-peptidergic neurons (Basbaum et al., 2009). The peptidergic small-diameter neurons express neuropeptides such as CGRP and Substance P (Basbaum et al., 2009). These markers have been used to study the distribution of peptidergic neurons. Meanwhile, non-peptidergic neurons express surface carbohydrates (α-galactose group) which bind to lectin molecule, IB4, of the *Griffonia simplicifolia* (Goldstein and Winter, 1999), thus referred to as IB4-positive neurons. The central projections of peptidergic neurons terminate in lamina I and outer lamina II (IIo) of the spinal dorsal horn while the non-peptidergic terminate in lamina I and inner lamina II (IIi) (Bradbury et al., 1998, Seal et al., 2009). Non-peptidergic neuron fibres that terminate in lamina IIi activate the central pathway to the hypothalamus and amygdala, which are mostly involved in the emotional and affective aspect of pain (Bernard et al., 1996). Other markers for non-peptidergic neurons include Mas-related G-coupled protein receptors (*Mrgprd)* (Rau et al., 2009, Zylka et al., 2005) and the P2X3 receptors (Luo et al., 2007, Staikopoulos et al., 2007). Sensory neurons that express *Mrgprd* are known to innervate the outermost layer of the epidermis, the stratum granulosum. Activation of *Mrgprd* has been associated with not only nociception but also itch sensation (Bader et al., 2014). Meanwhile, purinergic receptors are known to be involved in nociception in different parts of the body, including skin and viscera; reviewed in (Burnstock, 2009). In particular, the P2X3 is a purinergic receptor which was cloned in 1995 and found to be expressed in small nociceptive sensory neurons in DRG (Chen et al., 1995).

TRPV1 is another protein used as a neurochemical marker in categorizing small-diameter neurons. TRPV1 is a non-selective cation channel within the superfamily of transient receptor potential (TRP) ion channels. TRP superfamily comprises TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and the TRPA (ankyrin) groups; TRPV, TRPM and TRPA subfamily members are expressed in subpopulations of sensory neurons and responsible for a wide range of sensations such as thermal, mechanical and chemical stimuli and bind to different noxious ligands (Nilius et al., 2007). TRPV1 can be activated by noxious heat (>42°C) as well as by chemicals such as capsaicin (Caterina et al., 1997) which can be found in hot chili peppers. TRPV1 can also be activated by low extracellular pH (Tominaga et al., 1998), divalent cations such as Mg^{2+} and Ba^{2+} (Cao et al., 2014) and animal toxins (Bohlen et al., 2010, Yang et al., 2015). TRPV1 receptors act as polymodal sensors for a diverse physical and chemical stimuli, from extra- as well as intracellular environment. Another member of TRP channel family, TRPA1, is activated by a different set of painful of potentially harming stimuli including natural and exogenous electrophilic compounds such as wasabi and mustard oil, extreme temperatures and endogenous inflammatory mediators (Jordt et al., 2004, Laursen et al., 2014, Viana, 2016). Both TRPV1 and TRPA1 channels are often found in the same, small- and medium-sized neurons (Story et al., 2003, Kobayashi et al., 2005).

Another protein that is commonly used to label small neuron is peripherin. Peripherin is a 57 kDa type III neuronal intermediate filament that is widely expressed in the PNS. For this reason, investigations on peripherin expression has been used to define a specific population of small neurons in rat DRG (Deshmukh et al., 2016, Goldstein et al., 1991).

3.1.5 Glial markers

For centuries, neurons have been the focus of science of the nervous system, while glia were seen as only supporting structures of these vital cells. It was only in 1950s, when studies on glia began to discover scientific breakthroughs and conceptual shifts of its important role in the function and behaviour of the nervous system (Fan and Agid, 2018). According to current understanding, the number of glial cells in nervous system is comparable to that of neurons (Herculano-Houzel, 2014, von Bartheld et al., 2016).

Similarly to neurons, glial cells originate from neural tube stem cells during development (Rowitch and Kriegstein, 2010). There are many subtypes of glial cells; astrocytes, oligodendrocytes, microglia and radial glia mostly found in the brain (Zuchero and Barres, 2015), while Schwann cells and satellite cells are mostly found in the PNS (Jessen and Mirsky, 2005).

Anatomically, glia wrap around neurons in both CNS and PNS like a glue sticking on a structure; accordingly, the term “glia” is a Greek word meaning glue. Physiologically, glial cell functions are indispensable in neuronal development and for normal function of the nervous system. Glia also play an essential role during disease and injury to the nervous system (Barres, 2008, Fields et al., 2015).

In the PNS, DRG cell bodies are tightly wrapped by the satellite glia cells (SGC) (Pannese, 2010). Few changes in SGC have been reported following peripheral nerve injury; SGC divide, the number of its gap junction increases and glial fibrillary acidic protein (GFAP) expression increases (Ohara et al., 2009). Due to their anatomical structure, primary sensory neurons are more prone to injury compared with SGC, indeed their long axons are more likely to be injured if peripheral tissues are subjected to trauma. Thus the changes in SGC following trauma to the peripheral nerve can be secondary to and as an adaptation to the injured neurons which occurs via neuron SGC signalling. In response to trauma, sensory neurons release

inflammatory substances such as Substance P, nitric oxide and adenosine triphosphate (ATP). ATP released from DRG neuron somata has been shown to activate purinergic receptor P2X7 expressed in SGC, which could contribute to increased nociceptive neuron excitability (Zhang et al., 2007).

Realising the importance of glial cells in the studies of biological systems, several glial markers have been developed to substantiate the identification of these cells such as S100B, GFAP and glutathione synthase (GS) (Regan, 1988, Roots, 1981). The identification may help to further understand the contribution and their role in especially, pathological conditions.

3.1.6 Synaptic vesicular markers

Synaptic vesicles are small vesicles that are pooled at the presynaptic terminals. The number of synaptic vesicles determines the strength of the communication that occurs between cells. This communication occurs via neurotransmitters that are released by the synaptic vesicles. Thus synaptic vesicle markers have been used to study the activity of neurotransmitter release.

There are several markers for synaptic vesicles available for scientific research. Most of these markers are developed against the specific proteins present on the synaptic vesicle membrane. Synaptic vesicle 2 (SV2), synaptotagmin and synaptophysin antibodies are examples of markers that have been developed to identify the respective protein. Each of these proteins have their own important role during neurotransmitter release (some are explained in Chapter 4, section 4.1). In particular, SV2 is a synaptic vesicle protein which is found in all synaptic vesicles regardless of the neurotransmitter content (Feany et al., 1992). Three isoforms of SV2 exist, SV2A, SV2B and SV2C . SV2A is the most common isoform found in mouse brain (Crowder et al., 1999). Earlier during its discovery, SV2 was proposed to function as a universal transmembrane transporter of all types of neurotransmitters achieved by sodium-dependent membrane transporter (Feany et al., 1992). Decades

after its discovery, more specific functions of SV2 have been revealed including its role in transporting cytosolic Ca^{2+} into secretory vesicles that leads to neurotransmitter release (Wan et al., 2010). SV2 proteins are found ubiquitously in all synaptic vesicles, which justifies the use of SV2 antibody to study the presence of this neurotransmitter-releasing vesicles in DRG, paving the way to understanding the possible mechanism of GABA release therein.

3.2 Results

3.2.1 Optimisation of VGAT antibodies raised in rabbit and guinea pig

Immunohistochemical protocols for vesicular GABA transporter (VGAT) antibody raised in rabbit (VGATrab) and in guinea pig (VGATgp) were developed using DRG tissue sections fixed with 4% PFA. For optimisation, three different dilutions were used for VGATrab antibody; 1:500, 1:1000 and 1:2000 while for VGATgp two dilutions were used, 1:1000 and 1:2000. Via visual assessment, it was concluded that 1:2000 VGATrab and 1:1000 VGATgp dilution gave the best signal-to-noise ratio of immunofluorescence [**(Figure 3.2 (A) and (B)**]. The assessment was done by standardising the laser intensity and gain when imaging sections stained with the different dilutions. The 1:2000 and 1:1000 dilution of VGATrab and VGATgp respectively, showed clear VGAT staining on the DRG cell bodies with well-demarcated nuclei (**Figure 3.4)**. It also showed the least background noise under confocal imaging. Thus, the successive immunofluorescence investigation of VGAT expression using VGATrab and VGATgp was done with 1:2000 and 1:1000 dilutions, respectively. Co-staining of DRG sections with VGATrab and VGATgp antibodies showed a very similar pattern of staining (**Figure 3.3**), validating the use of VGAT raised in two different species for further co-localisation experiments with other neurochemical markers.

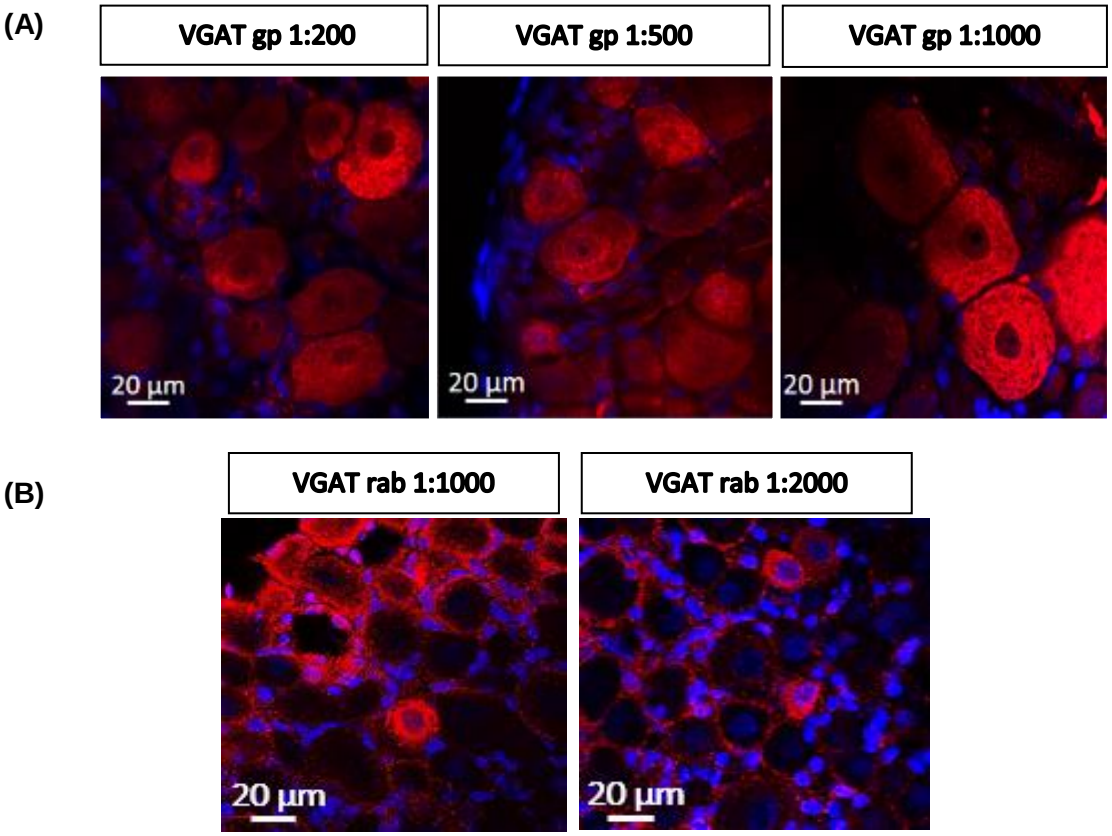


Figure 3.2 Optimisation of VGAT antibodies.(A) The expression of VGAT antibody raised in rabbit (VGATrab) in DRG neurons incubated in antibody dilutions 1:200, 1:500 and 1:1000 and (B) the expression of VGAT antibody raised in guinea pig (VGATgp) in DRG neurons incubated in antibody dilutions 1:1000 and 1:2000.

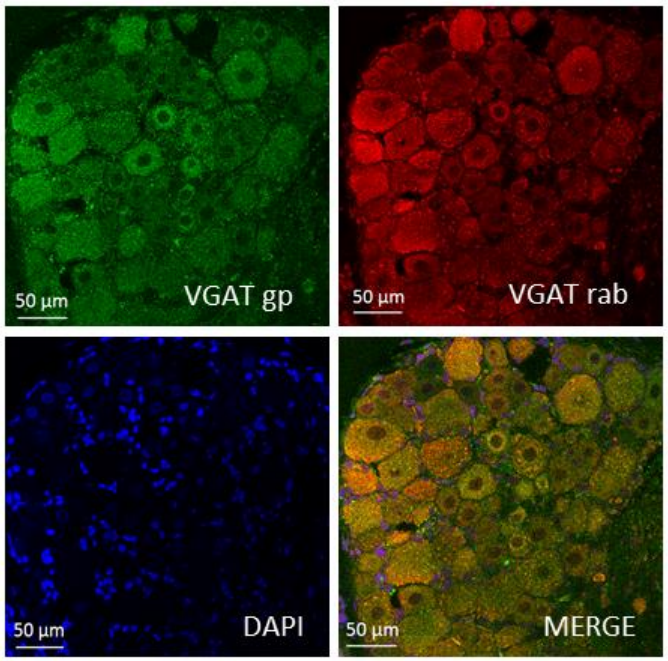


Figure 3.3 Co-localisation of VGATrab and VGATgp. Co-localisation of VGAT antibodies raised in two different species, rabbit and guinea pig (VGATrab and VGATgp) showing a similar pattern of staining.

3.2.2 Soma size analysis of the VGAT-positive DRG neurons

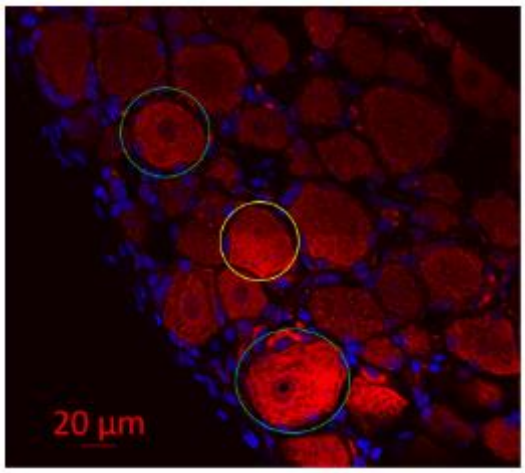


Figure 3.4 Examples of DRG neurons included for quantification. showing clear margins with visible nucleus. Neurons in green circle are examples of medium- and large-sized VGAT-positive neurons with visible nuclei, while the neuron in yellow circle is an example of those VGAT-positive neurons but with no visible nucleus.

Since DRG neuron somatic diameter correlates with somatosensory modality of the fibre, DRG neurons were categorised according to the diameter of the neuronal somata and characterised for VGAT expression. Generally, the soma or cell bodies of DRG neurons had pale cytoplasmic staining with centrally located nuclei and darkly stained nucleoli; darker colour of nuclei being produced by DAPI staining. Of note, neuronal nuclei showed noticeably a weaker DAPI staining compared to the glial cell nuclei. In some neurons nuclei were not seen, presumably because sections were cut above or below their mid-levels. A typical neuron has its nucleus located at the centre of the spherical cell body (Lee et al., 1986) thus the size of a neuron is best presented at its widest diameter where the nucleus is likely to be present. For this reason, measuring the diameter of a neuron with no visible nucleus present maybe an underestimation of its actual size. Thus, in this present study, I performed two different methods of analyses for the size distribution of DRG neurons and VGAT expression from the same dataset according to the presence or absence of nucleus in a neuron. One method is more accurate for the VGAT distribution in the DRG while another method is more truthful for DRG neuron size analysis. Accordingly, the

results of these analyses are presented in two different sections: i) analysis of 'all neurons' and ii) analysis of 'neurons with visible nucleus'.

3.2.2.1 Analysis of 'all neurons'

The total number of DRG neurons counted (regardless of the visibility of the nucleus) was 515. The size distribution of DRG neurons is skewed to the right indicating that most of the DRG neurons analysed were smaller in size while the distribution of the VGAT-positive neurons was more evenly distributed across the scales. The number of neurons was highest in the 25 – 30 µm diameter category, while the lowest was in the >50 µm diameter category [**Figure 3.5 (A)**]. When analysis was done according to small-medium-large cell size category, the percentage of DRG neurons was highest in the <32 µm category (51.8%) followed by medium- and large-sized neurons which were 25% and 23.1% respectively. Out of the total 515 sensory neurons imaged, 102 were stained positive with VGAT (19.8%). Within the population of VGAT-positive neurons, the highest expression was found in the small-sized neurons (44%) followed by medium- and large-sized neurons, 31% and 25% respectively [**Table 5 (A)**]. For ease of reference, this method will be referred to as 'method (i)' in the following discussion.

3.2.2.2 Analysis of 'neurons with visible nuclei only'

This analysis showed similar size distribution of DRG neurons to that of method (i); most of the neurons were of the smaller size. However, the size distribution of the VGAT-positive neurons has a slight preference towards smaller size neurons [**Figure 3.5 (B)**]. As only neurons with visible nuclei were considered for this analysis, the total number of neurons dropped to 127. When analysis was done according to small-medium-large cell size category, the percentage of DRG neurons was highest in the <32 µm category (43%), followed by medium- and large-sized neurons which was 29% and 28%, respectively [**Table 5 (B)**]. Out of 127 neurons included in the analysis, 84 cells were stained positive with VGAT (66.14%). Most of the small- and

medium-sized DRG neurons expressed VGAT (67% and 74%, respectively) and a slightly lesser proportion of large-size neurons (57%). Out of all DRG neurons labelled with VGAT, 44% were small-sized neurons followed by medium-sized (31%) and large-sized neurons (25%) [**Table 5 (B)**]. For ease of reference, this method will be referred to as ‘method (ii)’ in the following discussion.

These two different methods showed that regardless of the presence of a nucleus, the size distribution of DRG neurons demonstrated a similar pattern, that DRG neurons were mostly populated by small diameter neurons which results are consistent with findings from literatures (Deshmukh et al., 2016, Lawson et al., 1984, Lee et al., 1986).

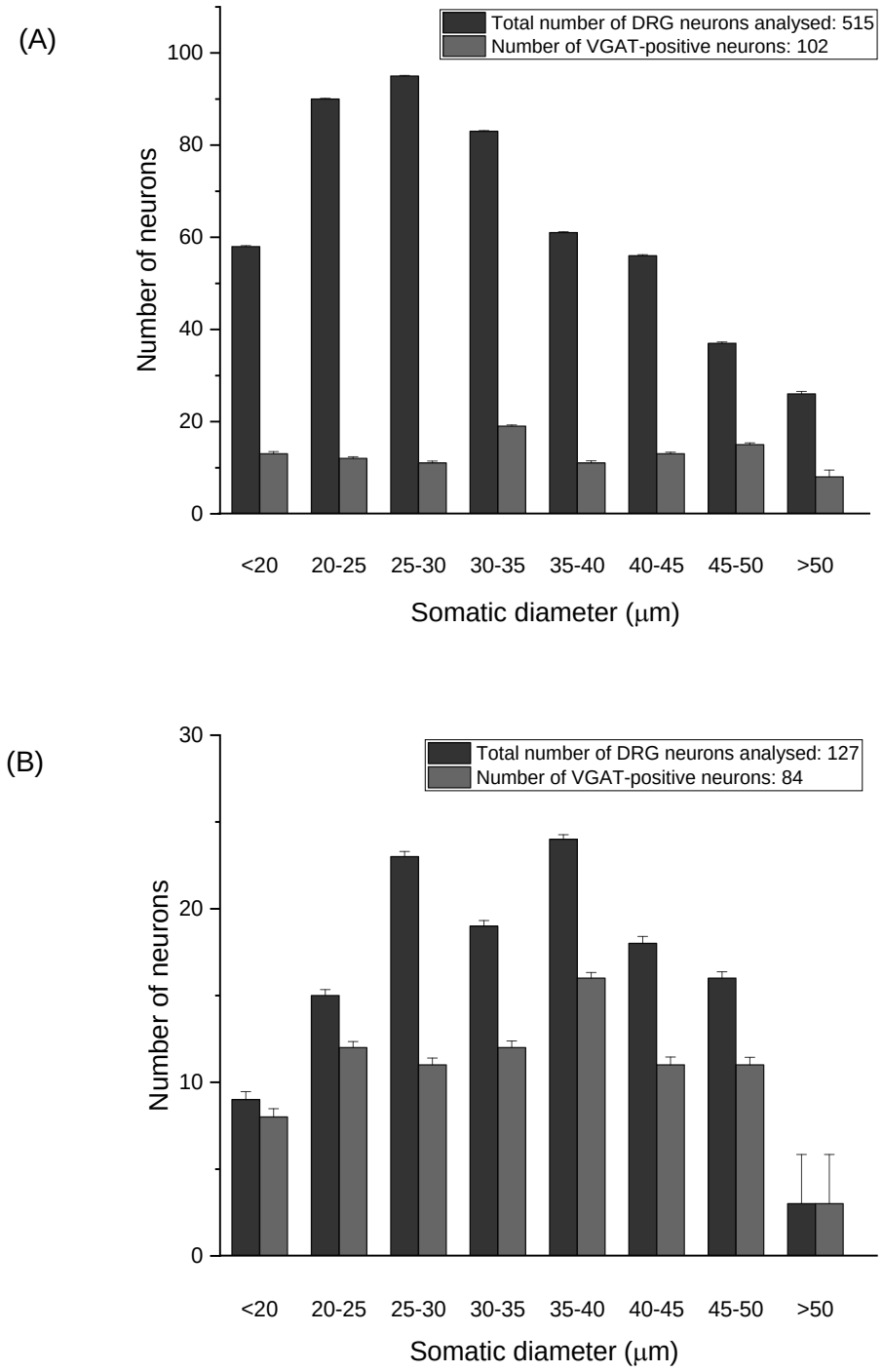


Figure 3.5 The distribution of VGAT in rat DRG neurons. Frequency distribution of the somatic diameter showing the total number of DRG neurons (black bars) alongside with the VGAT-positive neurons (gray bars). Data were obtained from analysis of neurons from six DRG tissue sections (2 sections per rat, N=3), neuronal numbers were summed, means per rat calculated, shown as mean ± SEM. **(A)** and **(B)** represent data from method (i)– all neurons' and method (ii)–'neurons with visible nuclei only', respectively.

Table 5 VGAT distribution in DRG neurons according to size classification. Neurons are classified into three size categories, the small (<32 µm); medium (32 – 40 µm) and large (>40), **(A)** and **(B)** represent data from method (i)– all neurons' and method (ii)–'neurons with visible nuclei only', respectively.

(A)

Cell body size	Total number of neurons	% of total neurons	Number of VGAT-positive neurons	% of VGAT-positive neurons within the size band	% of neurons of the given size within the population of VGAT-positive neurons
<32 µm	267	51.8	46	17.2	45.1
32-40 µm	129	25	19	14.7	18.6
>40 µm	119	23.1	37	31.1	36.3
Total number of neurons	515		102		

(B)

Cell body size	Total number of neurons	% of total neurons	Number of VGAT-positive neurons	% of VGAT-positive neurons within the size band	% of neurons of the given size within the population of VGAT-positive neurons
<32 µm	55	43	37	67	44
32-40 µm	35	29	26	74	31
>40 µm	37	28	21	57	25
Total number of neurons	127		84		

3.2.3 VGAT co-localisation with neurochemical markers

Double immunofluorescence labelling was performed to investigate the co-localisation of VGAT with markers known to be expressed in specific subpopulations of DRG neurons. IB4 and TRPV1 were used to label small-size neurons (**Figure 3.6)** while NF200 and trkC were used to label larger-size neurons (**Figure 3.7)**. Co-localisation incidence was analysed using two methods; i) as the proportion of the total number of neurons displaying co-localisation of VGAT with each of the neuronal markers over the total number of VGAT-positive neurons, and ii) as the proportion of the total number of neurons displaying co-localisation of VGAT with each of the neuronal markers over the total number of each of the neurons expressing a given marker. Accordingly, the analysis was performed using two different denominators (the total number of VGAT-positive neurons and the total number of marker-positive neurons) to determine the distribution of VGAT within the subpopulations of the DRG neurons. When VGAT was used as the denominator, the proportions of co-localisation of VGAT with NF200, trkC, IB4, TRPV1 and SV2 were: 25.67%, 32%, 55.33%, 54.54% and 68.61% respectively. When neuronal markers were used as the denominators, the percentage of their co-localisations with VGAT were 18.67% (NF200), 63.33% (TrkC), 45% (IB4), 92.43% (TRPV1) and 93.03% (SV2) (**Table 6)**. Regardless of the denominators, the co-localisation of VGAT with NF200 was significantly lower than the co-localisation of VGAT with TRPV1 and IB4 (Fisher's exact test with Bonferroni correction, $P<0.001$) (**Figure 3.6**). VGAT was also co-expressed with SV2, a synaptic vesicle marker with the highest level of co-localisation (**Figure 3.9**), suggesting a possible role of VGAT in exocytic GABA release mechanism into the extracellular space within the DRG. Fluorescence microscopy for the double immunolabelling of VGAT with S100B showed no co-localisation of these two antibodies (**Figure 3.10**). In summary, these experiments of the present study revealed that DRG neurons of any modality expresses VGAT with higher expression in small-diameter, TRPV1-positive DRG neurons and less with larger

neurons. Among the antibodies tested, the highest degree of co-localization was found between the VGAT and SV2.

Interestingly, EM imaging of VGAT stained with DAB and silver-enhancing FNG revealed what could be a VGAT presence, not only in DRG neurons, but more interestingly in SGC and intercellular space in exosome-like extracellular vesicles (**Figure 3.11**); this was not seen in our immunostainings. This discrepancy could arise from higher sensitivity of the EM. We used DAB and Nanogold-silver enhancement technique for the EM imaging, these methods are known to improve immunolabelling and hence also referred to as signal amplifiers (Hainfeld and Furuya, 1992, Weipoltshammer et al., 2000, Werner et al., 1996). **Figure 3.13** depicts the typical immunogold silver enhancement-based immunolabelling. A combination of these signal amplifiers has been shown to enable a use of ten-fold lower concentration of primary antibody (Köhler et al., 2000). Together, these studies show that using signal amplifiers, the antibody expression could be detected at a lower antibody concentration (than that used for IHC), this stands to reason the lack of VGAT expression in IHC (where VGAT expression was not seen in EC and SGC) compared to EM. On the other hand, this discrepancy between IHC and EM could also be contributed by the non-specific binding due to autonucleation (Thanh et al., 2014). In addition, gold-enhanced particles (gold grain) that undergo crystallisation and enlargement responsible for the signal amplification could cluster with the neighbouring gold grain; this leads to formation of larger gold particles (Weipoltshammer et al., 2000) which could be observed in **Figure 3.12**. Thus, further experiments are needed to test the presence of VGAT in SGC and extracellular vesicles.

Table 6 Percentage of VGAT co-localisation with five different neuronal markers. VGAT was co-localised with NF200 and TrkC (markers for large neurons), IB4 and VR1 (markers for small neurons) and SV2 (a marker for synaptic vesicles).

Antibody		Number of neurons with positive staining	% of co-localisation (per marker)	% of co-localisation (per VGAT)
TRPV1	TRPV1 positive	71	92.43	54.54
	VGAT positive	130		
	Co-localisation	66		
IB4	IB4 positive	127	45	55.33
	VGAT positive	105		
	Co-localisation	59		
NF200	NF200 positive	146	18.67	25.67
	VGAT positive	122		
	Co-localisation	24		
TrkC	TrkC positive	33	63.33	32
	VGAT positive	124		
	Co-localisation	20		
SV2	SV2 positive	138	93.03	68.61
	VGAT positive	204		
	Co-localisation	129		

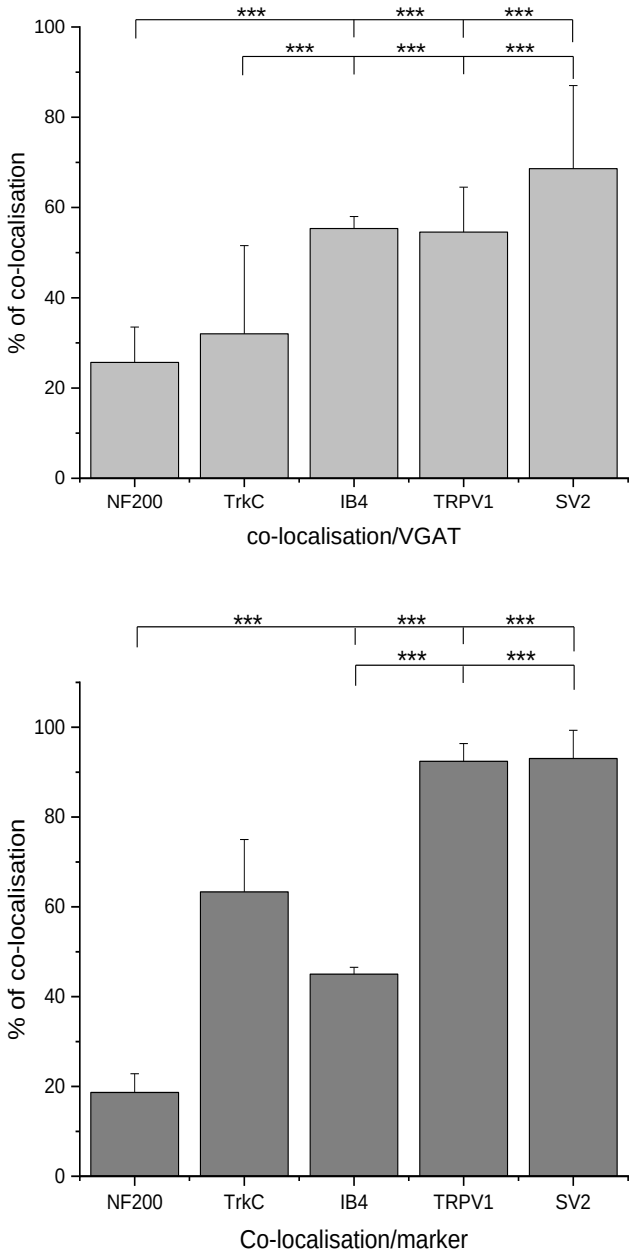


Figure 3.6 Percentage of VGAT co-localisation with NF200, trkC, IB4, TRPV1 and SV2. (A) Percentage of co-localisation when VGAT was used as the denominator and **(B)** neuronal markers were the denominators. VGAT was co-localised with NF200 and trkC (markers for large neurons), IB4 and TRPV1 (markers for small neurons) and SV2 (a marker for synaptic vesicles). Asterisks indicate statistical significance ($P<0.001$ as determined by Fisher exact test with Bonferroni correction) of percentage co-localisation among the five neuronal markers. The percentage of co-localisation was analysed with two different denominators: the total number of VGAT-positive neurons or the total number of marker-positive neurons. Data were obtained from analysis of neurons from three DRG tissue sections (1 section per rat, N=3), for each neuronal marker investigated. Percentage of co-localisation of VGAT with each of the neuronal marker determined, means per neuronal marker calculated, shown as mean ± SEM.

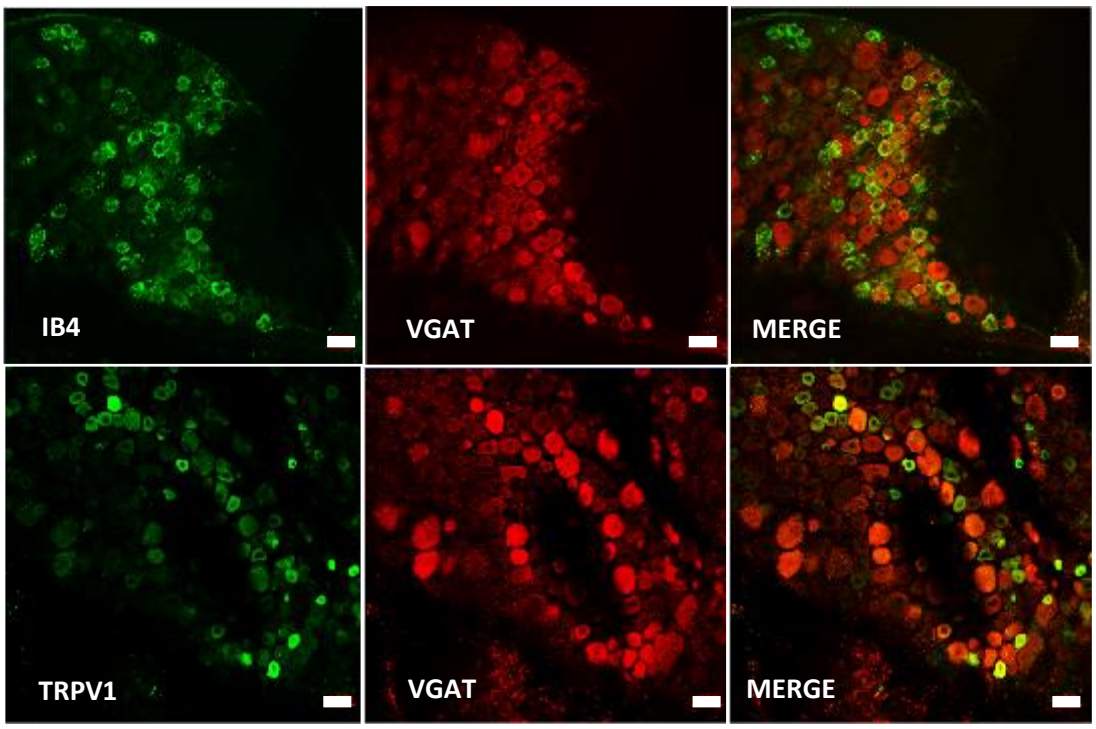


Figure 3.7 Co-localisation of VGAT with a non-peptidergic small neuron marker, IB4 and a nociceptive neuron marker, TRPV1. Scale bar 50 µm

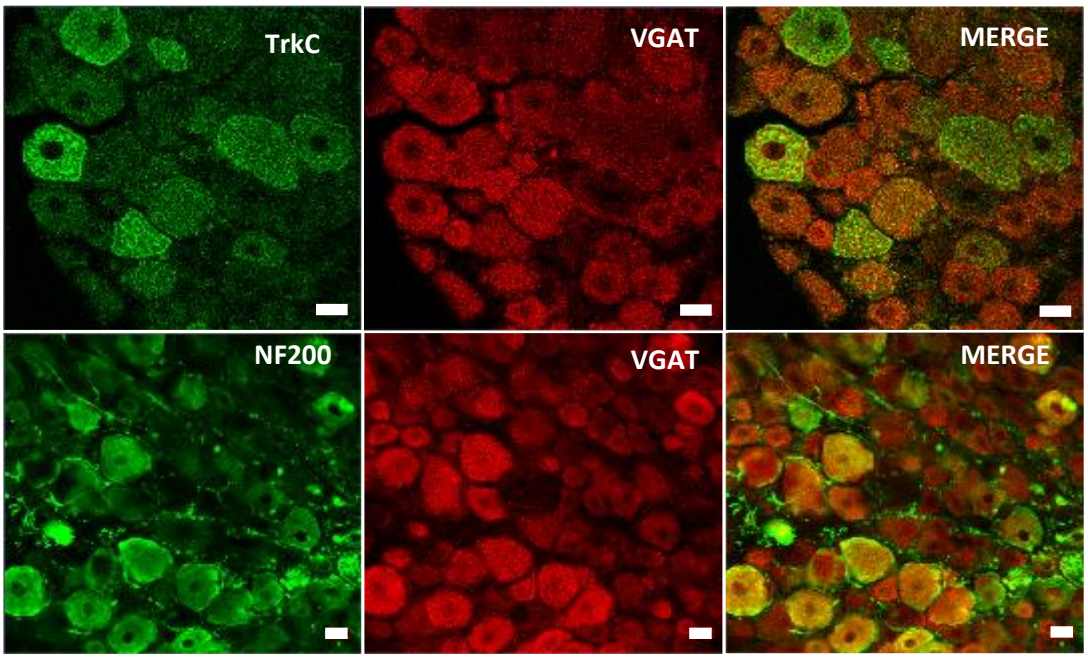


Figure 3.8 Co-localisation of VGAT with large neuron markers, NF200 and trkC. Scale bar 20 µm.

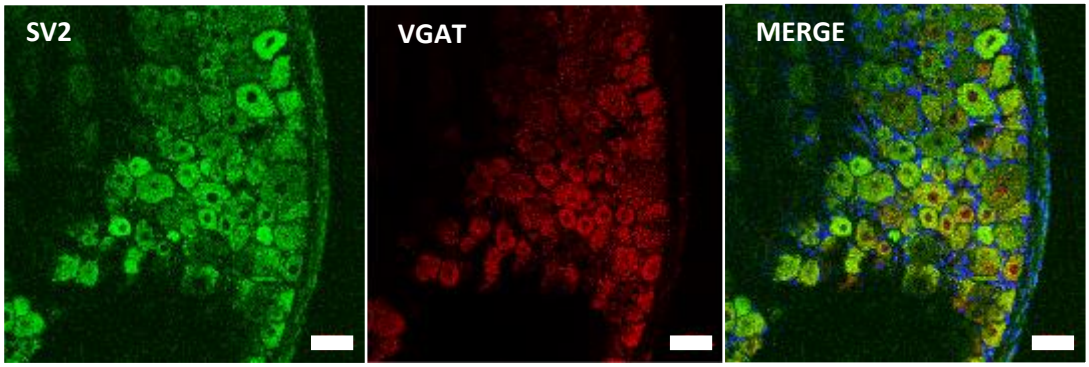


Figure 3.9 Co-localisation of VGAT with synaptic vesicle marker (SV2). Scale bar 50 µm.

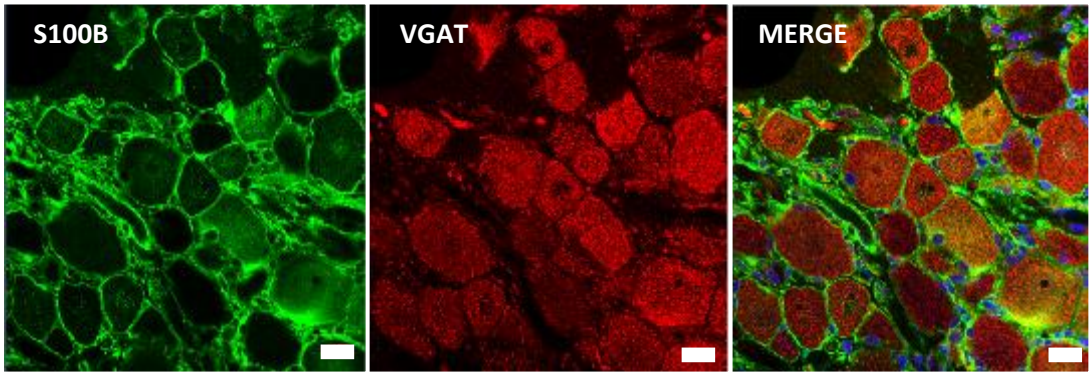


Figure 3.10 Co-localisation of VGAT with a glial marker, S100B. Scale bar 20 µm.

	Small (<32 µm)	Medium (32–40 µm)	Large (>40 µm)
NF200	26.53	44.90	28.57
TrkC	27.27	27.27	45.45
IB4	82.25	11.24	6.51
TRPV1	71.54	18.46	10

Table 7 Distribution of NF200-, trkC-, IB4- and TRPV1-positive neurons according to small-medium-large cell size category. TrkC are expressed mainly in large neurons. IB4 and TRPV1 are expressed in small neurons. Data were obtained from analysis of neurons from three DRG tissue sections (1 section per rat, N=3) for each of the neuronal marker studied. Numbers of neuronal marker-positive neurons for each small, medium and large neurons were summed over the total number of neurochemical marker-positive neurons in all sections to get the percentage of their distributions in different sizes of neurons.

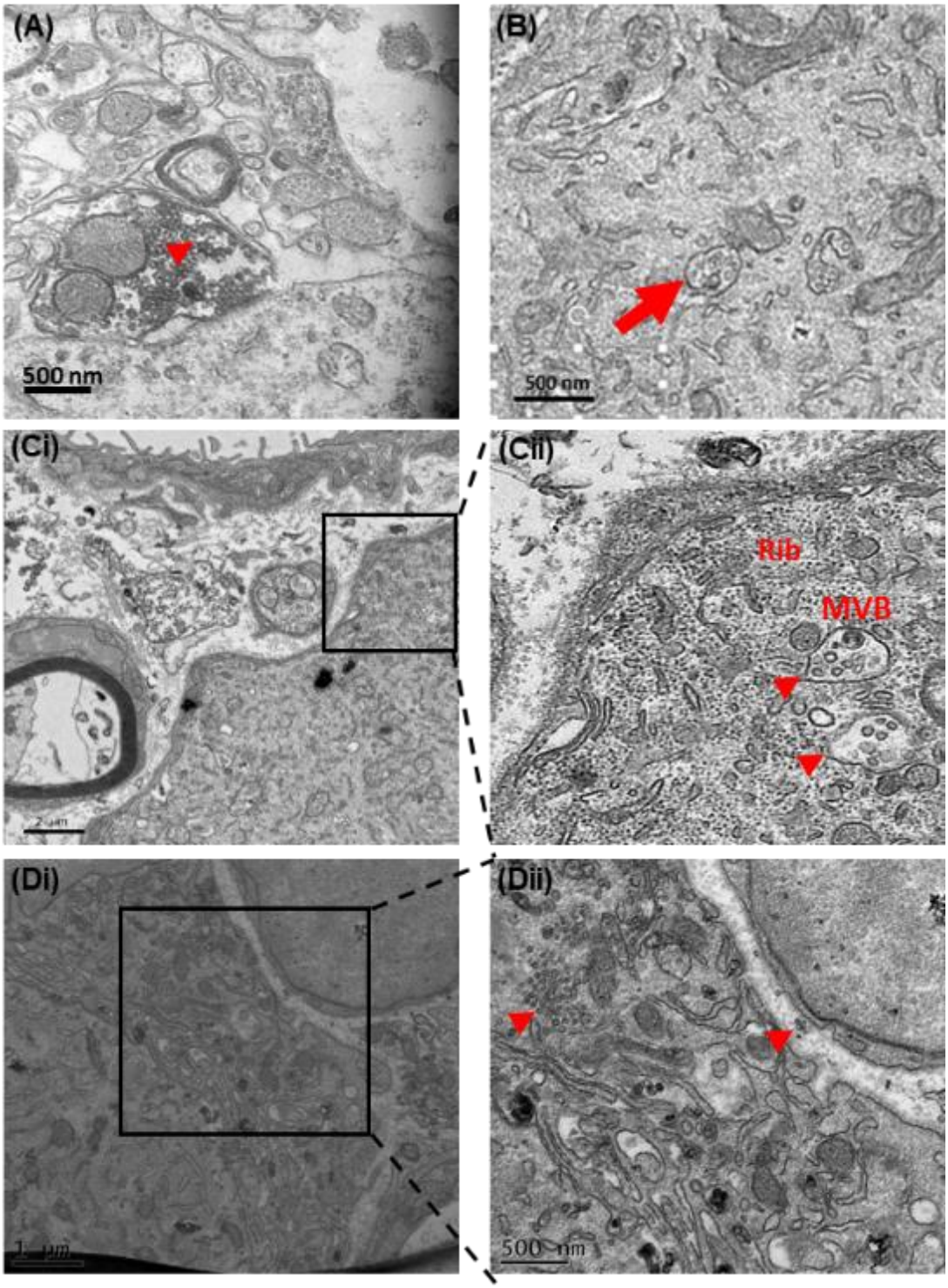


Figure 3.11 Electron microscopy of VGAT expression in rat DRG neurons stained with DAB. (A) VGAT expression in spinal cord (positive control); **(B)** DAB staining without VGAT antibody (negative control) in DRG neurons, **(Cii)** VGAT expression in DRG (magnified view from **(Ci)**), **(Dii)** VGAT expression in SGC and in exosome-like structure within the extracellular space (magnified view from **(Di)**). VGAT-positive labelling is shown with red arrowheads. Vesicles with no VGAT staining are shown with a red arrow. Rib: ribosomes; MVB: multivesicular bodies.

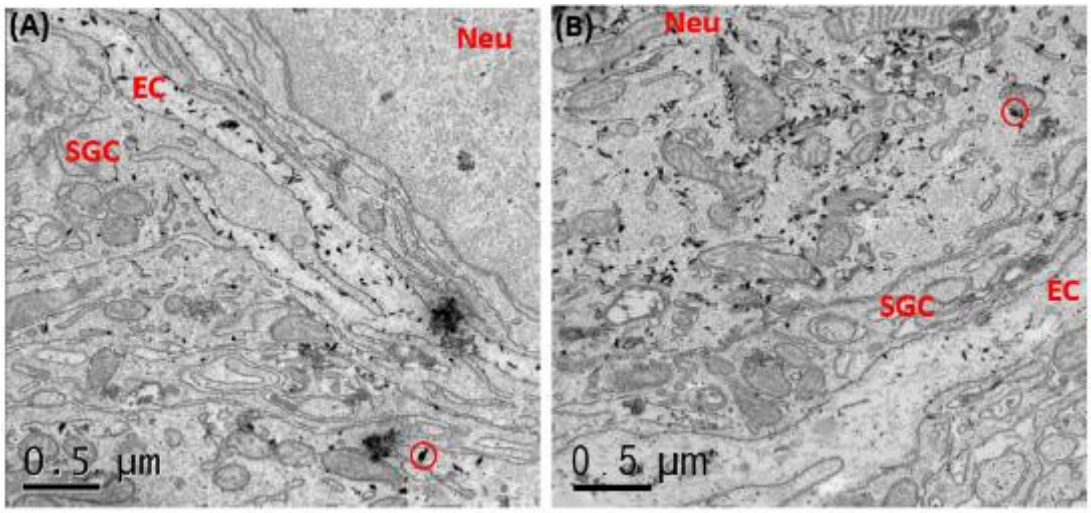


Figure 3.12 Electron microscopy of VGAT expression in rat DRG neurons stained with FNG. VGAT immunoreactivity in SGC and extracellular space **(A)** and DRG neuron **(B)**. Red circles in **(A)** and **(B)** indicate examples of larger gold particles formed by clusters of neighbouring gold grains.

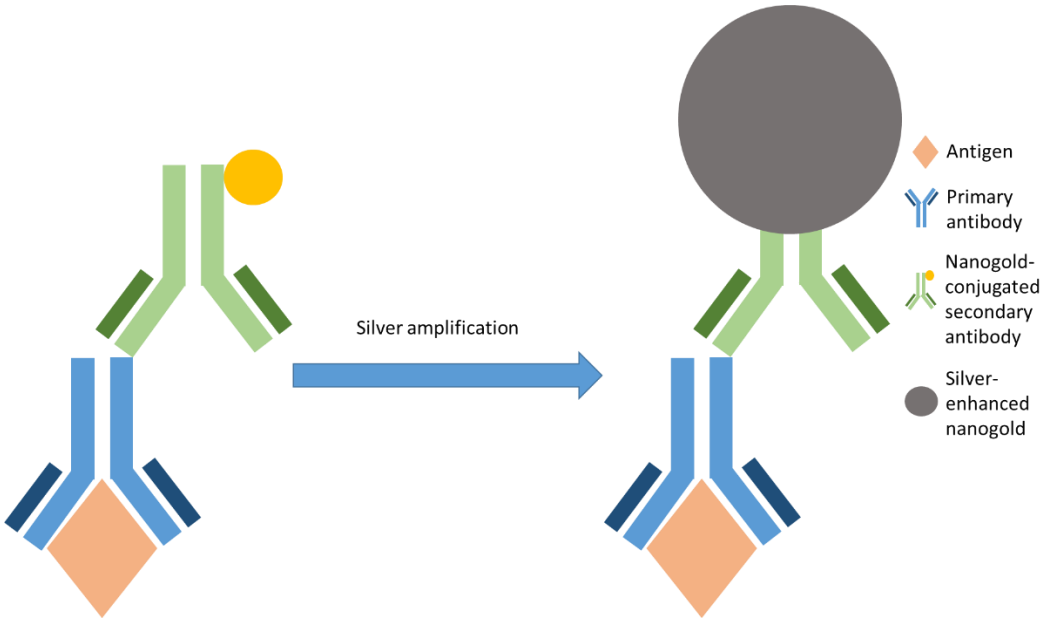


Figure 3.13 Typical immunogold silver enhancement-based immunolabelling. [Based on concept reported in (Liu et al., 2010)].

3.3 Discussion

3.3.1 DRG quantification

DRG house heterogeneous populations of somatosensory neurons. As mentioned earlier, there are many different classifications of somatosensory neurons including classification by size. In this study, the method for quantification of DRG neurons (cervical – lumbar) of the adult male rats were adopted from the classification method used by Ramachandra and colleagues in their study on the expression of $Na_V1.8$ channels in sensory neurons of adult male rats (Ramachandra et al., 2013). Similar to the data presented here, Ramachandra and colleagues categorised the cell body diameter into small (<32 µm), medium (32 – 40 µm diameter), and large neurons (>40 µm). Although the boundaries of this classification are right shifted compared to the earlier DRG morphology studies by Lawson and colleagues, who used DRG neurons from female adult rats (Lawson et al., 1984, Lawson et al., 1993), this classification is strongly supported by Deshmukh and colleagues who determined the classification of DRG neuron diameter using peripherin, a marker for small and medium neurons (Deshmukh et al., 2016). Similar to the present study, Deshmukh and colleagues also measured the entire DRG neurons of the adult male rats. They found that the maximum diameter of neurons which were peripherin-positive was 40 µm. Thus in this present study, neurons with diameter >40 µm were considered as large neurons. According to the classification by Deshmukh and colleagues, the small neurons are suggestive of unmyelinated C fibre neurons while the medium and large neurons are the myelinated Aδ and Aβ fibres, respectively. The difference of classification by Lawson and colleagues with the present study could be due to the different sex of animal used, Lawson and colleagues used female while the present study used male rats which morphologically can have a significantly bigger DRG neurons compared to females (Shen et al., 2006).

The results from the small, medium and large neurons were also consistent with the classification used for the neuronal diameter classification in the present study. Within

the population of IB4-positive neurons, >80% of neurons were small neurons. Similarly, the majority of TRPV1-positive neurons (~70%) were also small neurons. However, a small proportion of TRPV1-positive neurons were medium neurons which is in agreement with other literature (Kobayashi et al., 2005, Story et al., 2003). The percentages of NF200- and trkC-positive neurons which were mainly expressed in medium and large neurons, also correspond to this classification. The majority of NF200- and trkC-positive neurons were of the medium and large neurons (**Table 7**).

Results from the neuron quantification showed that the highest proportion of the DRG neurons were of the small-sized (51.8% and 43% for 'method (i) all neurons' and 'method (ii) neurons with only visible nuclei' respectively). Albeit a slight underestimation, this result is in agreement with other studies reporting that the most abundant type of DRG neurons are the small neurons which constitute more than 50-60% of the total number of primary afferent neurons (Deshmukh et al., 2016, Jardí et al., 2014)**.** The lesser proportion of small DRG neurons found in this present study compared with those from literature could be due to the methods for DRG neurons quantification used; method (ii) considered only neurons with visible nuclei (which limits the number of neurons counted). Also, inconsistent distribution of different sizes of DRG neurons was sometimes observed in different DRG sections during this study, which could cause an underestimation of the total number of small neurons quantified.

3.3.2 VGAT expression in DRG neurons

In this thesis, the size distribution of DRG neurons, VGAT distribution in DRG neurons and the co-localisation of VGAT with small and large neuronal markers as well as with SV2 are broadly consistent with these presented in our previous work (Du et al., 2017), albeit with some quantitative differences. The proportion of VGAT-positive neurons was higher in method (ii) than that of method (i). In method (i), regardless of the nuclei visibility, all neurons were counted for analysis which

reflected the higher total number of neurons in the whole DRG population. Also, VGAT labelling in method (i) was done via visual assessment compared to method (ii) which used mean fluorescence intensity. Visual assessment is more subjective and has the tendency to underestimate the VGAT labelling in the DRG neurons. Together, the higher total number of neurons and lower VGAT-positive neurons (identified via visual assessment) have led to lower percentage of VGAT positive neurons in DRG in method (i) compare to method (ii).

Table 8 Reasons for discrepancies in VGAT distribution in DRG neurons between results from 'method (i) all neurons, and 'method (ii) neurons with visible nuclei only'.

	Method (i)	Method (ii)
Method of assessment for VGAT-positive neurons	Visual assessment	Mean fluorescence intensity
Nucleus visibility	All neurons were counted for analysis regardless of the presence or absence of nuclei	Only neurons with visible nuclei were counted for analysis

Reasons for the differences in the results obtained from method (i) than that from method (ii) are presented in **Table 8**. The data presented in method (i) considered all neurons present including the ones without visible nucleus, in the six DRG sections used for the analysis (two sections from each of the three adult rats). Although this method is able to show a more precise number of neurons in the whole DRG, it may not be accurate for the measurement of size distribution of all neurons counted. As neuron somata are of spheroidal shape, and that the nucleus lies at the centre of the cell where the diameter is measured (as here it gives the maximum diameter), the absence of a visible nucleus in a neuron indicates that the section was cut below or above the centre level thus the size could be an underestimation from the true measurement. Thus in this thesis, another method of analysis was performed; only the neurons with visible nuclei were counted and analysed to determine the size distribution of the DRG neurons.

The VGAT distribution in this present study was analysed using two different methods depending on nuclei visibility. Nucleus visibility is an important consideration for determination of neuron size, however as mentioned above, it tends to underestimate the proportion of neurons which are positively stained with VGAT. Thus the following discussion will focus on the results from method (i) (where nuclei visibility was not considered).

In the present study, IHC investigation showed that VGAT was expressed in ~20% of the whole DRG neurons. This figure is somewhat higher than the expression of VGAT detected using the single cell reverse-transcriptase polymerase chain reaction (RT-PCR) (~7-8% of DRG neurons) reported by us earlier (Du et al., 2017). However, due to the non-specific binding of antibody in IHC (which can overestimate VGAT expression) and high false-negative in single-cell RNA analysis (which underestimate VGAT expression), the discrepancy between these two results is fairly justified.

The proportion of VGAT-positive neurons was highest in the large neurons. However, due to the lower proportion of large neurons compared to small and medium neurons (Deshmukh et al., 2016, Jardí et al., 2014), the total number of small, medium and large neurons were comparable and indeed the absolute number of VGAT-positive large-diameter neurons was lower than that of the VGAT-positive neurons of smaller diameters.

In this current study, VGAT expression was found in a subset of DRG somatosensory neurons of male adult rats. VGAT has been reported to have similar affinity towards GABA and glycine (Burger et al., 1991, Chaudhry et al., 1998, Gammelsaeter et al., 2004, Wang et al., 2009) and that these two neurotransmitters are also co-released via exocytosis (Wojcik et al., 2006). Although our current study did not include characterisation of these two neurotransmitters involved in VGAT activity, our previous electrophysiological findings have shown that capsaicin, bradykinin, elevated extracellular K^+- or ATP-induced depolarisation of DRG produced a robust

release of GABA into the extracellular space (Du et al., 2017). These findings suggest that GABA can be produced and released by several subtypes of DRG neurons (Du et al., 2017). Additionally, we and others have shown that the majority of neurons can respond to GABA (Du et al., 2017). These results are also in agreement with Hanack and colleagues who reported that nociceptive DRG neurons can produce GABA and release it from the peripheral terminals (Hanack et al., 2015). In their experiments, GABA was found in a blister obtained from human skin and extracted mouse corneal fluid at a concentration which was sufficient to produce physiological action (in that case – inhibition of TRPV1 sensitisation).

3.3.3 VGAT co-localisation with other neuronal markers

VGAT-positive neurons had a wide distribution amongst IB4-, TRPV1-, trkC- and NF200-positive cells which were the markers for non-peptidergic, capsaicin-sensitive, NT3-sensitive and myelinated fibres, respectively.

We defined small neurons as those having cell bodies with diameters <32 μm (Khasabova et al., 2004, Ramachandra et al., 2013). These are unmyelinated fibres which respond to temperature, tissue damage, chemical irritants and mechanical stimuli (Dubin and Patapoutian, 2010). Small neurons can be further divided into peptidergic (IB4-negative) and non-peptidergic (IB4-positive) neurons. IB4 is a neuronal marker for small, non-peptidergic neurons (Fang et al., 2006). The co-localisation analysis showed that approximately half of the VGAT-positive DRG neurons were also positive for IB4. To my knowledge, no other studies have reported the co-localisation of VGAT with IB4, however similar distribution of IB4-positive neurons has been reported for the whole DRG neuron population (where it was expressed in small- and medium-sized neurons) (Fang et al., 2006, Silverman and Kruger, 1990). In the present study, DRG neurons were not labelled with any of the peptidergic markers. However, considering that VGAT expression was found in all sizes of neurons, and that IB4-negative neurons include those neurons of small-,

medium- and large-sized (Fang et al., 2006, Lawson et al., 1984, Lawson and Waddell, 1991), it stands to reason that the remaining of the VGAT-positive neurons (which include all sizes of neurons) were populated by the IB4-negative neurons.

Both IB4-positive and IB4-negative neurons express TRPV1 receptors with higher expression on the former (Liu et al., 2004). TRPV1, a receptor which responds to noxious heat and capsaicin is known to be expressed in small DRG neuron cell bodies (Aoki et al., 2005). Thus I used both IB4 and TRPV1 antibodies to investigate and determine the distribution of VGAT expression in different subpopulations of primary sensory neurons. The results showed that more than 50% of VGAT was co-localised with both IB4- and TRPV1-positive neurons albeit slightly lower TRPV1 co-localisation than that of IB4-positive neurons. These data suggest that VGAT is widely expressed in non-peptidergic and capsaicin-sensitive small size neurons, supporting the role of VGAT-positive neurons in carrying the specific sensory modalities within the peripheral nociception system.

In this study, NF200 and trkC antibodies were used to label medium to large DRG neurons. Both of these markers showed less than 30% co-localisation with VGAT. NF200 is the marker for myelinated neurons expressed in Aδ, Aβ and Aα responsible for nociception, mechanoreception and proprioception respectively, while trkC receptors are highly expressed in proprioceptors (Kramer et al., 2006).

Together, these results demonstrate a higher co-localisation of VGAT-positive neurons with small-sized neurons and lower co-localisation with larger neurons. These findings suggest a more important role of VGAT-positive neurons in nociception.

3.3.4 Co-localisation of VGAT with glial marker

In the brain, presence of GABA receptors in glia has been well documented. GAD and monoamine oxidase B (MAOB) are among the key enzymes within the glia that are important for GABA synthesis (Yoon and Lee, 2014). Indeed, glia have been

shown to have GABAergic and GABA-ceptive activities (Yoon et al., 2012). Astrocytic GABA release (Barakat and Bordey, 2002, Lee et al., 2010, Yoon et al., 2011) and GABA receptors (Bovolin et al., 1992, Fraser et al., 1995, Riquelme et al., 2002) have been reported in rodents and human; astrocytic GABA receptor activation caused efflux of Cl^- (Hoppe and Kettenmann, 1989, MacVicar et al., 1989) leading to depolarisation (Backus et al., 1988, Hösli et al., 1990, Kettenmann and Schachner, 1985) which was mimicked by muscimol, GABA agonist and blocked by bicuculline, a GABA antagonist (Fraser et al., 1995).

When the glial marker S100B was co-stained with VGAT in DRG neuron somata, no co-localisation of these two antibodies was observed. However, images from EM analysis showed contradicting results. EM labelling performed with DAB staining and FNG showed a potential presence of VGAT in both SGC and more interestingly, in the extracellular space. These contradicting results could be due to the ability of the electron microscope to detect more detailed biological structures and proteins than that can be viewed via confocal microscopy. However, these EM data require further verification with an independent method. For instance, future experiments could test if GABA release from purified population of SGCs can be detected by HPLC.

S100B antibody is one of the antibodies used to label SGC. However, Albuerne and colleagues investigated the expression of S100B in several species including rat and human and found that S100B immunoreactivity was found not only on SGC, but was also clearly seen in all sizes of DRG neurons (Albuerne et al., 1998). In my experiments however, S100B staining was very clearly glial, with minimal presence in neuronal cell bodies.

VGAT is responsible for transporting synthesised GABA into the synaptic vesicle leading to GABA release via exocytosis. Although the investigation regarding the presence of GABA in the SGC was not performed in this study, its presence in DRG had long been reported back in the 1970s (Minchin and Iversen, 1974, Schon and

Kelly, 1974). Thus, the presence of VGAT in SGC from EM data could suggest a potential mechanism of GABA release in the SGC. Yet, further research is required to confirm this finding.

Glial cells have been known to be involved in signalling pathways of many cell types. Interestingly, in DRG, Rozanski and colleagues reported a transglial transmission between the DRG somata, which they referred to as ‘sandwich synapses’ (Rozanski et al., 2013). In a sandwich synapse, a single SGC (which wraps the neuron somata) is ‘sandwiched’ between two neuron somata to form a neuron somata-glial cell-neuron somata trimer and that communication occurs within these two junctions (Rozanski et al., 2013).

Indeed the study of GABAergic signalling in DRG is still lacking the mechanistic detail. However, studies on GABA release from glial cells in the brain have been emerging for the past few decades. Most of the studies reported a reversal of GABA transporters (GAT1/2/3) as the mechanism involved for GABA release in the glial cells of the brain (Barakat and Bordey, 2002, Lee et al., 2011, Wu et al., 2007). Barakat and Bordey studied the Bergmann glial cells of rat cerebellar slices and reported reversal of GAT-1 activity resulting in tonic GABA release into the extracellular space and activation of $GABA_A$ receptors of the same cells (Barakat and Bordey, 2002). The reversal of a transporter as a mechanism of GABA release was also supported by Richerson and Wu in their review on neurotransmitter transporters (Richerson and Wu, 2003). In human astrocytic cell culture, the reversal of the GABA transporter has also been reported by Lee and colleagues involving GAT1, GAT2 and GAT3 (Lee et al., 2011). Another mechanism of tonic GABA release has also been demonstrated by the same group (Lee et al., 2010). This mechanism involves Ca^{2+}-activated anion channel, referred to as Best1; GABA permeates through the plasma membrane via Best1 channels, allowing for tonic inhibition and causing reduction in neuronal excitability, synaptic transmission and motor performance in

mice (Woo et al., 2018). Future experiments are deemed necessary to test how (and if) the SGCs in the DRG also release GABA.

3.3.5 VGAT expression in DRG neurons via electron microscopy

EM is a method which uses electron density to detect protein expression in biological tissues (Harris, 2015). It is a remarkable tool to explore the microstructures of biological and physical properties that are difficult to be visualised via light or even the advanced fluorescence microscopes. Unlike immunofluorescence investigations in which resolution is not able to localise structures smaller than ~20 nm (Huang et al., 2009, Schermelleh et al., 2010), EM is capable of imaging structures up to approximately ~0.5 nm (Nellist et al., 1995).

To investigate the localisation of VGAT in DRG neurons in more detail, DAB staining was performed on rat DRG neurons. Labelling of VGAT by DAB was further confirmed by FNG labelling. Nanogold (NG), the electron-dense particle in FNG is an excellent tool for antibody labelling. With a particle size of ~1.4 nm, it penetrates cells readily, labelling antibodies at a higher density by conjugating the antibodies' Fab fragments rather than the whole IgG molecules. The high labelling density is achieved by the silver enhancement (Takizawa et al., 2015). DAB staining indeed is a specific method of antibody labelling, however the staining is more diffuse, and if left for too long in peroxidase, brown precipitation could form in the solution giving unwanted background staining.

Despite performing FNG labelling to support and confirm DAB staining of VGAT, the sections could not be imaged to the best of quality; the microstructures of DRG neurons could not be distinctly seen (**Figure 3.11**). During tissue fixation, DRG neurons were fixed with 4% PFA without glutaraldehyde. While glutaraldehyde is commonly used for its ability to preserve the proteins well, it interferes with VGAT binding to its epitope, hence fixation was performed only with 4% PFA, a fixative known to preserve antigenicity of proteins (Cheville and Stasko, 2014).

Regardless of the compromised quality of images, results from both DAB staining and FNG labelling showed VGAT immunoreactivity in DRG neurons which were consistent with the immunofluorescence labelling. Surprisingly and yet interestingly, VGAT labelling using both DAB and FNG was seen not only within the DRG neuron cell bodies (where it expectedly localised to vesicle-like cytosolic structures) but also in the extracellular space and within the SGC, a finding which was not seen in fluorescence labelling. Previously, VGAT expression on SGC or extracellular space within DRG has not been reported however, VGAT expression has been shown in astrocytes of the pineal gland of adult rats (Echigo and Moriyama, 2004). In this experiment, Moriyama and Echigo investigated the expression of VIAAT (another term for VGAT) in astrocytes, microglia and pinealocytes. Results from RT-PCR and immunoblotting showed molecular weight of 684bp and 53 kDa, respectively, which correspond to that of VIAAT's. VIAAT expression was also investigated via IHC labelling in astrocytes, microglia and pinealocytes in pineal gland section (Echigo and Moriyama, 2004). VIAAT expression was found in both astrocytes and microglia but not in pinealocytes. VIAAT expression on pineal gland culture also showed similar results, supporting the expression of VIAAT in glial cells. VIAAT was co-expressed with GABA, GFAP (a glial marker) and synapsin I (a marker for synaptic vesicle), and this was also not observed in pinealocytes. These co-expressions support the idea that astrocytes are the site for the vesicular GABA storage in pineal glands (Echigo and Moriyama, 2004).

The presence of multi-vesicular bodies (MVBs) and exosome-like structures observed under EM in the present study also suggests a possible mode of transmission or communication between neurons within the DRG. Exosomes are extracellular nanovesicles released from cells upon internal vesicle fusion with the plasma membrane. The internal vesicles known as the MVBs, are the endosomal precursor for the lysosomal degradation pathway (Klumperman and Raposo, 2014). Exosomes contain the molecular fingerprint of the cell they are released from. When

exosomes are taken up by a distant cell, they could induce a cellular response following the release of their content such as proteins, mRNAs and microRNAs into the intracellular environment (Raposo and Stoorvogel, 2013). Results from EM investigation also suggest the presence of VGAT within the external milieu. Thus, these exosomes could possibly contain VGAT and hypothetically could transport VGAT to a distant neuron. This indeed could be another possible mode of communication between neurons in the DRG.

Despite this inconsistency between IHC and EM in regard to the VGAT presence in satellite glia and the extracellular space, both methods report presence of VGAT in DRG neuron somata, and in SV2-positive vesicles specifically. Hence, the next step was to investigate the mechanism of GABA release from DRG neurons by utilising C terminal VGAT antibody in cultured neurons.

Chapter 4 Mechanism of GABA release from primary sensory neurons

4.1 Introduction

4.1.1 Vesicular GABA transporter and synaptic vesicle

Neurotransmitters are mostly synthesised in the cytoplasm. In particular, the peptide neurotransmitters are synthesised in the endoplasmic reticulum and are transported into synaptic vesicles by vesicular synaptic transporters before synaptic release can take place via a process known as exocytosis (Rizo and Xu, 2015). The VGAT, also known as vesicular inhibitory amino acid transporter (VIAAT), was discovered in 1997 via genetic and morphological studies of *Caenorhabditis elegens* orthologue with a mutant gene *unc-47.* The gene *unc-47* was found to be expressed in GABAergic neurons and protein encoded by *unc-47* co-localizes with synaptic vesicles (McIntire et al., 1997). C-terminus of VGAT folds into the synaptic vesicle lumen, while N-terminus extends into the cytosol (Martens et al., 2008). This discovery of VGAT-C terminus can help to elucidate the mechanism of GABA release via exocytosis during which the VGAT-C epitope is exposed to the extracellular environment, allowing its labelling by the VGAT-C specific antibody.

VGAT level in the somatosensory cortex of rat brain is low during the first week of life, slowly increased in the second week and reaches its adult level during the third week (Minelli et al., 2003). VGAT has been reported to be present in 16% of all synaptic vesicles isolated from rat brain (Takamori et al., 2000). IHC investigation of the rat cerebellum showed the highest VGAT expression in the basket cells followed by the granular cells and the least expression was found in the molecular cell layers (Takamori et al., 2000). Consistently, an interesting investigation on the differential expression of VGAT throughout the development of the mouse auditory forebrain via multimodal profiling approach [RNA seq, in situ hybridisation (ISH) and IHC] also reported similar findings to that of (Minelli et al., 2003): GABA expression during postnatal period increased towards adulthood. Hacketts and colleagues found that

the maturation trajectory of VGAT in the primary auditory cortex area 1 (A1) and medial geniculate body (MGB) was parallel to that of postsynaptic changes such as the inhibitory strength and $GABA_A$ receptor expression; VGAT immunoreactivity was observed to increase from seven days postnatal (P7) and reached a steady-state by P21 (Hackett et al., 2016).

In DRG neurons, VGAT expression investigated via IHC and RT-PCR has also been reported by our group (Du et al., 2017). VGAT has been shown to co-localise with subpopulations of DRG neurons of all sizes; the high co-localisation of VGAT with markers for small neurons (TRPV1 and IB4) suggests the involvement of VGAT in the peripheral GABAergic signalling pathway.

4.1.2 The life cycle of a synaptic vesicle

The nature of synaptic vesicles varies for different types of transmitters. Small-molecule neurotransmitters such as acetylcholine and amino acid transmitters such as GABA are packaged in small clear vesicles, 40 - 60 nm in diameter. The centre of these vesicles appears clear in electron micrograph, hence the name clear vesicle. Neuropeptides are packaged in larger vesicles, with a diameter of 90 – 250 nm. These vesicles are electron-dense in the electron micrograph, thus they are referred to as the large dense-core vesicles. Biogenic amines are also packaged in dense-core vesicles, but the vesicle size can be either small (40 – 60 nm) or irregularly-shaped and larger (60 -120 nm), depending on the type of a neuron (Purves et al., 2001). Regardless of its classification, synaptic vesicle functions are to take up and release neurotransmitters. For this reason, synaptic vesicles are usually present in largest densities at synaptic terminals. Action potentials arriving at a nerve terminal triggers release of synaptic vesicle contents via exocytosis. During action potential, depolarisation of the synaptic membrane opens the voltage-gated Ca^{2+} channels causing Ca^{2+} transient and exocytosis of the GABA-containing synaptic vesicles. Vesicular release is usually stimulated by Ca^{2+} via P/Q-($Ca_V2.1$) or

N-type Ca^{2+} channels and rarely via the $Ca_v2.3$ or R-type Ca^{2+} channels (Dietrich et al., 2003). In order for vesicular release to occur, synaptic vesicles cluster at the active zone, an event also known as docking. Subsequently, the synaptic vesicles undergo priming where the assembly of the fusion machine occurs. The fusion machines are composed of a synaptic vesicular protein synaptobrevin, and two of the plasma membrane proteins, the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNARE) complex and Munc18 (Hata et al., 1993). Following this fusion, Ca^{2+} binds to synaptotagmin (Syt), a Ca^{2+} sensor expressed on synaptic vesicle involved in neurotransmitter release. Ca^{2+} binding to Syt C2 domain triggers a fast simultaneous interaction of Syt with SNARE complex resulting in fusion pore opening (Südhof and Rothman, 2009).

Despite a complex molecular machine involved in the neurotransmitter vesicular release, it takes only ~50 µs following Ca^{2+} transient, for phasic synchronous neurotransmitter release to occur (Sabatini and Regehr, 1996) while a slower asynchronous release can last more than 1 sec (Atluri and Regehr, 1998). After exocytosis, the vesicles are endocytosed, recycled before undergoing exocytosis again. Vesicles can be recycled via three possible pathways; i) kiss and run, ii) kiss and stay, and iii) endosomal recycling (Südhof, 2004). “Kiss and run” and “kiss and stay” are fast pathways while the endosomal recycling, which is a clathrin-mediated endocytosis, is slower. **Figure 4.1** and **Figure 4.2** show the schematics of the synaptic vesicle release mechanism and synaptic vesicle recycle pathways, respectively.

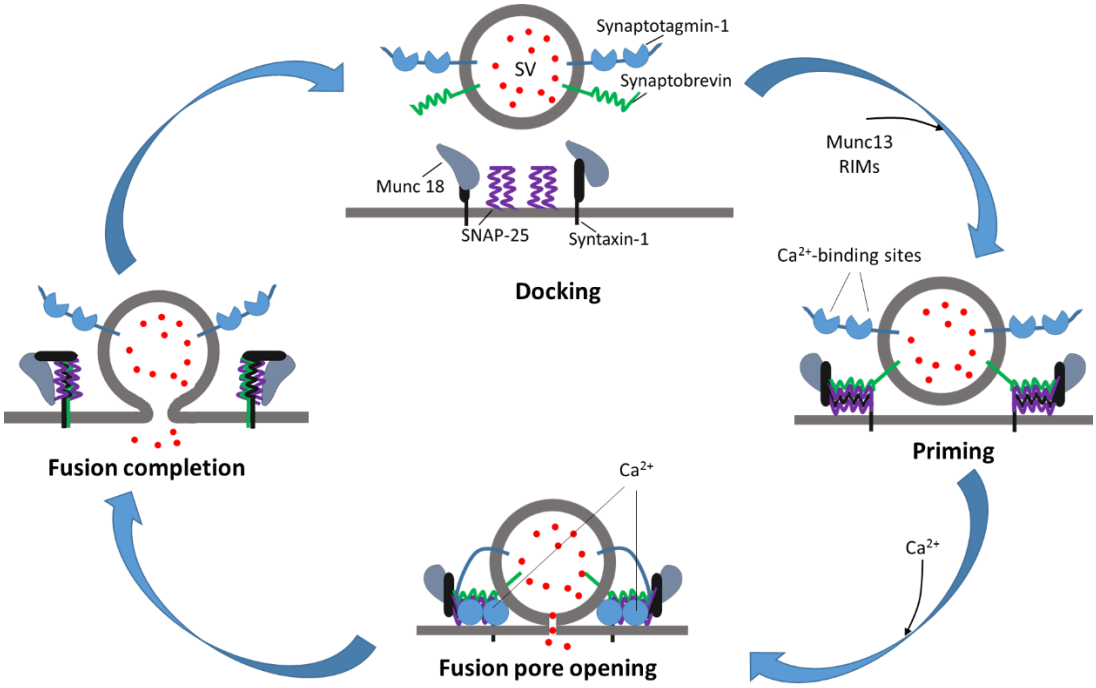


Figure 4.1 Neurotransmitter vesicular release mechanism. During docking, Synaptic vesicle (SV) is recruited to the active zone by RIMs protein and Munc 13. Following this, priming of the synaptic vesicle occurs where SNARE proteins (SNAP 25 and syntaxin-1) assemble with synaptobrevin and Munc 18. Ca^{2+} binding to C2 domains of the synaptotagmin 1 (during Ca^{2+} transient) results in binding of synaptotagmin 1 to the SNARE complex assembly. Synaptotagmin is a Ca^{2+} sensor for vesicular release. Upon depolarisation which leads to Ca^{2+} transient, Ca^{2+}-activated synaptotagmin binds to SNARE complex thereby triggering fusion pore opening [based on a concept reported in (Südhof, 2013)].

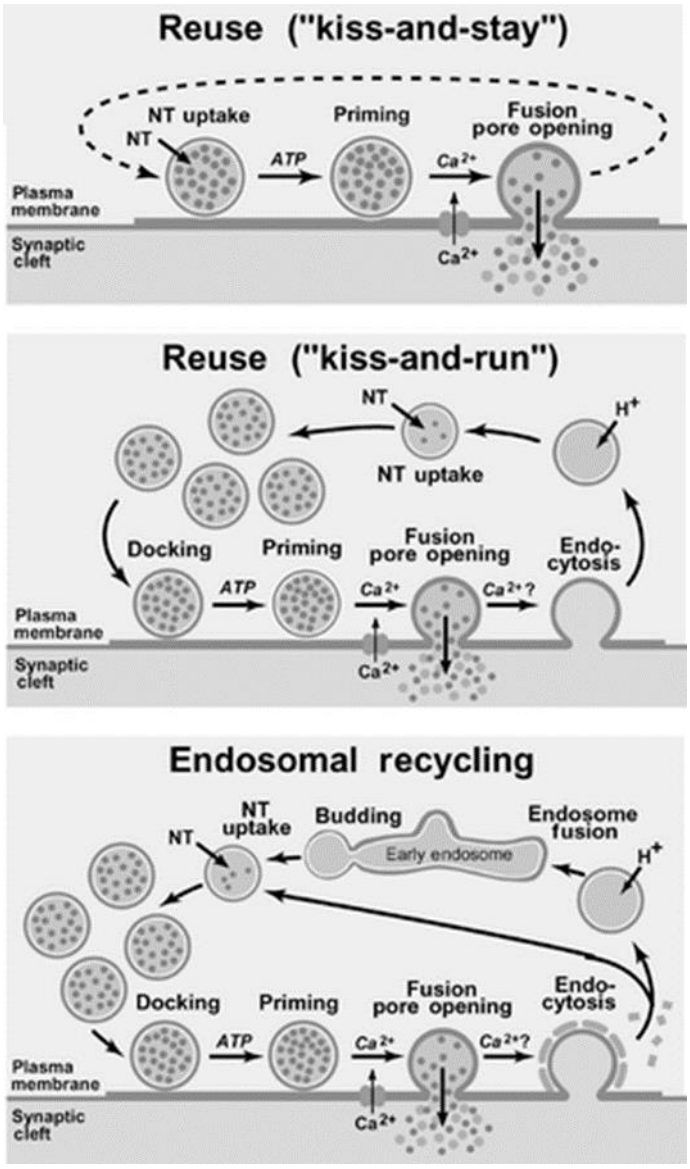


Figure 4.2 Synaptic vesicle recycling pathways. Vesicles can be recycled via three possible pathways; i) kiss and run, ii) kiss and stay, and iii) endosomal recycling. Kiss and run" and "kiss and stay" are fast pathways while the endosomal recycling, which is a clathrin-mediated endocytosis, is slower. (Südhof, 2004).

4.1.3 Mechanism of GABA release

Classically, GABA release occurs upon depolarisation of a presynaptic neuron which allows Ca^{2+} influx via VGCCs. Ca^{2+}-dependent secretion (CDS) is a well-established theory which secretion of neurotransmitters from synaptic vesicles occurs following Ca^{2+} influx through VGCCs. The Ca^{2+} influx in turn, is triggered by the action potentials following membrane depolarisation. In DRG neurons, Huang and Neher demonstrated robust Ca^{2+}-dependent exocytosis of synaptic vesicles upon depolarisation of DRG neuron somata (Huang and Neher, 1996). In their experiments, the involvement of Ca^{2+} in exocytosis was indicated by the increase in the membrane capacitance (*Cm*) (presumably reflecting fusion and exocytosis) with transient rise in intracellular Ca^{2+} in DRG neurons and the reduced exocytosis activity in Ca^{2+}-depleted conditions (Huang and Neher, 1996). Interestingly, this CDS theory has been extended by a group of researchers from Zhuan Zhou's laboratory who initially found a new type of action potential-mediated secretion, Ca^{2+}-independent but voltage-dependent-secretion (CiVDS) in primary DRG neurons. In CiVDS, the arrival of action potentials opens the voltage-gated N-type Ca^{2+} channel ($Ca_v2.2$) triggering a conformational change of $Ca_v2.2$-SNARE complex which in turn promotes CiVDS-vesicle fusion and ATP release even without a rise in intracellular [Ca^{2+}]. Here, ATP acts as a release cargo of CiVDS as evident by EM imaging and total internal reflection fluorescence imaging (TIRF) (Chai et al., 2017). These findings were supported by the earlier findings from the same group showing that the CiVDS exocytosis, measured by *Cm* recording was readily triggered by a brief membrane depolarisation in the absence of internal and external Ca^{2+} (Zhang and Zhou, 2002). This new mechanism of exocytosis was further confirmed by *Cm* recording, total TIRF and luciferase fluorescence assays; they found that blocking CiVDS using $Ca_v2.2$ specific blocker, ω-conotoxin GVIA inhibited CiVDS which was rescued by overexpression of $Ca_v2.2$ (Chai et al., 2017).

Alternatively, GABA can also be released non-vesicularly via the activity of GABA transporter 1 (GAT1). GABA transporters can be classified into six groups: A1/GAT1, A13/GAT2, A11/GAT3, A12/BGT1, A8/CT1, and A6/TauT (Scimemi, 2014). GAT1, GAT2, and GAT3 (particularly GAT1) are the most extensively characterized GABA transporters, all of these were found in DRG neurons (Du et al., 2017, Novak et al., 2011). Wu and colleagues studied the activity of GAT1 in mediating GABA release using CHO cells and cultured hippocampal neurons and demonstrated that if enough stimulation is given to a neuron, GAT1 activity can be reversed. This reversal occurs during high-frequency firing due to depolarisation and a rise in intracellular [Na^+] within presynaptic terminals. The reversal activity of GAT1 depends on the ambient extracellular [GABA] which determines the driving force of GAT1 (Wu et al., 2007). During reversal of GAT1, GABA flux remained coupled to two Na^+ and one Cl^-, thus the GAT1 reversal potential (GAT1 is at equilibrium) was measured at variable concentrations of Na^+, Cl^- or GABA in whole-cell patch-clamp electrode solutions. The lower the ambient (extracellular) [GABA], the lower the driving force which eventually becomes zero when extracellular [GABA] falls to the level at which GAT1 is at equilibrium (Wu et al., 2007). A fall in ambient GABA concentration below this equilibrium will cause GAT1 to reverse the direction of GABA transport. Instead of transporting GABA back into the cells, GAT1 will release GABA into the synaptic cleft, maintaining tonic postsynaptic inhibition by GABA (Wu et al., 2007).

4.1.4 Mechanisms of intersomatic communication within DRG

Neurons communicate with each other via various methods. The transfer of molecules from one neuron to another is important to ensure a continuous and sustained functional biological system. In the CNS the main type of neuron-to-neuron communication is by means of synapses (both chemical and electrical).

However, recent studies have suggested the possible mechanisms of how these neurons communicate with each other within the ganglia. In 2011 Braz, Ackerman and Basbaum used a neuronal tracer wheat germ agglutinin (WGA) to investigate the cell-cell communication within DRG *in vivo* and revealed that the WGA tracer from injured neurons was found on the neighbouring uninjured neurons. This tracer was also found in the SGC (Bráz et al., 2011). Although there were no experiments done to support the mechanism of the transfer, they also proposed that this transfer occurred via exocytosis and endocytosis of the injured and the non-injured neurons respectively. In another study, Rozanski, Li and Stanley reported similar cell-cell communication within DRG albeit *in vitro* (Rozanski et al., 2013). These authors showed that there were communications took place between two neuron somata mediated by SGC capsule wrapping each neuron. This phenomenon was referred to as a sandwich synapse. Together, these studies revealed that primary sensory neurons are not an anatomically isolated structure as previously assumed.

The ultrastructural investigation has demonstrated no detectable synapses in healthy DRG neuron (Zenker and Högl, 1976). Nonetheless, a recent study has reported the presence of an interesting feature in DRG neurons; sprouting neurites of IB4-, CGRP- and Kv4.3-positive nociceptors surrounding large DRG somata together with the sprouting neurites of tyrosine hydroxylase-positive neurons (a marker for sympathetic axons) in spinal nerve injury rat model (Cheng et al., 2015). These sprouting axons were positive for synaptic proteins' markers, PSD95 and synaptophysin suggesting established synapse-like structures formed during spinal nerve injury (Cheng et al., 2015). The sprouting axons, also known as Dogiel's nest first described in the

nineteenth century, are endings of sympathetic axons that resemble a nest enveloping individual DRG neurons (Garcia-Poblete et al., 2003). The axons with Dogiel's nest feature are positive for tyrosine hydroxylase; a marker used to label sympathetic axons and subpopulation of small DRG neurons (Brumovsky et al., 2006). Earlier studies support that sympathetic fibre only form nests around DRG neurons during conditions of neuropathic pain (Cheng et al., 2015, Chien et al., 2005, McLachlan et al., 1993, Xie et al., 2011). Sympathetic fibres are likely to enhance DRG neurons' excitability and drive DRG neurons spontaneous activity, a hallmark of neuropathic pain.

Another interesting finding on neuron-to-neuron communication within DRG was also reported by (Kim et al., 2016). Using imaging technique which allowed recordings of >1600 neurons/DRG, these researchers discovered a striking neuronal coupling phenomenon between adjacent neurons by means of gap junction following inflammation or nerve injury. This coupled activation involved both small-diameter nociceptive and large-diameter low-threshold mechanoreceptors (LTMRs). By blocking the gap junctions, there were reductions in neuronal coupling activation and mechanical hyperalgesia induced by complete Freund adjuvant (CFA) injection (Kim et al., 2016).

Previous findings from our lab showed that the inhibitory neurotransmitter GABA played an important role in controlling the peripheral nociceptive transmission (Du et al., 2017). Our previous behavioural studies clearly showed that GABA applied locally at the level of DRG attenuated pain stimuli in both acute as well as chronic constriction injury neuron which were not seen in control groups when GABA was replaced with saline (Du et al., 2017). Similarly, stimulation of GABA release in DRG via optogenetic and chemogenetic approaches also reduces pain transduction in transgenic mice (Du et al., 2017). Thus, our previous experiments have established that 1) GABA is released within the DRG in response to stimulation; and 2) GABA applied exogenously produces an analgesic effect. However, the mechanism of how

GABA released from one neuron gets across to another neuron, which responds to its release still remains elusive. Here, we used antibodies accessing either cytosolic (N-terminal) or vesicular (C-terminal) VGAT epitopes, to investigate further the possible mechanisms of GABAergic transmission in the peripheral primary sensory neurons.

As my IHC finding shows a high co-localisation of VGAT (N-terminal) antibody with a synaptic vesicular marker (SV2) in DRG neurons, synaptic vesicles could play a role in the storage and release of GABA into the intersomatic junction in the DRG. Thus I hypothesized that in DRG neurons, synaptic vesicular-like exocytosis could be at least, one of the mechanisms of GABA release.

To test this hypothesis, I utilised the vesicular domain of VGAT (VGAT-C) epitope located on the luminal side of the synaptic vesicle membrane. We incubated live DRG neuron cultures with VGAT-C antibody and depolarised them using a high concentration of extracellular KCl to induce membrane depolarisation that would lead to synaptic vesicle exocytosis. During synaptic vesicle exocytosis, the VGAT-C epitope would be exposed to the extracellular environment while the VGAT-N epitope would still be enclosed within the neuronal cytoplasm (**Figure 4.3**). Thus, exocytosis would allow VGAT-C antibody uptake while keeping VGAT-N antibody away from being able to bind to the cytosolic part of the VGAT. The subsequent labelling of VGAT-C antibody with fluorescence marker enabled its visualisation via fluorescence microscopy.

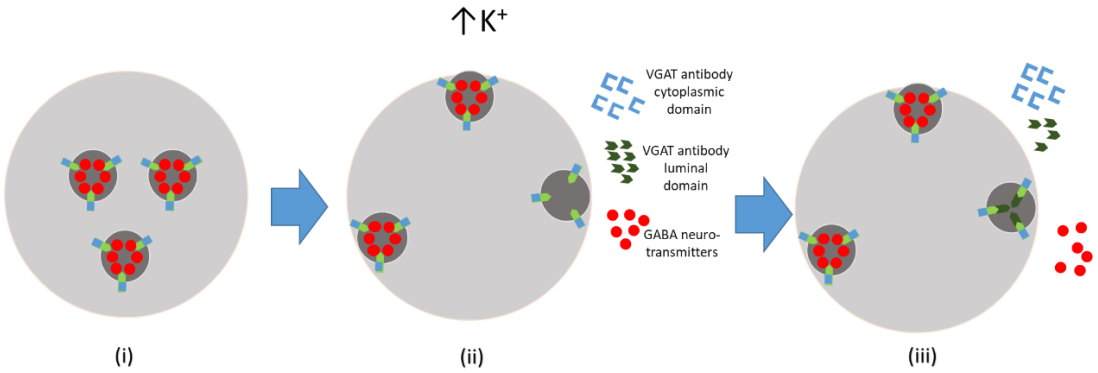


Figure 4.3 Schematic illustration for VGAT-C antibody uptake by DRG neuron during exocytosis. Live DRG neuron cultures were incubated with VGAT-C antibody and depolarised using a high concentration of extracellular KCl to induce membrane depolarisation that led to synaptic vesicle exocytosis (ii). During synaptic vesicle exocytosis, the VGAT-C epitopes were exposed to the extracellular environment while the VGAT-N epitopes were still be enclosed within the neuronal cytoplasm. Exocytosis allowed VGAT-C antibody uptake while keeping VGAT-N antibody away from being able to bind to the cytosolic part of the VGAT (iii).

4.2 Results

4.2.1 Optimisation of VGAT-C and KCl concentrations for incubation of live DRG neurons.

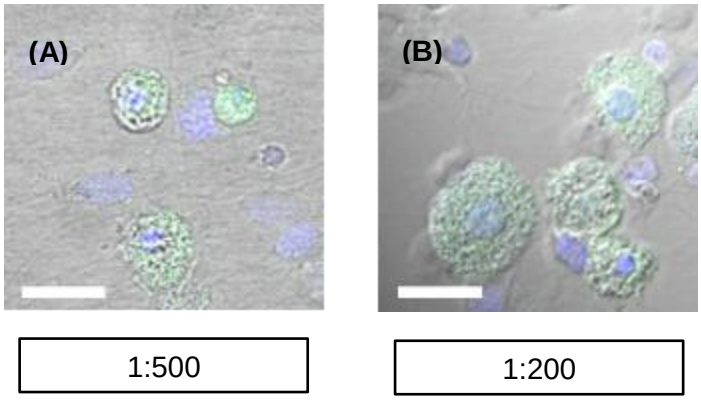


Figure 4.4 Optimisation of VGAT-C antibody concentration used for labelling live DRG neurons incubated in 100 mM KCl. **(A)** VGAT-C antibody uptake by live DRG neurons incubated with 1:500 VGAT-C antibody showed less marked immunofluorescence of DRG neurons compared to **(B)** VGAT-C antibody concentration 1:200. Scale bar 20 µm

Before performing VGAT-C antibody uptake experiment, the working dilution for VGAT-C antibody was determined. The DRG neuron cultures were incubated using 1:500 and 1:200 dilutions. Dilution 1:200 was used by Marten in their experiment on cortical GABAergic synapses (Martens et al., 2008). Indeed, clearer cytoplasmic staining of VGAT-C was seen using the dilution 1:200 than that using 1:500 (**Figure 4.4**), thus the following experiments were performed using 1:200 dilution.

4.2.2 Depolarization-induced uptake of luminal VGAT antibody (VGAT-C) by live DRG neurons

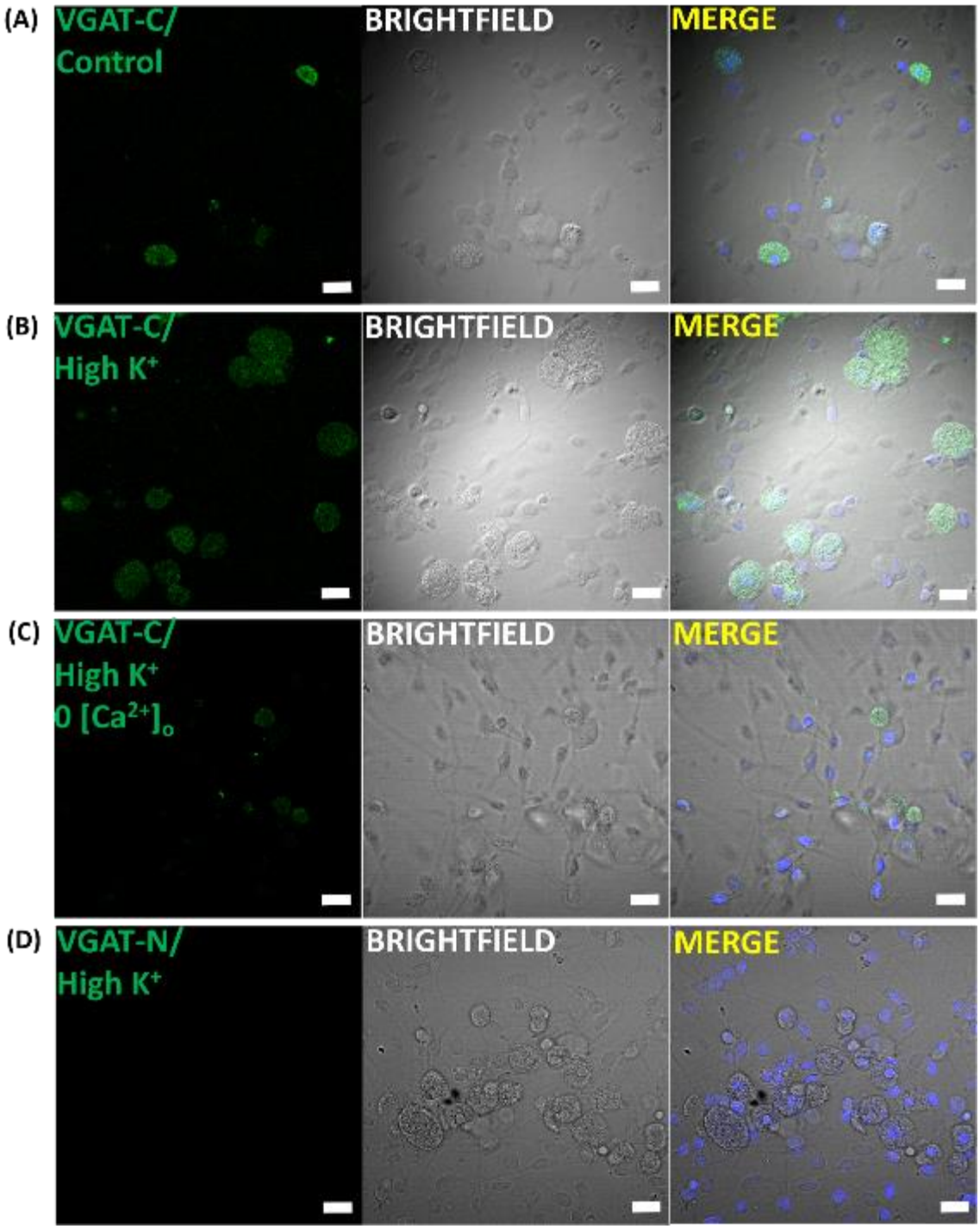


Figure 4.5 Uptake of VGAT-C and VGAT-N antibodies by live DRG neurons in culture. (A) Tonic VGAT-C antibody uptake by non-depolarised neurons; **(B)** VGAT-C antibody uptake via high K^+ (KCl) (100mM)-induced exocytosis. Incorporated antibodies were visualised after fixation and permeabilisation by indirect immunofluorescence; **(C)** VGAT-C antibody uptake by neurons stimulated with high KCl in Ca^{2+}-free extracellular solution and **(D)** absence of VGAT-N (cytoplasmic terminal) antibody uptake by depolarised DRG neurons**.** Scale bar 20 µm.

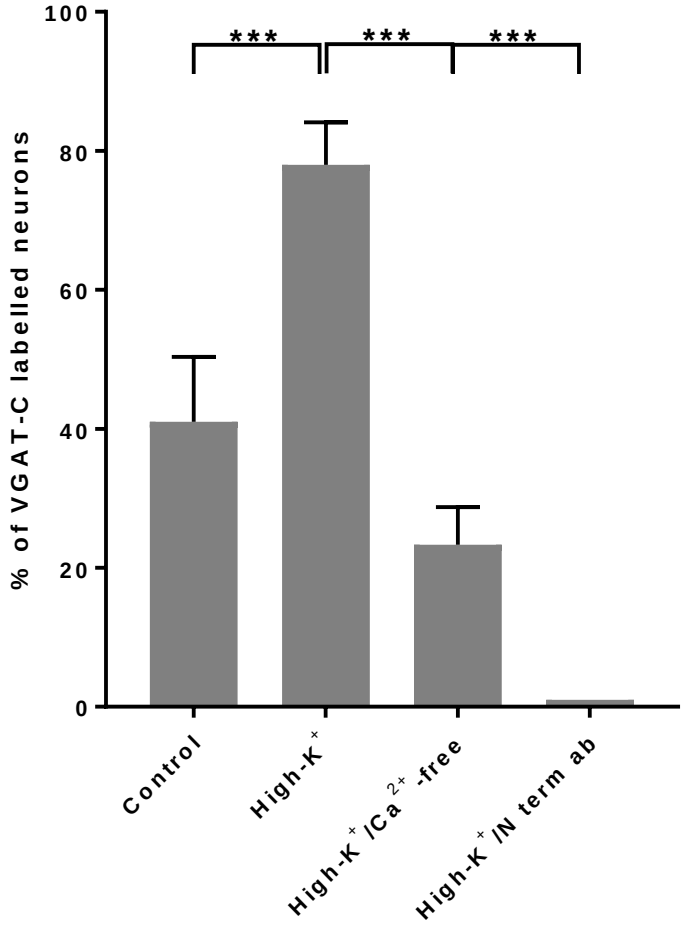


Figure 4.6 Proportion of VGAT-C-positive DRG neurons in VGAT-C antibody uptake experiment. The proportion of VGAT-C positive neurons were determined for each rat, means per rat were calculated and data presented as mean ± SEM. One way ANOVA with Bonferroni correction shows a significant difference in the proportion of VGAT-C positive neurons between the depolarized (High-K^+) and any of the other groups– the non-depolarized (control), depolarized in the absence of extracellular Ca^{2+} (High-K^+/Ca^{2+}-free) and the High-K^+/N term ab ($P<0.0001$). N=3, n(Control)=98, n(High-K^+)=170, n(High-K^+/Ca^{2+}-free)=107, and n(High-K^+/N term ab)=181.

Figure 4.5 shows the immunoreactivity of VGAT-C antibody in DRG neurons incubated in three different conditions: high KCl extracellular solution, normal extracellular solution, and high KCl solution in which extracellular Ca^{2+} has been excluded. We also incubated DRG neurons with the VGAT-N antibody in high KCl solution as a negative control group. The cultures were then fixed and subjected to secondary antibody staining (Alexaflour 488; see Methods). Almost 80% of DRG neurons incubated with VGAT-C antibody in high KCl extracellular solution showed VGAT-C antibody immunoreactivity (**Figure 4.5 B**). Meanwhile DRG neurons incubated in both normal extracellular solution and high KCl in Ca^{2+}-free extracellular solution showed a much lower proportion of VGAT-C antibody positive neurons (**Figure 4.5 A** and **C** respectively). Indeed the immunoreactivity of VGAT-C antibody in these two groups was significantly lower compared to high KCl group (41 ± 9% and 23 ± 5% respectively) (**Figure 4.6)**. DRG neurons incubated in anti-VGAT-N antibody did not show any immunoreactivity at all (**Figure 4.5 D**).

4.2.3 GABA controls peripheral nociception at the level of DRG–an *in vivo* study

With help from Mr Hao Han, a PhD student in Xiaona Du's laboratory (Gamper Lab collaborator), I sought to investigate further the GABAergic signalling in DRG by performing an extracellular *in vivo* electrophysiological recording on the spinal nerve and the dorsal root of L5 of adult rats with surgically exposed L5 DRG (**Figure 4.7**). This experiment

Our previous biophysical modelling (Du et al., 2017) and simulations from others (Du et al., 2014, Sundt et al., 2015) suggest that the axonal bifurcation (T-junction) within sensory ganglia influences the transmission of passing spikes where it filters the depolarizing $GABA_A$ currents at the somatic/perisomatic level. This filtering effect results in AP failure to propagate past the T-junction. Thus this experiment hypothesised that GABA released within the DRG contributes to this filtering function and inhibits the propagation of action potential from the periphery (spinal nerve) to the dorsal root. As action potential propagates from the spinal nerve to the dorsal root via DRG, any filtering effect exerted by DRG would be manifested in the difference of neuronal firings between the spinal nerve and the dorsal root. This experiment was designed to also support the phasic and tonic GABA release observed in the VGAT-C antibody internalisation (section 4.2.2). In the presence of tonic GABA activity, the neuron firing would show lower frequency in the spinal nerve than that in the dorsal root. Meanwhile, similar results observed after stimulating the peripheral nerve via intraplantar capsaicin injection would support the phasic GABA release and activity. We recorded the baseline firing of both dorsal root and spinal nerve which act as controls for this experiment. Interestingly, there was a lower nerve firing in the dorsal root than that of spinal nerve even before the injection of capsaicin (which acts as the baseline) (10.21 ± 5.93 and 17.91 ± 7.80 Hz for dorsal root and spinal nerve, respectively) suggesting an inhibitory mechanism that may take place within the DRG even at resting conditions. An injection of 10 µM 50 µl capsaicin (a ligand for TRPV1

receptor whose activation leads to nerve firing) onto the right hindleg increased the neuronal firing rate in the L5 spinal nerve from 17.91 ± 7.80 Hz to 30.12 ± 7.37 Hz (n=6, $p<0.05$) and L5 dorsal root from 10.21 ± 5.93 Hz to 20.38 ± 7.02 Hz (n=6, $p<0.05$) **Figure 4.8 (A)**. The increase in neuronal firing rate of the spinal nerve was slightly higher than that of the dorsal root however they were not significantly different from each other. Local GABA application 200 µM directly onto exposed L5 DRG decreased the frequency of nerve firing of dorsal root fibres from 20.38 ± 7.02 Hz to 10.61 ± 3.59 Hz (n=6, $p<0.05$) **Figure 4.8 (A)**. Strikingly, there was no decrease of nerve firing on the spinal nerve from 30.12 ± 7.37 Hz to 31.49 ± 7.20 Hz (n=6). This result strongly suggests that DRG-applied GABA acts specifically by inhibiting propagation of action potential through the ganglion.

Consistently, when $GABA_A$ receptor antagonist, bicuculline 200 µM was applied locally onto L5 DRG, the firing frequency increased significantly in the dorsal root from 12.89 ± 7.2 Hz to 24.13 ± 9.84 Hz (n=7, $p<0.05$). Similar to the local application of GABA, local application of bicuculline caused no significant changes in spinal nerve firing frequency from 27.77 ± 9.17 Hz to 30.71 ± 10.60 Hz (n=7, $p<0.05$). **Figure 4.8 (B)**. Taken together, these experiments established that i) tonic activity is present in the L5 (sciatic nerve) even in the absence of the peripheral stimulation; ii) this tonic activity is filtered at the DRG; and iii) this activity can be increased and decreased via $GABA_A$ receptor stimulation and inhibition, respectively.

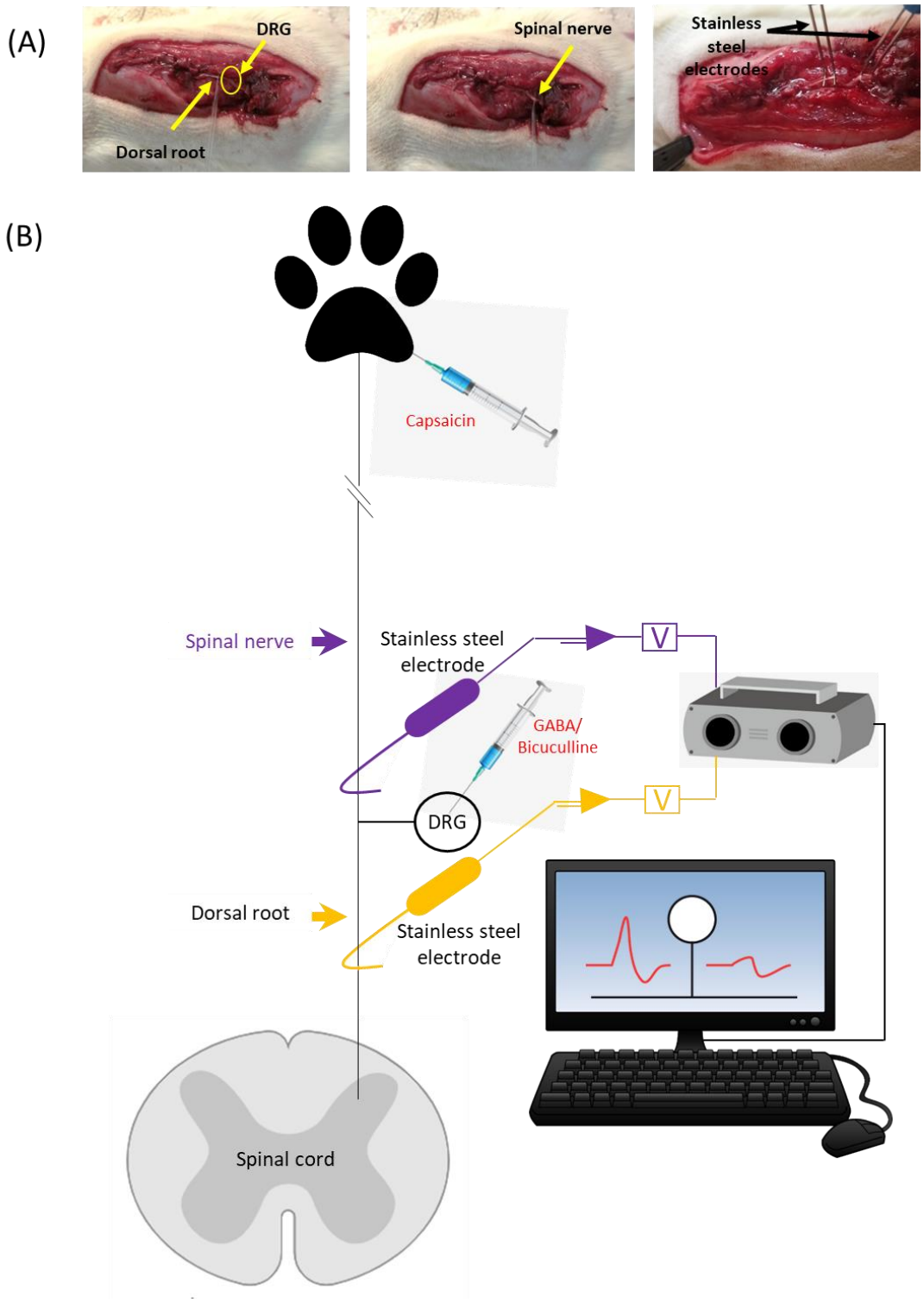
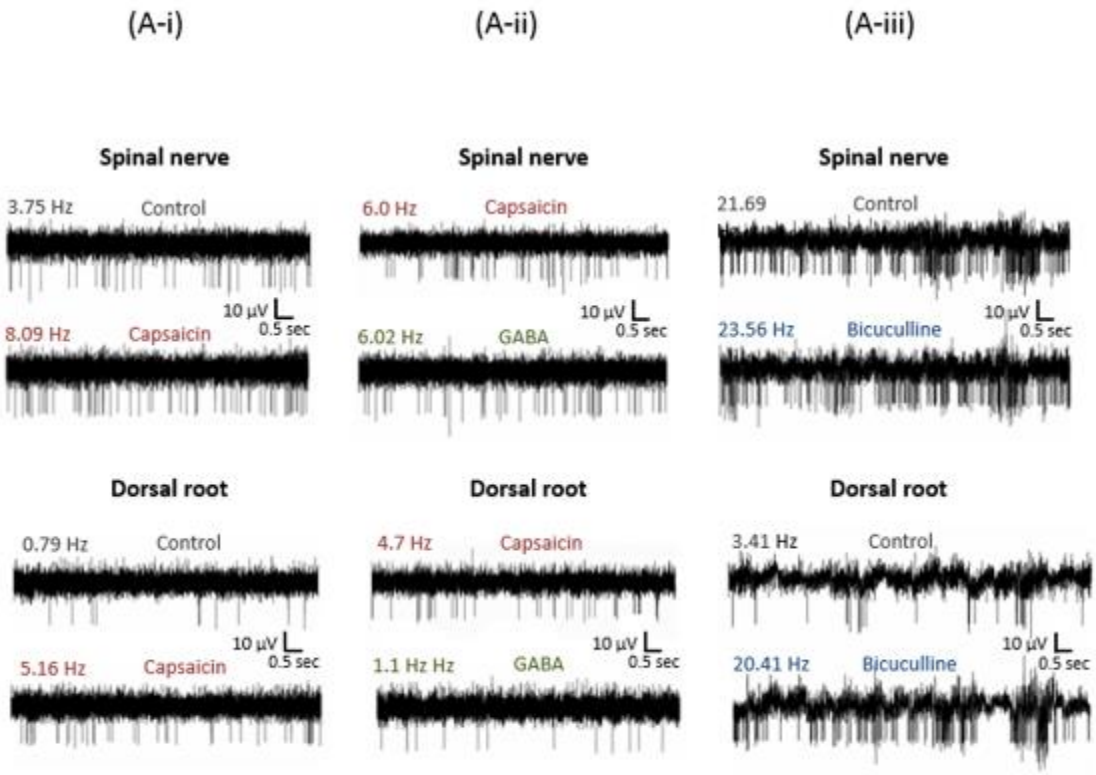


Figure 4.7 Surgical exposure of the L5 spinal nerve (left), L5 DRG and the dorsal root (right) in an anaesthetized rat (A). Schematic of the electrode placement on dorsal root and spinal nerve (B).


(A-i)
(A-ii)
(A-iii)
Spinal nerve
3.75 Hz
Control
10 μV
0.5 sec
8.09 Hz
Capsaicin
Spinal nerve
6.0 Hz
Capsaicin
10 μV
0.5 sec
6.02 Hz
GABA
Spinal nerve
21.69
Control
10 μV
0.5 sec
23.56 Hz
Bicuculline
Dorsal root
0.79 Hz
Control
10 μV
0.5 sec
5.16 Hz
Capsaicin
Dorsal root
4.7 Hz
Capsaicin
10 μV
0.5 sec
1.1 Hz Hz
GABA
Dorsal root
3.41 Hz
Control
10 μV
0.5 sec
20.41 Hz
Bicuculline

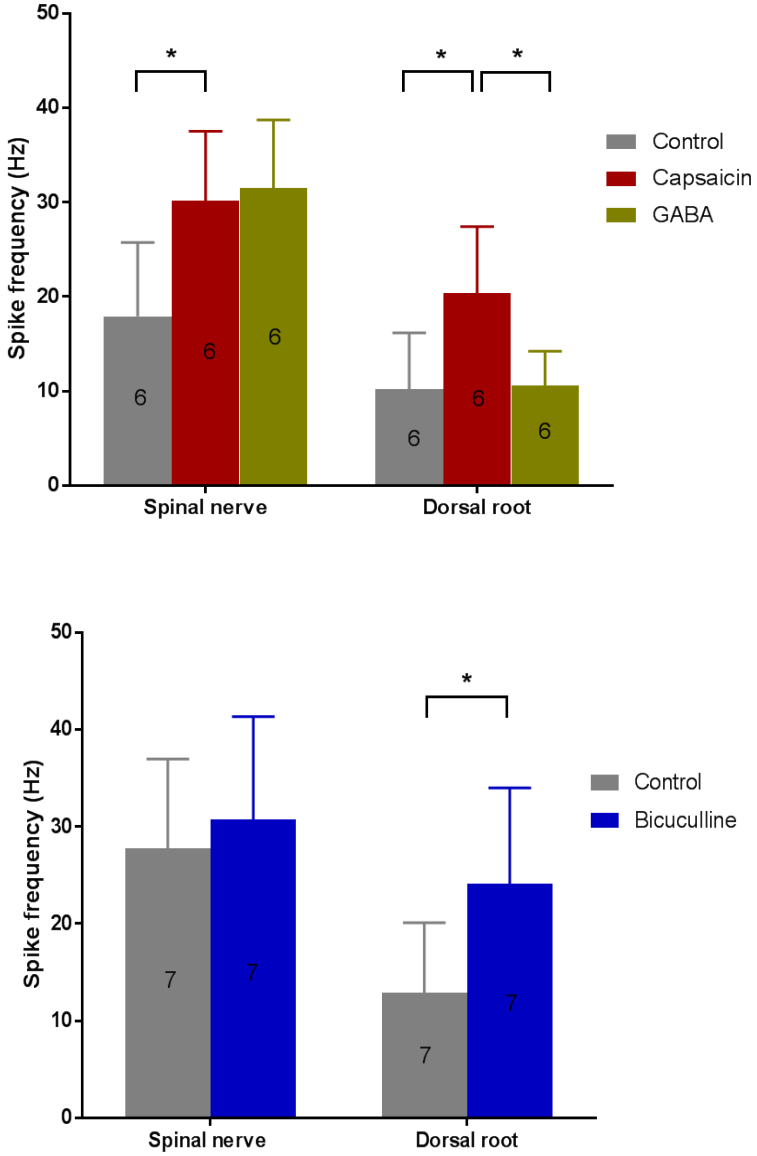


Figure 4.8 GABA filters peripheral nociception in DRG. Representative data of *in vivo* recordings of L5 spinal nerve and dorsal root: **(A-i)**, at baseline (control) and upon application of capsaicin (10 µM, 50 µl); **(A-ii)** upon application of capsaicin followed by GABA (200 µM, 3 µl) and **(A-iii)** upon application of bicuculline (200 µM, 3 µl). Hindpaw injection of capsaicin increased firing frequency in both spinal nerve and dorsal root branches of the nerve (compared with the basal activity in the upper traces). Application of GABA to DRG reduced capsaicin-induced firing frequency in the dorsal root but not in the spinal nerve (**A-ii**). **(B)** Summary of the recordings shown in **A**. Data were extracted from frequency of neuronal firing recorded on anesthesised adult rats at baseline and during applications of 10 µM, 50 µl capsaicin, 200 µM, 3 µl GABA and 200 µM, 3 µl bicuculline. Mean neuron firing frequency for each condition; control, capsaicin, GABA and bicuculline application was calculated, shown as mean ± SEM. Intraplantar injection of capsaicin increased neuronal firing on both the spinal nerve and dorsal root, while application of GABA on L5 DRG caused significant decrease in neuronal firing on L5 dorsal root. Bicuculline application on DRG caused significant increase of neuronal firing on dorsal root. Data on application of capsaicin and GABA were analysed using one-way ANOVA followed by Bonferroni correction. Data on bicuculline application were analysed using Wilcoxon signed rank test. (Capsaicin and GABA)=6, N(Bicuculline)=7.

4.3 Discussion

4.3.1 Tonic and induced GABA release in DRG

The results from this study suggest several important conclusions. i) There is a significant exocytosis of VGAT-positive vesicles, even in the absence of stimulation. This ties along well with the results presented in the previous chapter and with previously published data (Du et al., 2017). ii) Significantly lower VGAT-C antibody uptake in non-depolarised neurons and in neurons pre-treated with Ca^{2+}-free solution, as compared to the depolarization in Ca^{2+}-containing solution suggest that a significant fraction of vesicular exocytosis indeed was dependant on depolarisation and Ca^{2+} influx into the neurons. iii) Lack of the anti-VGAT-N antibody uptake demonstrates the robustness of the assay (note that this antibody stains permeabilised DRG neurons well, as evidenced in Chapter 3).

The present study reveals a potential mechanism of GABA release from the primary sensory neuron and its possible physiological significance. Primary sensory neurons were previously known to conduct action potentials from the sensory receptive field to central terminals without interruption. There was no known significant information processing occur therein. However, our recent data show that there is a functional GABAergic signalling pathway that is suggested to play a significant role in peripheral pain transmission (Du et al., 2017). Here, we investigated the mechanisms that may be involved in GABA neurotransmitter transfer within the DRG. Our previous results revealed that action potentials from peripheral nerve terminals could be attenuated within the DRG. In this chapter I demonstrate that i) there is significant somatic exocytosis of VGAT-positive vesicles in cultured DRG neurons, which has both spontaneous and induced components; ii) exogenous application of GABA to the DRG *in vivo* specifically reduces firing in the dorsal root aspect of the peripheral nerve, having no effect on the spinal nerve aspect; iii) inhibition of the endogenous $GABA_A$ receptors in DRG *in vivo* results in the increase in the tonic firing rate within the dorsal root, but not in the spinal nerve. This

phenomenon could be explained by the action of GABA present within DRG as shown by previous results from our lab (Du et al., 2017). The excitatory action of bicuculline is especially significant, as it strongly suggests the presence of the GABA tone within the DRG. Such GABA tone would explain lower basal firing frequency in the dorsal root as compared to the spinal nerve **Figure 4.8 (A-i)**. This is also in excellent agreement with the excitatory action of bicuculline in the dorsal root. In addition, GABA tone also agrees well with the results of the VGAT-C antibody uptake presented earlier in this Chapter. **Figure 4.9** shows a summary of the mechanism of GABA communication and release within DRG which was responsible for 'filtering' effect seen as reduced frequency of neuronal firing from the spinal nerve to the dorsal root.

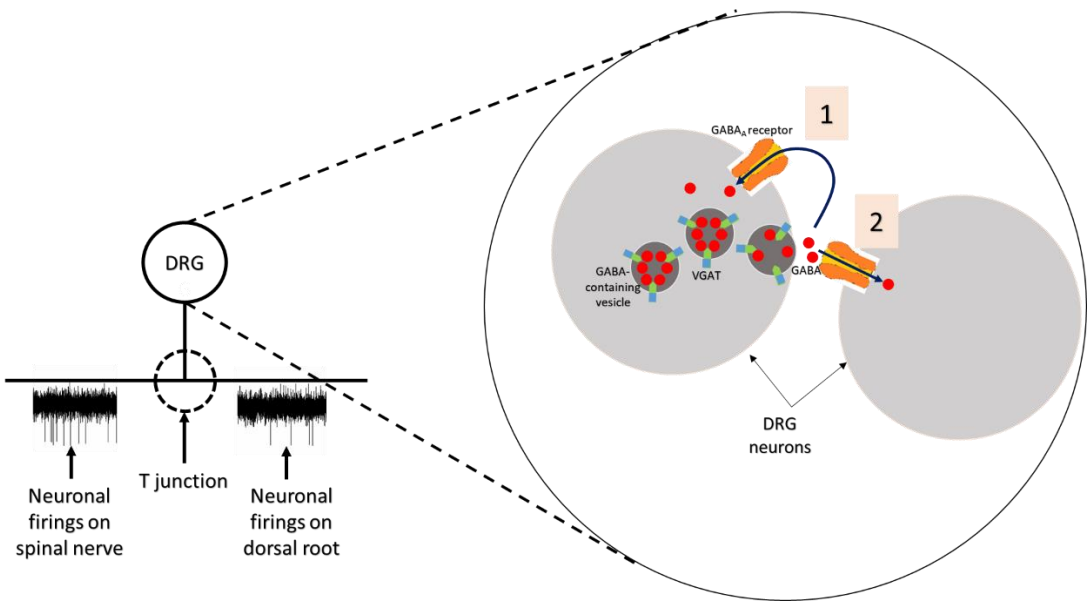


Figure 4.9 GABA communication method within DRG. Action potentials are propagated from the spinal nerve to the dorsal root via DRG. Within DRG, GABA is released via either autocrine (1) and/or paracrine (2) communication, filtering the signals from the spinal nerve by reducing the frequency of the neuronal firing on the dorsal root.

A significant proportion of cultured DRG neurons showed uptake of VGAT-C antibody without being stimulated with high KCl solution, demonstrating that some level of tonic exocytosis of potentially GABA-containing vesicles indeed take place.

An additional source of tonic GABA release could be the reversal of GAT1-mediated GABA transport, as discussed earlier in this chapter. There are several other proteins that could be responsible for the tonic GABA release such as Best1 (Lee et al., 2010) and LRRC8 (Lutter et al., 2017). Best1 is an anion channel that is activated by the intracellular Ca^{2+}. Lee and colleagues showed that in the Bergmann glial cells, Best1 was permeable to GABA and that its permeability was independent of intracellular Ca^{2+} concentration as observed in the identical reversal potential of GABA at different intracellular Ca^{2+} concentration measured at -80 mV (Lee et al 2010). In DRG neurons, expression of Best1 has been reported, whose expression was upregulated during nerve injury which in turn promoted the expression of CaCC (Boudes et al., 2009).

Another interesting channel that has been recently reported and associated with the flux of GABA is the volume-regulated anion channel (VRAC) (Lutter et al., 2017). VRAC is composed of LRRC8 heteromers; the obligatory subunit LRRC8A and at least one other LRRC8 isoform (LRRC8B, LRRC8C, LRRC8D or LRRC8E) (Voss et al., 2014). Recent evidence demonstrated a significant role of VRAC for transporting GABA; VRAC-dependent GABA transport was almost abolished in HEK cells lacking single LRRC8 subunits i.e. LRRC8A$^{-/-}$ and LRRC8D$^{-/-}$ (Lutter et al 2017). Preliminary immunohistochemical findings from our lab showed a high LRRC8A and LRRC8D immunoreactivity in small DRG neurons (unpublished data) which could suggest the role of VRAC in transporting GABA therein.

Despite the presence of spontaneous release possibly responsible for tonic inhibition within the DRG, the experiments with the VGAT-C antibody uptake revealed a significant activity-dependent component, which is in line with the ‘classical’ synaptic-like mechanism. These data are also in good agreement with findings previously published by us on the activity-dependent release of GABA from DRG detected by HPLC and ‘sniffing patch’ experiments (Du et al., 2017). This result is also consistent

with earlier studies which showed that the majority of vesicular release in DRG neuron somata occurs via CDS (Liu et al., 2011, Zhang and Zhou, 2002).

Yet, a small proportion of DRG neurons in my experiments took up VGAT-C antibody when the DRG culture was depolarized in the absence of extracellular Ca^{2+}. I hypothesize that this uptake may correspond to the CiVDS described by Zhuan Zhou's group (Chai et al., 2017). These authors reported that not only the action potential opens the voltage-gated Ca^{2+} channels causing CDS but in addition, it also opens voltage-gated N-type Ca^{2+} channel ($Ca_V2.2$) triggering a different pathway of transmitter secretion, the CiVDS. They further demonstrated that the $Ca_V2.2$ binds with SNARE proteins through synprint region on the plasma membrane of DRG neurons. $Ca_V2.2$ acts as the voltage sensor, SNARE proteins form the vesicle fusion pore while synprint works as the linker between the two. Further investigation into the contribution of CDS and CiVDS could shed light on the mechanisms of GABA release in DRG neurons that play an important role in controlling the peripheral nociceptive signalling.

4.3.2 GABA from DRG neuron modulates pain transmission

Following experiments on VGAT-C antibody uptake, we sought to investigate the role of GABA via extracellular *in vivo* electrophysiological recording of L5 dorsal root and spinal nerve. The L5 DRG was chosen due to its large size and the fact that the majority of the sciatic DRG neurons reside in the L4 and L5 DRG despite the components of the sciatic nerve in rats reported to range from L3 – L6 (Asato et al., 2000). During the electrophysiological recording, there was an increase in neuronal firing in the dorsal root following capsaicin injection to the rat's right hindpaw. Meanwhile, local GABA application onto the L5 DRG attenuated neuronal firings. Local application of $GABA_A$ receptor antagonist bicuculline was able to overcome the tonic activity of GABA in the DRG resulting in increased neuronal firings from the dorsal root. Indeed, the expression of $GABA_A$ receptors have been reported not only

in DRG but also in the dorsal root and peripheral nerves (Carr et al., 2010, Kingery et al., 1988, Liske and Morris, 1994) Thus, the local application of GABA and bicuculline on the DRG could dissipate to both the dorsal root and peripheral nerve, acting on its receptors. Indeed, the technique used in the present experiment could not rule out this possibility. However, only small amount of drugs were used for local application which would minimize the dissipation of these drugs to both nerve/root fibres and hence minimising the effect of extrasynaptic $GABA_A$ receptor activation. For future experiments, substituting local application with intra-DRG injection of GABA could overcome these biases in the results obtained, confirming (or otherwise) the antinociceptive effect of GABA on DRG.

Based on our previous results and modelling (Du et al., 2017) we hypothesized that the action potentials produced at the peripheral nerve endings and travelled within the spinal nerve towards DRG are filtered at the T-junctions within the DRG, where axons bifurcate to peripheral and central directions. As axonal bifurcation represents a low safety point for action potential propagation, some of these action potentials may fail to cross over the bifurcation and do not reach the dorsal root and, subsequently, the spinal cord. Our *in vivo* data support these findings, indeed, basal firing rates in the dorsal root were consistently lower than those in the spinal nerve. We further hypothesize that GABAergic system controls the filtering at the DRG, as was suggested by us earlier (Du et al., 2017). Indeed, inhibition of $GABA_A$ receptors in the DRG equalised the firing rates in spinal nerve and the dorsal root [**Figure 4.8 (A-iii)**], an effect equivalent to scaling down the filtering. Moreover, capsaicin-induced firing was specifically diminished by the injection of GABA into the DRG in the central, but not peripheral aspect of the nerve [**Figure 4.8 (A-ii)**], an effect equivalent to scaling the filtering up.

These data agree well with our earlier findings, that *in vivo* DRG application of GABA produced antinociceptive effect in acute and chronic pain conditions. Our data are also consistent with findings from Obradovic and colleagues (Obradovic et al., 2015).

These authors found that DRG neurons of the adult rats preferentially express $\alpha_2\beta_3\gamma_2$ $GABA_A$ subunits (Ma et al., 1993). As the α_2 subunit is an important functional subunit, Obradovic and colleagues looked into the role of α_2 in nociception. In their experiment, α_2 expression was significantly decreased during nerve injury (2 days post-injury) compared to the baseline on the ipsilateral side of the injury. They demonstrated that silencing the α_2 subunit of $GABA_A$ receptors in rat DRG enhanced nociception in sciatic nerve crush injury rat model (Obradovic et al., 2015) suggesting a major role of this subunit in anti-nociception post-traumatic injury. These authors also showed that DRG application of GABA is antinociceptive.

Data presented in this chapter strongly support the hypotheses that i) GABA is being produced and released by the DRG neurons and ii) that endogenous and/or exogenous GABA facilitate filtering of the throughput nociceptive conduction through the DRG. Yet, we are still largely in the dark in regard to the understanding of how neuron-to-neuron communication occurs within the spinal ganglia.

Studies have shown that there is indeed a degree of information transfer that occurs in DRG and that it can be modulated therapeutically, in particular for the treatment of chronic pain (Berta et al., 2017). However, the mechanisms that underlie these signalling pathways within the DRG still remain elusive. The presence of sandwich synapse within DRG has been reported by Rozanski and colleagues which involved a communication complex formed by a trimer structure– neuron somata-glial cell-neuron somata (Rozanski et al., 2013). Yet, even if such a dedicated communication device does exist in the DRG, there are still more questions than answers. How does GABA cross the glial septum? Could there be a more sophisticated process, whereby a ‘presynaptic’ neuron soma releases transmitter-1 to stimulate the glial septum, which then releases a transmitter-2 to act on the ‘postsynaptic’ soma? Does phasic GABA release play the major role in signal (especially nociceptive) transmission, or does it mostly involve tonic release and the subsequent activation of different composition of $GABA_A$ receptors located at extrasynaptic/perisomatic receptors?

These intriguing questions require future research, which should help to better understand and hopefully support the new ‘peripheral gating’ phenomena that our group has proposed and in the long run, contribute to chronic pain treatment.

In this chapter, the *in vivo* electrophysiological recordings have shown that $GABA_A$ receptors could be the receptor involved in the peripheral GABAergic signalling during tonic activity. To support this finding, the next chapter investigates the activation of $GABA_A$ receptors by GABA released from DRG neurons using a halide biosensor.

Chapter 5 Tonic GABA release from DRG neuron somata activates $GABA_A$ receptor on HEK293 cells transfected with α1, β2 and γ2 $GABA_A$ receptor subunits

5.1 Introduction

5.1.1 Tonic and extrasynaptic GABA release by the nervous system

Phasic release of neurotransmitter in CNS produces rapid transmission of information from presynaptic terminals to postsynaptic cells and is the main mechanism for neuron-to-neuron communication (Farrant and Nusser, 2005). However, neurotransmitters such as GABA that are involved in this rapid transmission has also been shown to participate in a slower form of signalling (Mody, 2001). This slower form of signalling could, therefore, involve tonic activation of receptors and may also reach extra-synaptic sites. Tonic current produced by GABA has been shown to be directly correlated with phasic current (Glykys and Mody, 2007b). In CNS, apart from GABAergic synapses, there is another role for GABA in neuron-to-neuron communication; termed spillover or volume transmission. These terms generally describe phenomena, where GABA escapes from the synaptic cleft and activates receptors in extrasynaptic membranes (Brickley et al., 2001), acts presynaptically (Trigo et al., 2008), or acts on adjacent synapses (Wei et al., 2003).

In rat cerebellar granule cells, tonic GABA release has been shown to be important to ensure persistent GABA receptor activation (Brickley et al., 1996). These GABA receptors were present in the somatic, dendritic and axonal regions of the neuronal membrane that were distant from sites of synaptic release (Kullmann et al., 2005). A variation of ambient GABA concentration from tens of nanomolar to few micromolar has been reported in different regions of the rat brain (Attwell et al., 1993, Kennedy et al., 2002, Lerma et al., 1986, Tossman et al., 1986). The different values could be due to the different methods used, also regional and temporal variations in extracellular GABA concentration in these different studies.

It is known that tonic GABA release activates $GABA_A$ receptors of different compositions than that of phasic release (Mody, 2001). A review on the phasic and tonic inhibition of $GABA_A$ receptors published in *Nature* reported that the predominant receptor subtypes involved in the phasic synaptic inhibition were the γ_2 subunit in association with α_1, α_2 or α_3 subunits. Receptors containing α_4, α_5 and α_6 subunits were predominantly extrasynaptic which mediate the tonic inhibition (Farrant and Nusser, 2005). Of particular importance, the different composition of $GABA_A$ receptor subunits exhibits different pharmacological characteristics (Luscher et al., 2011). Mortensen and colleagues looked into the potency of GABA at different $GABA_A$ receptor subunits, α_1 – α_6, by determining the EC_{50} values from the concentration-response curve established by measuring whole-cell currents (Mortensen et al., 2012). Of these six isoforms, GABA displayed the lowest potency at receptors containing α_2 and α_3, followed by α_1-, α_4- and α_5-containing receptors with intermediate potency; while the α_6 subunit-containing receptors exhibited the highest sensitivity to GABA. The sensitivity difference between α_2/α_3 and α_6-subunit-containing receptors was approximately 80-fold (Mortensen et al., 2012). Meanwhile, the sensitivity for β_1, β_2 and β_3 subunits was examined in receptors co-expressing α_1 and γ_2 subunits. There was a significant difference in the sensitivity among the three isoforms, with β_3-containing isoform being the most sensitive to GABA. Of interest, the extrasynaptic type $GABA_A$ receptors, the α_4 and α_6-subunit-containing receptors, higher potency was exhibited by the α_6 consistent with results from earlier studies (Brickley et al., 2001, Nusser et al., 1998).

5.1.1.1 The extrasynaptic GABA receptors in DRG neurons

In DRG, studies have shown that GABA-mediated currents in neuron somata are dominated by currents from the low-affinity receptors i.e. synaptic receptors, in acutely dissociated DRG neurons *in vitro* (Lee and Gold, 2012, Oyelese et al., 1997, Sung et al., 2000). Results from our previous work support these findings. Different low affinity receptor subunits– α, β and γ subunits of $GABA_A$ receptor were detected via RT-PCR, the most abundant subunits found were the α_{1-2} and γ_{1-2} (Du et al., 2017), in agreement with findings from the literature (Ma et al., 1993, Obradovic et al., 2015, Klinger et al., 2015). Despite the dominance of the low affinity currents in acutely dissociated neurons, Lee and colleagues revealed that increase in culture time (24 hours) caused an upregulation of the high-affinity $GABA_A$ receptors in cultured DRG neurons, partly mediated by the extrasynaptic δ subunit-containing receptors (Lee et al., 2012). In this experiment, also detected in a subpopulation of neurons were the increase of ρ_2 and ε-subunit mRNA in association with these high-affinity currents (Lee et al., 2012). Another study reported the involvement of δ -containing $GABA_A$ receptors in the analgesic effect of flupirtine in DRG neurons (Klinger et al., 2015). Klinger and colleagues investigated the effects of flupirtine, a centrally acting analgesic drug, on the native synaptic and extrasynaptic $GABA_A$ receptors, and revealed that flupirtine was more sensitive to δ-containing $GABA_A$ receptors than that of γ_2, with higher preference for δ subunit in the DRG than that of hippocampus (Klinger et al., 2015). Inflammatory mediators such as bradykinin and prostaglandin E2 have also been reported to potentiate the high affinity $GABA_A$ currents in rat DRG neurons (Lee and Gold, 2012). Together, these studies suggest that extrasynaptic $GABA_A$ receptors are more dominant than that of the synaptic in prolonged culture and pathological conditions in DRG neurons.

5.1.2 HEK293 cells

Human Embryonic Kidney 293 cells (HEK cells) are widely used in cell biology research to study protein and gene expression including ion channels such as $GABA_A$. HEK cells were generated from human embryonic kidney cells which underwent transformation with sheared fragments of human adenovirus 5 DNA (Ad5); an important technique of transforming human cells using adenovirus made by Frank Graham in the early 70s (Graham et al., 1977). HEK cells are widely used among electrophysiologists due to several advantages; HEK cells are easy to maintain and grow with high reproducibility (Graham et al., 1977), they are also very efficient for protein production and are accessible for transfection (Lin et al., 2014, Thomas and Smart, 2005). Plasmid vectors introduced into HEK cells effectively take control of HEK cells' protein machineries and force translation of the gene artificially incorporated into the plasmid. There are several other advantages of using HEK cells in the expression of recombinant neuronal proteins such as recombinant hetero-oligomeric ion channels; the subunit compositions can be studied in isolation from other receptors of the same or different family as being non-neuronal in origin, HEK cells do not normally express native neuronal proteins and receptors at high levels.

HEK cells are theoretically of endothelial, epithelial or fibroblast in origin. However, despite showing a low level of expression of native neuronal proteins and receptors, several studies have shown the expression of neurophysiological receptors and protein subunits detected as protein or mRNA endogenous to HEK cells which could interfere with the results of studies conducted. Channels like VGCCs, acid-sensing ion channel (ASIC1a), ligand-gated ion channels, GPCR and intracellular regulatory receptors have been detected in HEK cells [a review by (Thomas and Smart, 2005)]. Few studies have reported the endogenously expressed proteins in HEK cells which are summarised in **Table 9**. Depending on the relative amount of endogenous-expressed and transiently transfected proteins of the same family and action, these endogenously expressed proteins may or may not affect the outcome of the

investigation. For example, Schachter and colleagues reported the expression of endogenous P2Y1 and P2Y2 receptors in HEK cells (Schachter et al., 1997) which, similar to P2X receptors, are activated by ATP, thus using HEK cells is not a suitable vehicle to study P2X-receptor-mediated Ca^{2+} influx (He et al., 2003). Of particular interest is the endogenous expression of the β_3 subunit of $GABA_A$ receptor observed in non-transfected WSS-1 cells (HEK cells transformed to stably express α_1 and γ_2 $GABA_A$ receptor subunits) reported by Davis and colleagues (Davies et al., 2000). Using RT-PCR technique, they found that the β_3 mRNA was endogenously expressed in both WSS and HEK293 cells. As α_1 and γ_2 subunits do not form functional receptor, the GABA-induced currents in both WSS and HEKs-transiently transfected with α_1 and γ_2 subunits was highly suggestive of the presence of β_3 subunits that renders the $\alpha_1/\gamma_2/\beta_3$ subunits assembly functional (Davies et al., 2000). However, other studies had not been able to detect the expression of the β_3 subunit in HEK cells (Taylor et al., 1999, Wooltorton et al., 1997) which could be attributable to the specific clone of HEK cells or culture conditions (Fuchs et al., 1995).

Table 9 Important neurophysiological receptors or protein subunits detected in HEK293 cells.(Thomas and Smart, 2005)

Ligand-gated	G-protein coupled	Voltage-gated and other[a]
Muscle acetylcholine (AChR) δ	Muscarinic AChR (M3)	Potassium channels: voltage-gated (Kv(α) 1.1, 1.2, 1.3, 1.4, 1.6, 3.1, 3.3, 3.4, 4.1, and Kvβ) and calcium-activated SK1
Nicotinic acetylcholine (nAChR): α7 and α5	Metabotropic (m) GluR: 1β and 4	Sodium channel: BNaC2 and β1A
Glutamate (GluR) 3	$GABA_B$ R1A	Voltage-gated calcium channel: $\alpha2_{\beta}$, $\alpha2_{\delta}$ isoforms I and β
γ-Aminobutyric acid ($GABA_A$ R): β_3, γ_3 and ε	5-Hydroxytryptamine: (5-HT) 1_D, 6, and 7_B	Protein synthesis kinase
Glycine (GlyR) β	Dopamine D2	PKA: catalytic and regulatory (R) II subunits
Acid Sensing Ion Channel Ia	Corticotropin-releasing factor 1 (CRF1)	PKC: α and δ
	Somatostatin type 2	Clathrin light chain α and β
	Bradykinin	Synaptotagmin
	Sphingosine-1-phosphate	Huntington interacting protein (HIP2)
	β_2 adrenoceptor	Trp1, 3, 4,6 (mediators of store-operated calcium entry)
	Purinergic (ATP/ADP) receptor $P2Y_{1 \text{ and } 2}$	Ryanodine receptor
	Adenosine A_{2B}	MKα (neurite outgrowth promoter)

R: receptor

[a] Includes mRNAs usually exclusive to neuronal cells

5.1.3 Cl^- ion in living cells

Cl^- is the most abundant intracellular anion in living animal cells (Berend et al., 2012). The homeostasis of $[Cl^-]_i$ is crucial for sodium secretion, neurosynaptic transmission and cell volume regulation (Powers, 1999). Cl^- channel dysfunction has been implicated in many diseases such as dystrophia myotonica; a muscular disorder caused by *DMPK* gene mutation (inhibition of the Cl^- conductance due to loss of CIC 1 channel activity), cystic fibrosis; a disorder caused by *CFTR* gene mutation affecting mostly the lungs (defective Cl^- transport into the epithelial cells) (Puljak and Kilic, 2006) and epilepsy (Cl^- accumulation due to intense $GABA_A$ receptor activation) (Raimondo et al., 2015). The measurement of $[Cl^-]_i$ could facilitate the diagnosis of diseases implicated by its imbalance. Of particular importance, neuronal Cl^- regulation has been demonstrated to play an important role in pain pathways (Price et al., 2009); a depolarising shift of E_{Cl^-} can contribute to hyperexcitability and an increase in nociceptive signalling. Thus, the measurement of neuronal $[Cl^-]_i$ is imperative in understanding the pathophysiology of pain.

Recent decades have seen the development of different tools used to measure $[Cl^-]_i$ in living cells. Various chemical and genetically encoded biosensors have been reported to probe intracellular Cl^- levels; of particular interest, fluorescent optical sensors have been engineered. Indeed, the fluorescence quenching-based optical sensors such as the green fluorescent mutant protein– YFP-H148Q/I152L offer a powerful tool for non-invasive monitoring of the anionic conductance via an opened channel.

5.1.4 Halide biosensor based on the green fluorescent protein

The green fluorescent protein (GFP) has become a widely used protein in biological and medical research. GFP was derived from the jellyfish *Aequorea Victoria* and has the ability to emit green fluorescence when exposed to light in blue to ultraviolet range (Remington, 2011). GFP and its variants have been used in a wide range of experimental applications as markers for gene expression (Chalfie et al., 1994), protein localisation and folding (Feilmeier et al., 2000) and as biosensors (Ibraheem and Campbell, 2010).

The GFP fluorescence is produced by chromophores that undergo covalent modifications of three of its amino acids: Ser 65-Tyr 66-Gly 67 (Cody et al., 1993). Chromophore is formed via four distinct processes: folding, cyclisation, oxidation and dehydration (Craggs, 2009). The building block of chromophore is made up of 11 β-strands that fold to form a β-barrel. The β-barrel resembles a cylinder of 42 A by 42 A (**Figure 5.1),** which is bound at either end by α-helix with chromophore located in the central (Craggs, 2009).

The opportunity to evaluate Cl^- fluxes using GFP-based indicators was brought to attention when it was discovered that the fluorescence of a mutant GFP; the yellow fluorescent protein (YFP) depends on the environment concentration of halogens; the higher the halide concentration, the dimmer is YFP fluorescence observed (Wachter and Remington, 1999). YFP was engineered via four-point mutations of GFP: S65G, V68L, S72A, T203Y. YFP sensitivity towards halides was further improved by mutations at H148Q and I152L (Galietta et al., 2001). Mutations at H148Q and I152L yield highly fluorescent protein with higher binding affinity to halides (Wachter et al., 2000). Activation of anion channels allows flux of halides such as I^-,NO_3^-, Br^- and Cl^- into cells transiently transfected with EYFP H148Q/I152L protein, which quench and reduce the EYFP fluorescence. A simultaneous transfection of YFP H148Q/I152L with anion-permeable ion channels yields a high degree of co-expression thus enabling optical measurement of anion channel

activation (Kruger et al., 2005). The high co-expression of the mutant YFP H148Q/I152L with ion channels is a major advantage; when used as a biosensor for $GABA_A$ receptor activation, the need to use a stable cell line which requires a longer time to be produced can be overcome.

In this experiment, I hypothesised that GABA released from DRG neurons bind to $GABA_A$ receptors leading to activation and opening of this Cl^- channel. As $GABA_A$ receptor is permeable to I^- (Robertson, 1989), I investigated the activation of $GABA_A$ receptor by studying the movement of I^- that goes through it. In this experiment, I utilised the genetically modified green fluorescence mutant protein EYFP H148Q/I152L which has the ability to bind to small anions leading to its fluorescence quenching. I transiently co-expressed EYFP H148Q/I152L with α_1, β_2 and γ_2 subunits of $GABA_A$ receptors into HEK cells to study $GABA_A$ receptor channel activity through I^- influx. As the binding affinity of anions to EYFP-H148Q/I152L showed anion sensitivity sequence $I^->NO_3^->Br^->Cl^-$ (Galietta et al., 2001), and that the I^- concentration in mammalian cells is negligible thus I used extracellular NaI to study the activity of $GABA_A$ ion channel. The movement of I^- was observed by measuring the quenching of EYFP H148Q/I152L in HEK cells transiently transfected with EYFP H148Q/I152L and α_1, β_2 and γ_2 subunits of $GABA_A$ receptors after exposure to NaI bath solution. The aim of the experiments discussed in this chapter was to use HEK cells transfected with $GABA_A$ receptors and EYFP-H148Q/I152L as 'indicator cells' for detection of GABA release from the cultured DRG neurons.

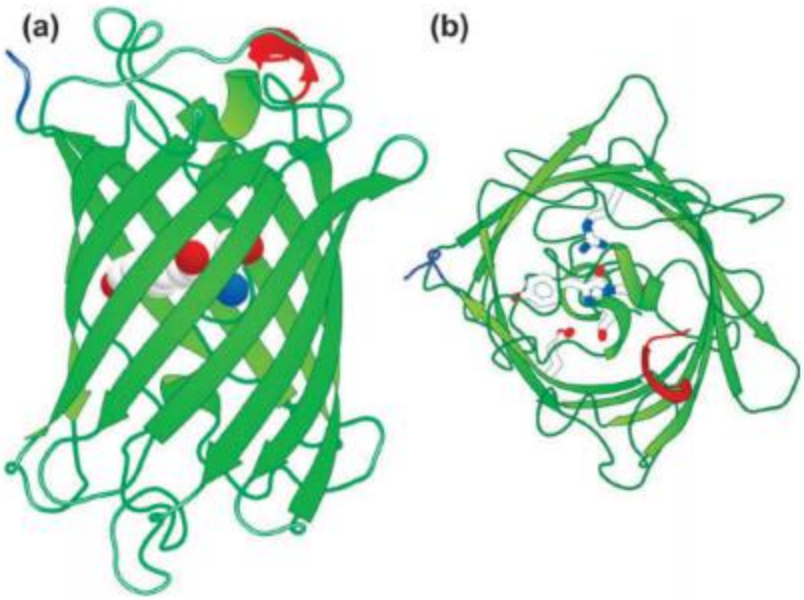


Figure 5.1 The tertiary structure of avGFP: Carbon atoms are shown in white, nitrogen in blue and oxygen in red. The N- and C- termini are coloured red and blue, respectively. (a) The β-barrel fold with the chromophore depicted as space-filled spheres. (b) The view from the top of the barrel, showing the chromophore and the catalytically important residues R96 and E222 (Craggs, 2009).

5.2 Results

5.2.1 GABA is released tonically by DRG neuron somata in culture

Previous results from our group showed that GABA was released by DRG neuron somata upon exposure to depolarising agents i.e. elevated K^+ level, bradykinin, capsaicin, ATP (Du et al., 2017). Sniffer patch-clamp recordings from HEKGABA$_A$ cells which were juxtaposed with small DRG neurons in co-culture showed robust inward current from HEK$_{GABAA}$ indicator cells in response to stimulation of the adjacent DRG neurons with capsaicin. While patch-clamp electrophysiology is considered the 'gold standard' for studying ion channels, it is often limited in throughput. Thus we employed an EYFP H148Q/I152L fluorescence assay to study GABA$_A$ receptor activity. The rationale for the experiment was as follows: if DRG neurons release GABA, then such GABA release can produce the opening of GABA$_A$ channels in co-cultured 'indicator' HEK cells. If the measurement is performed in the presence of extracellular NaI, then I^- influx through the activated GABA$_A$ channels would produce quenching of the EYFP H148Q/I152L fluorescence in the indicator cells, hence, the quenching of the fluorescence in the indicator cells could be used as a measure of the GABA release from DRG.

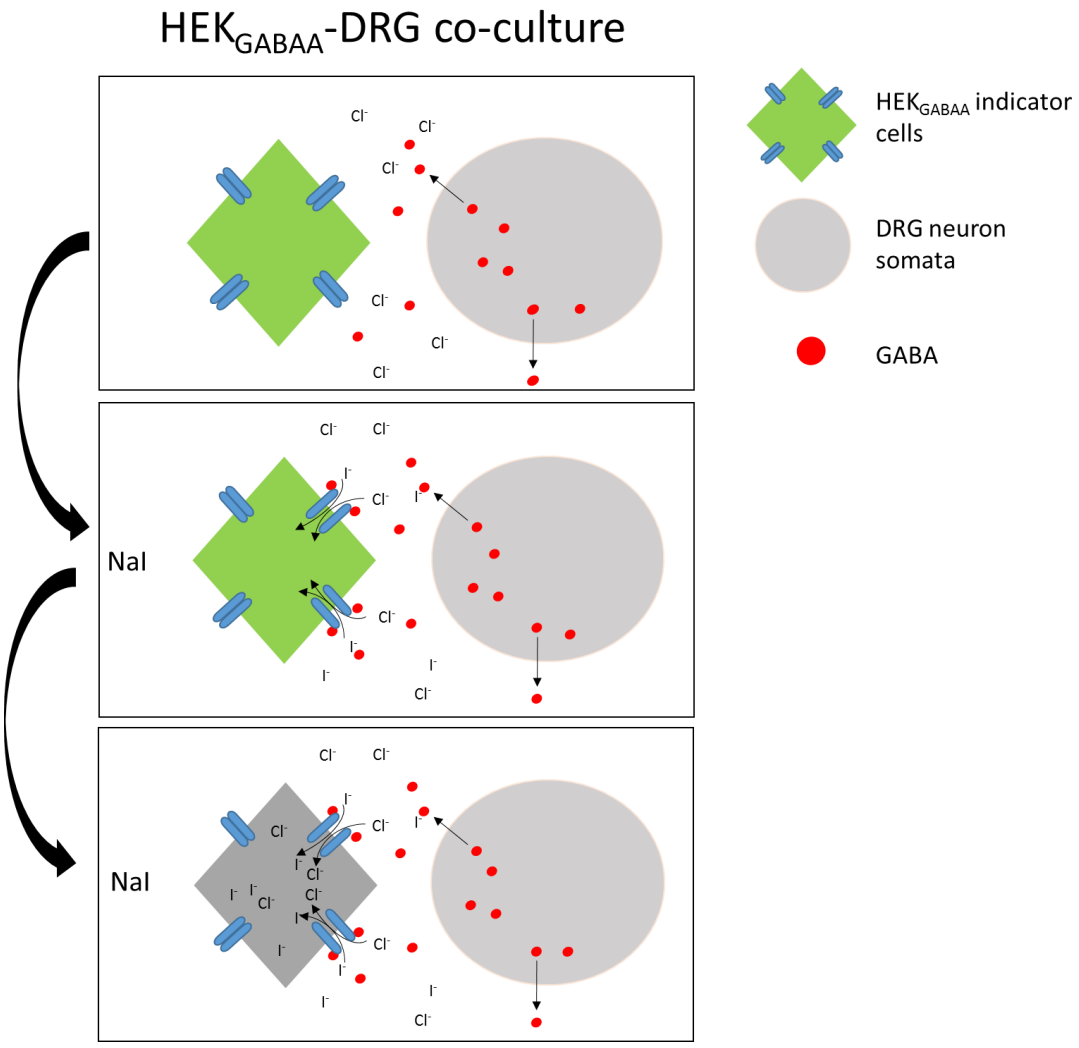


Figure 5.2 A schematic of EYFP H148Q/I152L quenching in HEK_{GABAA}-DRG co-culture system. GABA released from DRG neuron binds to $GABA_A$ receptor on HEK_{GABAA} cell. This binding leads to activation and opening of $GABA_A$ receptor allowing I^- and Cl^- to enter the HEK_{GABAA} cell, quenching the EYFP fluorescence. (HEK_{GABAA} indicator cell: HEK cells transiently transfected with EYFP H148Q/I152L and $GABA_A$ receptor subunits α_1, β_2 and γ_2).

Prior to performing HEK cells – DRG neurons co-culture, we first transfected HEK cells with an I^--sensitive EYFP mutant (H148Q/I152L) and the α_1, β_2, and γ_2 $GABA_A$ receptor subunits (these transfected HEK cells will be referred to hereafter as 'HEK_{GABAA} indicator cells'). I^- has been reported to be the most potent halide for EYFP quenching (Galietta et al., 2001), thus we used extracellular solution supplemented with 5 mM NaI to study the $GABA_A$ receptor activity. Using the appropriate I^- concentration is very important in determining the activity of agonist studied as high I^- concentration can produce agonist-independent quenching of the EYFP (Johansson et al., 2013, Jin et al., 2013). While establishing YFP-based assay to measure $GABA_A$ ion channel activity, Johansson and colleagues showed that 5 mM and 10 mM NaI generated minimal basal quench while 20 mM and 40 mM NaI showed a significant agonist-independent YFP fluorescence quench (Johansson et al., 2013). Thus 5 mM NaI was chosen as the I^- concentration used in this study.

Application of 5 mM NaI to HEK cells transfected with EYFP H148Q/I152L but not the α_1, β_2, and γ_2 $GABA_A$ receptor subunits (these transfected HEK cells will be referred to hereafter as 'HEK indicator cells'), produced a small rundown of EYFP H148Q/I152L fluorescence, amounting to 0.8 ± 0.16% quenching over 100 sec (**Figure 5.3**); we consider this value as agonist–independent quenching due to background anion conductance. Application of a potent and selective $GABA_A$ agonist muscimol to HEK indicator cells resulted in EYFP H148Q/I152L fluorescence quenching, indistinguishable from the agonist-independent quenching (1.8 ± 0.2% over 100 sec; **Figure 5.4**). In contrast, application of muscimol to HEK_{GABAA} indicator cells resulted in significantly stronger quenching (14 ± 2.4% over 100 sec; **Figure 5.5**). This experiment validated the optical detection of $GABA_A$ receptor activation in HEK_{GABAA} indicator cells; additionally, it also confirmed the absence of measurable endogenous $GABA_A$ conductance in HEK indicator cells. Based on our previous experiments with Ca^{2+}-activated Cl^- channels (Jin et al., 2013), quenching of EYFP

upon 10 μM muscimol perfusion by ≥10% was regarded as the benchmark for successful transfection.

In the next experiment, we co-cultured HEK_{GABAA} indicator cells with DRG neurons (hereafter will be referred to as 'HEK_{GABAA}-DRG co-culture') from preweaner rats (5-11 days old). We then attempted to induce GABA release from DRG using depolarizing extracellular solution containing 50 mM KCl (50 mM KCl). In these experiments, 5 mM NaI was perfused for 30 sec prior to perfusion of 50 mM KCl in the presence of 5 mM extracellular NaI to enable I^- to reach optimum concentration upon $GABA_A$ receptor activation. During this experiment, indeed, a robust EYFP fluorescence quenching was observed suggesting the presence of GABA in this co-culture system (quenching by 26 ± 3.8% over 100 sec; **Figure 5.6**). However interestingly, we also observed that the robust EYFP fluorescence quenching in HEK_{GABAA}-DRG co-culture started immediately with the onset of the 5 mM NaI perfusion. To confirm whether the presence of NaI alone could cause EYFP fluorescence quenching in these conditions, we perfused the HEK_{GABAA}-DRG co-culture with only 5 mM NaI and surprisingly, a robust EYFP fluorescence quenching was produced (quenching by 36.63 ± 2% over 100 sec; **Figure 5.7**). The EYFP fluorescence quenching in the HEK_{GABAA}-DRG co-culture in the presence of 5 mM NaI and 50 mM KCl was similar to that in the presence of 5 mM NaI only. These results hint at the presence of ambient GABA in the HEK_{GABAA}-DRG co-culture. Indeed, a tonically released GABA could induce 'leak' of I^- into the HEK_{GABAA} indicator cells even in the absence of stimulation.

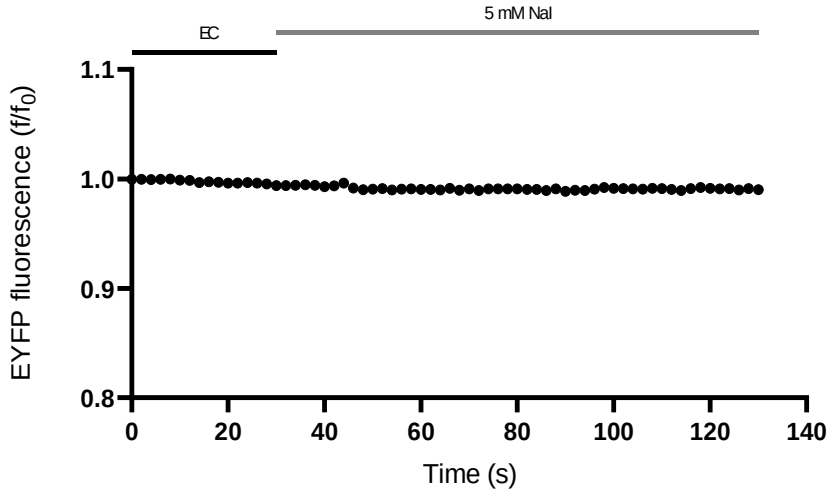


Figure 5.3 Mean EYFP H148Q/I152L quenching of HEK cells transfected with EYFP H148Q/I152L only but not $GABA_A$ receptors, in response to 5 mM NaI. Data are shown as mean ± SEM (3 biological replicates, n=19).*Where error bars are not seen, they are shorter than the symbols used to plot the EYFP fluorescence.

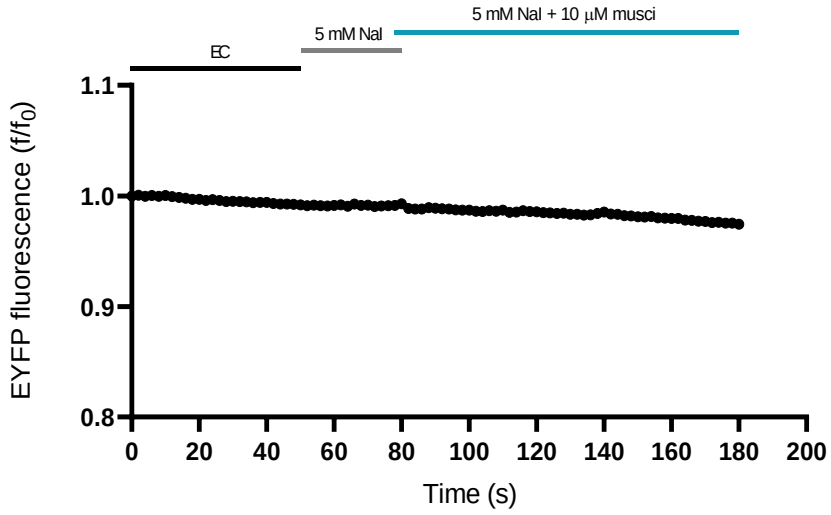


Figure 5.4 Mean EYFP H148Q/I152L quenching of HEK cells transfected with EYFP H148Q/I152L only but not $GABA_A$ receptors, in response to 10 μM muscimol. Data are shown as mean ± SEM (3 biological replicates, n=17).*Where error bars are not seen, they are shorter than the symbols used to plot the EYFP fluorescence.

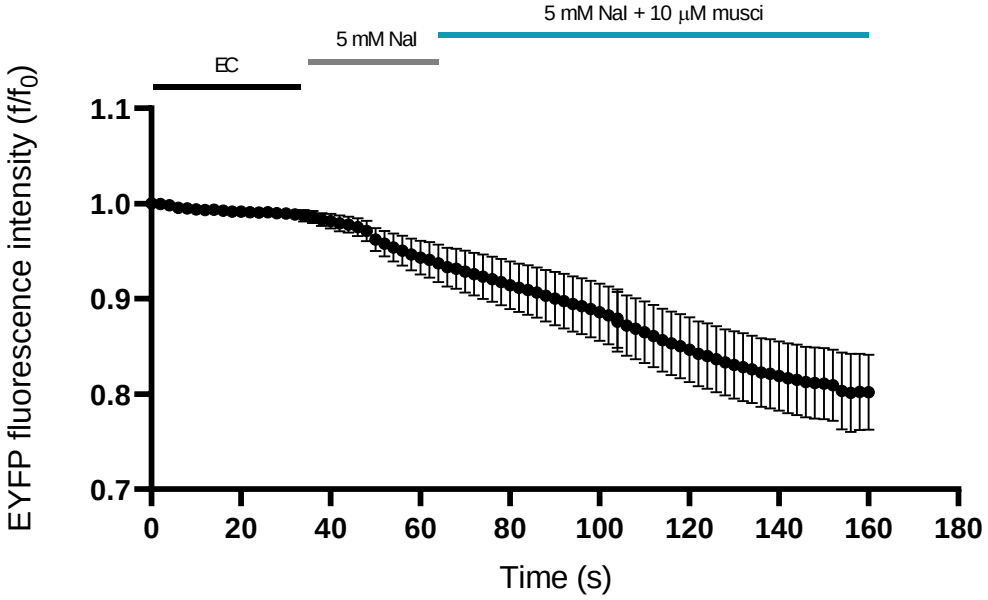


Figure 5.5 Mean EYFP H148Q/I152L quenching of HEK$_{GABAA}$ indicator cells in response to 10 μM muscimol. Data are shown as mean ± SEM (3 biological replicates, n=16).*Where error bars are not seen, they are shorter than the symbols used to plot the EYFP fluorescence.

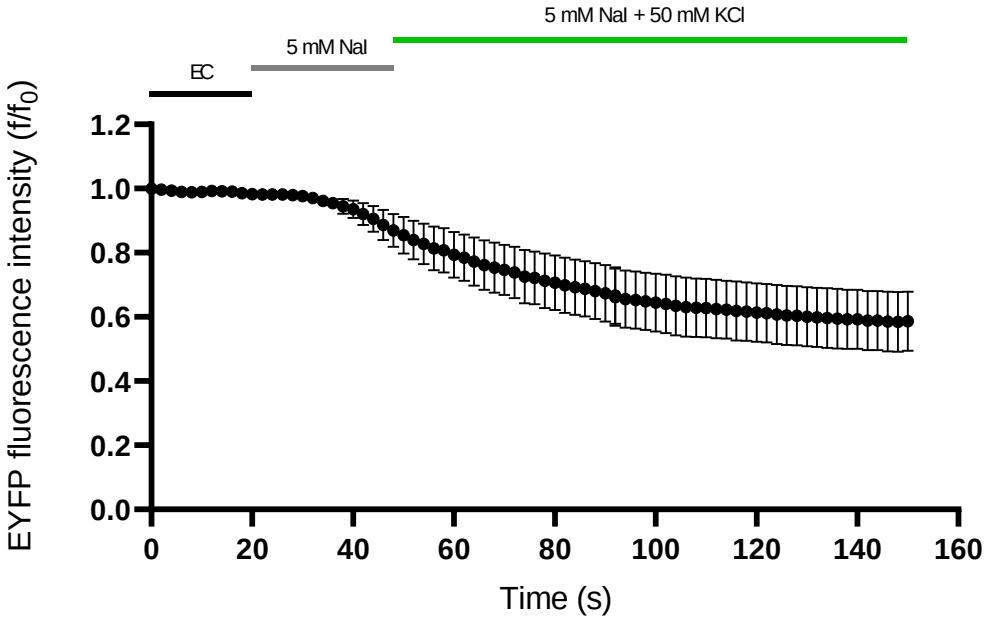


Figure 5.6 Mean EYFP H148Q/I152L quenching in response to 50 mM KCl in HEK$_{GABAA}$-DRG co-culture. EYFP quenching was observed during NaI 5 mM-only perfusion, before perfusion of 50 mM KCl to induce GABA release. Data are shown as mean ± SEM (3 biological replicates, N=3, n=8).*Where error bars are not seen, they are shorter than the symbols used to plot the EYFP fluorescence.

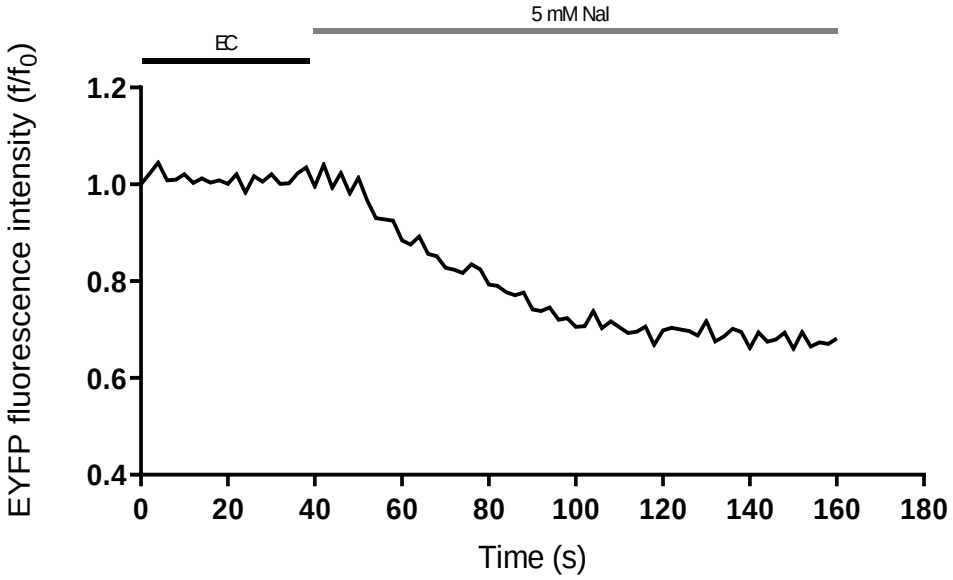
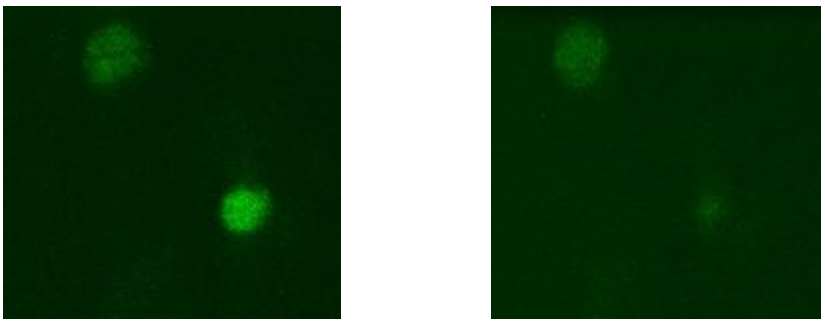


Figure 5.7 Representative trace of EYFP H148Q/I152L quenching in HEK$_{GABAA}$-DRG co-culture perfused with 5 mM NaI. (3 independent experiments, N=3, n=23).



Before 5 mM NaI perfusion After 2-min of 5 mM NaI perfusion

Figure 5.8 Images of EYFP quenching upon perfusion of 5 mM NaI in HEK$_{GABAA}$-DRG co-culture.

5.2.2 Spontaneous quenching of EYFP-transfected HEKGABAA indicator cells in the presence of DRG neurons was blocked by GABA antagonist.

As strong EYFP fluorescence quenching was observed in HEK_{GABAA}-DRG co-culture even in the absence of stimulation of the latter, we sought to confirm that $GABA_A$ receptors were indeed involved in the I^- influx that led to EYFP fluorescence quenching. To achieve this objective, HEK_{GABAA}-DRG co-cultures were perfused with either 5 mM NaI solution or in 5 mM NaI + 50 µM bicuculline, a competitive $GABA_A$ receptor antagonist. Perfusing the HEK_{GABAA}-DRG co-cultures with bicuculline should block $GABA_A$ receptor producing lower EYFP fluorescence quenching compared with the control group. Indeed that was the case; when the co-cultures were perfused with 5 mM NaI + 50 µM bicuculline, the EYFP fluorescence quenching was halved (compared to that in the presence of NaI alone; **Figure 5.7)**. We also analysed the kinetics of EYFP fluorescence quenching in the presence and in the absence of bicuculline. We analysed the time constants (tau, Ƭ) generated from exponential decay of the EYFP fluorescence quenching in HEK_{GABAA}-DRG co-cultures after perfusion with 5 mM NaI without or with bicuculline; these Ƭ values were 62.42 ± 5.86 sec (5 mM NaI alone; n=23) *vs*. 98.47 ± 8.24 sec (5 mM NaI + 50 µM bicuculline; n=52; $P<0.05$, Mann Whitney U test). These results strongly suggest that the $GABA_A$ receptors in the HEK_{GABAA} indicator cells are indeed tonically active in the presence of DRG neuron culture.

Figure 5.10 summarises the mean EYFP fluorescence quenching from three different conditions: i) the HEK_{GABAA} indicator cells alone perfused with 5 mM NaI only; ii) HEK_{GABAA}-DRG co-culture perfused with 5 mM NaI only and iii) HEK_{GABAA}-DRG co-culture perfused with 5 mM NaI and 50 µM bicuculline. Kruskal Wallis ANOVA demonstrated a significant difference of EYFP fluorescence quenching between all groups. Data obtained from these experiments suggest that DRG neurons release GABA via tonic activity which in turn is able to activate $\alpha_1\beta_2\gamma_2$

subunits of $GABA_A$ receptors as indicated by the EYFP fluorescence quenching in HEK_{GABAA} indicator cells.

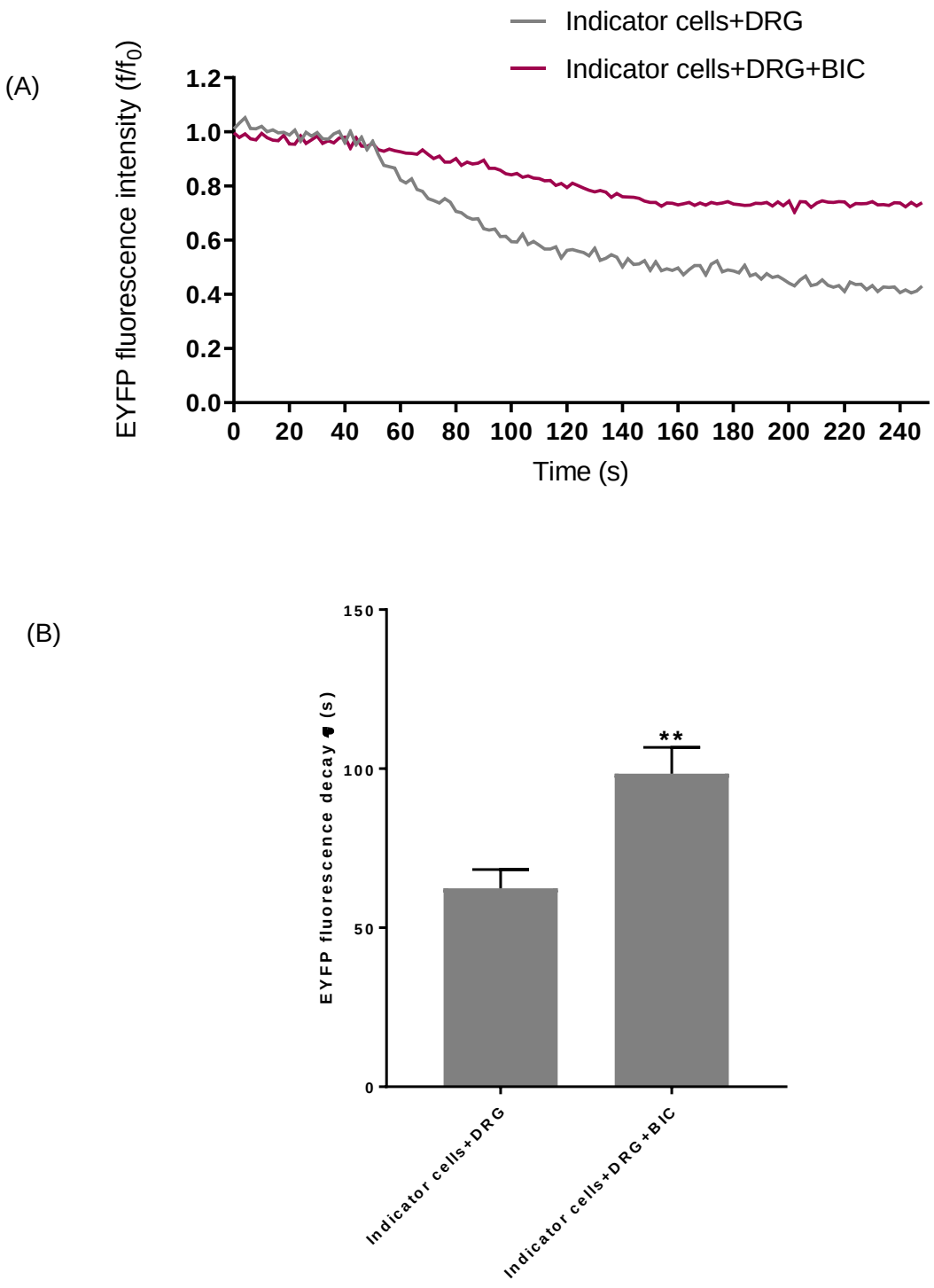


Figure 5.9 EYFP quenching of HEK$_{GABAA}$ indicator cells in the presence or absence of GABA$_A$ antagonist bicuculline. (A) Representative traces of EYFP (H148/152L) in HEK$_{GABAA}$ indicator cells after exposure to either 5 mM NaI or 5 mM NaI+50 µM bicuculline. **(B)** Mean time constant (T) of EYFP quenching in the presence of NaI alone (n=23) or NaI+bicuculline (n=52). Data were analysed by calculating the mean EYFP H148Q/I152L fluorescence decay (T) of HEK cells per rat and per transfection for HEK cell-DRG neuron co-cultures (one transfection represents one biological replicate). Data are shown as mean ± SEM. Data were analysed using Mann-Whitney U test ($P<0.05$). (N=3, biological replicate=3).

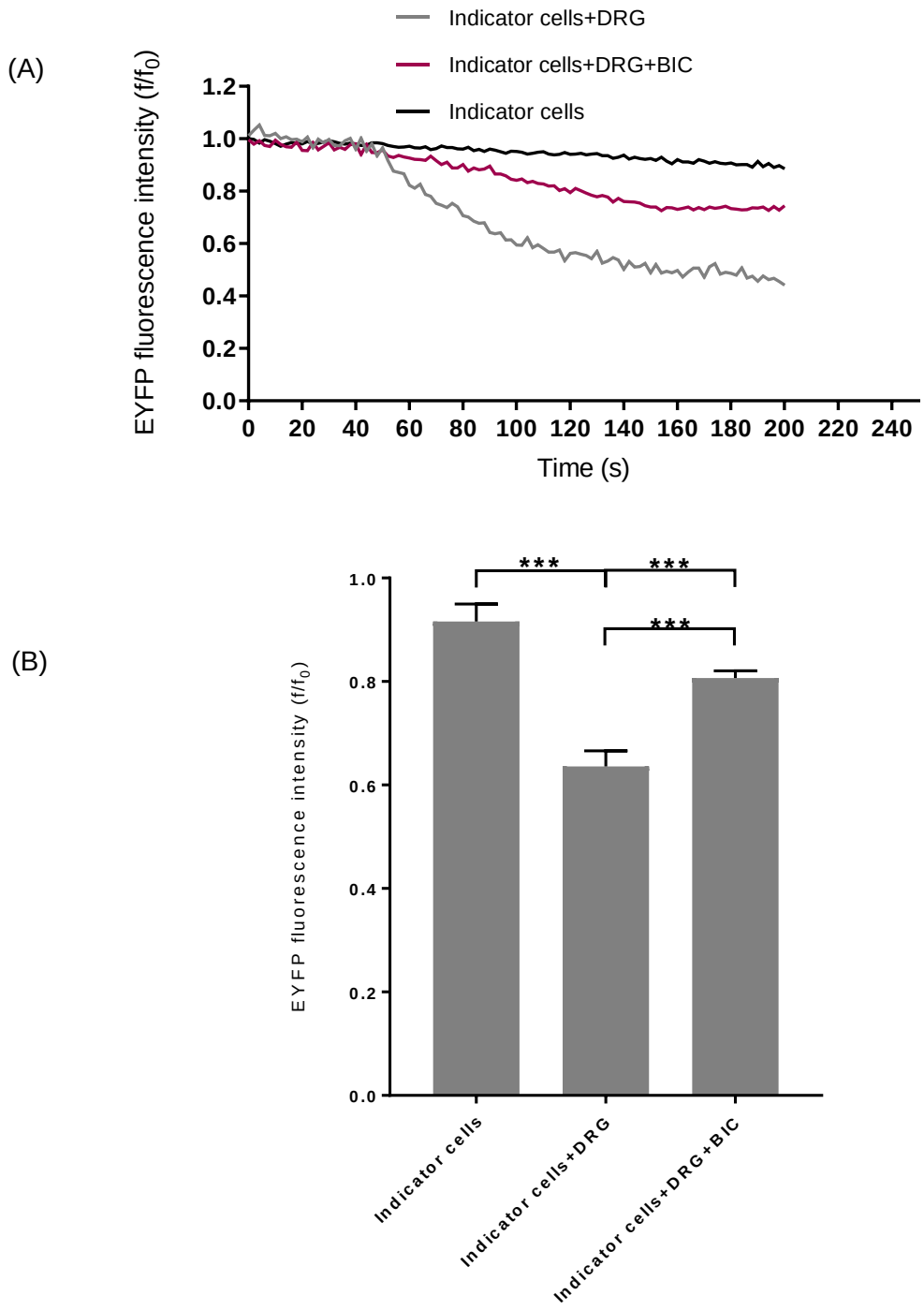


Figure 5.10 Summary of EYFP quenching in three different groups. (A) Representative traces from three group of cells tested: i) HEK_{GABAA} indicator cells in monoculture perfused with 5 mM NaI only; ii) HEK_{GABAA} indicator cells-DRG co-culture perfused with 5 mM NaI only; iii) HEK_{GABAA} cells-DRG co-culture perfused with 5 mM NaI + 50 µM bicuculline. **(B)** EYFP fluorescence intensity were analysed by calculating the mean EYFP H148Q/I152L fluorescence intensity of HEK cells per transfection (one transfection represents one biological replicate) and per rat and transfection for HEK cell-DRG neuron co-cultures. Data are presented as mean ± SEM. EYFP fluorescence intensity was significantly different among the three groups (Kruskal wallis ANOVA, $p<0.05$), [N=3, biological replicate=3, n(control)=37, n(NaI)=23, n(NaI+Bic)=52].

5.3 Discussion

5.3.1 Endogenous Cl- ion channel expressed in HEK cells

As mentioned in a previous chapter, there are other anion channels that serve as a passage for Cl^- to move into and out of the cells. These anion channels could be responsible for the minimal EYFP quenching observed in HEK_{GABAA} indicator cells single culture, where DRG neurons being the source for GABA were absent. Of particular interest are the ion channels that were reported to be endogenously expressed in HEK cells, glycine receptors (Thomas and Smart, 2005). However, without its specific ligand, the glycine neurotransmitter binding to the receptor to open the ion channel, it is very unlikely that Cl^- flux could occur in the co-culture system in this experiment. NKCC1 co-transporters which mediate the coupled movement of Na^+, K^+ and Cl^- into most mammalian cells are also endogenously expressed in HEK cells (Isenring et al., 1998). However, they demonstrate a significantly lower affinity for the cations than that expressed in other mammalian cells. Despite the low affinity to cations, they could also contribute to the minimal EYFP quenching of HEK_{GABAA} indicator cells (Isenring et al., 1998).

Nonetheless, spontaneous openings in the absence of agonist have been observed in a few cases with native receptors (Jackson, 1994), although it is more commonly seen in some mutants. Indeed spontaneous opening of glycine receptor has been reported in D97R α1 glycine receptor mutant in the presence of ethanol and volatile anaesthetics (enflurane and chloroform). Other endogenous HEK cells' Cl^- channels have also been indicated by Zhu and colleagues (Zhu et al., 1998). Via patch-clamp, they found five outward Cl^- currents that were all voltage-dependent, Ca^{2+}-independent but otherwise were distinct from each other in terms of channel properties and voltage sensitivities (Zhu et al., 1998). Volume-regulated anion channels (VRACs), another class of Cl^- channels have also been reported to be endogenous to HEK cells (Helix et al., 2003) and thus, may contribute to background activity in this experiment.

5.3.2 Spontaneous activation of $GABA_A$ receptor

GABA is a major inhibitory neurotransmitter in the CNS. Many studies of $GABA_A$ channels have been conducted on the primary sensory neurons, however, whether GABA could also be an inhibitory neurotransmitter therein still remains poorly understood with few recent studies pointing to such a possibility (Du et al., 2017, Hanack et al., 2015, Obradovic et al., 2015). In this study, we report a robust iodide uptake by the HEK_{GABAA} indicator cells in the presence but not in the absence of co-cultured DRG cells; this uptake was sensitive to $GABA_A$ inhibitor, bicuculline. These results suggest the presence of ambient GABA within the co-culture environment. Indeed, GABA is the most common agonist to activate $GABA_A$ receptors on HEK_{GABAA} cells. However, it is worth to note that GABA receptors can also undergo spontaneous activation as shown by (Birnir et al., 2000) and (O'Neill and Sylantyev, 2018). Birnir and colleagues found that 48% of the cell-attached patches on pyramidal neurons in the CA1 region of rat hippocampus were the spontaneously opening Cl^- channels; whose opening was inhibited by bicuculline and enhanced by diazepine; these results further concluded that these spontaneously opening Cl^- channels were $GABA_A$ receptors (Birnir et al., 2000). O'Neill and Sylantyev demonstrated that in the dentate gyrus when the extracellular concentration of GABA was matched to the ambient levels measured *in vivo*, the majority (~90%) of tonic inhibition occurred due to spontaneously opening $GABA_A$ receptors (s-$GABA_A$Rs) (O'Neill and Sylantyev, 2018). They distinguished the spontaneously-opening $GABA_A$ receptors from the conventional $GABA_A$ receptors as having shorter average open-time and lower opening probability albeit similar in their conductance. As s-$GABA_A$Rs do not require binding of GABA to enter an active state, they are resistant to block by GABA competitive antagonist [SR-95531 (SR)] but can be inhibited by an open-channel blocker, picrotoxin. These authors used the differences in the mechanism of action of SR [a GABA competitive antagonist (McCartney et al., 2007)] and picrotoxin (open-channel blocker) to distinguish

between GABA-dependent and GABA-independent effects of $GABA_A$ receptors activation (O'Neill and Sylantyev, 2018). Briefly, SR abolished $GABA_A$ receptor activity induced by GABA binding i.e blocking the conventional GABA-dependent $GABA_A$ receptor activation while picrotoxin bound to the inside (intracellular region) of the $GABA_A$ receptor channel blocking all $GABA_A$ receptor channels that entered active state; thus applying SR+picrotoxin blocked both the activation of conventional $GABA_A$ receptors and spontaneously-opening $GABA_A$ receptors as demonstrated in their study (O'Neill and Sylantyev, 2018). While spontaneous activity of $GABA_A$ receptors in the HEK_{GABAA} indicator cells cannot be excluded, the notion that the I^- uptake was dramatically enhanced in the presence of HEK_{GABAA}-DRG co-culture still indicates that it is the presence of the DRG-derived cells, that stimulates the exogenous $GABA_A$ channel activity in the HEK_{GABAA} indicator cells with the release of GABA from DRG neurons being the most simple explanation.

5.3.3 DBI as potential endogenous $GABA_A$ receptor activator

It is also worth to note that GABA is not the only endogenous compound that can activate $GABA_A$ receptors. The endogenous $GABA_A$ positive and negative modulators have been described in a previous chapter. Of particular interest, the diazepam binding inhibitor (DBI), is a small protein of 10 kD that binds to the benzodiazepine binding site of the $GABA_A$ receptor. DBI has been reported to be endogenously expressed in both central and peripheral nervous system; of particular interest is the expression of DBI in the satellite glia in the DRG (Karchewski et al., 2004). Expression of DBI has also been reported in the brain and spinal cord.

A more recent report on DBI expression and effects on neural stem cells showed that DBI interacts with GABA by inhibiting GABA feedback signalling in subventricular zone (SVZ) thereby promoting neurogenesis in the niche (Dumitru et al., 2017). Even though the exact mechanism of DBI action on $GABA_A$ channels is currently poorly understood, the release of DBI from satellite glia could also be considered as a

mechanism for $GABA_A$ receptor activation in this HEK_{GABAA}-DRG co-culture system. Immunohistochemical findings from our lab demonstrated that DBI was expressed in SGC but not in DRG neurons (unpublished data), which could suggest the involvement of this protein in modulating nociception at the level of DRG. Thus, targeting DBI for future experiment could help to confirm or to rule out the involvement of this protein in this DRG GABAergic signalling system.

5.3.4 Use of preweaner rat in co-culture system

5.3.4.1 Chloride homeostasis during development

As mentioned in the previous chapter, $[Cl^-]_i$ changes from developmental period to adulthood. In neonatal rat brain, it has been demonstrated that $[Cl^-]_i$ was high and only decreased significantly at the end of the first month of life (Frederikse and Kasinathan, 2015, He et al., 2014, Zhang et al., 2006). However, $[Cl^-]_i$ in the PNS is maintained above electrochemical gradient irrespective of their phenotypes and postnatal ages (Mao et al., 2012). The persistence of NKCC1 expression with the lack of expression of KCC2 in DRG neurons irrespective of postnatal age, explain why, unlike CNS neurons, DRG neurons do not exhibit $[Cl^-]_i$ shift (Mao et al., 2012). Thus, studying Cl^- flux using preweaner rats should demonstrate similar results to that using adult rats. In this experiment, DRG neurons were extracted from preweaner rats (5 – 11 days old) for the HEK cell-DRG neuron co-culture. However, it is worth to note that this experiment looked into the activity of $GABA_A$ receptors transiently transfected in HEK293 cells, where DRG neurons served as the source for GABA. Thus, the influx of Cl^- into HEK_{GABAA} indicator cells was independent of the E_{Cl^-} of the DRG neurons. The absence of $[Cl^-]_i$ changes during development (in rodents) could be useful for future potential experiments when investigating the activity of $GABA_A$ receptors in DRG neurons.

Understanding GABAergic signalling within DRG neurons would be more interesting and affirmative if recording the activity of $GABA_A$ receptors on DRG neuron could be

performed with overexpressed EYFP H148Q/I152L. This indeed was our initial aspiration, however, due to a very low transfection efficiency of post-mitotic neurons in culture (Ohki et al., 2001) including DRG, these experiments were unsuccessful.

5.3.4.2 Use of preweaner rats

Prewener rats were chosen over adult rats as they provide a better quality of DRG culture, and that culturing adult neuron requires trophic factors for neuron survival (Brewer, 1997). Although the use of growth factors such as NGF and BDNF in neuron culture enhance axonal regeneration growth of adult sensory neuron (Gnavi et al., 2018, Santos et al., 2016, Yasuda et al., 1990), these trophic factors such as BDNF interferes with GABAergic signalling pathway (Kim et al., 2017b, Xiao and Le, 2016); BDNF acts via trkB receptor which inhibits phosphorylation of GABA receptor (Jovanovic et al., 2004) reducing its conductance (Rivera et al., 2002). Thus, only preweaner rats were used for experiments involving DRG neuron culture.

Chapter 6 General Discussion

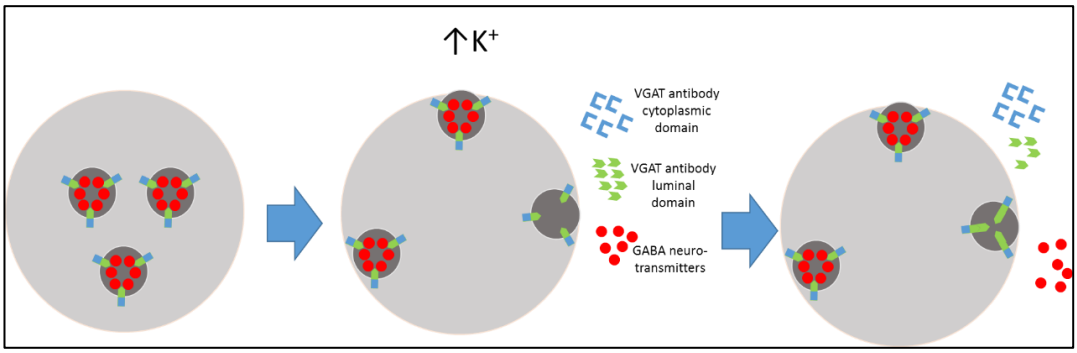
6.1 The role GABAergic mechanisms in peripheral nociceptive signalling

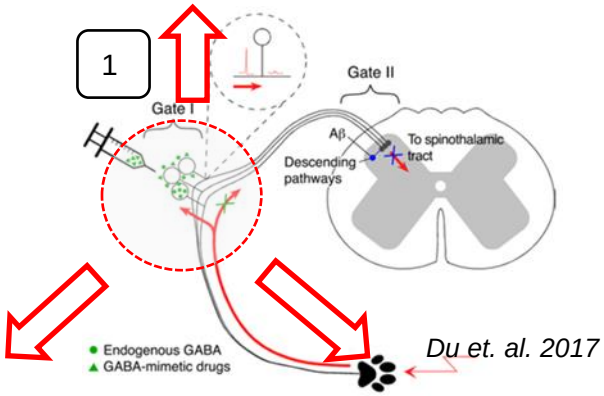
The discovery of GABA and its signalling mechanisms was made more than half a century ago. However, despite being a major inhibitory neurotransmitter in the CNS where it balances and controls the temporal fidelity of excitatory transmissions, its inhibitory role in PNS is only beginning to emerge. Our recent findings (including these presented in this thesis) demonstrate that GABA and its production, packaging and release machineries (i.e. GAD65 and GAD67, GABA transporters and VGAT) are present in the DRG. Moreover, we found a hitherto underappreciated role of GABA in controlling peripheral nociception; this was demonstrated by both *in vivo* and *in vitro* experiments conducted in rodents and HEK cells respectively. Together, our previous results (Du et al., 2017) showed that the nociceptive transmission could be gated more peripherally at the level of DRG before it reached the spinal cord. Thus, this thesis continued to explore the GABAergic signalling at the level of DRG. In Chapter 3 I explored the nature of GABAergic neurons in DRG using immunohistochemistry, I then focused on the mechanisms of GABA release that might be responsible for the resulting inhibition of the nociceptive transmission to the spinal cord and the higher centres together with a more direct *in vivo* evidence for the GABAergic gating at the DRG. I also sought after the involvement and activation of $GABA_A$ receptor by the released GABA from DRG neurons. The first objective was achieved via investigating the VGAT expression and its co-localisation with several neuronal markers in the DRG neurons (Chapter 3).The second objective was achieved via the investigation of the VGAT-positive vesicle exocytosis and by extracellular *in vivo* sensory nerve fibre recordings in adult rats (Chapter 4); and the

third objective was achieved via the fluorescence biosensor imaging of HEK_{GABAA} indicator cells (Chapter 5).

In summary, we found that i) the intracellular GABA transporter VGAT was expressed in more than half of the small and medium diameter, nociceptive DRG neurons, ii) VGAT was highly co-expressed with SV2, iii) Ca^{2+}-dependant vesicular release played a major role in GABA release in DRG neurons, iv) both tonic and phasic GABA release occurred in DRG neurons and, v) inhibition of the propagation of action potentials by both ambient and stimulated release of GABA was mediated by $GABA_A$ receptors. **Figure 6.1** depicts the schematics of protocols and findings from the immunohistochemistry, vesicular VGAT antibody uptake, halide sensor imaging and extracellular *in vivo* sensory nerve fibre recordings in adult rats from the present study supporting the findings from our previous published work.

1. Phasic GABA release





Du et. al. 2017

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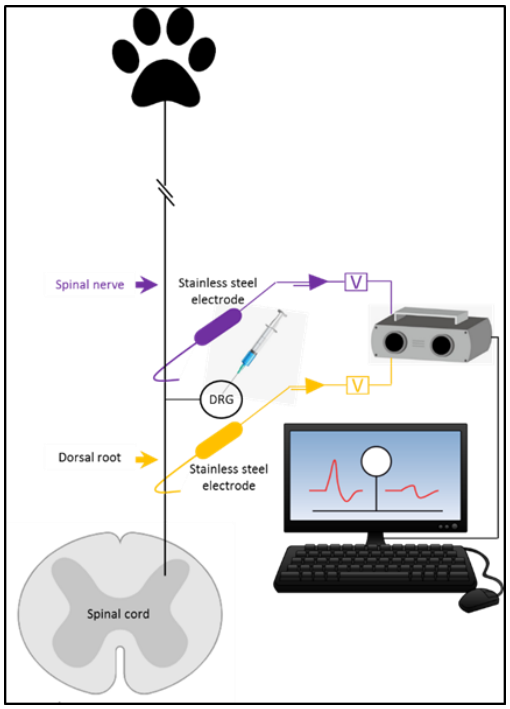
2. GABA released from DRG neurons attenuates neuronal firings from the periphery



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3. Ambient GABA in DRG activates $GABA_A$ ion channel

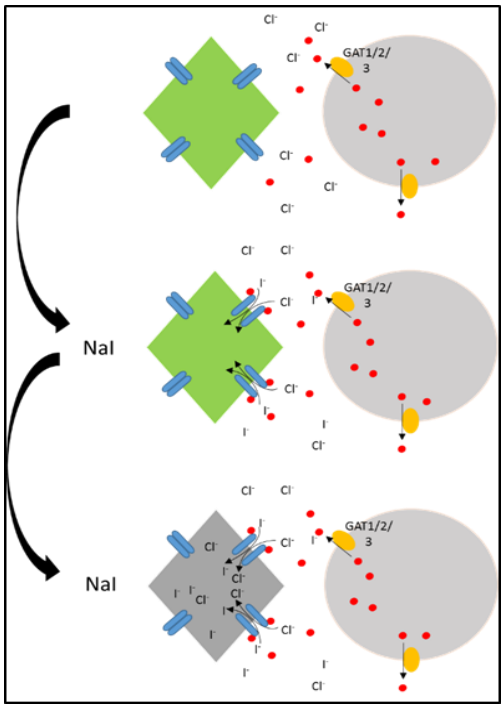

Figure 6.1 Mechanisms of GABA release and signalling in DRG neurons.

6.2 Synaptic-like vesicular release plays a crucial role in the mechanism of GABA release in DRG neurons

Neurons communicate with each other via electrical and chemical release of neurotransmitters into synaptic junction. Most neurotransmitters are released into the extracellular milieu via synaptic vesicular exocytosis. In agreement with Chaudhry and colleagues who investigated the expression of VGAT in association with synaptic vesicles in adult rat CNS (Chaudhry et al., 1998), the co-localisation of VGAT-N with the synaptic vesicle marker SV2 in the present study showed high co-expression of these two proteins in the DRG neuron somata. This finding suggests the important role of synaptic vesicles-like structure in the mechanism of GABA release in DRG neurons. It is assumed that the main neurotransmitter produced and released by the DRG neurons is glutamate (Wanaka et al., 1987), some subpopulations of DRG neurons also release peptide neurotransmitters, such as substance P and CGRP (Keast and Stephensen, 2000). The main site of the release of these neurotransmitters is the first synapse within the dorsal spinal cord. Neuropeptides are also released peripherally at the nerve ending areas in the skin and viscera in response to depolarization during a phenomenon called ‘neurogenic inflammation’ (Richardson and Vasko, 2002). Some DRG neurons also release ATP and this could happen both at the spinal synapses (Sawynok and Liu, 2003) and at the somatic level (Matsuka et al., 2008). All the above mentioned neurotransmitters are excitatory. Yet, accumulating evidence suggests that inhibitory neurotransmitters, such as GABA and glycine are also being produced by the DRG (Du et al., 2017, Rozanski et al., 2013, Schlösser et al., 2015). However how and where these are released by the peripheral neurons remain poorly understood.

The uptake of VGAT-C antibody by the live DRG neuron somata revealed that both phasic (activity-dependent) and tonic exocytosis of VGAT-positive vesicles can be detected; the exocytosis of VGAT-positive vesicles could be responsible for liberating

GABA into the extracellular milieu. Previous experiments that utilised VGAT-C were performed on different cellular targets in the brain (Chaudhry et al., 1998, Martens et al., 2008). This experiment is, to my knowledge, the first study to utilise VGAT-C to investigate the neurotransmitter vesicle exocytosis in DRG neuron somata. Our previous work has demonstrated that GABA was released from DRG neurons with a functional role in controlling the peripheral nociceptive signalling. Thus the current method suggests that GABA is released, at least partly via vesicular mechanism, similar to synaptic release.

Synaptic vesicular release of neurotransmitter requires calcium transient which in turn is dependent on the arrival of action potential and depolarisation of the neuronal membrane. However, results from the present study also found a subpopulation of DRG neurons that showed VGAT-C antibody uptake in the absence of Ca^{2+}. Electrophysiological investigations on adult rats by Zhuan Zhou group also revealed that Ca^{2+}-independent neurotransmitter secretion occurred in subpopulations of DRG neurons and that this bona fide release mechanism involved three major components: the Cav2.2 channel that functions as the voltage sensor, SNARE complex as the fusion pore and synprint as the linker that links voltage sensor with the fusion pore (Chai et al., 2017).

6.3 Potential non-vesicular release of GABA via channel-like mechanism

Neurotransmitters can be liberated from neurons not only via exocytic release from synaptic vesicle but also through channel-like mechanisms including connexin hemichannels and pannexins (Montero and Orellana, 2015), P2X7 receptors (Duan et al., 2003), bestrophins (Lee et al., 2010) and the volume-regulated anion channels (VRAC) (Kimelberg et al., 1990, Pasantes-Morales et al., 1994). Of particular interest is the VRAC which comprises heteromers with LRRC8A being the obligatory subunit, and any of at least one of the other isoforms (LRRC8B, LRRC8C, LRRC8D and

LRRC8E) (Voss et al., 2014). Although an earlier study showed no clear evidence for VRAC-dependent transport of GABA (Franco et al., 2001), a recent finding by Lutter and colleagues who investigated properties of VRAC subunits in HEK293 cells revealed that LRRC8 was involved in the transport of both inhibitory and excitatory neurotransmitters including GABA, with LRRC8D being the most permeable to GABA amongst the LRRC8 family (Lutter et al., 2017). Interestingly, preliminary results from our lab showed that VRAC isomers LRRC8A and LRRC8D are expressed in rat DRG neurons, with more than 50% co-localisation of LRRC8D with TRPV1. This co-localisation suggests that LRRC8D is expressed in subpopulation of TRPV1-positive nociceptors of primary sensory neurons which although is not directly indicative of its involvement in releasing GABA from DRG, could shed light to its role in nociception. This hypothesis requires further investigation.

6.4 Potential other GABAergic mechanisms in DRG neurons

The released GABA neurotransmitter binds to $GABA_A$ receptors which in turn can be modulated by positive and negative modulators. Benzodiazepine is one of the positive modulators that bind to extracellular domain of the $GABA_A$ receptor, between α and γ subunits. DBI, a 10 kD endogenous polypeptide that was discovered in the 80s, but physiological function of which remains largely unknown, has the ability to displace diazepam from its binding domain on the $GABA_A$ receptors and was suggested to play a role of 'endogenous benzodiazepine' in CNS (Christian et al., 2013). DBI has been shown to have a role in modulating nociception in both the peripheral (spinal cord) and CNS– an intrathecal injection and intracerebroventricular administration of DBI have been shown to increase hindpaw withdrawal latency (HWL) in adult rats (Wang et al., 2002). However, co-administration of DBI with GABA did not influence the increase in HWL in both spinal cord and brain (Wang et al., 2002) which suggests a non-GABA related antinociceptive effect of DBI in the CNS.

The function of DBI in PNS is currently unknown, but DBI mRNA has been shown to be highly expressed in the non-neuronal cells of the DRG (Karchewski et al., 2004). Recent findings from our lab (unpublished) are also in agreement with this; DBI is highly expressed in SGC surrounding DRG neuronal somata thus could be a candidate for $GABA_A$ receptor activator or modulator.

In the present study, $GABA_A$ receptor activation was observed during a co-culture of indicator HEK_{GABAA} cells and DRG cells (neurons and glia). The activation was registered by a bicuculine-sensitive iodide influx, via the quenching of halide-sensitive EYFP fluorescence. We interpreted this signal as evidence of $GABA_A$ receptor activation, presumably by a release of GABA from the DRG neurons present in co-culture, which is a logical assumption, given the wealth of other evidence pointing to GABA release from DRG. Yet, activation of $GABA_A$ receptors could have been produced by other neuromodulators existing in the co-culture system, e.g. by DBI released from the satellite glia.

The role of $GABA_B$ receptors in peripheral nociceptive signalling is also worth mention. Although in the CNS, the discovery of $GABA_B$ receptor was made decades after the discovery of $GABA_A$ receptors, the use of $GABA_B$ receptor agonists as therapeutic agents in the treatment of nervous system disorders preceded the use of $GABA_A$-related compounds. The $GABA_B$ receptor agonist baclofen, is used to treat plasticity in the multiple sclerosis (Smail et al., 2006) and spinal cord injury (Loubser et al., 1991), as well as chronic pain associated with spinal cord injury (Cardenas and Jensen, 2006) and trigeminal neuralgia (Chole et al., 2007). In light of the use of baclofen in the treatment for chronic pain, our previous study also tested the effect of local application of baclofen on bradykinin-induced nocifensive behaviour in rat DRG. Our findings showed that baclofen did reduce the nocifensive behaviour however, it was significantly less than that of GABA (Du et al., 2017). A study on the interaction of $GABA_B$ and TRPV1 receptors in transgenic mice also revealed that under

physiological condition, GABA limited TRPV1 mediated hyperalgesia (Hanack et al., 2015). Results from the present study are in agreement with these findings; neuronal firings caused by injection of capsaicin, a ligand for TRPV1 receptor, into the hindpaw of adult rat was attenuated by a local GABA application onto DRG.

6.5 The role of GABA in the progress of pain

Results from our previous study revealed that stimulation of GABA system in rat DRG relieved both acute and chronic pain (Du et al., 2017). In the mature CNS GABA acts as an inhibitory neurotransmitter, where activation of $GABA_A$ receptor results in inhibition of the generation of action potentials due to Cl^- influx-mediated hyperpolarization. Due to the different expression of Cl^- transporters NKCC1 and KCC2 in the brain than that in DRG, activation of $GABA_A$ receptor in DRG causes Cl^- efflux and depolarisation of the neuronal membrane. Moreover, release of inflammatory mediators such as NGF, bradykinin and PGE_2 further increased intracellular Cl^- concentration corresponded to the increase in NKCC1 phosphorylation (Funk et al., 2008). Thus activation of $GABA_A$ receptors during inflammation is likely to cause even greater Cl^- efflux and depolarisation.

How can we reconcile GABA-induced depolarization of the neuronal membrane with antinociceptive (hence inhibitory) action of GABA in the periphery? Studies in rat sensory neurons suggested that GABA-induced depolarisation promoted the inactivation of voltage-gated Na^+ channels (Price et al., 2009), activation of K^+ channels (Zhu et al., 2012) and neuronal membrane shunting (Price et al., 2009) resulting in decreased glutamate release hampering the nociceptive signalling to the second order neuron within the dorsal horn of the spinal cord.

Our recent study (Du et al. 2017) suggests that similar mechanism of GABAergic inhibition could exist in the DRG, but with a twist: we have suggested that in the DRG, the main inhibitory action of GABA occurs at the site of axonal bifurcation (T-junction),

a point where neurite emanating from the DRG somata splits into the peripheral and central branches. This T-junction has intrinsically low safety factor for action potential propagation (Sundt et al., 2015) due to reduced impedance at the branching point and, thus, naturally lands itself into a filtering mechanism for the action potential passing from the periphery to the dorsal root. Here, DRG somata is likely to provide a voluminous source of GABA while the T-junction is likely to be responsible for the AP failure which occurs upon activation of $GABA_A$ receptor. In the presence of painful stimulation, AP is generated and propagated from the periphery towards the spinal cord. Our previous findings showed that this AP gets filtered at the T-junction resulting in AP failure.

During chronic pain conditions, several mechanisms of reduced GABAergic signalling have been reported in rodent's peripheral sensory neuron (Moore et al., 2002, Obradovic et al., 2015) and human thalamus (Henderson et al., 2013). In nerve injury rat models, the loss of inhibition could be due to the down-regulation of GAD65 and GAD67 enzymes, essential for the synthesis of GABA (Kami et al., 2016, Moore et al., 2002) or a decrease in the expression of the α_2 subunit (Obradovic et al., 2015) which is abundantly present in DRG neurons (Ma et al., 1993). Together, findings from these studies highlighted the important role GABAergic machinery plays during acute and chronic pain conditions, providing new avenues for the treatment of chronic pain.

6.6 GABAergic signalling in peripheral nociceptive pathway: future hope for pain management

Our pioneer publication on the functional GABAergic nociceptive signalling in primary sensory neurons has shed light on the nociception gating system that could be modulated more locally and peripherally at the level of DRG. Over the years, the treatment for chronic pain has evolved from general administration of drugs to a more localised drug application. The focus on more localised drug delivery ensures a more

precise management of chronic pain which minimises the side effects of generally administered drugs.

The management of chronic pain requires a multidisciplinary approach. This includes psychological, physiological, pharmacological as well as non-pharmacological treatment that will ensure a holistic management and better prognosis of pain improvement. The pharmacological component by itself is crucial especially in giving an immediate relief or at least lessening the pain a patient is suffering from. Thus it is imperative to develop drugs that could target and give therapeutic relief more locally for a more immediate pain relief. Due to the increasing number of individuals suffering from chronic pain worldwide, and the multifaceted problems it poses specifically to the patient and generally to the society and the government, it is of urgent need that a better treatment is developed and made accessible.

Pharmacological treatment targeting DRG is not new, a review by Krames (Krames, 2015) on targeting DRG for pain relief reported different techniques that have been targeted on the DRG to treat chronic pain: transforaminal epidural steroid injection for the treatment of lumbosacral neuropathy, pulse radiofrequency for spinal pain, ganglionectomy for occipital neuralgia and neuromodulation for chronic neuropathic pain (Krames, 2015). Except for the pain that comes from the orofacial region (which information relays in the trigeminal ganglion), DRG is the relay centre for almost all sources of pain: the cutaneous, visceral, muscular and the joint-related pains. Thus targeting DRG for the treatment of chronic pain would be of utmost relevance for this purpose. Providing more evidences via further investigations involving species of higher taxonomic hierarchy would further confirm this promising potential of GABA in the treatment of chronic pain.

Several GABA-related targets could be considered for therapeutic interventions at the DRG level. Thus, GAT1 inhibitor (tiagabine) which is used to treat epilepsy, has been shown to inhibit excitatory neurotransmitter release in the spinal cord via a

GABAergic mechanisms exerting antinociceptive effect at the spinal cord level (Daemen et al., 2008, Smith et al., 2007). Another GABAergic machinery that could be targeted to treat chronic pain is VGAT. In the present study, VGAT has been shown to be expressed in DRG neurons suggesting its role in GABAergic signalling in the peripheral nociceptive pathway. In our previous and the present studies, VGAT level was not assessed during acute and chronic pain conditions. However, VGAT has been shown to decrease in different animal models of epilepsy. Investigating VGAT level in different pain conditions could be useful in determining its significance in chronic pain which if proven, could be a candidate for therapeutic target for this condition. Different targets within the GABAergic signalling pathway with potential therapeutic benefit are shown in **Figure 6.2**.

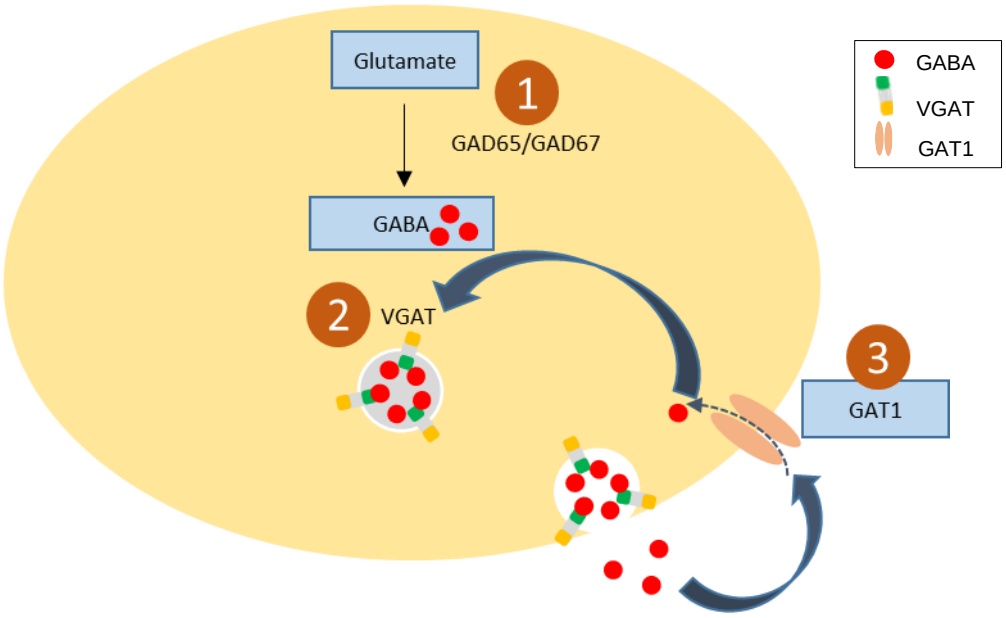

Figure 6.2 Three potential therapeutic targets within GABAergic signalling pathway in DRG neuron. The inhibitory action of GABA in DRG could be potentially enhanced by increasing GABA concentration in the extracellular environment, which could be achieved in three ways; (1) by increasing the activities of GAD65 and GAD67, the enzymes for GABA synthesis, (2) by increasing the activity of VGAT, the transporter that transports synthesised GABA into the synaptic vesicle, or (3) by inhibiting GAT1, a GABA transporter which removes GABA from the extracellular environment.

6.7 Experimental problems, alternative strategies and future directions

6.7.1 Iodide binding assay to evaluate GABA release and binding

The result for the somatic GABA release were conducted on a culture system with $GABA_A$ receptor subunits (α_1, β_2 and γ_2) transfected into HEK cells to simulate the *in vivo* DRG environment. The results of these study could be made more accurate by accessing and recording responses directly from the DRG neurons. Attempts to transfect DRG neurons with fluorescent protein using different methods; nucleofection method using Amaxa™ Rat Neuron Nucleofector™ Kit and BacMam system (transforming Premo™ Halide Sensor into DRG using *baculovirus*) had come to no avail. The failure could be due to the vulnerability of DRG neurons; when transfecting the DRG neurons using electroporation method, very few cells survived (less than 3/coverslips) and even those neurons with successful transfection were only weakly fluorescent and displayed significant rundown of fluorescence, making them unsuitable for accurate measurements. Similar difficulties were experienced with BacMam delivery system albeit to a lesser degree. BacMam-mediated gene delivery system reported by Levin and colleagues (Levin et al., 2016) was reported to produce high transduction efficiency (~80%) in both juvenile and adult rat neurons, however our lab could not obtain similar results using a similar gene delivery system (Premo™ Halide Sensor #P10229, Thermo Fisher Scientific, Life technologies Corporation, Oregon, USA). Our low transduction efficiency could be due to the different BacMam preparation used. Levin and colleagues generated the recombinant *baculovirus,* and its titres were determined before transducing it into DRG neurons (Levin et al., 2016). In my experiment, a readymade BacMam gene delivery system was used; thus the *baculovirus* titre could not be determined before performing the transduction. Thus, my failure of obtaining high transduction efficiency could be attributed to low *baculovirus* titre used for the gene delivery into the DRG neurons. The fact that the DRG neurons are post-mitotic cells also imposes a

challenge during the transfection as even if a neuron is successfully transfected with an exogenous cDNA, the number of transfected cells will not increase during incubation. Increasing the number of DRG neurons for every transfection could be one of the ways that could be considered when using this transfection method.

6.7.2 Different composition of $GABA_A$ receptor subunits

In iodide binding assay, $\alpha_1\beta_2\gamma_2$ combination of $GABA_A$ receptor subunits was chosen to study the $GABA_A$ receptor activation. This combination is indeed the most common $GABA_A$ receptor subunit composition found in the brain (Sieghart and Sperk, 2002). As our previous work on sniffer patch-clamp used this combination, we used the same subunit combination to ensure the data obtained was comparable to the findings from previous work. However it has been reported that the $\alpha_2\beta_3\gamma_2$ subunit composition is preferentially expressed in rat DRG neurons (Ma et al., 1993), thus, in order to better mimic the DRG responses, different indicator HEK cells overexpressing $\alpha_2\beta_3\gamma_2$ subunits can be used in the future. A study comparing kinetics between the α_1 and α_2 subunits on HEK cells demonstrated that α_2-containing receptors open twice as fast, with the current decay six to seven times slower than that of α_1-containing receptors (Lavoie et al., 1997). Silencing α_2 subunit of $GABA_A$ receptors in rat DRG has also been shown to effectively ameliorate neuropathic pain in sciatic-crush-pain model (Obradovic et al., 2015). Thus, comparing the kinetics of these α_1 and α_2 subunits in DRG neurons could be helpful and important in targeting a potent $GABA_A$ subunit with promising therapeutic benefit.

6.7.3 In vivo electrophysiological recordings

In this thesis, the *in vivo* recording involved anaesthetising the rat with pentobarbital via intraperitoneal injection to allow for the humane surgical exposure to be carried out on the rats. While this method provides 'no or less pain' to the rats, it could also affect the GABAergic signalling. As mentioned in a previous chapter, depending on its concentration, pentobarbital can potentiate, activate or block $GABA_A$ receptors (Muroi et al., 2009). Thus, the responses elicited from the *in vivo* recordings could be contributed by the pentobarbital injected intraperitoneally to achieve anaesthesia. To avoid the use of anaesthesia, the electrophysiological recordings could be carried out on a decerebrate animal. In decerebrate animal, the cerebrum is removed thus the animal is free from pain and distress (Silverman et al., 2005) avoiding the use of anaesthesia.

6.7.4 Single fibre recording using microneurography

The electrophysiological *in vivo* recordings conducted in the current project were obtained from the whole dorsal root and spinal nerve fibres. Thus the neuronal firings recorded were a combination of currents travelling via different types of somatosensory fibres; both A and C fibres. In this respect, microneurography allows an *in vivo* recordings of single-unit action potentials from different types of myelinated and unmyelinated nerve fibres. Using this method, prolonged simultaneous recordings can be performed on several functional fibres such as the C fibres (Serra et al., 2010). Data obtained from those recordings could be used to evaluate the effect of drug treatments in physiological and pathophysiological conditions.

6.7.5 GABAergic signalling in chronic pain conditions

In this thesis, experiments on GABA were done on acute inflammatory pain conditions. While acute pain is a physiological body response to noxious stimuli and gradually resolves within several days as the injured tissue heals, chronic pain goes

beyond the normally expected course relative to the stimulus. Thus it is imperative to also evaluate the effect of GABA on chronic pain conditions. Performing the *in vivo* electrophysiological recording on a chronic pain model such as chronic constriction injury and CFA could determine and support our previous work and results in the current thesis on the role of GABA in chronic pain.

6.7.6 Potential therapeutic targeting

The current thesis only focuses on the nociceptive fibres in DRG. Yet it is well known that DRG contain not only nociceptive fibres, but also others carrying sensations for noxious heat and noxious cold, innocuous mechanical and proprioception. Whilst the results in this thesis further support the role of GABA in controlling nociception at the DRG level, the effect of GABA on fibres carrying the other sensory modalities still remains elusive. As mentioned earlier in Chapter 1, the different sensory modalities such as touch and proprioception carried by primary afferent fibres can be gratifying and help the body to ‘communicate’ with the external environment. Thus, it is crucial to ensure that the action of GABA is nociception-specific and not compromising the other sensory modalities carried by their respective sensory fibres that are present in the DRG. Further investigations are deemed important to determine and rule out the effect of GABA on other sensory modalities before its antinociceptive action can therapeutically be benefited.

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