

Neuronal control of cardiac excitability in pro-hypertensive states



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Abstract

Hypertension is associated with marked cardiac sympathetic over-activity and end organ hyper-responsiveness. The sympathetic dysfunction is caused by aberrant calcium (Ca^{2+}) handling resulting in enhanced neurotransmission. However, it remains unclear whether the sympathetic neuron or the myocytes is the primary driver behind the initiation and maintenance of the autonomic phenotype. The work in this thesis characterises the Ca^{2+} dysfunction and regulation at the membrane level. Further, it employs physiologically coupled sympathetic neurons and ventricular myocytes to determine the cellular driver of cardiac dysautonomia in the pro-hypertensive state.

Chapter 1 provides a general overview of the field of autonomic hypertension with a specific focus on the sympathetic control of cardiac excitability. In particular, the role of Ca^{2+} and cyclic nucleotides in the facilitation of neurotransmission are explored.

Chapter 2 details the methods used in this thesis. It provides rationale for the approaches taken to record membrane Ca^{2+} currents, cyclic adenosine monophosphate (cAMP) levels and cAMP-activated protein kinase (PKA) activity, and the development and uses of a co-culture of coupled sympathetic neurons and ventricular myocytes.

Chapter 3 describes the successful development of an effective voltage clamp method to isolate whole cell Ca^{2+} currents in sympathetic neurons. It details the issue of space clamp problem when using this technique on peripheral neurons and provides experimental guidance on how to quantify and limit these issues.

Chapter 4 identifies that the pro-hypertensive four-week old neurons from the spontaneously hypertensive rat (SHR) have significantly larger whole cell Ca^{2+} currents when compared to normotensive (Wistar Kyoto-WKY) neurons, that are largely N-type in nature. Restoring the cGMP cyclic nucleotide dysfunction seen in these cells, rescues the ion channel phenotype and bring the Ca^{2+} down to levels seen in the normotensive WKY neuron. Further, it identifies that phosphodiesterase (PDE) 2A inhibition differentially affects the currents in the WKY and SHR, further supporting the notion of PDE2A dominance.

Chapter 5 identifies the presence and functional relevance of cGMP cross-talk with the cAMP-PKA pathway in sympathetic neurons. This cross talk is significantly altered in the pro-hypertensive state, via the differential involvement of PDEs. It functionally identifies the presence of PDE3 and PDE2A and provides further evidence that these enzymes could be dysregulated in pro-hypertensive neurons.

Chapter 6 describes the use of a co-culture model of ventricular myocytes and sympathetic neurons. Physiological stimulation of the sympathetic neuron with nicotine whilst monitoring cAMP levels in the myocytes confirms that the cellular phenotypes seen in the individual cells are functionally present in the co-culture. Using cross-cultures, it identifies the neuron as the principal driver behind the cardiac sympathetic responses observed in pro-hypertension. The results provide evidence for a dominant role played by the neuron in driving the adrenergic phenotype seen in cardiovascular disease and highlights the potential of using healthy neurons to turn down the gain of neurotransmission, akin to a smart pre-synaptic β -blocker.

Chapter 7 forms the concluding discussion that summarises the main findings of this thesis and attempt to place it in a clinical context, and highlights avenues of further research. In particular, the possibility of using a cell therapeutic approach to treat sympathetic hyperactivity.

Awards and Prizes arising from this thesis

NCARnation award 2016 for best oral presentation given at the NCAR (Neural Control and Autonomic Regulation) session at Experimental Biology 2016, San Diego, USA

Pfizer prize 2014 for the best oral presentation given by an affiliate member at the Physiological Society 2015 annual conference: Physiology 2014, London, UK.

Best oral presentation IUPS (International Union of Physiological Sciences) International Early Careers Symposium, 2013.

Physiological society travel awards in 2013 and 2015

Wellcome Trust 4-year D.Phil award. OXION, University of Oxford 2011-2015

The Graduate Science Scholar at St Catherine's College 2011-2014

Awarded place at the 30th Microelectrode techniques for cell physiology, Plymouth 2013.

Publications arising from this thesis

Journal articles:

Larsen, HE., Lefkimiatis, K., Paterson, DJ. Sympathetic neurons are a powerful driver of myocyte function in cardiovascular disease.
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Larsen, HE., Bardsley, EN, Lefkimiatis, K., Paterson, DJ. Dysregulation of neuronal Ca²⁺ channel, linked to impaired cyclic nucleotide signalling contributes to the heightened sympathetic transmission in hypertension.
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Bardsley EN., **Larsen HE.**, Paterson, DJ.
Impaired cAMP-cGMP cross-talk during cardiac sympathetic dysautonomia.
Channels, 2016 Nov 11:1-3

Burton, RAB*, **Larsen, HE***., Bilton, S, Stephens, G, Li, D, Schmidt, C, Kramer, H, Robinson, C, Paterson, D.J, Bub, G. Spatio-temporal changes in cardiac cultures in response to extracellular cues. * joint first authorship.
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To be submitted

Lu C-J, Hao, G. Nikiforova, N. **Larsen, H.E.**, Liu, K., Cabtree, M.J., Li, D., Herring, H., & Paterson, D.J. (2015). CAPON modulates neuronal calcium handling and cardiac sympathetic neurotransmission during dysautonomia in hypertension.
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Larsen, HE, Lefkimiatis, K., Paterson, DJ. Dysregulation of Cardiac cAMP in Nicotine Stimulated Sympathetic Neuronal-Myocyte Co-Cultures from Hypertensive Rats: Are Sympathetic Neurons the Primary Driver of Autonomic Hypertension? Experimental Biology 2016, San Diego, USA. *The FASEB Journal* vol. 30 no. 1 Supplement 1006.7

Larsen, HE., Mann, Ed., Paterson, DJ. A novel neuronal channelopathy could provide the trigger for Ca²⁺ dysregulation and sympathetic impairment seen in the pro-hypertensive rat. International Society for Autonomic Neuroscience (ISAN) annual meeting 2015, Italy. *Autonomic Neuroscience: Basic and Clinical* 192 (2015) 73.

Kalla, M., Bub, G., Burton, RAB., **Larsen, HE.**, Paterson, DJ., Herring, N. The sympathetic co-transmitter Neuropeptide Y is a novel pro-arrhythmic trigger even in the

presence of maximal beta-blockade. International Society for Autonomic Neuroscience (ISAN) annual meeting 2015, Italy. *Autonomic Neuroscience: Basic and Clinical* 192 (2015) 74

Burton, RAB., Tomek, J., Aslam, S., Bilton, S., **Larsen, HE.**, Sharkey, A., Stephens, G., Mann E., Paterson, DJ., Bub, G. Sympathetic stellate neurons increases cardiac conduction velocity and alters wave patterns in a myocyte-neuron co-culture model system. International Society for Autonomic Neuroscience (ISAN) annual meeting 2015, Italy. *Autonomic Neuroscience: Basic and Clinical* 192 (2015) 72-73.

Hao, G.*, **Larsen, HE.***, Liu, K., Li, D., Mann, E., Paterson, DJ. Enhanced neuronal calcium conductance in cardiac stellate neurons from young pro-hypertensive rats: A neuronal channelopathy? *joint first authorship. Physiology 2014 London, UK & Channels and Transporters meeting Erice, Italy 2014. *Proc Physiol Soc* 31.

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Burton, RAB, Stephens, G, Sharkey, A, Bilton, S, **Larsen, HE**, Kramer, H, Schmidt, C, Molina, C, Li, D, Mirams, G, Robinson, C, Paterson, DJ, Bub, G. Spatiotemporal transitions in cardiac neuronal co-cultures. *Biophysical Journal*, 2014/106(2):630a.

Burton, RAB, Schmidt, C, Lu, C, **Larsen, HE**, et al. Label-free proteomic analysis uncovers early molecular link to abnormal cardiac neurotransmission in pro-hypertension rats. 2014 *The FASEB Journal*. vol 28 no1 supplement 856.2

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Larsen, HE, Burton, RAB, Stephens, G, Bilton, S, Sharkey, A, Bub, G, Mann, E, Paterson, DJ. Developing methods for electrophysiological assessment of cell-cell communication between cardiac myocytes and neurons using co-culture model systems. The International Union of Physiological Sciences (IUPS). *Proceedings of the Physiological Society* 2013

Burton, RAB, Bilton, S, Stephens, G, **Larsen, HE**, Li, D, Paterson, DJ, Bub, G. High speed macro imaging of co-cultures. IUPS 2013. *Proceedings of the Physiological Society* 2013

Additionally, various posters have been presented at the Wellcome Trust, OXION, Oxford Medical Science, BHF centre of excellence events in the years 2012-2015.

Invited oral presentations

“Using FRET to investigate neuronal-cardiac signalling dynamics: Identifying the cardiac sympathetic neurons as the driver behind autonomic hypertension”

University of California, Los Angeles UCLA autonomic neuroscience group meeting, 2016

“Dysregulation of Cardiac cAMP in Nicotine Stimulated Sympathetic Neuronal-Myocyte Co-Cultures from Hypertensive Rats: Are Sympathetic Neurons the Primary Driver of Autonomic Hypertension?”

Experimental Biology, Data NCARNation, San Diego, 2016

Talk awarded the NCARNation award 2016

“A novel neuronal channelopathy could provide the trigger for Ca²⁺ dysregulation and sympathetic impairment seen in hypertension”

University of Oxford Medical Sciences D.Phil day, 2015

Talk awarded second place

“Investigating the role of the voltage gated Ca²⁺ channel in the sympathetic impairment seen in pre-hypertension”

University of Oxford, Burdon Sanderson Cardiac Research Centre for excellence, speed talks 2015

“Neuronal control of the heart”

STEM outreach talk Bishop Douglas School, London 2015

Resulted in on-the-spot job offer

“Enhanced neuronal Ca²⁺ current in cardiac stellate neurons from young pro-hypertensive rats: a neuronal channelopathy?”

Physiology society annual meeting, London 2014.

Talk awarded the 2014 Pfizer Prize

“Neuronal control of cardiac excitability in hypertension - A multi model approach”

St Catherine’s College Biomedical Society, 2014

“Developing methods for electrophysiological assessment of cell – cell communication between cardiac myocytes and neurons using a co-culture system”

International Union of Physiological Sciences (IUPS), Birmingham 2013. International Early Careers Symposium

Talk awarded best oral presentation

OXION mini seminars 2012-14

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Statement of contribution to this thesis

Whilst the majority of the experimental design, execution, analysis and interpretation was done by me I would like to acknowledge the help and contribution made by others.

Chapter 2: The creation of the co-culture was initiated by Prof Paterson, Dr Burton and Dr Bub, but was developed jointly by Dr Burton and I during the first year of my D.Phil. Figure 2.3A and 2.4 were created by Dr Burton. The gold electron microscopy work was conducted by Dr Burton (figure 2.6).

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List of abbreviations

AC	=	Adenylate Cyclase
Ach	=	Acetylcholine
nAChR	=	Nicotinic acetylcholine Receptor
ATP	=	Adenosine TriPhosphate
BR	=	Beat Rate
BP	=	Blood pressure
cAMP	=	cyclic Adenosine Mono Phosphate
cGMP	=	cyclic Guanosine Mono Phosphate
CN	=	Cyclic nucleotide
EGTA	=	Ethylene Glycol Tetraacetic Acid
FBS	=	Fetal Bovine Serum
FRET	=	Forster Resonance Energy Transfer
GC	=	Guanylate Cyclase
HBSS	=	Hanks Balanced Salt Solution
HF	=	Heart Failure
IBMX	=	3-isobutyl-1-methylxanthine
MAO	=	Monoamine oxidase
NA	=	Noradrenaline
NCX	=	Na ⁺ /Ca ²⁺ exchanger
NET	=	Noradrenaline reuptake transporter
NGF	=	Nerve Growth Factor
nNOS	=	neuronal Nitric Oxide Synthase
NO	=	Nitric Oxide
PBS	=	Phosphate Buffered Saline
PDE	=	Phosphodiesterase
PGSN	=	Post ganglionic stellate neuron
PKA	=	Protein Kinase A
PKG	=	Protein Kinase G
PLB	=	Phospholamban
SD	=	Sprague Dawley
SERCA	=	Sarcoplasmic Reticulum Ca ²⁺ -ATPase
sGC	=	soluble Guanylyl Cyclase
SHR	=	Spontaneously Hypertensive Rats
SR	=	Sarcoplasmic Reticulum
TEA	=	Tetraethylammonium
TH	=	Tyrosine Hydroxylase
VGCC	=	Voltage Gated Ca ²⁺ channel
WKY	=	Wistar Kyoto
RC	=	Resistor-Capacitor
RMP	=	Resting membrane potential
pA	=	picoAmpere
pF	=	picoFarad
mV	=	millivolts
FRET	=	Förster resonance energy transfer
8b-cGMP	=	8-bromo-cGMP

CHAPTER ONE

1 General introduction

1.1 Hypertension

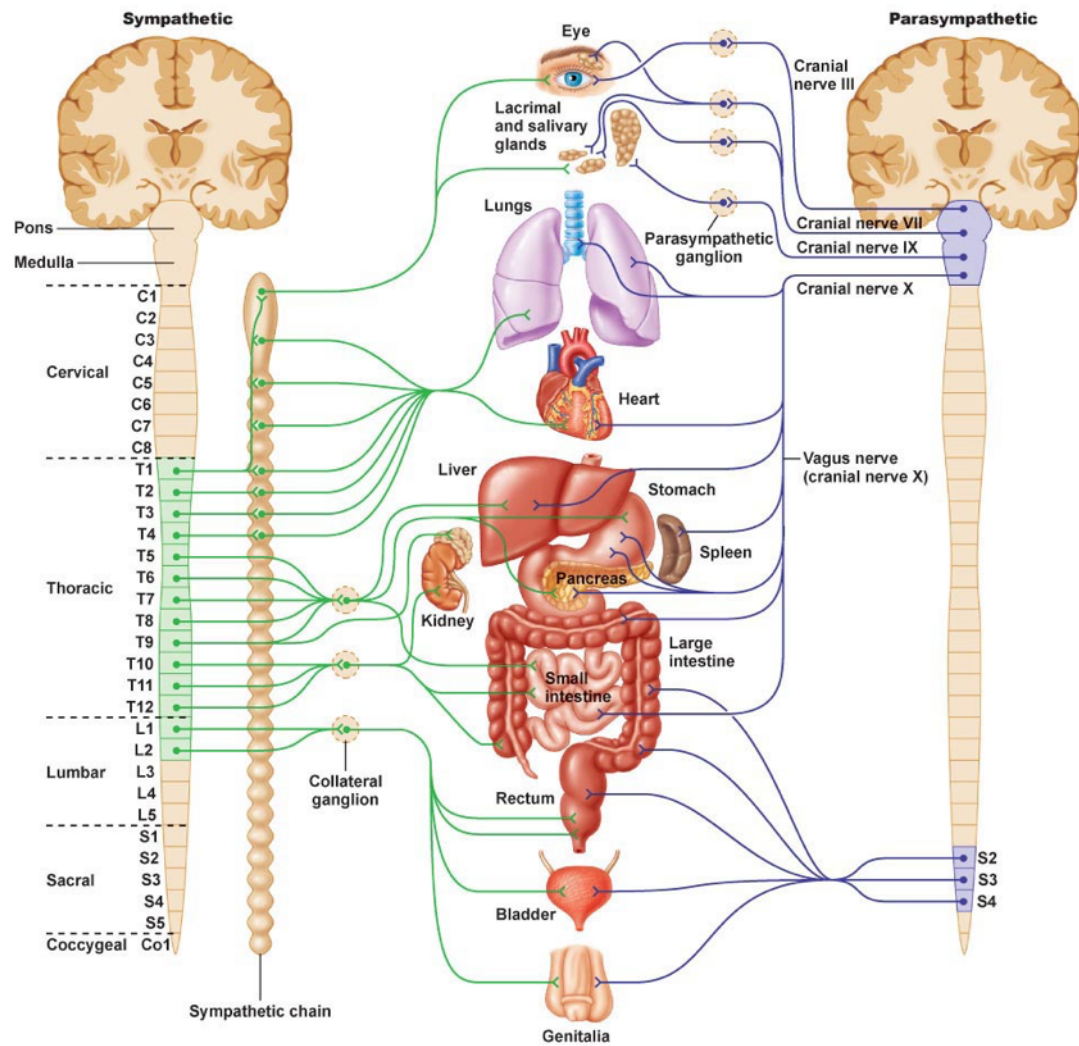
A normal blood pressure ensures appropriate perfusion of organs in the body and maintains their function. High blood pressure (hypertension), defined as a maintained systolic/diastolic blood pressure of $\geq 140/90$ mmHg, is a major health problem in today's society and a significant burden on healthcare systems globally. In 2008 a World Health Organisation (WHO) report announced that 40% of people over the age of 25 had hypertension – a number that has increased significantly in the years that followed. Hypertension often presents itself as asymptomatic, leading to many people going undiagnosed. This is a significant problem as hypertension is a major predisposing condition for other cardiovascular disorders such as arrhythmias, stroke and myocardial infarctions, that often prove lethal ¹⁻³. In fact, hypertension caused 7.5 million deaths world wide in 2013 – that is 12.8% of total deaths ³. As such, hypertension represents a significant burden both to individuals and the economy of today's society – a fact that is reflected in the increasing scientific study of the condition.

Hypertension is a multi-organ disease that involves the vasculature, kidneys and nervous system ⁴⁻⁶. In contrast to secondary hypertension where a direct causal factor can be found, essential hypertension accounts for the vast majority (~95%) of hypertensive cases ⁷ and has puzzled the medical community for centuries due to its unclear aetiology. However, early clinical work demonstrated the clinical benefits of sympathectomy in the treatment of hypertension ⁸ suggesting the autonomic nervous system played a more prominent role than previously thought ⁹. Recently the role of the

autonomic nervous system in the pathophysiology of essential hypertension has gained much attention. Its dysfunction is now accepted as an early hallmark of the disease^{2,7,10-12} and it is increasingly being recognised as a causative factor due to its occurrence prior to the onset of clinical symptoms^{13,14}. Hypertension is associated with significant sympathetic hyperactivity and vagal impairment leading to end organ hyper-responsiveness¹³. This sympathetic hyperactivity predisposes patients to significant organ damage² lethal arrhythmias¹⁵ and is associated with increased mortality¹⁶⁻¹⁸. This thesis centres around the sympathetic control of the heart in hypertension. In particular, the molecular aspects that contribute to sympathetic hyperactivity observed in the post ganglionic stellate neurons (PGSNs) innervating the heart.

1.2 Autonomic control of the the heart

The heart is able to sustain its own beating and strictly speaking does not require any input from the brain. However, it is strongly modulated by the autonomic nervous system (ANS) which is critically important for its normal function and its ability to respond to daily changes in demand (see figure 1.1). The ANS is divided into the sympathetic and parasympathetic branches which generally have opposing effects on the heart (see figure 1.2). In short, stimulation of the sympathetic branch results in the release of noradrenaline which stimulates cardiomyocytes via its action on the β_1 -adrenergic receptors. In contrast, the parasympathetic vagus nerve lowers the innate rate of the pace making cells and exerts a β -blocking effect to lower the heart rate⁴ (see figure 1.3). Physiological changes in heart rate and blood pressure (BP) are achieved by reciprocal changes in both the parasympathetic and sympathetic neural activity acting directly at the level of the heart but also through feedback mechanisms involving the baroreflex, and the kidneys.



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Figure 1.1: The autonomic nervous system. The sympathetic and parasympathetic branches of the autonomic nervous system work in conjunction (often with opposite effects) to finely tune the autonomic input and control of the organs in the body. The sympathetic neurons that predominantly innervate the heart reside in the thoracic region (T1 to T4) of the spinal column. Figure is reproduced from <https://droualb.faculty.mjc.edu>

1.2.1 The autonomic balance

A delicate balance exists between the sympathetic and parasympathetic branches of the ANS that allows the heart to adapt to everyday changes in demand. Many cardiovascular diseases are associated with an altered autonomic balance, often manifested in increased sympathetic innervation and/or activity and vagal withdrawal and/or impairment^{1,19,20}. Moreover, following insults to the heart such as myocardial infarction and ischaemia the re-innervation that occurs is often disordered and unbalanced²¹. Such neuronal remodelling and regional hyper-innervation has been linked to the generation of arrhythmias and sudden cardiac death^{1,15,22}.

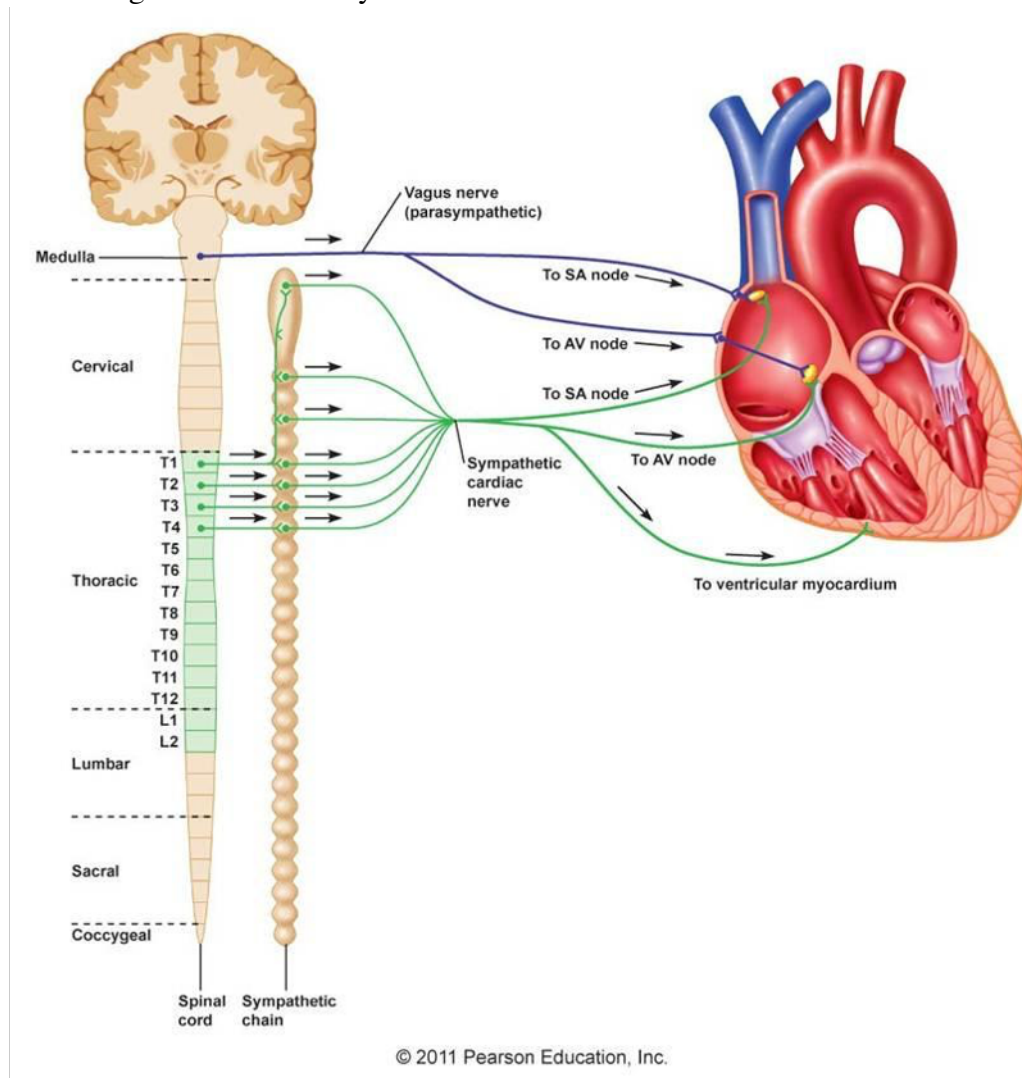


Figure 1.2: Autonomic innervation of the heart. The sympathetic ganglia extend from the spinal cord (T1-T4) and extend to richly innervate the atria and ventricles. They work in conjunction with the vagus nerve to finely tune cardiac responses. Figure is reproduced from <https://droualb.faculty.mjc.edu>

1.3 The sympathetic nervous system and its control of blood pressure

The sympathetic nerves that innervate the heart are composed of preganglionic neurons that are located in the intermediolateral columns of the grey matter and extend through the upper thoracic segments (T1-T4) of the spinal cord (see figure 1.2 and 1.3). The neurons pass through the thoracic sympathetic ganglia and ascend via the neck to the paravertebral sympathetic chain where they synapse onto post ganglionic nerves. These neurons converge to form the post ganglionic stellate neurons (PGSNs) that directly innervate the heart. In the sympathetic nervous system, the activation of the pre ganglionic cell results in the release of acetylcholine which activates the post ganglionic neuron (PGSN) via the nicotinic acetylcholine receptor (nAChR). The activated PGSN releases a range of neurotransmitters including neuropeptides, ATP (adenosine triphosphate) and of particular relevance to this thesis, noradrenaline (NA)^{4,19}.

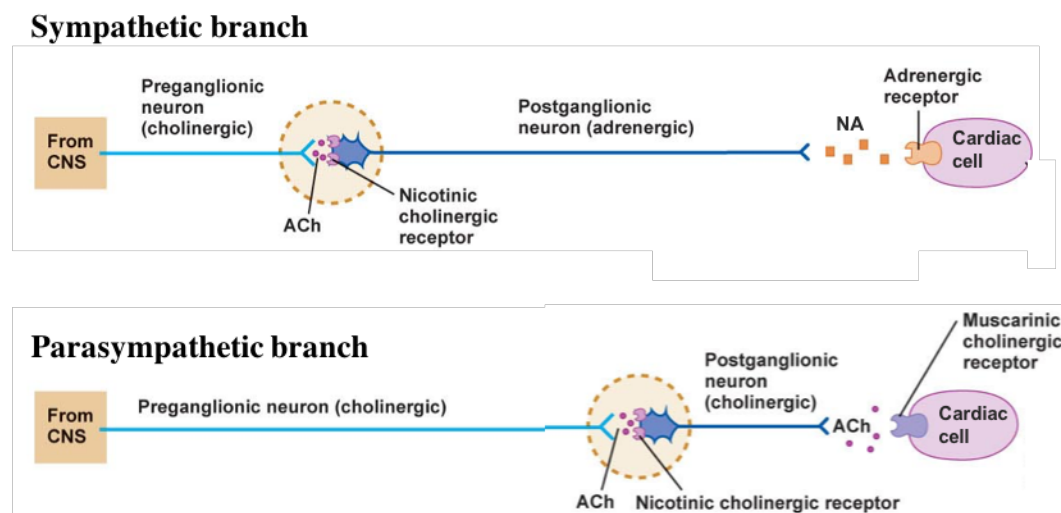


Figure 1.3: Sympathetic and parasympathetic neurotransmission. Two branches of the autonomic nervous system work in conjunction (often with opposite effects) to finely tune the autonomic input and control of the organs in the body. At the level of the heart, the preganglionic cells release acetylcholine (ACh) that binds to the nicotinic Acetylcholine receptor on the postganglionic neurons, resulting in its activation. In the sympathetic branch this activation results in the release of noradrenaline (NA) which acts on the heart via the adrenoreceptors to stimulate the heart. Conversely the activation of the postganglionic parasympathetic neuron results in further release of ACh. Its action on muscarinic cholinergic receptors results in inhibition of the heart.

Whilst BP fluctuates significantly in response to changes in behaviour, the 24 hour average BP remains fairly constant²³. BP is a function of vascular resistance and cardiac output, the latter of which is dependent on heart rate, end diastolic volume and cardiac contractility. These parameters are all controlled by the sympathetic (and parasympathetic) nervous system, which on a short time scale (seconds to hours), adjusts these parameters to cope with daily change in demand. These behaviour-related changes in BP are facilitated and modulated by the baroreflex. Further control is achieved from the thermo-, chemo- and gluco-sensitive afferents that sense and respond to central and peripheral changes in chemical and thermal states^{4,7,23,24}.

1.4 Sympathetic neurotransmission

1.4.1 Sympathetic neuron activation and the release of noradrenaline

The activation of the PGSN via the nAChR receptor results in membrane depolarisation and the influx of Ca^{2+} from voltage gated ion channels and intracellular stores²⁵⁻²⁷. The resulting Ca^{2+} elevation triggers the exocytotic machinery in the neuron^{28,29} (see figure 1.4). Following noradrenaline (NA) synthesis, NA is transported into vesicles that cluster near the active zone of the synaptic terminal. The vesicle are docked and primed for release, awaiting the triggering signal. Rapid increases in Ca^{2+} following neuronal activation triggers Ca^{2+} dependent fusion of the vesicles with the membrane, and pore opening, resulting in noradrenaline NA release into the synaptic cleft³⁰⁻³². The molecular mechanisms that facilitate this highly controlled membrane fusion are complex, but involves the binding of membrane and vesicle proteins that create the SNARE complex. Vesicular SNARE proteins (i.e. synaptobrevin) bind to target membrane SNAREs (syntaxin and SNAP-25) to pull the membranes together and induce membrane fusion^{30,32}.

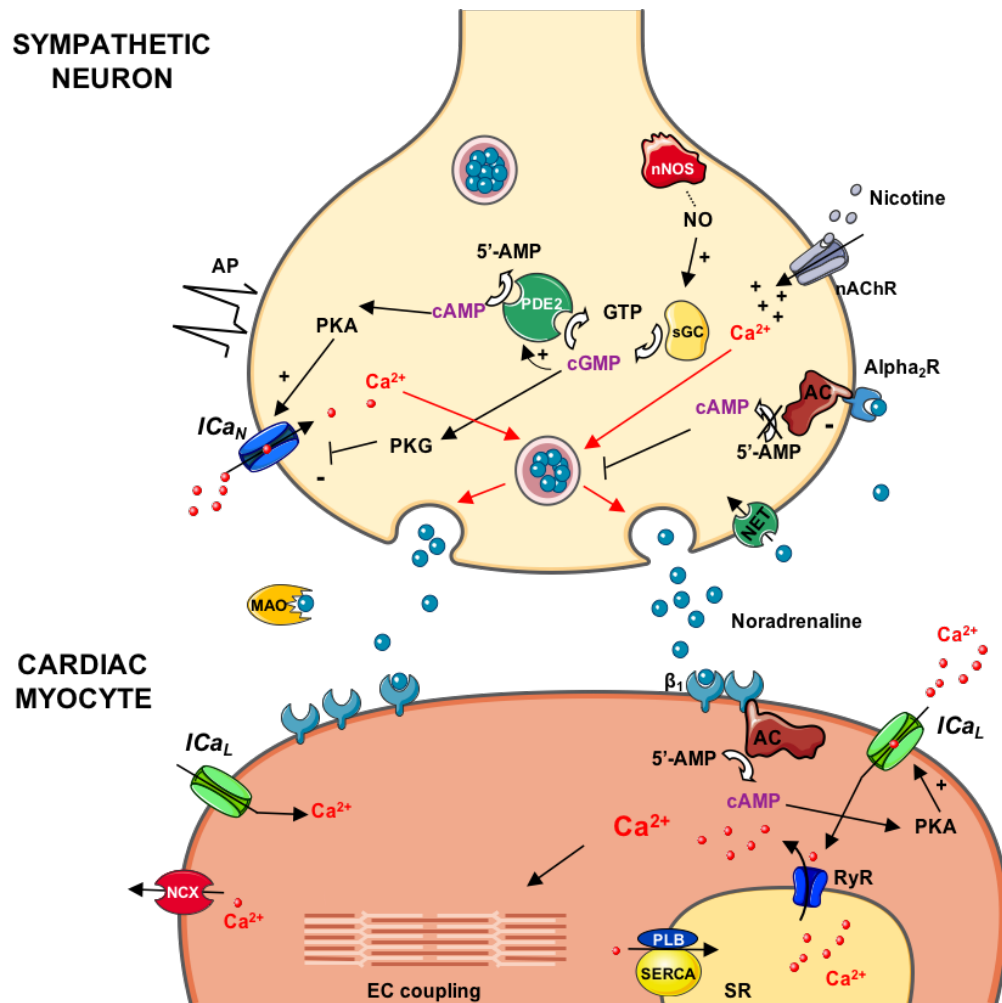


Figure 1.4: Sympathetic neurotransmission. The arrival of an action potential in the synaptic nerve terminal of the PGSN, leads to Ca^{2+} entry via voltage gated Ca^{2+} channels. This triggers exocytosis of noradrenaline (NA) into the synaptic cleft where it acts on the β_1 adrenoreceptor on the myocyte, generating cAMP. cAMP facilitates Ca^{2+} through L-type Ca^{2+} channels. This triggers further release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR) which triggers excitation-contraction (EC) coupling. Cytoplasmic Ca^{2+} is taken back up into the SR via the SERCA pump or extruded from the cell by the NCX transporter. NA signal termination is facilitated by the reuptake of NA through the noradrenaline transporter (NET) and its breakdown by monoamine oxidases (MAO). NA also act on presynaptic α_2 receptors to inhibit its own release. In hypertension, nNOS expression and the β_1 subunit of soluble guanylyl cyclase expression is down, resulting in lower levels of cGMP. Coupled to enhanced PDE2A activity, this results in increased neurotransmission.

NA acts on the β_1 adrenoreceptors on the myocyte membranes, triggering a signalling cascade coupled to biochemical messengers that regulate cardiac excitation contraction coupling. In the myocytes, the activation of the β_1 adrenergic receptor results in the activation of adenylyl cyclase (AC) and the generation of cyclic adenosine

monophosphate (cAMP). cAMP modulates a variety of intracellular pathways linked to myocyte excitability³³. Additionally NA acts on presynaptic α_2 receptors to modulate its own release^{4,28}. The α_2 adrenoreceptor is coupled to AC and its activation leads to inhibition of AC and a reduction in cAMP levels inside the neuron. cAMP levels are critically related to NA exocytosis and so the net result of α_2 activation is a reduction in NA release³⁴⁻³⁸. For a detailed review please see^{28,39}. The inhibition of these presynaptic receptors results in increases in blood pressure^{40,41}. Indeed, α_2 agonists, such as rimenidine, are being developed for use in the treatment of hypertension. Following its release from the sympathetic neurons, NA signalling is rapidly terminated by its neuronal reuptake via the norepinephrine transporter (NET) and its metabolism by monoamine oxidases (MAO)⁴².

1.4.2 Cellular actions of the β_1 adrenergic-cAMP pathway in myocytes

The β_1 adrenoreceptor is coupled to a stimulatory G protein (G_s). β_1 adrenergic activation results in the replacement of GDP with GTP on the the G protein, which activates the stimulator α subunit of G_s resulting in the activation of AC and the generation of cAMP^{4,42,43}. Increases in second messengers such as cAMP and cGMP act as important points of signal amplification thereby allowing the cell to respond appropriately to a given stimulus. The resulting cellular actions of cAMP in the myocyte span all the way from gene expression to cell signalling and are significantly compartmentalised to ensure signal fidelity^{42,44}. Of particular relevance to the beta adrenergic pathway is cAMPs direct modulation of the hyperpolarisation-activated cyclic nucleotide gates (HCN) channels, and its PKA dependent ability to modulate L-type Ca^{2+} channels⁴⁵, the sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) and the ryanodine receptor, resulting in increased intracellular Ca^{2+} levels that elicit changes in the contraction of the myocytes⁴². Broadly speaking this result in

increased heart rate (chronotropy), contractility (ionotropy) and conduction velocity (dromotropy) ^{4,42,43,46}.

1.5 Sympathetic dysfunction in the hypertensive state

Increased activity of the sympathetic nervous system is now established as a major contributing factor to the pathophysiology of human ^{2,9,10,12,13,24,47-50} hypertension and that observed in the spontaneously hypertensive rat (SHR) ⁵¹⁻⁵⁴. More than a decade's worth of work has shown that this autonomic dysfunction is present at many distinct autonomic nuclei ⁷.

1.5.1 Central sympathetic dysfunction

The brainstem forms the principal site for the regulation of central sympathetic outflow ^{7,55}. It has been postulated that oxidative stress linked to inflammation, elevated vascular resistance and hypoperfusion of the brainstem contributes to the hypertension observed in the SHR ^{56,57}. This results in an increased sympathetic outflow to the periphery including the heart, kidneys, vasculature and skeletal muscle ⁷. What is more, it has been postulated that the significant hyperactivity of the carotid body chemoreceptors facilitates this enhanced sympathetic activity ^{6,58,59}. This suggests afferent modulation of efferent signalling could play a significant role in the generation and progression of hypertension ⁶⁰.

1.5.2 Molecular changes in the postganglionic sympathetic neuron

The post ganglionic sympathetic neurons innervating the heart ⁶¹ have significant intracellular ⁶²⁻⁶⁸ and membrane level ^{69,70} molecular changes that contribute to the sympathetic phenotype. Coupled to significant vagal impairment this results in end organ hyper-responsiveness which is associated with increased mortality ^{2,16,17,71}. In the SHR, the signalling between the sympathetic preganglionic and postganglionic neurons is significantly heightened in the SHR resulting in greater activation of the

postganglionic cell ⁷². However, on the postganglionic cell innervating the heart, there is no difference in nAChR activation between the SHR and their normotensive Wistar Kyoto (WKY) control neurons ⁷³ suggesting the dysfunction lies further inside the cell. In fact, postganglionic sympathetic stellate neurons from the SHR have greatly dysregulated intracellular Ca^{2+} levels when compared to WKY neurons. This is manifested by significantly larger membrane Ca^{2+} currents ⁶⁴, which coupled to greatly increased Ca^{2+} transients in response to KCl stimulation ⁶³ result in excess NA release ⁵³. Moreover, these neurons also display significantly impaired NA reuptake through the noradrenaline reuptake transporter (NET) contributing to elevated levels of NA in the neuro cardiac synapse ^{53,54,69,74,75} (see figure 1.4). Increased NA turnover and reduced reuptake has also been observed in the human condition ⁷⁶⁻⁷⁸.

1.5.3 Resulting over excitability of the end organ

This heightened sympathetic activity leads to over excitability and responsiveness of the myocyte. This is manifested in increased basal heart rate ⁵³ and heart rate responses after both electrical stimulation and noradrenaline ^{53,79,80} and significantly greater baseline and isoproterenol stimulated L-type Ca^{2+} currents ^{80,81}. Moreover the SHRs portray significantly increased tachycardia following stimulation of the stellate ganglion ^{53,79}. A multitude of experimental and some emerging clinical evidence suggest that reversing this autonomic imbalance significantly reduces neurotransmission and could improve survival rates ^{10,12,54,66,82-84}.

1.5.4 Sympathetic dysfunction in the pro-hypertensive state

Interestingly, humans in the very early stages of hypertension, including those with a family history, but who are yet to develop clinical symptoms, show significantly increased sympathetic activity ¹⁴. This is in line with what we see in the SHR where the molecular changes associated with the increased sympathetic drive are also present in

the young animals ⁵³ – hereafter referred to as the pro-hypertensive animals. The Ca^{2+} dysfunction ^{63,64} resulting in excessive NA release and heightened neurotransmission ^{53,69} occur at a developmental stage (4-weeks) where the mean arterial BP is not different between age and weight matched SHR and WKYs when measured during awake states ⁸⁵, under general anaesthesia ⁵³, or following surgical implantation of a telemetry device ⁸⁶. In these pro-hypertensive animals, the only observed evidence of the high sympathetic tone is the slight tachycardia seen at rest and in response to activity⁵³. This suggests that the dysregulation of the sympathetic nervous systems is one of the early hallmarks of the disease and could play a larger role in the initiation of the condition.

1.6 Critical role of calcium in the post ganglionic stellate neurons

Though the exact mechanisms underlying the sympathetic hyperactivity remains unknown, it is becoming clear that the dysregulation of Ca^{2+} is central to the facilitation of sympathetic hyper-excitability in the PGSNs. We know this, as restoring the Ca^{2+} imbalance rescues normal neurotransmission ^{54,64,65}. Therefore, understanding the cause of the Ca^{2+} impairment is critical in directing targeted therapies.

1.6.1 Ca^{2+} as an important biochemical messenger

The importance of Ca^{2+} in mammalian physiology was first realised and described by Sydney Ringer in 1883, who identified it as a crucial factor for cardiac contraction ⁸⁷. Ca^{2+} ions are unique in that they not only function as a passive relayer of electrical information, they also serve as important biochemical messengers within the cell ⁸⁸. Ca^{2+} is both able to pass on electrical information and change the conformation of proteins – arguably the two universal instruments of signal transduction, making it one of the only molecules known to date that is able to act as both a first and second messenger ⁸⁹. In fact, Ca^{2+} is often referred to as the ‘third messenger’ ^{90,91}. Ca^{2+} ions regulate some of the most important processes within a cell, from fertilization and normal signalling to

apoptosis, via short and long term changes in cellular behaviour ⁹². It is therefore vital that Ca^{2+} signalling is tightly controlled, as any changes to Ca^{2+} handling in disease is likely to affect multiple processes within the cell ⁹³. Cells expend vast amounts of energy to control their Ca^{2+} levels, at the same time creating a 20 000-fold concentration gradient across the membrane (nM intracellular vs mM extracellular). This allows for Ca^{2+} signals to be finely tuned and allows the cell the ability to effect large changes in intracellular Ca^{2+} . Elevation of intracellular Ca^{2+} occurs via both Ca^{2+} entry across the membrane, and its release from intracellular stores. In the cytosol, it is tightly controlled and buffered by intracellular Ca^{2+} binding proteins. During signal termination, the Ca^{2+} is removed via reuptake into cellular compartments and extrusion from the cell ^{88,92}.

1.6.2 Voltage gated Ca^{2+} channels

Ca^{2+} channels act as important signalling molecules as well as a way of Ca^{2+} entry into the cell. Each channel is able to conduct roughly one million Ca^{2+} ions per second, which coupled with the presence of a few thousand channels per cell enables the cells to effect a >10 fold increase in its intracellular Ca^{2+} levels within milliseconds ⁸⁸. The voltage gated Ca^{2+} channels are heteromeric proteins composed of a subtype defining $\alpha 1$ subunit, and ancillary β and $\alpha_2\delta$ subunits. These $\alpha 1$ subunits often have multiple splice variants and are able to combine in different conformations, which coupled with differential association with ancillary subunits create distinct channel behaviours ⁹⁴. For example, the presence of the β subunit significantly affects the gating of the channels ⁹⁵ whilst the $\alpha_2\delta$ subunit is important for channel trafficking and surface expression ^{96,97}. The tissue-specific expression of the various subunits adds to channel diversity and results in an arsenal of different currents with specific biophysical and pharmacological properties, allowing each cell to acutely control its Ca^{2+} entry. Ca^{2+} channels are subdivided into subclasses (see table 1.1) depending on the $\alpha 1$ subunit that is expressed.

Each of these channels are specifically blocked by certain drugs and toxins^{98,99} and their currents can thus be pharmacologically separated.

Table 1.1: Ca²⁺ channel nomenclature and function. Adapted from Catterall 2011			
Current type	α_1 subunit	Specific blocker	Principal physiological function
L	Ca _v 1.1	DHPs	Excitation contraction coupling in skeletal muscle and regulation of transcription
	Ca _v 1.2	DHPs	Cardiac excitation-contraction coupling, neuronal Ca ²⁺ transients, regulation of transcription
	Ca _v 1.3	DHPs	Endocrine secretion, cardiac pacemaking, auditory transduction
	Ca _v 1.4	DHPs	Visual transduction
P/Q	Ca _v 2.1	Omega agatoxin	Neurotransmitter release and dendritic Ca ²⁺ transients
N	Ca _v 2.2	Omega conotoxin GVIA	
R	Ca _v 2.3	SNX-482	
T	Cav 3	None	Repetitive firing in cardiac myocytes and thalamic neurons
DHP: Dihydropiridines			

1.6.3 Neuronal voltage gated ion channels and synaptic transmission

Voltage gated Ca²⁺ channels are critical for neuronal function and signalling in the brain as well as in the periphery. Centrally, the Ca²⁺ channels facilitate synaptic plasticity associated with learning and memory and fast synaptic transmission^{98,100-102}. In the periphery, the Ca²⁺ channels are critically responsible for neurotransmitter release^{26,28,103,104}. The opening of these channels leads to depolarisation of the cell membrane and an influx of Ca²⁺ via Ca²⁺ induced Ca²⁺ release (CICR), which triggers vesicle

fusion and the exocytosis and release of neurotransmitters. For a detailed review see ^{105,106}. This release of NA from sympathetic PGSNs governs the dynamic control of cardiac excitability. Of the subtypes of Ca^{2+} channels, the N-type channel has been shown to play a major role in the NA release from sympathetic neurons in the human heart ^{104,107}; the neurons innervating the carotid artery ²⁵, the aorta ¹⁰⁸ and various arteries ^{103,109}. It is characterised by a high single channel conductance, allowing the cell to generate a large and temporally precise calcium influx ¹¹⁰. The T-type channel has been shown to be involved at higher sympathetic stimulation, whilst the role of the L-type channel is more contentious ²⁶. This is likely due to its expression away from the synapse ¹¹¹ and its activation in response to different stimuli ¹¹².

1.6.4 Ca^{2+} channel regulation

The behaviour of the voltage gated Ca^{2+} channels are extensively regulated by intracellular second messenger proteins (reviewed extensively in ^{98,113,114}). The majority of this regulation is done via phosphorylation of the channels by various kinases, but also through negative feedback via G-Protein coupled receptors (GPCRs) ^{113,115,116}.

1.6.4.1 Cyclic nucleotide regulation of Ca^{2+} channels.

Of particular importance to the cardiovascular system is the Ca^{2+} channel regulation by the cyclic nucleotides (CNs) cAMP and cGMP (see section 1.8 for details). Elevation of these second messengers results in the activation of protein kinases (PKs) and the phosphorylation of the channel ^{113,117,118}. The phosphorylation of the channel by the cAMP dependent protein kinase (PKA) results in channel activation, whereas cGMP dependent protein kinase (PKG) phosphorylation inhibits the channel ^{98,119,120}. The relative balance between the channel PKA mediated stimulation and the PKG mediated inhibition governs the channel's response to membrane depolarisation. What is more the CNs are able to cross-talk via the activation and inhibition of cellular

phosphodiesterases (PDEs) ¹²¹⁻¹²⁵. These PDEs acutely control the intracellular levels of both cAMP and cGMP and thus have significant secondary effects of the phosphorylation and hence activity of the Ca²⁺ channels.

1.7 The importance of the NO-cGMP pathway in hypertension

The exact mechanisms behind the autonomic dysfunction associated with hypertension, remains somewhat unclear, but evidence suggests it could be directly linked to the disruption of the NO-cGMP pathway by oxidative stress ¹²⁶, leading to significant disruption of NA release in the peripheral sympathetic nervous system ¹²⁷ and in central autonomic nuclei ¹²⁸.

1.7.1 NO regulation of Ca²⁺ signalling

Neuronal nitric oxide synthase (nNOS) is a powerful modulator of myocyte and neuronal function through the release of nitric oxide (NO) and its resulting effects on intracellular pathways. Centrally, NO plays an important role in the cardiac neuronal axis where it acts as a brake in the periventricular nucleus, maintaining appropriate central sympathetic outflow ^{7,129}. In the sympathetic PGSNs nNOS catalyses the formation of NO resulting in the activation of soluble guanylyl cyclase (sGC). sGC facilitates the synthesis of cGMP - an important modulator of intracellular pathways acutely linked to Ca²⁺ regulation ¹³⁰. cGMP activates PKG resulting in Ca²⁺ channel inhibition ^{114,131,132}, and the inhibition of neurotransmitter release ^{119,130,133}. What is more, cGMP also acts through the activation of PDE2 ^{130,134,135} to lower cAMP levels resulting in less PKA mediated activation of channel currents ^{113,122,124}. As such NO is a powerful modulator of both the stimulation and inhibition of neuronal excitability via its action on the presynaptic Ca²⁺ channels.

1.7.2 Significance of the disruption of the nNOS-NO-cGMP pathway

There is an emerging theory of NO dysregulation and oxidative stress in cardiovascular diseases^{127,136,137} and research has shown a significant reduction in the bioavailability of NO in hypertension¹³⁸ that is associated with progression of the disease¹³⁹. The sympathetic PGSNs of the SHR show reduced nNOS activity and down regulation of sGC – a key precursor to the downstream effects of NO^{65,140}. As a result, the SHR PGSNs have significantly lower intracellular cGMP levels when compared to normotensive controls⁵⁴. Gene transfer of nNOS and its adaptor protein CAPON into PGSNs restores the expression of cGMP⁵⁴, significantly reduces intracellular Ca²⁺ transients^{64,65,134} and the sympathetic output of the hypertensive neurons^{54,141}. Similarly, gene transfer of nNOS also decreases excitability in single sinoatrial node cells by reducing the L-type Ca²⁺ current⁸⁰. Further, the application of a membrane permeable cGMP analogue (8 bromo cGMP) decreases the heart rate responses to sympathetic stimulation that is reversible with nNOS inhibition¹⁴². The observation that NO attenuates the myocyte responses to β adrenergic stimulation under normotensive conditions^{131,143,144} suggests that NO acts as an important brake on neuro-cardiac excitability. It is now clear that the loss of this inhibition¹²⁸ adds to the adrenergic hyperactivity observed in hypertension which has been linked to increased mortality^{18,145}.

On the parasympathetic side, nNOS also reverses aspects of the vagal impairment seen in the hypertensive state^{82,146} suggesting it not only has the ability to reverse the (para)sympathetic diseases phenotypes, but that that is also perhaps plays a role in the maintenance/restoration of normal autonomic balance¹³⁷.

In addition, NO has pronounced effects on vascular tone – an important regulator of blood pressure. However, in a recent human study looking at the effects of vascular NO in the progression of hypertension, no evidence of NO-dysregulation was identified in the hypertensive state ¹⁴⁷. This provides further evidence that NO dysregulation at the level of the autonomic ganglia plays a key role in the pathophysiology of human hypertension.

1.8 Cyclic nucleotide signalling

The CNs cAMP and cGMP have diverse and critically important functions in both myocytes and neurons.

1.8.1 The cyclic nucleotides cAMP and cGMP

cAMP and cGMP are important intracellular second messengers that mediate a variety of intracellular functions (see ^{148,149} for detailed review) and are also implicated in disease. cAMP and cGMP are generated by the enzymes adenylate and guanylyl cyclase in response to activation by intracellular mediators and/or membrane receptors. Increases in these second messengers act as important points of amplification of an initial cellular signal, allowing the cell to respond appropriately to a given stimulus. The cyclic nucleotides have three main modes of action within the cells; they modulate the function of the PKA and PKG leading to the phosphorylation of downstream protein targets ^{150,151}; they directly activate CN gated ion channels ^{152,153}; they activate exchange protein directly activated by cAMP (Epac) ^{154,155}. Signal termination is achieved by the phosphodiesterases (PDEs), the hydrolytic activity of which is controlled by the CNs themselves ^{122,156}, and protein phosphatases which dephosphorylate the PKs target proteins ¹⁵⁷.

1.8.2 Phosphodiesterases

In order to maintain an appropriate cellular response, signal termination is perhaps as important as signal induction. The effects of cyclic nucleotide signalling are controlled and terminated by the PDEs that catalyse the hydrolytic degradation of cAMP and cGMP ¹⁵⁸. The PDEs form a superfamily of enzymes of which there are currently 11 members (PDE 1-11, each with different splice variants) ^{156,159,160}. The PDEs are often classified by their substrate specificity (see table 1.2); into cAMP specificity, cGMP specificity, or dual specificity. The PDEs roles in the brain and in cardiac myocytes has been relatively well established, but their roles in the autonomic nervous system is only just coming to light. Work by our group in 2001, suggested PDE3 was important for the release of acetylcholine ¹⁶¹, and only recently has this enzyme been functionally identified in peripheral neurons ¹⁶². Similarly the importance of PDE2A in the control of sympathetic neurotransmission is gaining interest ⁷⁰.

Table 1.2: List of mammalian phosphodiesterases (PDEs) and their substrate specificities	
Substrate specificity	PDE
cAMP	4, 7, 8
cGMP	5, 6, 9
Dual specificity (cAMP and cGMP)	1, 2, 3, 10, 11

1.8.3 Cyclic nucleotide cross-talk

The cGMP and cAMP pathways are able to affect changes in each others signalling via the activation and/or inhibition of PDEs ^{122,123,130,160,163-165}. Much of the work characterising this cyclic nucleotide cross-talk has been conducted in cardiac myocytes ¹⁶⁴ where it plays a critical role in functionally coupling of two critical and opposing pathways in the human heart ⁴⁶: The β_1 adrenergic-cAMP pathway that increases cardiac function; and the NO-cGMP pathway that reduces it ^{122,164}. Here, cAMP and cGMP have

opposing effects and so their functional coupling via the PDEs (mainly PDE2 and 3) allows great signal fidelity and regulation of normal processes. The intracellular molecular mechanisms regulating this CN cross-talk in neurons and its discrete role in the control of sympathetic neurotransmission remains poorly understood.

1.8.4 Compartmentalisation of signalling

This signal fidelity is insured by the degree to which the β_1 adrenergic³³ and the NO-cGMP¹⁶⁶ pathways (and indeed their cross-talk) are organised into distinct subcellular compartments⁴⁴. The signals must be coordinated and kept secure from confounding signals if the cell is to correctly respond to given stimuli. The compartmentalisation is achieved at all levels of the signalling pathway, via the anchoring¹⁶⁷ of the ACs that generate the CNs^{168,169}, the PDEs that break them down^{163,165,170,171} and the downstream targets, to distinct subcellular compartments^{44,171,172}. This physical segregation of the signalling components allows the cell fine-control over its spatial and temporal signalling.

1.9 Cyclic nucleotide dysfunction in hypertension

It has been demonstrated that a significant causal factor for Ca^{2+} dysfunction seen in the hypertensive state is the reduction in cGMP. The relative importance of cGMP is illustrated from the multitude of experiments showing that NO's action as a neuromodulator of sympathovagal neurotransmission are mediated through direct or indirect increases in cGMP^{54,64,65,70,80,127}. The low levels of cGMP coupled to this increased activity of PDE2A is thought to facilitate increased PKA mediated activation and reduced PKG mediated inhibition of the neuronal voltage gated Ca^{2+} channels. This has been postulated to significantly contribute to the Ca^{2+} dysfunction seen in the hypertensive state and has been hypothesised to act as the trigger. As such, these enzymes could prove to be emerging central players in the control of sympathetic

excitability¹⁷³. The PGSNs of the pro-hypertensive SHRs have significantly higher PDE2A activity when compared to the normotensive controls⁷⁰. What is more, overexpression of PDE2A in the normotensive neurons significantly increased intracellular Ca^{2+} transients resulting in greater NA release⁷⁰, suggesting a powerful role for this enzyme in the hypertensive states¹⁷³. The PDEs are critically responsible for the hydrolysis of both cAMP and cGMP¹⁵⁸. Moreover, as they are activated by both cAMP and cGMP they form an important point of communication between these, often opposing signalling pathways via their cross-talk. With the critical role of cGMP in sympathetic neurotransmission and the heightened activity of PDE2A, it is conceivable that such cross talk could play a role in the PGSNs too, however this remains to be established experimentally.

1.10 The need for a physiological system to study CV disorders

It is clear that the disease phenotype associated with hypertension is present on both the sympathetic PGSNs^{63,64,69,174}, and the myocytes^{53,79,80} they innervate. The (patho-) physiology of the individual ion channels and signalling molecules in the single neuron and myocytes as separate systems are well described, but relatively little is known about the cell-to-cell interaction that takes place. Tao et al modelled the interaction using bioinformatics on single cell data to reassemble the cell network from individual ion channels¹⁷⁵. Nevertheless, the predictive power of this modelling is limited because it has not been validated against physiologically coupled neurons and myocytes.

1.10.1 The use of co-cultures to study the neuro-cardiac signalling.

Co-cultures have long been used to study intercellular signalling however, much of today's work still centres around the use of pharmacology on single cells and organs. The use of physiologically coupled cells, maintains the integrity of the synapse and provides us with a way of stimulating one cell and recording from the other, preserving

the special control of transmitter action. When neurotransmitters are applied globally to a cell, rather than via the synapse, it is conceivable that its response may be different. This is especially relevant with the increasing realisation that cell signalling occurs in distinct subcellular compartments^{44,176}. Furthermore, the significance of the co-release of multiple neurotransmitters from PGSNs and their critical importance for cardiac function is now coming to light^{19,174,177}. Such effects simply cannot be measured in single cell preparations. The functional development of sympathetic neurons is dependent on the presence of appropriate target cells¹⁷⁸. An early study using a co-culture of ventricular myocytes and sympathetic neurons reported significant functional development of the neurons that were in direct contact with cardiac myocytes, and that this was absent when the neurons were innervating skeletal muscle¹⁷⁹. This points towards the importance of studying sympathetic neurons in a more physiological setting if we are to fully characterise their function.

The presence of sympathetic neurons significantly increases the beat rate and contractile responses of myocytes in culture¹⁸⁰⁻¹⁸² in a β adrenergic dependent manner^{183,184}. The only reported co-culture work done in hypertensive animals was done in the early 90s. Here, two studies using ventricular myocytes and thoracolumbar sympathetic neurons showed increased cell growth of SHR vs WKY myocytes when innervated¹⁸⁵ and increased contractile responses¹⁸⁶. It is now clear that disease phenotype is present on both the sympathetic neurons^{63,69,174} and the myocytes^{53,80} they innervate. However, to my knowledge, no studies of the molecular aspects of these responses have been conducted in the SHR.

It is well established that the neurons signal to myocytes, but little has been done to characterize the signalling going the other way and to what extent this is altered in disease. The neurons form specialised junctions with the myocytes that are enriched in the signalling molecules linked to cardiac behaviour - molecules we know are highly impacted in cardiac disease. In these junctions there is an enrichment of β_1 adrenoreceptors directly beneath the neuronal membrane ¹⁸⁴. The presence of these neurons also leads to an up-regulation of cardiac calcium channels ¹⁸⁷. Taken together this suggests there is perhaps more going on in the synapse than what's been previously realised. Could the core control of signal fidelity and regulation lay at the level of the synapse?

1.10.2 The highly organised and functionally controlled synapse

The term synapse was first coined in 1897 by Charles Sherrington ¹⁸⁸ who used it to describe the “intercellular junction”. His work on the function of neurons and the communication occurring in these synapses earned him the Nobel Prize for Physiology or Medicine in 1932. Synapses function as relayers of signalling and as signal processors. It is now clear that the structure and function of synapses are core to the appropriate function of the nervous system as well as for the functional innervation of organs, such as the neuro-cardiac junction. These cellular junctions are highly structurally organised allowing them to serve particular physiological functions. A good example is the synapse between the pre- and post ganglionic neurons of the autonomic nervous system. The pre-ganglionic neurons from the sympathetic and parasympathetic branches both release acetylcholine, however the postganglionic response to this stimulus is different between the sympathetic and parasympathetic branch of the ANS – mostly due to the different molecular structure of the synapse. In the sympathetic branch, acetylcholine binds to the nACh receptor present on the post ganglionic cell

resulting in the release of NA. Conversely, in the parasympathetic branch, acetylcholine binds to the muscarinic acetylcholine receptors found on the postganglionic neuron, resulting in acetylcholine release.

These synapses can be significantly altered in disease, both with respect to structure, quantity, location and synapse strength. This has been described in the the brain ¹⁸⁹ and in neuromuscular disorders such as congenital myasthenic syndrome ^{190,191}. Of interest, the activity of a synapse can significantly affect its strength (plasticity) ¹⁹² – something that is often correlated with changes in the receptor expression of the synapse itself ¹⁹³. Could this be a result of the sympathetic hyperactivity seen in CV diseases?

1.10.2.1 The neuro cardiac synapse

It is now clear that the neuro cardiac junction is a highly specialised zone both from functional and physical evidence. NA release occurs within <1ms of neuronal stimulation producing significant elevation of heart rate within 80-130ms ¹⁹⁴. With a reported NA diffusion rate of $1\text{ms} < 20\text{ }\mu\text{m}^2/\text{s}$ and β -adrenergic receptor activation taking 50-60 ms ¹⁹⁵, the membranes between the PGSN and the myocyte must be in close proximity. In fact, early electron microscopy work postulated that the synaptic cleft was indeed very small – between 5 and 110 nm ^{196,197}. The neuro cardiac synapse therefore allows for both fast and specialised signalling. Interestingly, in addition to the structural changes in Ca^{2+} channels and β -receptors mentioned above, it has been demonstrated that the presence of myocytes affects the transmitter release and calcium homeostasis in sympathetic neurons ¹⁷⁹, though the mechanisms remain unclear. This was not present in cultures using skeletal muscle or sensory neurons, suggesting the neuro-cardiac synapse is unique.

1.10.2.2 NO as a paracrine agent?

It is clear that nNOS is a powerful modulator of sympathetic activity and cardiac excitability^{126,127,136,139}. Although a lot is now known about some aspects concerning micro domain signalling of nNOS¹⁶⁶ in neurons and myocytes, these have been based on seeing the action of NO working in an autocrine manner and not communicating between cells as a paracrine agent. This is largely due to the lack of understanding of the communication between the neuron and myocyte. NO's intracellular role has been well described both in the myocytes and sympathetic PGSNs (see section 1.7), but could its paracrine function play a larger role than previously thought? NO is critically important for synaptic plasticity in the brain and the appropriate activity of central neurons^{198,199}. Could such activity dependent plasticity also play a role in the peripheral nervous system? The answers to these questions require the use of dual cell models.

1.11 Aims of the thesis

- Is the intracellular Ca^{2+} dysfunction seen in the pro-hypertensive PGSNs present at the membrane at the level of the voltage gated Ca^{2+} channel?
- Which channel type is the major player?
- Does restoration of the cGMP-centred cyclic nucleotide dysfunction in the PGSNs restore normal Ca^{2+} channel function?
- Does the cGMP dysfunction in the PGSNs also interfere with cAMP levels via phosphodiesterase mediated cyclic nucleotide cross talk?
- Establish a co-culture system that will allow for the physiological study of PGSN-myocyte signalling.
- Is the PGSN the primary driver behind the cardiac sympathetic hyperactivity seen the pro-hypertensive state?

- Can diseased PGSNs induce a hypertensive phenotype in an otherwise healthy cardiomyocyte?

CHAPTER TWO

2 Materials and methods.

2.1 *Solutions and reagents*

All solutions, drugs and reagents used in this thesis were obtained from Sigma unless otherwise stated. Exact solutions are given in each experimental chapter.

2.2 *Animal models*

2.2.1 **The Spontaneously Hypertensive Rat (SHR)**

The SHR is the most studied animal model of genetic essential (primary) hypertension based on number of publications²⁰⁰. However, it is important to realise it is not the only model present, and only represents the essential hypertension that is inherited in a Mendelian way. It was created by Okamoto and colleagues in 1960 who started breeding WKY rats with high blood pressure^{201,202}. The reason the strain is used in this study is because it portrays significant sympatho-excitation and is currently the best model to study the autonomic influence on hypertension in isolation. This is due to the lack of potentially interfering comorbidities and pathologies present in the other models such as the Dahl salt sensitive rats or the two kidney-one clip model²⁰³. Early studies demonstrated that the SHR is associated with haemodynamic changes that are analogous to that seen in the human condition at multiple developmental stages²⁰⁴. More recently it has been demonstrated that the SHR has a marked autonomic imbalance coupled to altered cardiac responsiveness to autonomic mediators, which either results from or contributes to the hypertensive phenotype¹⁷⁴. These pathological changes are also found in the human conditions and together make the SHRs an ideal model to study the effects of sympathetic innervation on the myocytes in hypertension^{24,203}. Due to a demand problem, the SHR litters were bred in-house according to Home

Office Guidelines, UK (PPL 30/2360 and 30/3131, David J. Paterson) and University of Oxford ethical guidelines.

2.2.2 Wistar Kyoto (WKY)

The WKY rat is the genetic control that the SHR_s were bred from and was used as the normotensive controls in the majority of experiments. In Chapter 5 the Wistar rat was used for some experiments.

2.2.3 Sprague Dawley (SD) rats

The methods development was performed using SD rats as this proved most cost-efficient. In this study, four-week old (70-95g) males were used for the neuronal cultures. Male and female littermates were used at P3 for the myocyte cultures.

2.2.4 Schedule-1

All animals were killed in accordance with the Home Office (Scientific Procedures) Act 1986 (UK). Schedule-1 was performed as follows: the four week animals via anaesthesia with isoflurane and pentobarbital overdose before the hearts were removed resulting in exsanguination. Cervical dislocation and decapitation was performed prior to tissue harvest and culture from P3 pups. All animals were sourced from Harlan or Charles River (UK) and kept for three days in a local holding facility for familiarisation prior to experiments.

2.3 *Cell isolation and culture:*

2.3.1 Coverslip coating

Six mm glass coverslips were cleaned in acid over night and rinsed thoroughly in sterile water. Coverslips were coated with 0.1 mg/ml Poly-D-Lysine over night at 4° degrees in a solution containing 4.75 mg/ml Borax and 3.1 mg/ml Boric acid in sterile H₂O. Coverslips were rinsed six times in sterile water before being spread out on sterile mesh and allowed to dry. They were stored at 4° degrees until use, and thrown out after three

months. On the morning of the culture, the coverslips were placed into a four well plate (Nunc Delta Surface, Thermo Scientific, UK) (see figure 2.1) and coated with laminin (in L-15 media) for three hours in a CO₂ free oven held at 37°C degrees. The laminin solution was removed prior to cell plating.

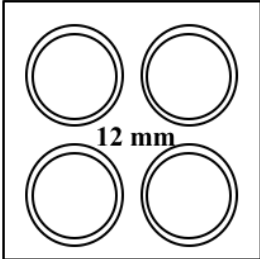
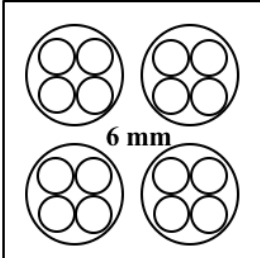
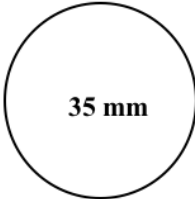
Plating surfaces		Cell densities	Experiment
12 mm Poly-D-Lysine/ Laminin coated coverslips (BD Bioscience)		78 000 cells/well	Immunofluoresence
6 mm Poly-D-Lysine/ Laminin coated coverslips (self coated)		78 000 cells/well	FRET Voltage Clamp
35 mm Poly-D-Lysine coated dishes (BD Biosciences)		235 000 cells/well	Micro/macrosopic behavioural studies.

Figure 2.1: Plating conditions of experiments. Cell densities reported are for the myocytes which were counted using a haemocytometer. The sympathetic neurons were too few to effectively count and were plated on top as best controlled by the experimenter.

2.3.2 Isolation of sympathetic postganglionic stellate ganglion neurons (PGSNs) ⁶⁹:

The left and right stellate ganglion that specifically innervate the heart were dissected out from between ribs 1-3 and placed into ice cold Hanks Balanced Salt Solution (HBSS). The fibroblast sheet was removed (de-sheathing) before the tissue was chopped into 1 mm³ pieces and placed into 15 ml Falcon tubes containing 2 ml of the appropriate enzyme (in PBS) for digestion. The tissue was incubated for 18 minutes in collagenase

(1 mg/ml in HBSS) in a rotating water bath kept at 37°C. The collagenase was replaced with trypsin (2 mg/ml in HBSS) and incubated for a further 22 minutes.

Following digestion, the tissue was placed in warm blocking media (see table 2.1) for three minutes. This was repeated three times before the tissue was washed twice in neuronal plating media. Cells were dissociated by pipetting through a 2 mm followed by a 1 mm glass pipette. After each round of pipetting, the tissue was allowed to settle, 50% of the supernatant (containing the dissociated cells) was removed and placed into a 35 mm dish. After the final dissociation, the PGSNs were plated onto six mm Poly-D-Lysine/Laminin coated coverslips (four in each well, four well plate) and maintained at 37°C, 5% CO₂. Media was changed 24 hours post isolation and thereafter every 48 hours.

Table 2.1: Cell isolation and culture medias used to isolate and maintain sympathetic neurons, neonatal cardiomyocytes and co-cultures		
<i>Neuronal Plating media</i>	<i>Neuronal Blocking media</i>	<i>Myocyte media</i>
90% L15 media	96.8% L-15	17% M199
24 mM NaHCO ₃	0.6% glucose	68% DMEM
38 mM Glucose	2 mM L-glutamine	n/a
1% Penicillin/streptomycin	1% Penicillin/streptomycin	1% Penicillin/streptomycin
50 ng/mL NGF	10% FBS	5% FBS
10% FBSS	n/a	10% Horse serum
<i>Co-culture media: 50 ng/mL NGF was added to the myocyte only media to form the co-culture media</i>		

2.3.3 Isolation of ventricular cardiomyocytes.

Hearts were excised from neonatal (P3) littermates and places in ice-cold HBSS. The atria were removed before the ventricles were minced into 3 mm³ pieces before being placed into cold filter sterilised Trypsin (TRL) solution (1 mg/ml) and placed on a shaker at 4°C for three hours. Following the digestion, the tissue was washed in 20 mL cold HBSS before the tissue was incubated with 1 mg/mL collagenase for two minutes

in a shaking water bath. The supernatant from the first round of the collagenase was discarded without disrupting the cells. A further five, two minute incubations in collagenase was completed. After each one, the tissue was titrated 15 times (one/second) with a plastic Pasteur pipette cut to allow a five mm smooth end, before the supernatant containing individual cells was transferred to a tube containing cold HBSS. New collagenase was then added for the next digestion. After the five titrations, 95% of the tissue had disappeared. The cell suspension was filtered through a 40 μm filter (EASYstrainer, Greiner bio-one, UK) to remove any cell clusters before the cells were centrifuged at 1000 rpm for eight minutes to pellet the cells. The pellet was re-suspended in 5 ml of myocyte plating media and placed into two 35 mm non-coated petri dishes. Importance was placed on the speed of this process as getting the cells out of the enzymes and into media as quickly as possible was desired in order to preserve cell health. The cells were pre-plated for one hour at 37°C, 5% CO₂ to allow the fibroblasts to settle. Cells were then counted using a haemocytometer and plated onto Poly-D-Lysine/Laminin coated surfaces (see figure 2.1). 24 hours post plating, the medias were changed to fresh media containing 1 μM Cytosine Arabinoside (Ara-C) to prevent any remaining fibroblasts from proliferating. This media was kept for a further 24 hours before the cultures were maintained in normal myocyte media that was changed every 48 hours.

2.3.3.1 Myocyte phenotype and electrical connectivity

Post culture, 99% of the cells in the dishes were myocytes as evidenced by their positive labelling for sarcomeric α actinin (see figure 2.2A) and their spontaneous beating. They grew as a thick monolayer with evidence of clustering in some areas. The cells were electrically connected and beat in synchrony (figure 2.2B). Figure 2.2A show positive

punctate staining for the gap junction protein connexin 43 indicative of healthy connectivity between the cells.

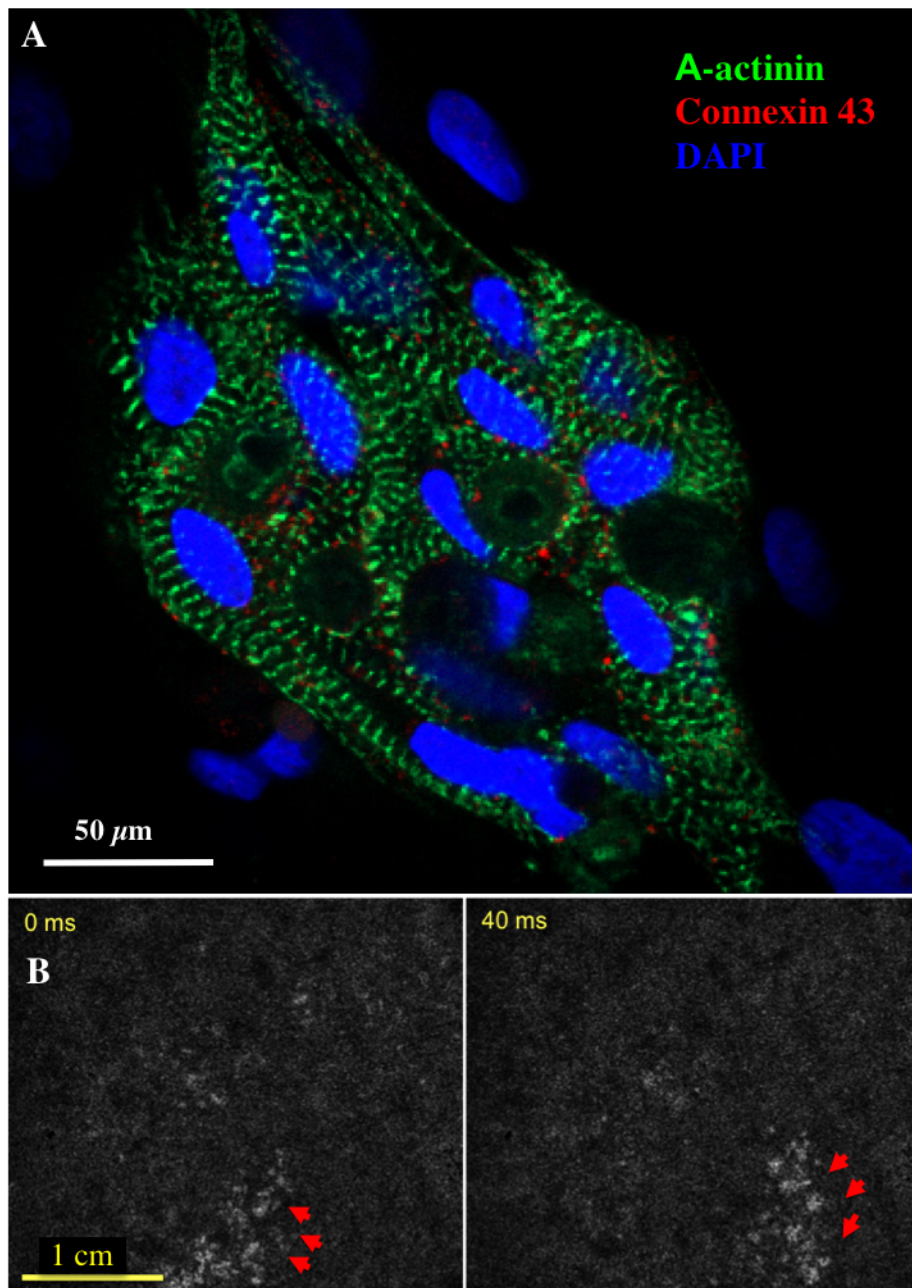


Figure 2.2: The myocyte phenotype. The myocyte phenotype was confirmed by their spontaneously beating phenotype and their positive staining for sarcomeric alpha actinin. The myocytes link up with each other via gap junction connections (A). The cells grew on top of each other - panel A depicts a confocal Z slice through such a cluster. Surrounding cells were out of focus and thus appear dark. The culture is electrically connected as evidenced by the spreading of electrical waves (B) measured by off axis illumination. The wave fronts are depicted with red arrows.

2.3.3.2 Dye-free imaging of myocyte monolayer

To image the macroscopic (~3.5 cm field of view) behaviour of the cultures, a dye-free imaging techniques was used. The use of dye-free methods is advantageous as it allows the culture to remain undisturbed throughout the experiment. Furthermore, it avoids the potential cytotoxic effects of using Ca^{2+} and voltage dyes with high powered illumination. Myocytes were very sensitive to motion and temperature changes and as such, in order to obtain accurate measures of beating behaviour, it was important that the cells were kept in a tightly controlled environment. To ensure this the cells were kept in an Okolab (Indigo Scientific, UK) stage incubating chamber controlled for both heat and humidity (37°C, 5% CO_2). The cells were allowed to equilibrate in this chamber for 30 minutes before recordings were made. The cells were imaged using an Olympus MVX10 Macroscope with an MVplanar 1x lens, a 1x objective with 1x zoom. and an Andor Neo sCMOS camera (2,560 × 2,160 pixels, 6.5 μm pixels), fitted with a 580 nm longpass filter. The minute contractions of the individual myocytes and the propagation of the waves across the macroscopic plate were detected and visualized by using off axis illumination (figure 2.3A) using a LED fitted with a band-pass filter (580 ± 20 nm). The frame rates were set between 25 and 100 fps²⁰⁵. An image processing filter (figure 2.3C) was applied to the resulting video that allowed the experimenter to observe the motion of the cultures by eyes. The intensity of each pixel was compared with the pixel in previous frames to get a digital measure of movement. Regions of interest were drawn using a custom built script in Gview (Figure 2.3B). The resulting beating behaviour was plotted to allow for ease of analysis (Figure 2.3D) using a custom written software in Gview. The double peaks are due to the software detecting both the contraction and relaxation of the cell.

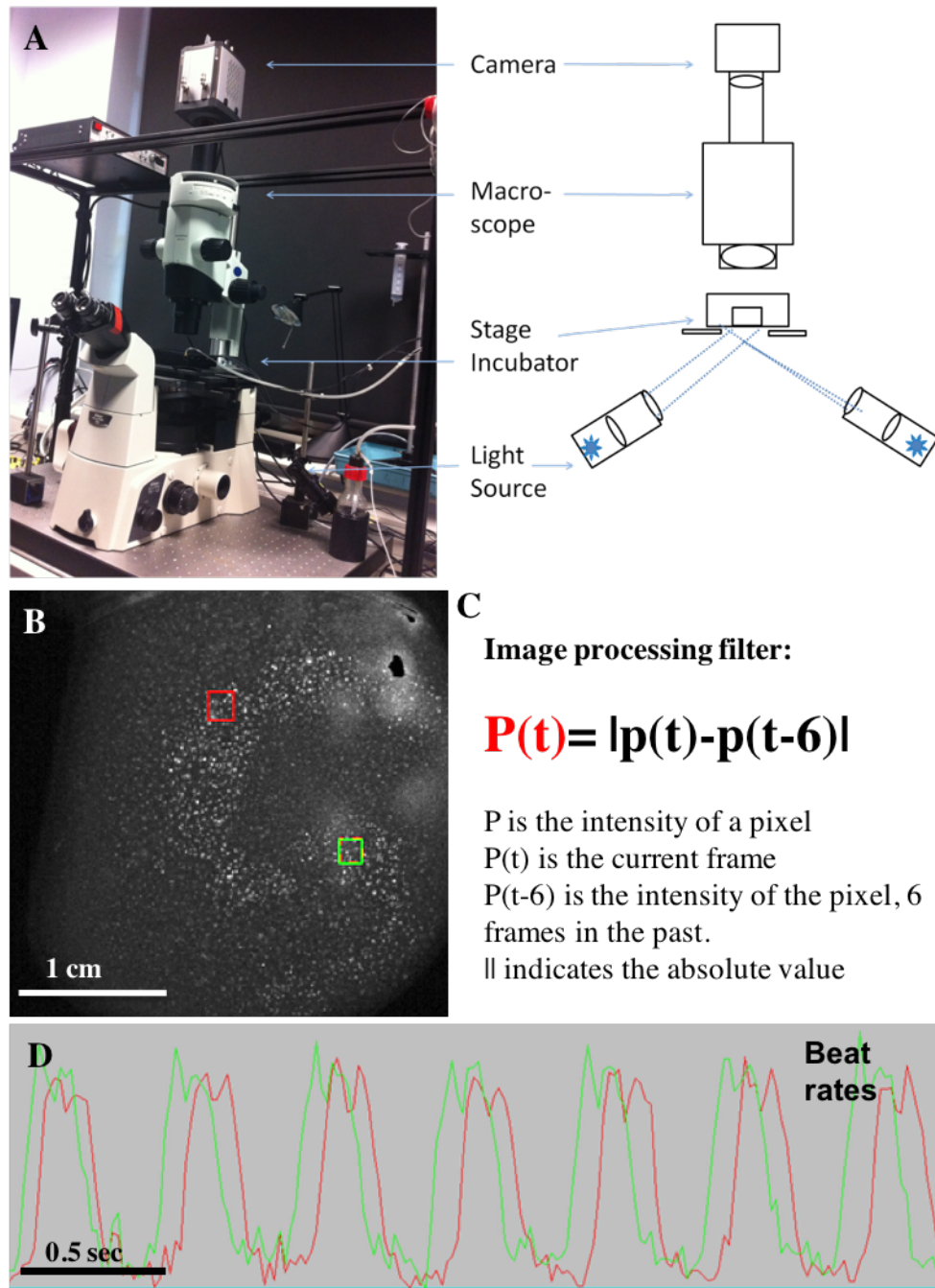


Figure 2.3: Dye free macroscopic imaging of co-cultures. The cells were imaged using a custom built macroscope using off-axis illumination (A). Regions of interest were applied to the image (B) and an image processing filter (C) was applied to enable the beating behaviour to be made out without the use of intracellular dyes. Briefly, as the cells moved in and out of focus, their pixels changed intensity. The filter compared the intensity of a pixel at t_0 with its intensity six frames before, thereby allowing visual representation of the spreading waves (see video at <https://youtu.be/DK9grhh3-TY>). The resulting information was plotted using a custom written program in gView to allow for ease of analysis (D)

2.3.3.3 Myocyte behaviour

The cells in culture portray distinct macroscopic behaviours that fall into three groups; target waves, spiral waves and wavelet behaviour. These are analogous to what has been reported in other studies^{206,207}. The cultures could therefore be described as good, simplified models of cardiac behaviour (Figure 2.4). Such models are needed to fully understand how individual cells communicate and link up to form a monolayer, but perhaps more importantly to screen potential drugs for lethal cardiac effect before they reach clinical trials. Deleterious effects on the cardiac action potential remains one of the most common reasons why drugs fail in trials and also have accounted for many drug retractions⁴³.

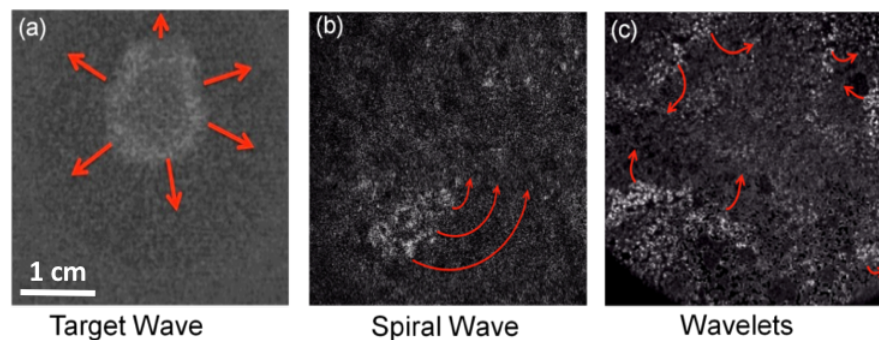


Figure 2.4: The co-culture is a good, simplified model system of cardiac behaviour. The culture portrays distinct behaviour that can be grouped into three classes: target waves (a); spiral waves (b); and wavelets (c). These behaviours are analogous to what has been described by other groups in other tissue models (Bub et al. 1998 and 2002) Figure reproduced with permission from Dr. Rebecca Burton.

2.3.4 Co-culture of sympathetic neurons and ventricular myocytes

Co-cultures have long been used to study the interaction between two or more cell types. Co-cultures of rat myocytes and sympathetic neurons have revealed that the neurons play a significant role in modulating both the behaviour and membrane receptor of the myocytes in culture¹⁸⁰⁻¹⁸⁴. As such, these cultures form critical points of reference to our understanding of physiology.

2.3.4.1 Co-culture formation

To create the co-cultures, the myocytes were plated as described above, with the cultures intended to be co-cultures being placed in co-culture media (see table 2.1). The isolation of the PGSNs took place after the myocyte isolation was complete, allowing the myocytes to settle for approximately three hours prior to the additions of the neurons. The freshly isolated PGSNs were distributed evenly across the dishes. It was important that the ratio of neurons to myocytes in co-cultures accurately represented the *in vivo* state. Various ratios have been described in literature, ranging from 1:5 – 1:20^{184,208}, though it is worth noting that many studies do not comment on the ratio used. In this study a ratio of 1:20 neurons to myocytes was attempted as this is thought to represent the *in vivo* state. The cells were counted using a haemocytometer, using trypan blue to identify dead cells. The co-cultures were treated with 1 μ M Ara-C as described above to eliminate fibroblasts.

2.3.4.2 Co-culture stimulation

In vivo the PGSNs are activated by acetylcholine released from the preganglionic neuron⁴ acting on the nicotinic acetylcholine receptor (nAChR)²⁰⁹. In an attempt to keep the co-culture in an as physiological setting as possible, the neurons were stimulated with nicotine (nAChR) agonist. Research has shown that the activation of the nAChR leads to Ca^{2+} transients in the sympathetic varicosities²⁷ resulting in the release of NA^{36,210,211} and an increased beat rate of the myocytes¹⁸⁴, analogous to that seen *in vivo*. To my knowledge it is unknown how much acetylcholine is released during such stimulation. With nicotine being a potent agonist, it becomes difficult to determine what is the most physiologically accurate nicotine concentration to use. Literature reports the use of concentrations ranging from 1 μ M to 500 μ M^{27,184}. In this study 1, 10, 100, 300 and 500 μ M nicotine was used. In many of the original (and current) studies, electrical

pulses were used to stimulate the neurons²¹²⁻²¹⁴. This method was deemed imperfect due to the potential unintended excitation of the myocytes in the co-culture.

2.3.4.3 Co-culture phenotype

When plated two hours after the myocytes, the PGSNs lay on top and reach out their axons to connect the myocytes in its vicinity (figure 2.5A). It was important to plate the neurons on top, as the neurons get damaged and often die from being stuck between an immobile culture surface and a moving myocyte layer. In the vast majority of cultures, all the myocytes in the vicinity of one neuron portrayed the same beating phenotype with identical beat rates. Figure 2.5B confirms the cellular phenotypes with sarcomeric α -actinin in red and TH positive neurons in green. Confocal imaging allows close investigation of the neuronal branching and the resulting innervation of the myocytes. Zoomed in, it is apparent that the neuronal processes are interwoven with the myocytes in a similar manner to that seen in vivo²¹⁵.

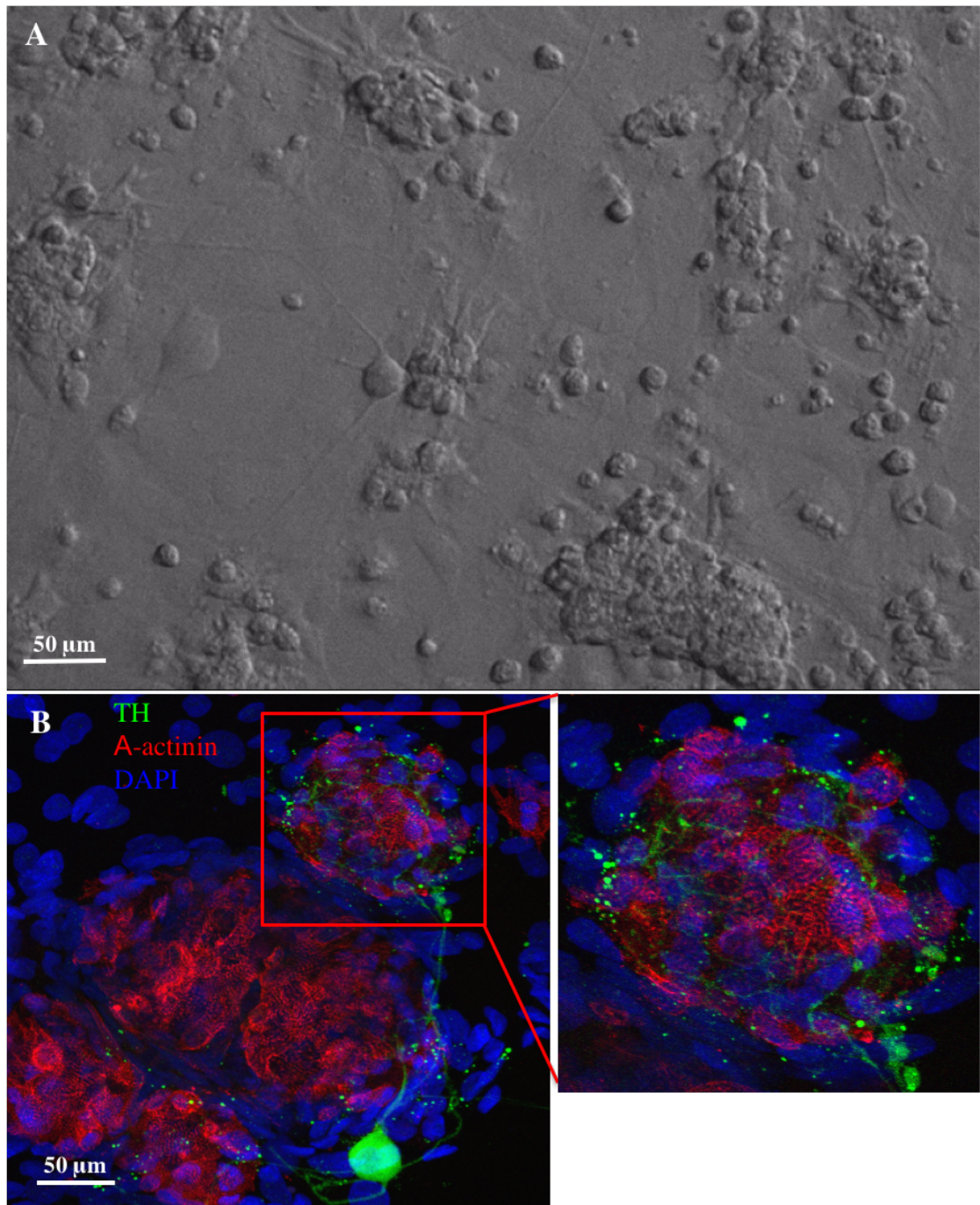
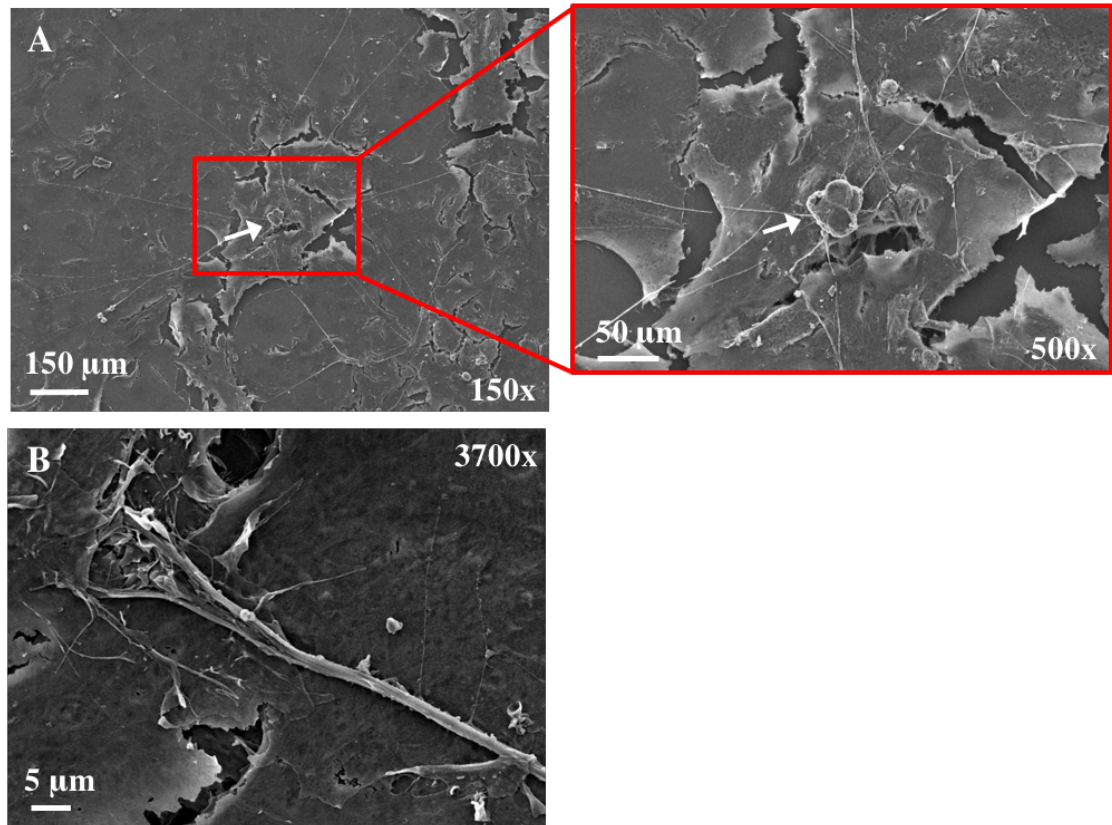


Figure 2.5: The co-culture phenotype. A snapshot of a video of the co-culture (A - see <https://youtu.be/bFKNKz-aaa0>) depicting how the neurons are located on top of the myocytes. The neuronal processes extend out from the cell body and touch the surrounding myocytes. All myocytes that were innervated by the same neuron beat in synchrony, suggesting functional connections were made. The cellular phenotypes were confirmed by immunofluorescent staining (B and inset). Sarcomeric alpha actinin marks the myocytes (red), Tyrosine hydroxylase (TH, green) is a sympathetic neuron marker. The neuronal processes naturally interweaved with the myocytes (inset) resulting in a rich innervation of the tissue.

2.3.4.4 Gold labelling electron microscopy detailing synapse formation

Co-cultures were prepared for Scanning Electron Microscopy (SEM) using a protocol adapted from ²¹⁶. Briefly, the cells were grown on 12 mm Poly-D-Lysine coated glass coverslips for five days. The cells were rinsed and fixed in 2.5% glutaraldehyde, and 1% osmium tetroxide at 4°C for one hour. The sample was taken through an ethanol dehydration series before it was dried for three minutes with hexamethyldisilazane. Cover-slips were mounted onto SEM stubs with a conductive carbon backing and coated with approximately eight nm gold. The samples were imaged on a JEOL-6390 SEM. It was found that the neurons portray extensive branching that spread out over the myocyte membrane (2.6A and inset). Processes extend out from the main axon that terminate on the myocyte membranes (2.6B). These are thought to be the sites of synapse formation (Burton et al 2016, unpublished).

Figure 2.6: Electron microscopy (EM) gold labelling of co-culture. EM imaging was done to investigate the structural aspects of the synapse formation between the sympathetic neurons (arrows) and the cardiac myocytes (underneath the neuron). It was found that the neurons portrayed extensive branching that spread out over the myocyte membrane (A and inset). Processes were sent out from the main axon, terminating on the myocyte membrane (B). These are thought to be the sites of synapse formation. The small invaginations seen on the myocyte membranes are T-tubules (communication with Dr. Burton). The black areas/tears are tissue damage that occurred during processing of the sample. Figure courtesy of Dr. Burton



2.3.5 Cross-culturing

When investigating two cell types grown together in a culture, it becomes difficult to pharmacologically affect one cell without also affecting the other. To investigate which cell was the driver of the phenotype and to which extent one cell could rescue or indeed induce a phenotype in the other, cross-cultures were created (see figure 2.7 for schematic). Briefly, SHR PGSNs were plated on healthy WKY myocytes and healthy WKY PGSNs were plated on diseased SHR myocytes. These cultures were then compared to the WKYn/WKYm and SHRn/SHRm cultures.

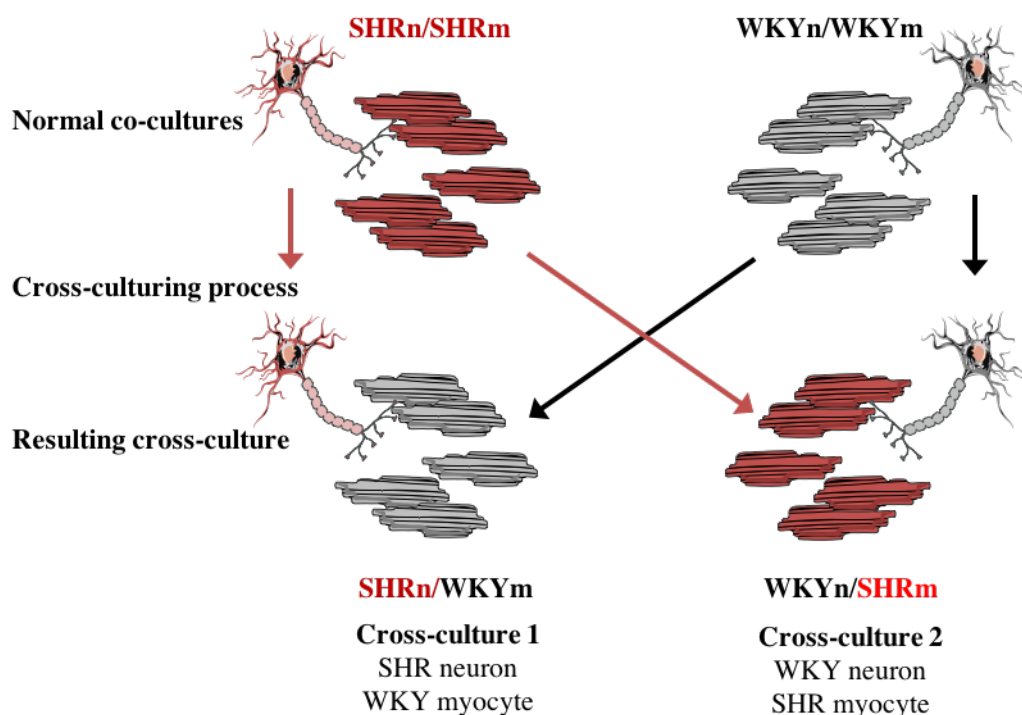


Figure 2.7 The cross-culture setup.

Schematic for the cross-culture formation. Healthy WKY neurons were plated on diseased SHR myocytes and diseased SHR neurons were plated on healthy WKY myocytes. These cultures were exposed to the same experimental protocols and compared to the WKYn/WKYm and SHRn/SHRm co-cultures.

2.3.6 Medias used

A variety of medias were designed to keep the cells viable in culture (see table 2.1).

Table 2.1: Cell isolation and culture medias used to isolate and maintain sympathetic neurons, neonatal cardiomyocytes and co-cultures		
<i>Neuronal Plating media</i>	<i>Neuronal Blocking media</i>	<i>Myocyte media</i>
90% L15 media	96.8% L-15	17% M199
24 mM NaHCO ₃	0.6% glucose	68% DMEM
38 mM Glucose	2 mM L-glutamine	n/a
1% Penicillin/streptomycin	1% Penicillin/streptomycin	1% Penicillin/streptomycin
50 ng/mL NGF	10% FBS	5% FBS
10% FBSS	n/a	10% Horse serum
<i>Co-culture media: 50 ng/mL NGF was added to the myocyte only media to form the co-culture media</i>		

2.4 Immunofluorescence

Immunofluorescence (IF) experiments were conducted on the PGSNs in culture to determine the cell phenotype and to assay the receptor expression on the cell surface.

For IF experiments the cells were grown on 12 mm ready coated Poly-D-Lysine/Laminin coverslips (BD Bioscience) and grown for five days. The media was removed and the cells were fixed for 10 minutes in 4% Paraformaldehyde. The cells were washed in PBS before being permeabilised and blocked for one hour using a solution containing 10% goat serum, 0.3% bovine serum albumin and 0.1% Triton X in PBS. The blocking media was replaced with the antibodies at the appropriate dilution (see table 2.2) and incubated over night at 4°C. The coverslips were washed in cold PBS 5-6 times over the next hour, before secondary Alexafluor conjugated antibodies were incubated for 2 hours in the dark. PBS washes were repeated before the coverslips were mounted using a DAPI containing soft mount Vectashield (Vector labs). The slides were sealed with nail polish and kept at 4°C in the dark until imaging. Imaging was done using a Live Cell Olympus confocal microscope.

Table 2.2: Overview of the immunofluorescent antibodies employed in this thesis			
Antibody	Catalogue #	Dilution	Description
Mouse anti-Tyrosine hydroxylase	T1299 Millipore	1:250	Sympathetic neuronal marker
Rabbit anti-Tyrosine hydroxylase	AB152 Abcam	1:250	Sympathetic neuronal marker
Choline acetyl transferase	Ab144P Abcam	1:500	Parasympathetic neuronal marker
Cav 2.2	ACC-002 Alomone	1:500	N-type Ca ²⁺ channel antibody
Connexin 43	Ab1728 Millipore	1:100	Gap junction protein
Sarcomeric α -actinin	A7811 Sigma	1:650	Myocyte specific marker.
Anti – mouse and rabbit Alexa - 647	General. See	1:1000	Secondary fluorescent antibody
Anti - mouse and rabbit Alexa - 488	Invitrogen webpages	1:1000	Secondary fluorescent antibody

2.5 Electrophysiology

2.5.1 Voltage clamp of sympathetic postganglionic stellate neurons

Whole cell voltage clamp experiments were performed to investigate the Ca^{2+} currents in the PGSNs of the normotensive WKY and pro-hypertensive SHRs. Experiments were conducted using the Axopatch 200B amplifier and the Clampex software on a computer linked to a conventional Nikon microscope with a 40x objective. The neurons were held at -90 mV before a set of 50 ms voltage steps from -50 to +50 mV were applied with 10 mV, 5 sec intervals. The bath was grounded using a Ag/AgCl silver pellet wire connected to the bath via a 3M/agar salt bridge. Cells were perfused with Tyrode or Ca^{2+} isolating solution using a custom built perfusion system, into a heated microscope stage chamber maintained at 37°C degrees.

Access to the cell was obtained in normal tyrode solution (in mM; 135 NaCl, 4.5 KCl, 11 glucose, 20 HEPES, 1MgCl₂, 2 CaCl₂, pH 7.40), before the solution was changed to the Ca^{2+} isolating solution containing no Na⁺, and K⁺ channel blockers (TEA and Cs⁺) with Ba²⁺ as the charge carrier in order to isolate the Ca^{2+} currents (in mM; 135 TEACl, 10 HEPES, 4.5 KCl, 1 MgCl₂, 4 Glucose, 1 NaHCO₃, 2 Ba²⁺, pH 7.40) either in the presence or absence of drugs. Ba²⁺ was also used to avoid Ca^{2+} dependent current inactivation²¹⁷. The internal solution contained (in mM; 140 CsCl, 10 HEPES, 0.1 CaCl₂, 1 MgCl₂, 4 MgATP, 1 EGTA, pH 7.30). All solutions had osmolarities of 300mOsm/L. 75-90% series resistance compensation was applied to all cells. Peripheral neurons portray significant axonal branching, making an effective voltage clamp difficult to achieve²¹⁸. Chapter 3 details the experimental work done to minimise these errors. Briefly to minimise space clamp errors: the cells were held at -90mV; low concentrations of the charge carrier was used; the cells were patched acutely using large

bore electrodes (1.5-2 M Ω); strict exclusion criteria were employed. Only cells that has a resting membrane potential of more negative than -40 mV, a series resistance of <10 M Ω throughout the recording, a holding potential of >-300 pA and cells displaying no visible contusion of the currents, were included in this study.

The voltage clamp of the PGSNs provided significant technical challenges due to the issues relating to obtaining effective space clamping. A considerable amount of experimental time was spent developing this technique in the lab, the results of which are described in full in Chapter 3.

2.5.2 Voltage clamp of neurons in a beating co-culture

One of the earlier studies of neuronal and myocyte co-cultures demonstrated that when grown in the presence of sympathetic neurons, the myocyte L-type Ca²⁺ current was significantly larger, than when the myocytes were grown in isolation¹⁸⁷. It was therefore of interest to see if the sympathetic neurons also portrayed a different Ca²⁺ channel current phenotype when grown in the presence of myocytes.

The voltage clamp experiments were conducted as described in section 2.5.1. The co-culture voltage clamp was performed using a 10x objective to enable the visualisation of the surrounding, innervated myocytes (2.8 A). Great care had to be taken to find a neuron that was innervating a group of myocytes in the vicinity, whilst at the same time being immobile on the coverslip and not affected by the movement of the myocytes. It was found that the myocytes surrounding the neurons hyper contracted when the patch pipette came near the neuron of interest. This is likely due to the KCl or ATP diffusing from the patch electrode upon approach to the neuron. Preloading of the tip of the electrode with an internal solution not containing KCl and ATP did not solve the

problem. In most cases this led to the neurons being ripped off the plate and moving along with the myocytes (see figure 2.8C). This became a problem as it was extremely challenging to effectively voltage clamp a moving cell. The integrity of the innervation could also be questioned. In some cases, this violent contraction also resulted in myocyte death due to sheer stress. To prevent this from occurring, efforts were made to arrest the heart cells using pharmacological agents such as blebbistatin, but as an important criterion for a healthy innervation of the myocytes was their beating phenotype, this was not ideal. Moreover, it was hoped that the activity of the neurons could be controlled using neuronal patch clamp techniques and to observe its effects on the beat rate and synchrony of the directly innervated myocytes and the ones surrounding it. This could shed light on to what extent the neurons themselves could form the substrate for arrhythmias.

Some recordings were however obtained, and it soon became evident that the space clamp issues were a huge obstacle in this work (see Chapter 3 for details on the space clamp issue). When grown in the presence of myocytes, the neurons increase their axonal branching in attempts to find their targets. This is in part due to the myocytes secreting NGF which attracts the neurons ²⁰⁸. As a consequence of this branching, an effective space clamp was not possible to obtain in these experiments (figure 2.8B) and so efforts were turned to another way of studying the co-cultures.

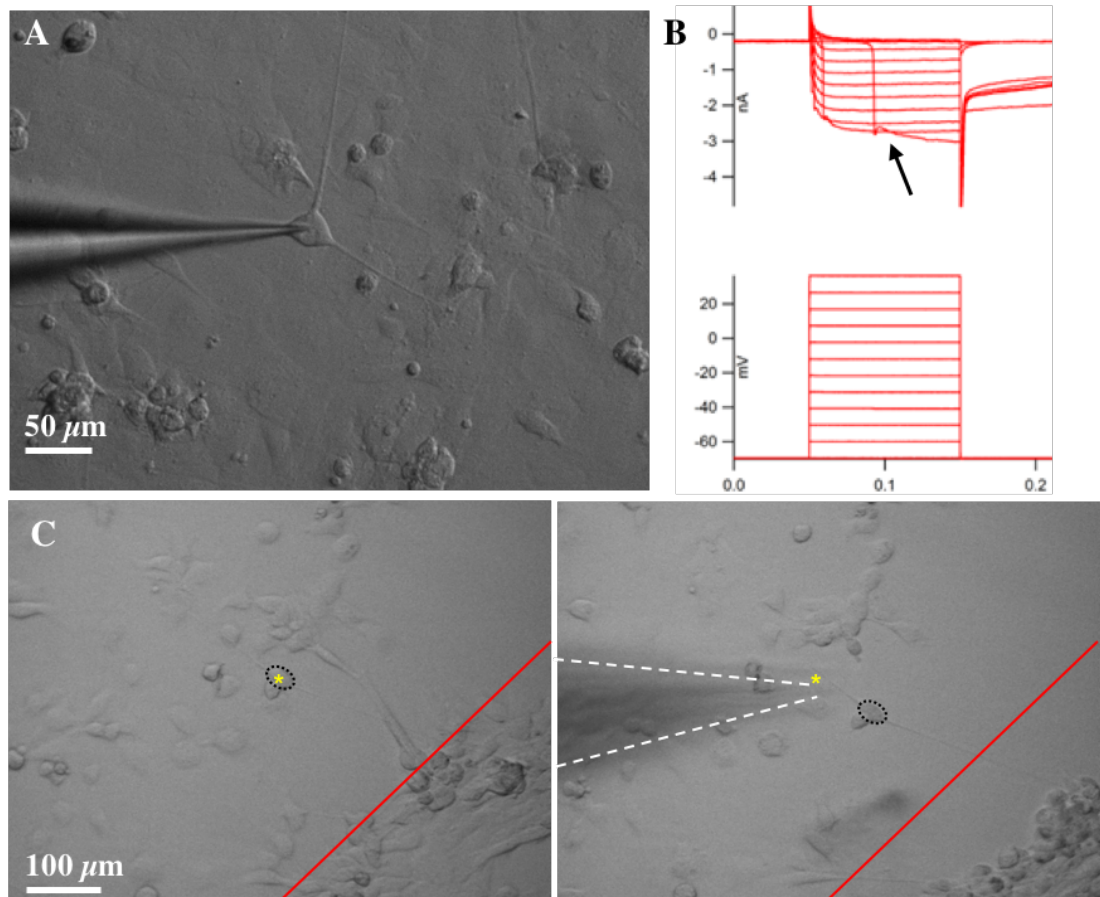


Figure 2.8: Voltage clamp of sympathetic neurons in a co-culture. The neuronal electrophysiological work was repeated on the co-cultures to test whether the presence of myocytes could alter the neuronal Ca^{2+} currents. Successful voltage clamp of the co-cultures only occurred in limited attempts (A) and often the resulting currents were marred by inadequate space clamp (B). The black arrows indicate the late activating currents, a characteristic of poor space clamp. Furthermore the myocytes went into hyper-contraction when the neuron was approached by the patch electrode leading to displacement of the neuron. (C) The dotted black ring denotes the current neuronal position relative to the original position (yellow asterisk).

2.6 Using FRET sensors to monitor intracellular second messengers

Since the discovery of the second messengers cAMP²¹⁹ and cGMP²²⁰, cyclic nucleotide signalling is rapidly becoming the focus of intense scientific research²²¹ due to their involvement in a multitude of important intracellular signalling pathways and their implication in disease. A breakthrough in their study came with the development of a range of intracellular fluorescent FRET sensors that are able to detect small changes in the intracellular cyclic nucleotide levels²²²⁻²²⁴. These sensors rely on the concept of

Förster resonance energy transfer (FRET)²²⁵ whereby energy from a donor fluorophore can be transferred to an acceptor fluorophore if they are in close proximity (<10 nM). The binding of a molecule such as cAMP to the sensors results in a conformational change bringing two fluorophores into close proximity resulting in a signal that can be detected using a simple fluorescence microscope^{222,226}. A variety of sensor molecules with different fluorophores have now been developed that can rely on either the loss or the gain of FRET²²⁷.

2.6.1 cAMP sensors

The first sensors to detect cAMP were developed in the early 2000s^{222,223} with subsequent optimisation of the sensors in 2004²²⁸⁻²³⁰. These are based on the cAMP binding sequence of epac (exchange protein directly activated by cAMP) sandwiched between a cyan (CFP) and yellow (YFP) fluorescent protein.

A new generation of cAMP sensors have recently been developed that use the Venus and mTurquoise2 fluorophores²²⁴, which makes for a much brighter and photostable sensor (its is virtually pH and ATP insensitive) with a significantly increased range (from >10 fold (30% max FRET change in epac^{H30} to 350% max change in Epac-S^{H187}). The sensor employs a point mutation that increases Epac's affinity for cAMP to levels similar to PKA²²⁴. This prevents buffering of the initial cAMP signal by its binding to native PKAs in the cell before the sensor, significantly increasing the sensitivity of the sensor.²²⁴ A significant limitation of the early cAMP FRET sensors, was their pH sensitivity. In excitable cells such as neurons, changes in membrane excitability often result in changes in pH levels²³¹ in the cell which can have detrimental effect on the fluorescent probe²³². The development of the pH insensitive, high sensitivity sensors

was therefore important for the study of excitable cells that portray limited or unknown changes in cAMP levels, such as the neurons in this thesis.

Figure 2.9A illustrates the molecular structure of the Epac^{H187} – one of the sensors used in this thesis to detect cAMP levels ²²⁴. When cAMP levels are low, this sensor is at its max FRET, with the two fluorophores in close proximity. The binding of cAMP to the sensor as it increases in the cell leads to a conformational change of the sensor, resulting in the loss of FRET signal ²³³. This loss of FRET is a measure of the dynamic change of cAMP levels in the cell. It is important to note that zero or max FRET is not a reflection of the actual cAMP levels in the cells – the sensors only measure relative and dynamic changes in cellular cAMP.

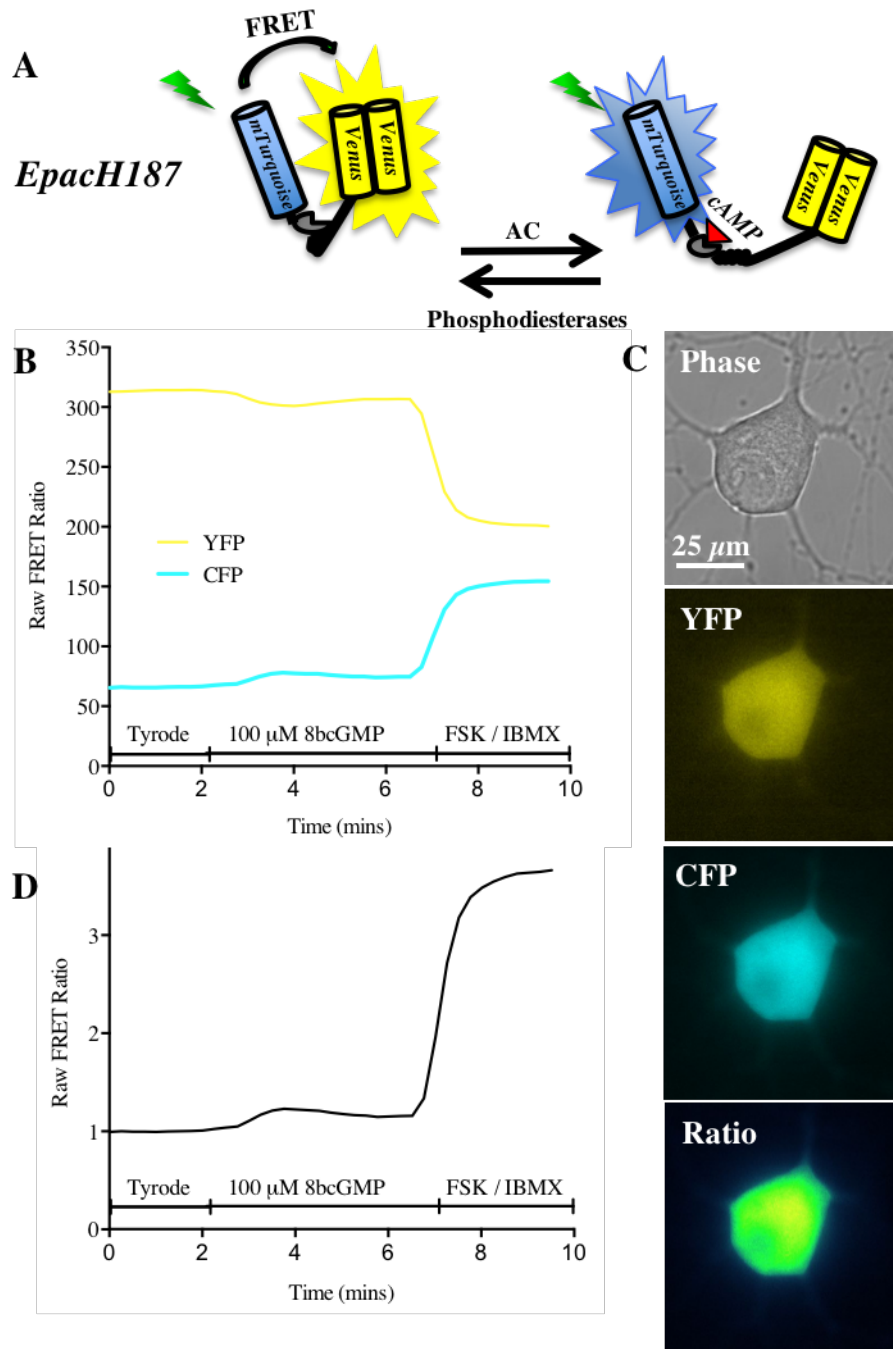


Figure 2.9: The molecular structure and function of the EpacH187 cAMP sensor. The sensor is comprised of two fluorescent proteins with overlapping excitation and emission spectra. When the two fluorophores are in proximity, the emission of one excites the other leading to Förster Resonance Energy Transfer (FRET). The binding of cAMP to EpacH187 (A) causes a conformational change in the sensor that moves the fluorophores apart, thereby resulting in loss of FRET. This can be detected experimentally by recording the CFP and YFP fluorescence intensities (B and C) and plotting the ratio (D) over time. Forskolin (FSK-an AC activator) and IBMX (PDE inhibitor) is added at the end of the experiment to record the max response of the sensor. The example trace illustrates the response of a Wistar neuron to the application of 100 μ M 8 bromo cGMP (which increases cAMP through cross-talk with PDEs). Panel A is adapted from reference 233.

2.6.2 PKA sensor

Figure 2.10A illustrates the molecular structure of the AKAR4 sensor used in this study to monitor PKA activity. At basal levels this sensor is at its minimum with respect to the FRET signal. When PKA activity inside the cell increases, an intramolecular portion of the sensor is phosphorylated by PKA leading to a conformational change. This results in a ‘gain of FRET’ signal indicative of PKA activity^{172,233}.

2.6.3 cGMP sensors

Sensors also exist for cGMP, but these are under construction/validation and have not been used in this study.

2.6.4 Adenoviral generation

Much of the work using FRET sensors have employed plasmids to deliver the sensor to the cells. As neurons are notoriously difficult to transfect²³⁴, the incorporation of the FRET sensor into a viral delivery system was desired. The AKAR4 PKA sensor already existed in an adenoviral delivery vector¹⁷² and the details on the construction of the H187 sensor was as follows: The complete open reading frame of the cAMP sensitive FRET-based sensor H187 was obtained from pcDNA3 using double digestion with the restriction enzymes EcoRI and HindIII and introduces into the pDual vector under a CMV promoter. This cloning was performed by Dr Konstantinos Lefkimmiatis (University of Oxford), but the generation and amplification of the viral particles were outsourced to Vector Biolabs, USA. In addition to the above mentioned sensors, one experiment in chapter 5 was performed using the Epac H30 sensor²³⁰.

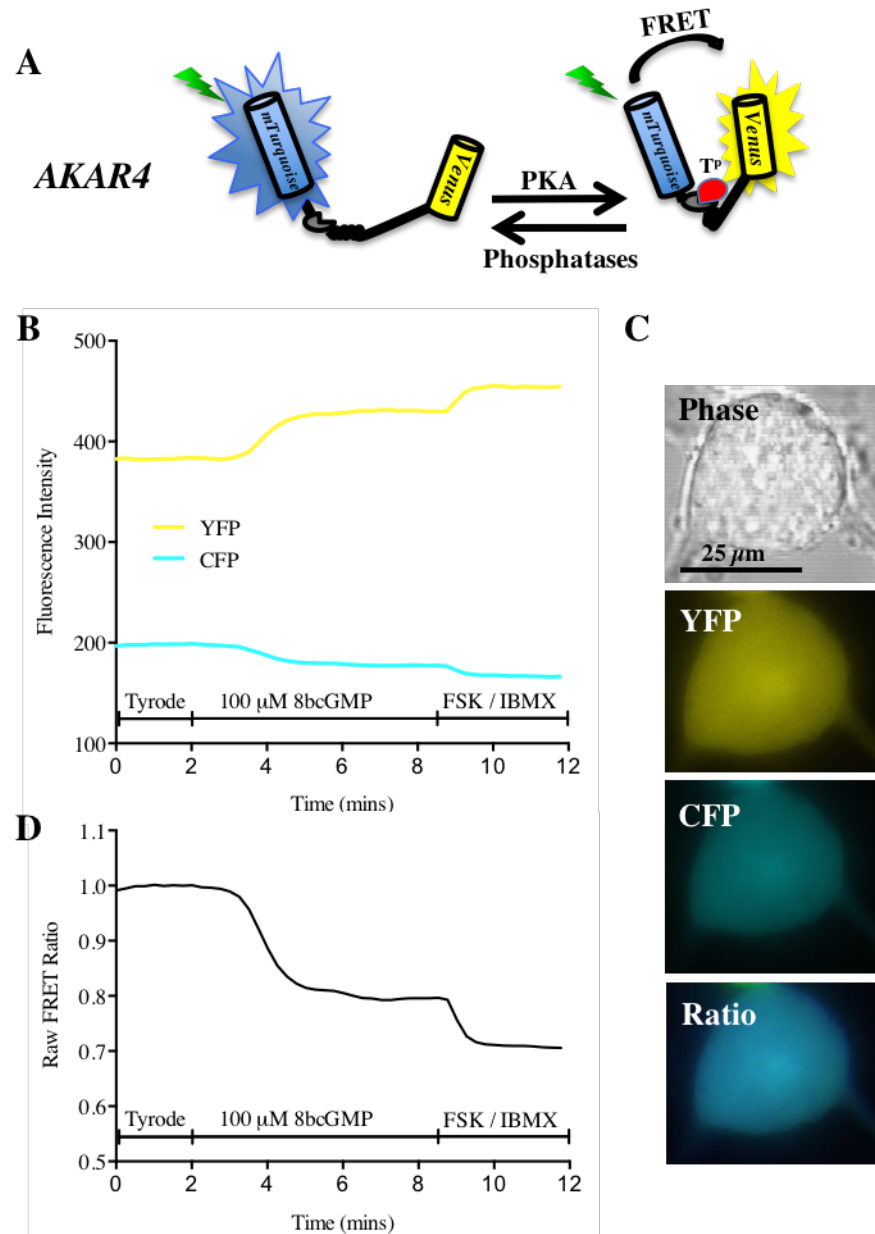


Figure 2.10: The molecular structure and function of the EpacH187 (cAMP) and AKAR4 (PKA) sensors. The sensor is comprised of two fluorescent proteins with overlapping excitation and emission spectra. When these two fluorophores are in proximity, the emission of one excites the other leading to Förster resonance energy transfer (FRET). The phosphorylation of the AKAR4 (A) sensor by PKA causes a conformational change in the sensor, bringing the fluorophores together resulting in the gain of FRET. This is detected experimentally by recording the CFP and YFP fluorescence intensities (B and C) and plotting the ratio (D) over time. The example trace illustrates the response of a four week Wistar neuron to the application of 100 μ M 8 bromo cGMP which activates PKA through cAMP elevation via cross-talk with PDEs. Figure adapted from reference 233.

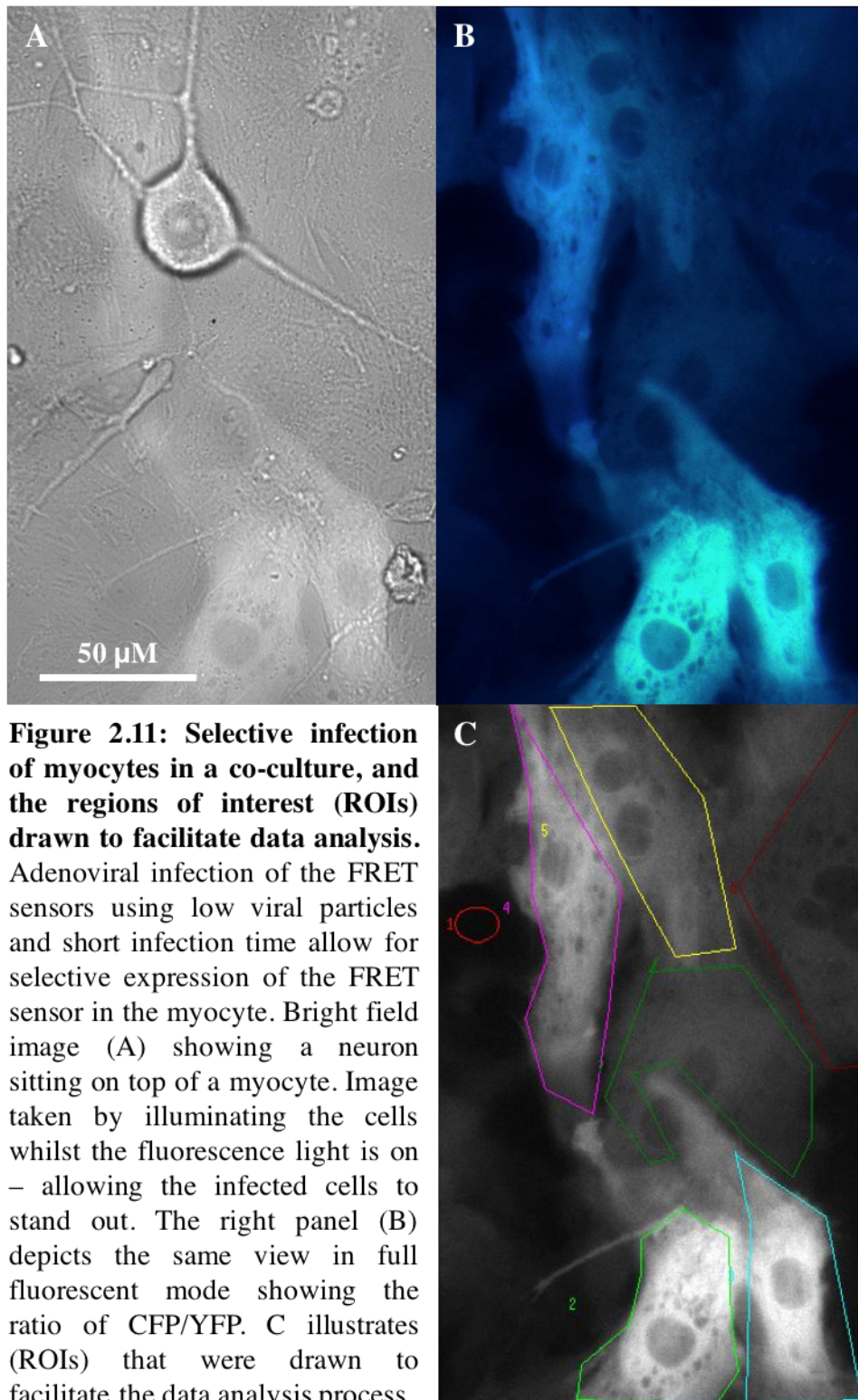
2.6.5 Viral infection of FRET sensors

2.6.5.1 PGSNs

One hour after culturing, the media was changed before the cells were infected with the relevant adenoviral fret sensor virus in a total volume of 200 μL . H187: 1.4×10^7 particle forming units (PFUs), AKAR4: 4×10^9 . The cells were infected for 24 hours before the cells were washed and fresh media applied. The cells were kept in a separate viral incubator and kept at 37° degrees at 5% CO_2 .

2.6.5.2 Myocytes and co-cultures

The coxsackie- and adeno-virus receptor (CAR) is a known site for viral attachment and entry. The expression of CAR on neonatal myocytes has been shown to be important for cardiac development²³⁵. This is taken advantage of by designing an adenoviral FRET²³⁶ sensor allowing for a high infection rate of the sensors, using minimal virus particles. The myocytes and co-cultures were infected for two hours with 3.6×10^5 PFUs of H187, as compared to 1.4×10^7 in the neuron. This low viral load and short infection time not only ensured adequate expression in the myocytes, but it also prevented the neurons in the culture from getting infected (see figure 2.11), ensuring that the FRET signal recorded is of pure myocyte origin.



2.6.6 Microscope setup

FRET sensor-expressing neurons were imaged three to four days post-isolation on an inverted Nikon microscope. This was connected to an OptoLED fluorescence imaging system (Cairn Research Ltd) equipped with a 40x oil-immersion objective, a CoolSnap HQ2 digital CCD camera (Photometrics) and a beam-splitter (DV2, Photometrics) which included the emission filters for CFP and YFP acquisition (dichroic mirror 505DCXR). The cells were excited at 430 nm for 100ms, every 15 seconds. CFP and YFP emissions were measured and the ratio between 480/535 nm fluorescent emission intensities was automatically calculated by the Optofluor software.

Spectral bleed-through and cross-excitation are two important factors that could introduce errors into measurements^{226,237}. However, these two components are dependent only on the microscope set-up. Such errors are eliminated if these components are kept constant between all experiments. For this reason, the exact same microscope setup was used for all experiments in this thesis.

2.6.7 FRET data analysis

The CFP and YFP channel images were separated before regions of interest were drawn (Figure 2.11C). Background fluorescence was subtracted from emission intensities and intensity ratios were calculated using the following equation for the cAMP sensor:

$$ratio = \frac{mean\ CFP\ intensity - CFP\ background}{mean\ YFP\ intensity - YFP\ background}$$

and

$$ratio = \frac{mean\ YFP\ intensity - YFP\ background}{mean\ CFP\ intensity - CFP\ background}$$

for the PKA sensor. The FRET ratio was normalised to the mean baseline, defined by the 2-6 minutes prior to drug treatment. The raw FRET values were expressed in percentages from baseline and plotted against time. Both peak levels and plateau phases

were used for analysis. Cells were only excluded from the study if they failed to respond to the 100 μ M IBMX/25 μ M forskolin step to maximize the sensor (see figure 2.12).

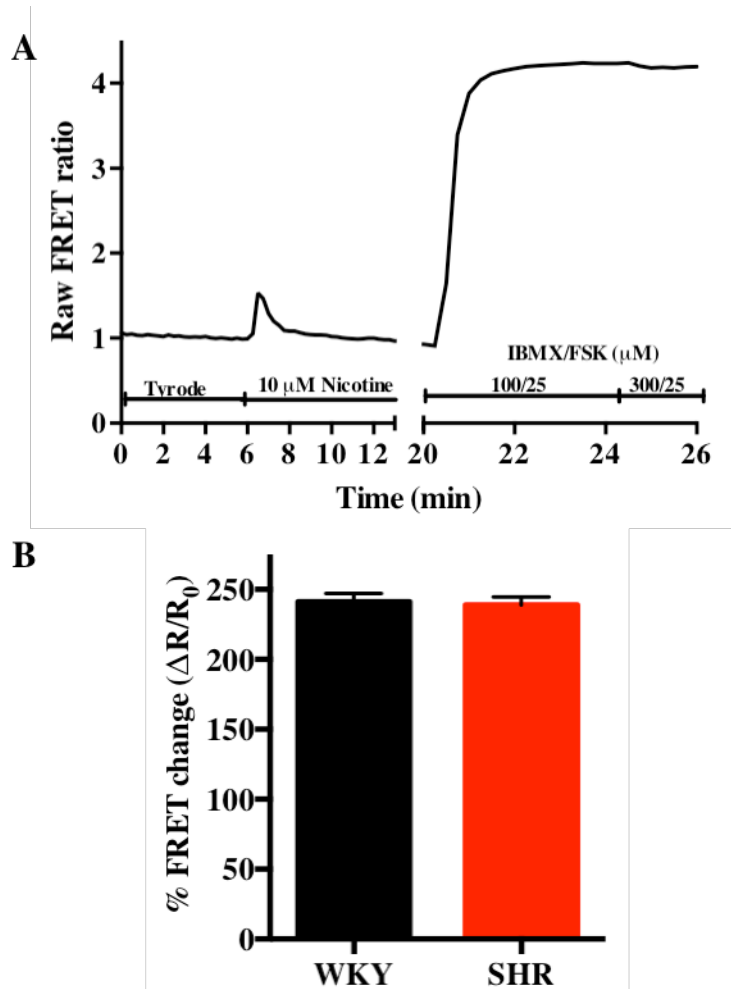


Figure 2.12: The H187 sensor reaches max FRET change with 100 μ M IBMX and 25 μ M forskolin (FSK). It was important to record the max FRET change of the sensor to ensure the cells were responding in a similar manner to the sensor, so that appropriate data analysis can be performed. Post nicotine exposure, the neuron was exposed to saturating concentrations IBMX/FSK (PDE inhibitor/Adenylyl cyclase activator) reported in literature to max out both neurons and myocytes. When the response had reached a plateau, the IBMX concentration was increased three fold. No changes were seen in the FRET response, indicative of the max being reached at 100 μ M IBMX and 25 μ M FSK. Similar results were found in the myocytes and co-cultures. As a result, this concentration was employed throughout the thesis. No changes were found in the max FRET response (B) between the WKY and the SHR (241.2% $\pm$ 5.94 SEM n=76 vs 239.0% $\pm$ 5.74 SEM n=87, p=0.79).

2.6.8 FRET imaging of nicotine stimulated co-cultures

As described in section 2.3.4.2 the co-cultures were stimulated using nicotine. FRET was used to study the cell-to-cell communication in the co-cultures of sympathetic PGSNs and ventricular myocytes. As described in section 2.3.4.2, the neurons were stimulated with nicotine leading to the release of noradrenaline into the synaptic cleft. 1, 10, 100, 300 and 500 μM concentrations were used to stimulate increased sympathetic drive. The resulting noradrenergic activation of the β_1 receptors results in increased cAMP levels in the cell which was picked up by an intracellular FRET cAMP sensor selectively infected into the myocytes (figure 2.13).

2.6.8.1 Nicotine stimulates the sympathetic stellate neurons via the nicotinic acetyl choline receptor (nAChR)

To ensure that the responses seen were as a result of nicotinic activation of the acetyl choline receptor (nAChR) on the neuronal membrane, the neurons were exposed to nicotine both in the absence and presence of the specific nAChR antagonist mecamylamine (10 μM). As can be seen from figure 2.14, the neurons were infected with the H187 cAMP sensors and upon nicotine stimulation displayed the loss of FRET in response to the elevation of cAMP in the neurons (2.14A). This response was completely blocked by 10 μM mecamylamine (n=8) strongly suggesting the neuronal activation was nAChR mediated. Immunofluorescence confirmed the expression of the nAChR on the neuronal membrane of a WKY neuron. The same was found in the SHR neurons (results not shown). The exact mechanisms behind this increase in cAMP in the neurons remains unclear (see Chapter 5 for discussion), as is whether this increase in cAMP directly contributes to noradrenaline release. But for the purpose of this experiment, it confirms that the observed effects of nicotine on the neurons are mediated via nAChR.

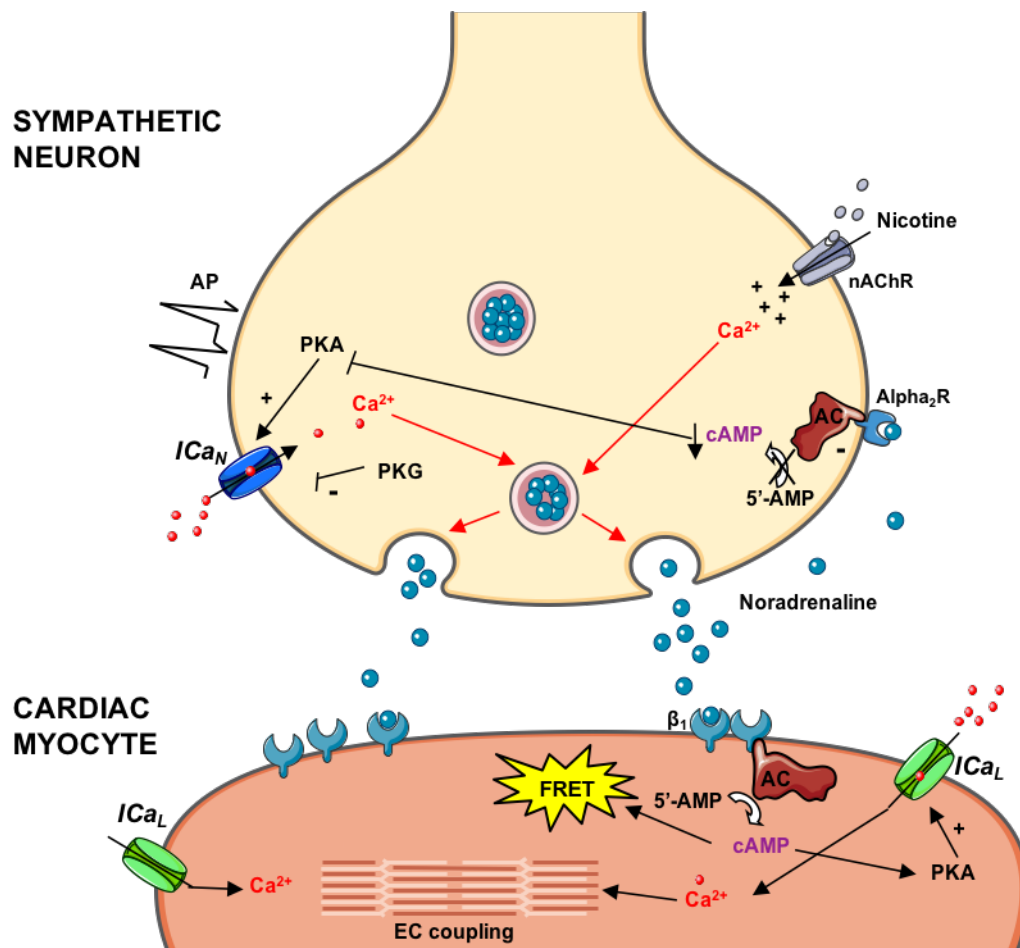


Figure 2.13: FRET imaging of the co-cultures. The arrival of an action potential in the synaptic nerve terminal, leads to Ca^{2+} entry via voltage gated Ca^{2+} channels. This triggers exocytosis of noradrenaline (NA) into the synaptic clefts which acts on the β_1 adrenoreceptors, altering myocyte behaviour. To stimulate the co-cultures, nicotine was applied which allowed native release of NA into the synaptic cleft. The activation of the β_1 adrenoreceptor results in the activation of adenylate cyclase (AC) resulting in the formation of cAMP. The dynamic changes in cAMP levels were recorded using the cAMP sensitive FRET sensor H187.

2.6.8.2 The nicotine evoked elevation of myocyte cAMP in the co-cultures is mediated by the β_1 adrenergic receptor

Similarly, to ensure that the elevation of cAMP in the myocytes, as a result of the nicotine stimulation of the neuron was due to noradrenaline acting on the β -adrenergic receptors, the co-cultures were exposed nicotine in the absence and presence of the β_1 receptor antagonist metoprolol. As can be seen from figure 2.15, nicotine application led to an immediate increase in myocyte cAMP levels (B). In all experiments (n=60) this response was entirely blocked by 10 μ M metoprolol, strongly suggesting that the cAMP elevation in the myocytes are the result of noradrenaline acting on the β_1 adrenergic receptor. Nicotine was also added to the myocytes alone to test for non specific effects of nicotine on the myocytes alone, and to test the potential presence of contaminating intra-cardiac neurons in the culture. Nicotine has no effect (n=30) on the WKY and SHR myocytes (see Chapter 6 for details).

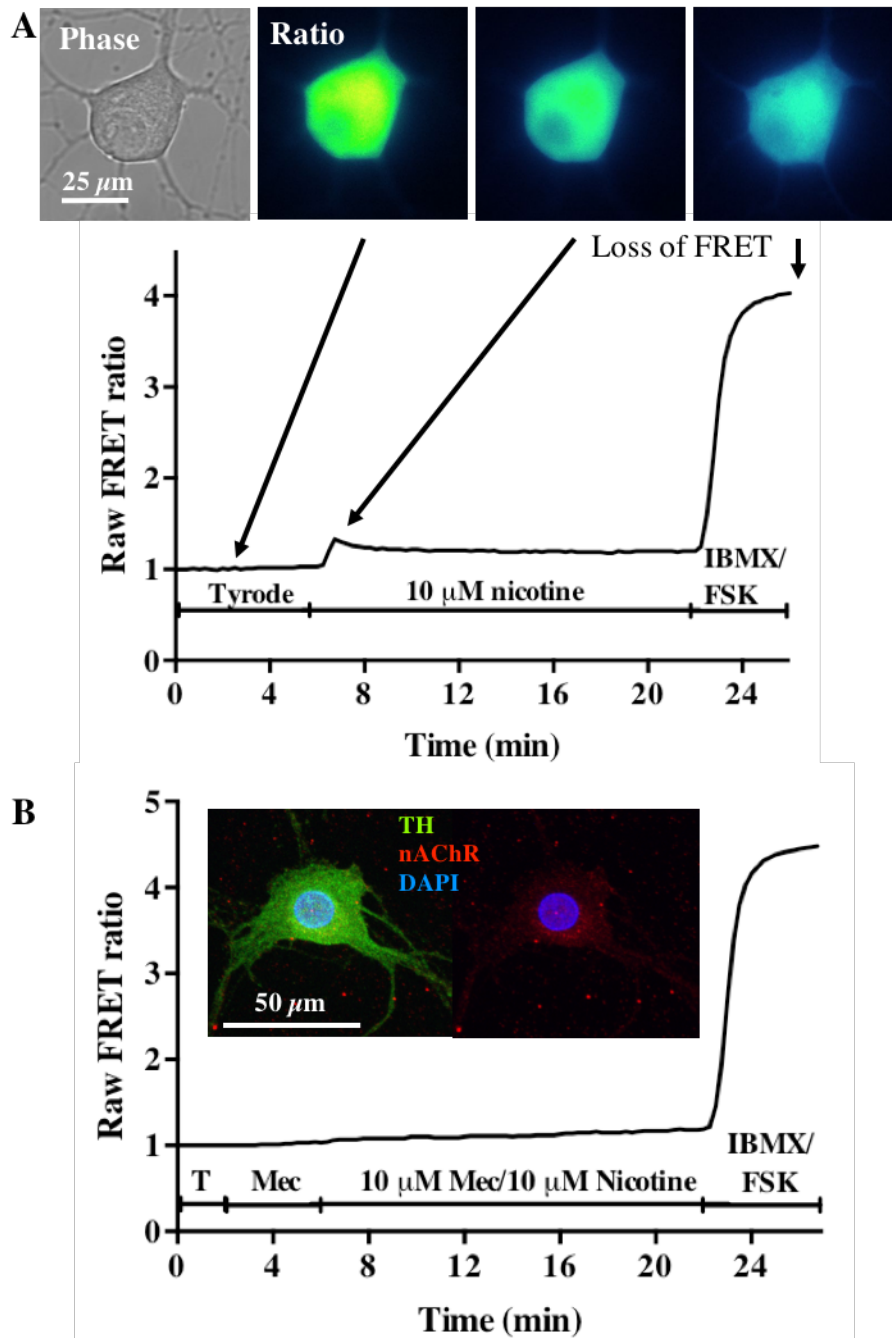
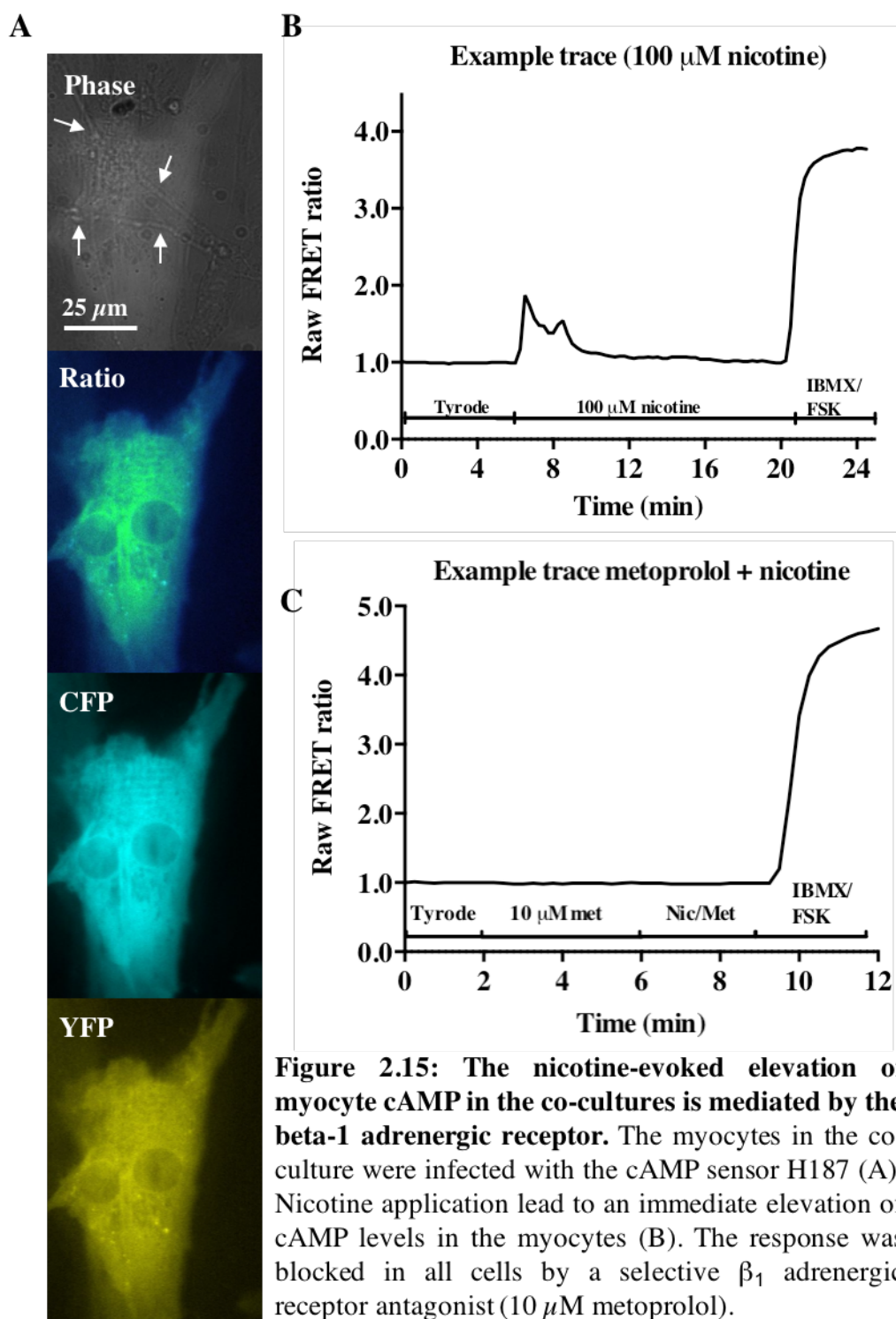


Figure 2.14: Nicotine responses are mediated via the neuronal nicotinic acetylcholine receptor. A WKY neuron was infected with the H187 cAMP FRET sensor and exposed to nicotine (A) resulting in an immediate marked cAMP spike. The elevation of cAMP levels within the cell leads to the loss of FRET which can be clearly seen from the images in A. The application of 10 μM mecamylamine (Mec) blocked the cAMP elevation in all cells ($n=8$) (B), confirming that the nicotine responses seen were mediated by the nicotinic acetylcholine receptor. Immunofluorescence confirms the presence of this receptor on the neuronal cell membrane.



2.6.9 Experimental protocols and drugs used

PGSNs, myocytes and the co-cultures were exposed to a range of experimental protocols. Specific details on these are described in chapters 5 and 6, but a brief summary can be found in table 2.3 and below.

All cells were perfused continuously with tyrode solution (in mM 135 NaCl, 4.5 KCl, 11 glucose, 1 MgCl₂, 2 CaCl₂, 20 HEPES and adjusted to pH 7.40.). Flow rate was controlled at 2.5 ml/min. Pharmacological agents were diluted in tyrode and perfused as appropriate for the respective experiment. Table 2.3 illustrates the drugs for the relative experiments. In all experiments, the maximal FRET change of each cells was recorded by exposing the cells to saturating concentrations of an AC activator and a PDE inhibitor (25 μ M forskolin and 100 μ M IBMX respectively) to ensure the cells responded similarly to the sensor. All FRET experiments were conducted at room temperature.

2.7 Statistical analysis

Electrophysiological data: Where the data passed normality testing (Kolmogorov–Smirnov), two tailed unpaired t-tests were used. Where the data was not normally distributed, the relevant statistical test used is reported in the figure legends. All data are expressed as the mean $\pm$ SEM. Statistical significance was accepted at P values <0.05 . Data was analysed using Graphpad Prism. Figures were created using Servier Medical Art.

Table 2.3. Drugs used for FRET experiments			
	Drug name	[Conc.]	Function
Neuron	Nicotine	1-500 μ M	Stimulate/depolarise neuron and induce NA release
	Mechamylamine	10 μ M	nAChR antagonist
	Forskolin	0-25 μ M	Dose response curve to assay cAMP generation
	IBMX	1-10 μ M	Dose response curve to assay global PDE activity
	8 bromo cGMP	100 μ M	Membrane soluble cGMP analogue
	BAY 60-7550	10 μ M	PDE2 selective blocker
	Cilostamide	10 μ M	PDE3 selective blocker
	Milrinone	10 μ M	PDE3 selective blocker
Myocyte	Isoprenaline	0.1, 1, 3, 10, 30, 100 μ M	β_1 adreno receptor agonist
Co-culture	Nicotine	1-500 μ M	Stimulate the neuron to release NA
	Metoprolol	10 μ M	β_1 adreno receptor antagonist
All	IBMX Forskolin	100 μ M 25 μ M	To maximally activate the FRET sensors.

CHAPTER THREE

3 Optimal development of single cell voltage clamp of peripheral neurons

3.1 Introduction

This chapter aims to give an overview of the field of electrophysiology and provide details on the voltage clamp experiments employed in chapter 4. Sympathetic PGSNs portray extensive axonal branching in culture that presents a set of unique challenges to the experimenter relating to obtaining an effective space clamp. This chapter will therefore highlight the method development that was required to generate a reproducible and effective voltage clamp of these cells.

3.1.1 Historical perspective

Long before the development of modern medicine, it was realised that electrical phenomena are part of physiology. The realisation that electric stimuli through the spinal cord of a frog could elicit muscle contraction, paved the way for what we know as modern electrophysiology ²³⁸. Prior to the knowledge of the existence of membrane potentials and ion channels, action potentials ²³⁹ and synaptic currents were first recorded from a giant squid axon ^{240,241} using a crude intracellular glass electrode. In 1952 Hodgkin and Huxley explained and developed a mathematical model of the ionic basis of how neuronal action potentials are initiated and propagate. This work earned them the Nobel Prize in 1963 in Physiology or Medicine. Later, Neher and Sakmann modified these techniques by using large bore pipettes that instead of puncturing the cell, formed a seal with the cell membrane, allowing them to record from single channels in distinct membrane patches ^{242,243}. They conclusively proved that ion channels exist in cell membranes and first described their function. This work led them to receive the Nobel Prize in 1991. Over the years, further refinement of the technique and the realisation of its full use has led to the current array of methods in the field of

electrophysiology^{244,245}. These electrophysiological methods can be combined with other techniques such as fluorescent imaging, cell injection and cell labelling to produce elegant and powerful work²⁴⁴.

3.1.2 Electrical properties of cell membranes

Mammalian cell membranes consist of a phospholipid bilayer that allows selective molecular crossing via ion channels, exchangers and transporters imbedded in the membranes. These are evolutionary conserved molecules that are highly regulated by both external and intracellular substances, allowing for fine-tuning and control of cellular homeostasis. The net result of the movement of charged species is the cellular resting membrane potential (RMP) – a potential voltage difference across the cells due to the varying amounts of positive and negative charge carriers on either side. In most mammalian cells, the intracellular environment holds a potential of -70 mV with respect to the extracellular environment. The difference in voltage and ion species sets up the electrochemical gradient that dictates in part the preferential movement of ions across the membrane²⁴⁶.

3.1.3 Ion channels

Ion channels are broadly classed by the ions they conduct and in some cases the agents that act on them (for example ligands/drugs, mechanical, stretch and heat stimuli). Cells express an arsenal of different ion channels in order to maintain cellular homeostasis and to appropriately respond to external and internal signals. For example in the pacemaker cells of the heart, six different K⁺ channels govern the repolarisation of the cardiac action potential, all of which are crucial for appropriate functioning of the heart^{246,247}. In most cases these currents can be separated using pharmacological agents or by taking advantage of their biophysical properties. Ion channels control a wide range of

crucial cellular functions and as such they are attractive drug targets and important foci for scientific study.

3.1.4 Electrophysiology to study ionic currents

Ion movement through selective ion channels represent the major players of the electrical properties of human cells, and it results in a variety of biological responses ranging from the maintenance of normal function, to pathology²⁴⁸. The study of these ion channels – that is the currents resulting from ion channels opening and closing and the resultant effect on the membrane potential - can be measured using the current clamp; where you inject current and measure the resultant changes in membrane potential and the voltage clamp; where the voltage is clamped and the resulting changes in current, or ion flow is measured. A variety of electrophysiological conformations exist, depending on whether the experimenter is interested in the behaviour of single channels or the response of the whole cell. In this thesis the method employed throughout is the whole cell voltage clamp, the details of which is explained in the below sections.

3.1.5 The whole cell voltage clamp

The membrane potential affects ion channel function due to changes in driving force; the voltage dependency of certain ion channels and the voltage dependent block of ion channels. It is therefore often desirable to directly control the membrane potential, so that the ion channels themselves can be studied. The single electrode voltage clamp (V_{clamp})²⁴⁹ occurs through an electronic feedback system where the potential set up by the experimenter (the holding potential or the command voltage) is compared to the measured potential (see figure 3.1). Any deviation of the recorded potential from that of the holding potential is instantly and continuously corrected by a compensation current. This compensation current is equal, but of opposite sign, to the ion current flowing

across the membrane under investigation. The command voltage can be made to vary over time, from simple voltage steps to a more complex wave pattern depending on the ion channel properties you wish to study^{244,250}.

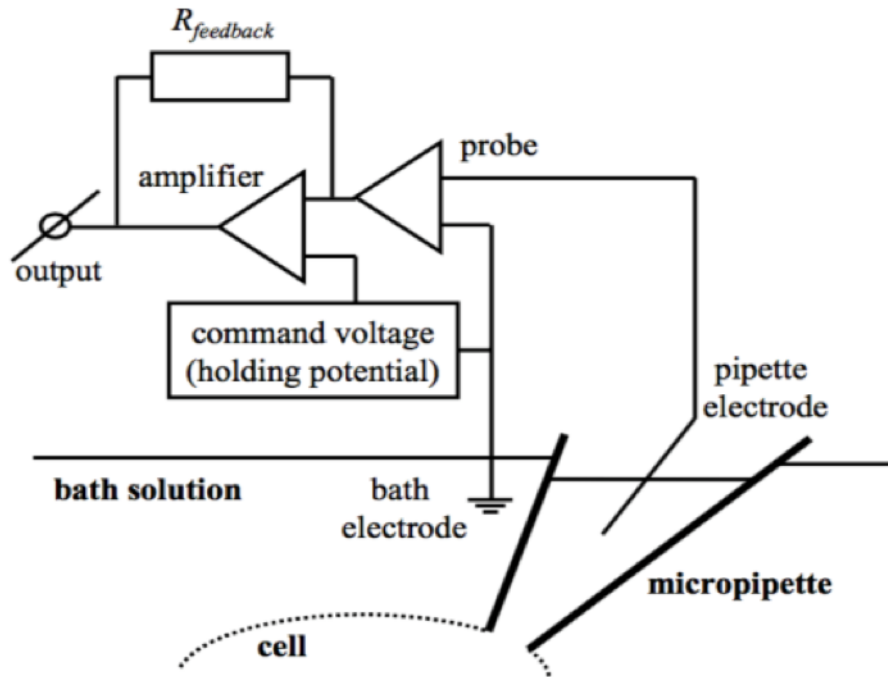


Figure 3.1: The whole cell voltage clamp. A voltage command is applied to the cell via the micropipette, and compared to the measured potential via an amplifier. Any difference is immediately compensated for by current injection, resulting in current traces that are equal but of opposite sign to the ion channel currents under investigation. Figure taken from Molleman, 2003.

3.1.6 Capacitance and series resistance

The membrane is an effective barrier to the movement of ions and in electronic terms it functions both as a resistor as well as a capacitor; something that stores charge. The same applies to the glass pipette used to probe the cell. Therefore, any amplifier induced changes in voltage applied to the cell must first charge up the pipette and the membrane before it can affect the cell. Together a resistor and a capacitor in series form a resistor-capacitor (RC) circuit (see figure 3.2), and the speed of which the capacitor is charged depends on the resistors in series with it - the series resistance (R_{series}). The R_{series} is made

up of the access resistance (R_{access}) and the membrane resistance (R_{membrane}). The R_{membrane} is by far the largest and simply put represents the ion channels, and so changes in R_{membrane} is a measure of the total current flowing through the ion channels as they open and close. The R_{series} and the capacitance impart a significant delay of the voltage command, and also the resulting recording. This forms the limiting factor in the speed of action potential propagation and so efforts need to be made to ensure the R_{series} is as low as possible to enable fast charging of the membrane. Any remaining R_{series} and capacitance needs to be compensated for using the electronic feedback system of the amplifier. Fuller detail can be found in ^{244,250}.

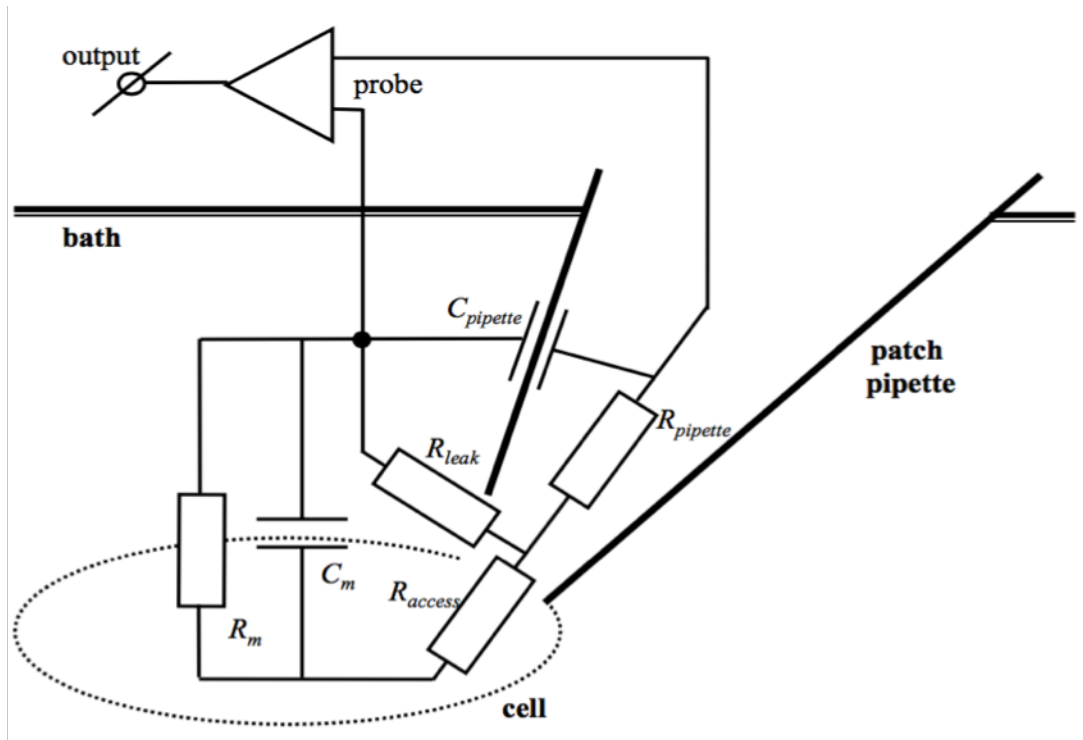


Figure 3.2: The equivalent circuit for the whole cell configuration. The whole cell voltage clamp forms an resistor-capacitor (RC) circuit. Before reaching the cell, the current has to cross a series of resistors and capacitors. These need to be kept to a minimum so that the membrane resistance, which is a measure of ion channel activity, can be recorded. Figure taken from Molleman, 2003.

Finally, the membrane capacitance is directly related to the surface area of the cell (assuming that the average cm^2 has a capacity of about $1 \mu\text{F}$). This number is important

for comparing membrane currents from different whole cell experiments, as it is assumed that the larger the cell, the more ion channels and the larger the whole cell current. It is for this reason most voltage clamp experiments report their current as the ratio of total whole cell currents to the cell capacitance (pA/pF).

3.1.7 Electrophysiology equipment setup

Most electrophysiological methods are performed using a conventional microscope placed inside a Faraday cage on an air table (to reduce electrical noise and vibrations). An electrode (a fine tipped glass pipette with a chlorided silver wire) connected to a feedback amplifier is controlled by a micro manipulator. The cell is approached and Giga Ohm ($G\Omega$) seal is obtained with the cell (cell attached configuration). Gentle suction is applied to the back of the electrode, selectively breaking the membrane directly underneath the electrode tip whilst retaining the tight electrical contact with the cell membrane (see figure 3.3) An intracellular solution with a make up similar to that of the inside of the cell keeps the electrical connection with the cytosol and prevents the deleterious effects of the intracellular dialysis that occurs once the cell membrane is broken. Immediately following membrane rupture, a transient switch is made to the current clamp ($I=0$) mode on the amplifier to get a reading of the cell's resting membrane potential – an important indicator of cell health.

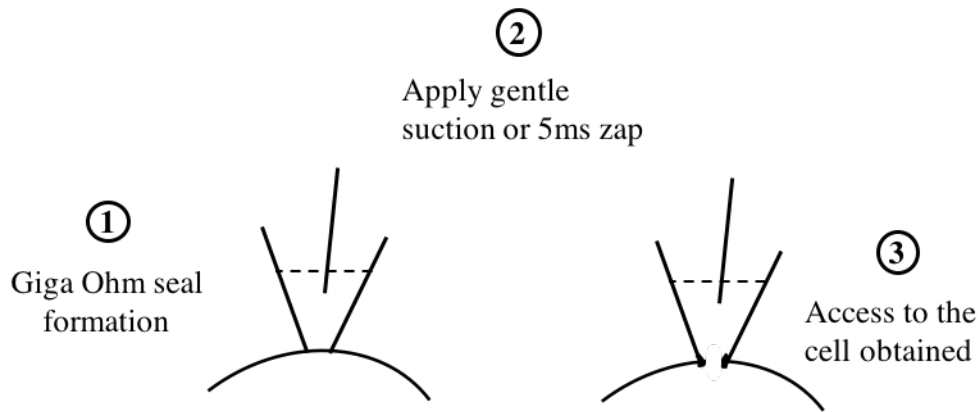


Figure 3.3: Three-step process to seal and break-through the cell membrane. 1: Cell approach and the release of the pipette pressure upon touchdown allows $G\Omega$ seal formation. 2: Gentle suction is applied to break through the membrane. Note how the membrane moves up into the pipette and creates a seal. 3: Access to the cell has been achieved. For tougher membranes a five ms current pulse can be used to aid break-through.

3.1.8 Space clamp issues in peripheral neurons

Voltage clamping of branched cells such as the PGSN is challenging due to a limiting factor commonly described as space clamp issues or isopotentiality^{218,244}. In order to get accurate information about channel behaviour, membrane currents need to be recorded from a group of channels that are all experiencing the same voltage at the exact same time. However, the intracellular solution does not conduct electricity as well as metals, which in practice means that the intracellular solution can be considered as multiple resistors in series with each other. This is in part due to the charge being carried by ions rather than electrons. Thus, in cells with a relatively large volume of intracellular solution spread over large distances, such as neurons with extensive axonal branching, space clamp issues can occur. In spherical cells, the resistance the intracellular solution imposes is very small in comparison to the membrane resistance (a measure of ion flow through channels) resulting in an effective voltage clamp. In neurons however, the resistance imposed by the intracellular solution becomes significant in relation to the membrane resistance, making an effective voltage clamp difficult, if not impossible to

obtain. During voltage clamping of such cells, the voltage is only controlled at the membrane in close proximity to the patch electrode. In areas distal to the electrode, the clamping is impaired by significant voltage drops over the volume of the intracellular solution. This leads to the opening of voltage gated ion channels at different time points, resulting in significant distortions of the currents recorded. A second distorting factor that contributes to the space clamp problem is the membrane capacitance associated with the membranes lining the axonal branches. This capacitance imparts a significant delay in any applied voltage.

The net result of the lack of a sufficient space clamp is significant distortions of both the amplitude and time course of the voltage activated current. This leads to a misrepresentation of biophysical properties of the channels including their voltage dependency, kinetics and conductance density ²⁵¹. This problem can be hard to detect and is often not recognised by experimenters which could lead to erroneous conclusion. Three seminal papers were published in 2003 ²⁵² and 2008 ^{218,253} that for the first time attempt to quantify the space clamp errors and suggest ways to limit them. This was done through both simultaneous recordings from the soma and dendrites, and mathematical modelling.

3.1.9 Liquid junction potentials

When two different salt solutions meet (notably the internal and external solutions) a junction potential arises. This liquid-liquid junction potential is created by the differences in concentrations and thus mobility of the ions in the two solutions. If high-precision measurements are required (for example in kinetic studies), this offset must be corrected for. Due to the space clamp issues mentioned in section 3.1.8, such precise measurements were unobtainable. The point of the study in Chapter 4 was to elucidate

whether the Ca^{2+} current of the SHR was larger than the WKY at any voltage. As such the junction potential (in this study calculated to be -14 mV) was identical between the two species and so the junction potential was not corrected for. This needs to be kept in mind when interpreting the data.

3.1.10 Aims of the chapter

This chapter aims to develop a method of voltage clamping cardiac sympathetic neurons that minimize the issues of space clamp irregularities.

3.2 Methods

3.2.1 Cell isolation

The cardiac stellate neurons that innervate the heart were isolated as described in Chapter 2. Briefly, the stellate neurons from four-week old pro-hypertensive SHR and normotensive WKY were dissected out and digested with collagenase (1 mg/ml) and trypsin (2 mg/ml) to create a single cell suspension. The cells were then grown on 6 mm Poly-D-Lysine/Laminin coated coverslips in an incubator maintained at 37°C 5% CO₂. All animal work was done in accordance with the Home Office Animals (Scientific Procedures) Act 1986 (UK).

3.2.2 Solutions and current separation

The objective of the V_{clamp} experiments described in this thesis concern the study of the neuronal Ca²⁺ channels and the method below concerns the special design of both the intracellular and extracellular solutions to enable this. Similar methods can be applied to the separation of other currents.

3.2.2.1 Protocol design

If the nature and voltage dependency of the currents you want to study are well known and characterised, it is possible to obtain current separation by special design of the voltage step protocol. This takes into account the reversal potentials and voltage- and time dependencies of the channels and uses specific voltage steps to both activate and eliminate the contribution of certain currents. As the currents in the cardiac sympathetic stellate neurons of the SHR and WKY have not yet been fully characterised, this method is not applied in this thesis.

3.2.2.2 Current subtraction and pharmacological separation

Current isolation can also be obtained by subtraction to isolate the current of interest. A whole cell recording in normal physiological solution and a recording using a specific

channel blocker, can be subtracted from each other and used to isolate a single current type.

3.2.2.3 Ionic substitution

Perhaps the most common method of current separation is ionic separation. Substitution of all other ions than the charge carrier of interest, ensures that only current type is present. This does not however isolate the current subtypes. Often a combination of the methods above is employed.

In order to separate out the Ca^{2+} current a range of ion substitutions and drugs were employed to either block or prevent the flow of ions through the other channels Table 3.1 detail the substitutions made and why. Ba^{2+} was chosen as the charge carrier to avoid Ca^{2+} dependent inactivation of the Ca^{2+} channels²¹⁷. Ba^{2+} ions are very similar to Ca^{2+} ions and its movement through the Ca^{2+} channels form a suitable representation of the native Ca^{2+} currents. An excellent account of the various ion substitutions and blocking mechanisms can be found in²⁴⁸.

In a normal voltage clamp experiment, the solutions should be designed to mimic the ionic composition of the solution the plasma membrane is exposed to - replicating the extracellular and intracellular environment of the cell. It is crucial that the pH of the two solutions is as close to what you find *in vivo* as many cellular processes are acutely affected by changes in the pH. The osmolarity of the solution – both the absolute, and the difference between the two solutions must be controlled to avoid cell shrinkage/swelling and cell death. However, a slight difference can be exploited to aid seal formation (see table 3.2).

Table 3.1: Comparison of the replacement made to the tyrode solution to create the current isolating extracellular solution		
Tyrode	Current isolating solution	Reasoning
140 mM NaCl	145 mM TEACl	Removal of Na ⁺ removes the Na ⁺ currents. TEA is a K ⁺ channel blocker
4 mM KCl	4.5 mM KCl	
2 mM CaCl ₂	2 mM BaCl ₂	Ba ²⁺ is used as the charge carrier to avoid Ca ²⁺ dependent inactivation of channels. Ba ²⁺ is also a K ⁺ channel blocker (ATP dependent and the inward and delayed rectifier)
1 mM MgCl ₂	1 mM MgCl ₂	To maintain the extracellular environment
10 mM HEPES	10 mM HEPES	
11 mM Glucose	11 mM Glucose	
n/a	1 mM NaHCO ₃	
300-320 mOsm	300-320 mOsm	
pH 7.40 using NaOH	pH 7.40 using Sigma base 7-9	

Table 3.2: The intracellular solution	
Component	Reasoning
140 mM CsCl	Cs is a K ⁺ substitute and a K ⁺ channel blocker
10 mM HEPES	pH regulation
0.1 mM CaCl ₂	To maintain the intracellular environment
1 mM MgCl ₂	
4 mM MgATP	
1 mM EGTA	
290-300 mOsm	The lower osmolarity of the intracellular solution aids in seal formation
pH 7.30 with CsOH	

3.2.3 Electrodes

3.2.3.1 Ground electrode

A silver pellet attached to a silver wire was coated with chloride by immersing it in bleach for one hour. An Agar bridge was created by immersing the ground electrode in a 3 M KCl Agar solution and allowing it to dry. This agar salt bridge provides an electrical connection to the bath but limits the movement of ions or solutes resulting from the electrical environment.

3.2.3.2 Pipette electrode

Borosilicate glass tubes (GC150-10, Harvard Apparatus) were heated and pulled apart using a P-87 pipette puller (Sutter Instruments). This generated two identical hollow and sharp glass tubes with a tip resistance of 1.6 – 2 M Ω when filled with the internal solution. The R_{series} is dependent of the size of the pipette tip and as such, larger sizes were preferred. However, larger pipettes make an adequate seal formation hard to obtain and so efforts must be made to find the optimal middle ground for the cell type in question. The puller settings are unique to each machine and dependent on the condition and shape of the heating filament and must be optimised and routinely monitored to ensure reproducibility (for more information the reader is directed to the pipette cookbook by Sutter Instruments²⁵⁴). The pipette is filled with internal solution before it is mounted onto the electrode holder where a Cl⁻ coated silver wire connects it to the amplifier.

3.2.4 Whole cell V_{clamp} protocol

The voltage clamp experiments were conducted using an Axopatch 200B amplifier and the Clampex software. Once the cell preparation was placed in the bath, positive pressure was applied to the pipette before immersing it into the bath. This was done to reduce contamination and blockage of the tip as it moved towards the cell. Cell

approach was evident by a relative increase in the pipette resistance and a visible dent appearing on the cell. The positive pressure was released and a $G\Omega$ seal created as the membrane bounced back. The pipette potential was briefly changed to a value near the resting membrane potential of the cell (-50 mV) to aid seal formation and to prevent sudden depolarisation when going whole cell. Gentle negative pressure was applied to breakthrough the membrane and get access to the intracellular environment of the cell. Following break through, the cells were held at -90 mV.

From this holding potential a 10 mV pre-pulse was applied at a voltage where no voltage dependent currents are active (-90 to -80 mV) in order to obtain a trace of the leak currents that could be used for leak subtraction. A set of 50 ms voltage steps from -50 to +50 mV were applied with 10 mV, 5 sec intervals to elicit ion currents. Cells were perfused with Tyrode or Ca^{2+} isolating solution using a custom built perfusion system, into a heated microscope stage chamber maintained at 37°C.

3.2.5 Run down

Due to the large volume of the pipette solution with respect to the intracellular solution, intracellular dialysis occurs relatively quickly. This was evident through the same voltage steps rendering smaller currents over time. There are many proposed reasons for why this occurs however, the common consensus is that dialysis occurs and/or exhaustion of internal molecules important for cell function such as ATP occurs. Attempts were made both to investigate the time- and activity dependence of the rundown, and to prevent it using an ATP regenerative internal solution (see table 3.3).

Table 3.3: The ATP regenerative internal solution	
110 mM CsAcetate	Phosphocreatine, MgATP and Creatine phosphokinase allow recycling of the ATP hydrolysis products (ADT and AMP) into ATP.
15 mM CsCl	
10 mM HEPES	
20 mM Phosphocreatine disodium	
50 U/ml Creatine Phosphokinase	
0.1 mM CaCl_2	
4 mM MgATP	
1 mM EGTA	Lower osmolarity of the intracellular solution aids in seal formation
290-300 mOsm	
pH 7.30 with CsOH	

3.2.6 Series resistance

All experiments were conducted in an unpaired manner (one cell acting as a control, the other receiving drug treatment) due to an inability to stabilise the R_{series} over longer time periods and a significant and unpredictable run down of the current. The inability to stabilise the R_{series} was likely due to frequent resealing of the cell, protein aggregates forming under the pipette tip and/or washout of the intracellular proteins. Efforts were made to reduce this by applying slight positive pressure to the pipette during recordings and altering the intracellular solution however, sufficient stability was not obtained and thus unpaired experiments were conducted.

3.2.7 Space clamp issues of peripheral neurons

The PGSNs show extensive axonal branching in culture and as such, the space clamp problem was a significant challenge in this study. A variety of efforts were made to reduce these errors;

- Using acutely isolated cells that are patched two hours post isolation, rather than cultured cells to avoid extensive axonal sprouting.
- Keeping the R_{series} as low as possible by altering the shape of the electrode²⁵⁵.
- Keeping the concentration of the charge carrier as low as possible as errors are amplified with larger currents.
- Keeping the holding potential at more negative potentials to ensure the cell is inactive during the holding intervals. In this study -90 mV was chosen.

3.2.8 Data analysis and inclusion criteria

Data were analysed using the pClamp software. Leak subtraction was performed using a scaled up voltage step that did not generate any active currents (-90 mV to -80 mV). Filtering made no difference to the pooled results and so was not applied. Peak and steady state currents were recorded and normalised to the cell capacitance as measured electronically via the amplifier. Only cells that adhered to the following criteria were included in the study:

- No apparent delay in the start-peak of the current in any of the IV steps
- Peak current occurring at -10 mV
- Resting membrane potential of <-40 mV
- Holding current >-300 pA at resting membrane potential of -90 mV
- R_{series} remaining low (<10 M Ω) and constant throughout the experiment

These criteria were developed partly from literature searches, but perhaps foremost from conversations with leading electrophysiologists at the two-week Microelectrode Course

for Cell Physiology I attended in 2013 at the Marine Biological Association in Plymouth, UK.

3.3 Results

3.3.1 Cell types

Upon investigation of the cultured PGSNs, four distinct cell types were noted (see figure 3.4), each of which had a different current profile. This data was not sufficient for quantitative analysis and will not be mentioned further in this thesis. The cells with marked axonal branching (as expected from peripheral postganglionic neurons *in vivo*²¹⁵) were selected in this study (Figure 3.4D). Only cells that portrayed a healthy phenotype both before and after the recordings were included. Figure 3.5 illustrates cells of different health that can be found on a coverslip two hours post isolation.

3.3.2 The space clamp problem

The PGSNs that innervate the heart show extensive axonal branching after only a couple of hours in culture (see figure 3.6A-B). As a result, significant space clamp problems were observed in cells that had been allowed to settle for more than five hours. The space clamp was evident through the appearance of late activating currents (figure 3.6C-E), often with varied closing times with respect to the timing of the voltage command. These delayed notches of inward current were due to channels in the distal regions experiencing a different membrane voltage when compared to the proximal areas which could result in the unexpected opening of ion channels in the distal areas that then propagate a depolarisation along the entire cell. This is often apparent in a very abrupt rise in the IV curve and can also be seen from the channels remaining open despite the voltage command from the amplifier having ended.

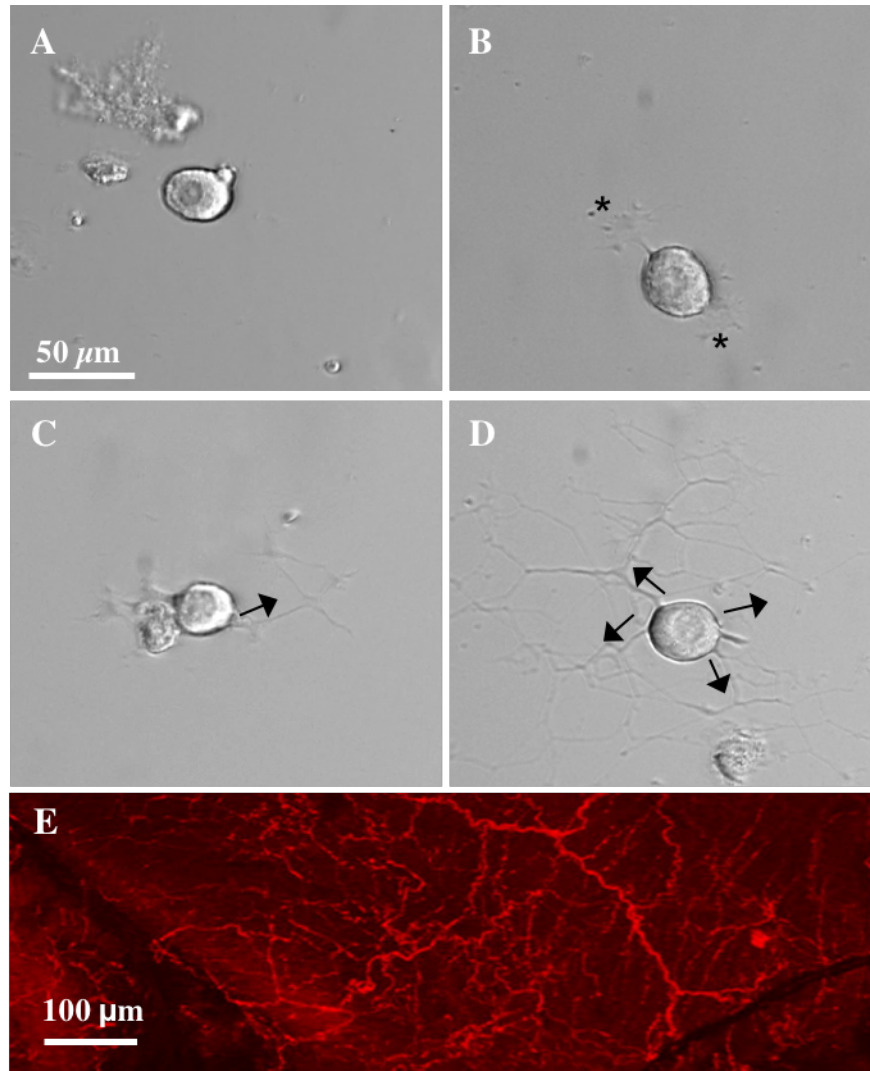


Figure 3.4: Neuronal morphology. Four different neuronal morphologies were present in the culture (A-D) that all portrayed electrical activity. Cells in panel A appeared without axonal branches. Cells in panel B had no axonal branching but presented lamellipodium like projections (asterix). Cells in panel C were associated with one axonal outgrowth (arrow), whilst cells in panel D had multiple outgrowths (arrows). Cell type D was chosen in this study as the neuronal activity and behaviour of neurons is linked to axonal length and thus channel expression. Peripheral neurons *in vivo* show such extensive axonal branching to allow innervation of the myocytes. Panel E is modified from Habecker *et al.* 2008. and depicts the sympathetic innervation of the atria of a wild type (C57BL) mouse.

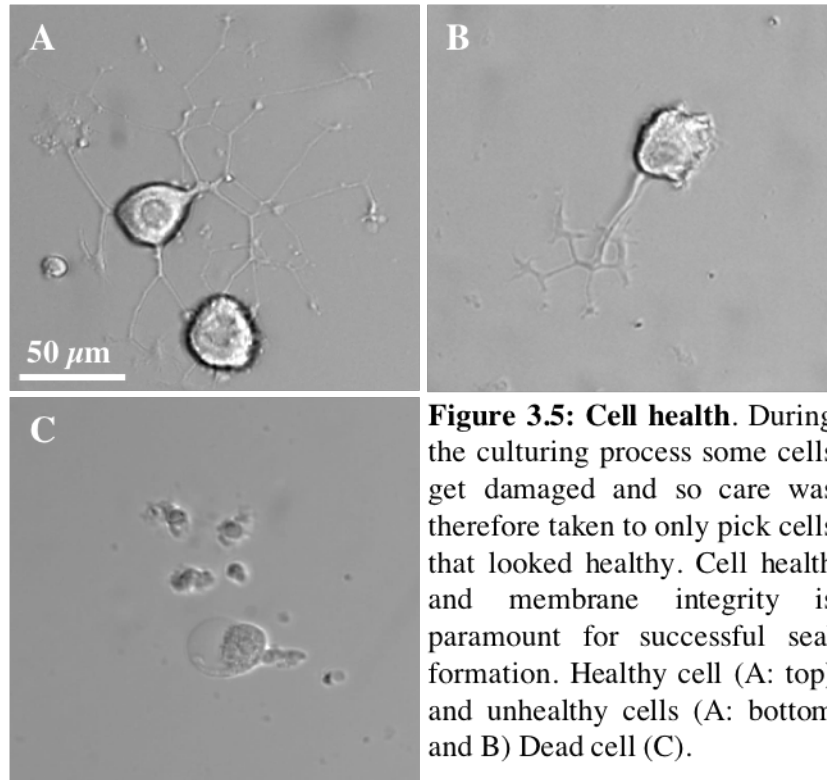


Figure 3.5: Cell health. During the culturing process some cells get damaged and so care was therefore taken to only pick cells that looked healthy. Cell health and membrane integrity is paramount for successful seal formation. Healthy cell (A: top) and unhealthy cells (A: bottom and B) Dead cell (C).

3.3.3 Reducing the space clamp

3.3.3.1 Reducing the amplitude of the current

The degree of space clamp errors is linked to the size of the currents and so reducing the concentration of the charge carrier from 10 mM (figure 3.6D) to 2 mM (figure 3.6E) significantly reduced the errors.

3.3.3.2 Acutely isolated vs cultured cells

Further reduction in the space clamp errors were achieved through conducting the experiments before the axonal branching had become too extensive. All the cells in this thesis were therefore patched between two and six hours post culture.

3.3.3.3 Reducing the series resistance

Before the cell senses the exact applied voltage command, this voltage needs to travel through the R_{series} , which often imparts a delay. Thus, the speed and effectiveness of the changes in the membrane voltage is dependent on the value of the R_{series} . It is therefore

desirable to keep the R_{series} as low as possible to allow quick changes in the voltage. The most effective way to reduce the R_{series} is to increase both the tip diameter and the shape of the shank of the pipette. A large bore, large tip will reduce the resistance but also make seals more difficult to obtain and potentially place a heavy load on the cell membrane. A multitude of pipette sizes and shapes were tested in this thesis, some of which are outlined in figure 3.8. The most consistent results were obtained using the pipette in figure 3.8D.

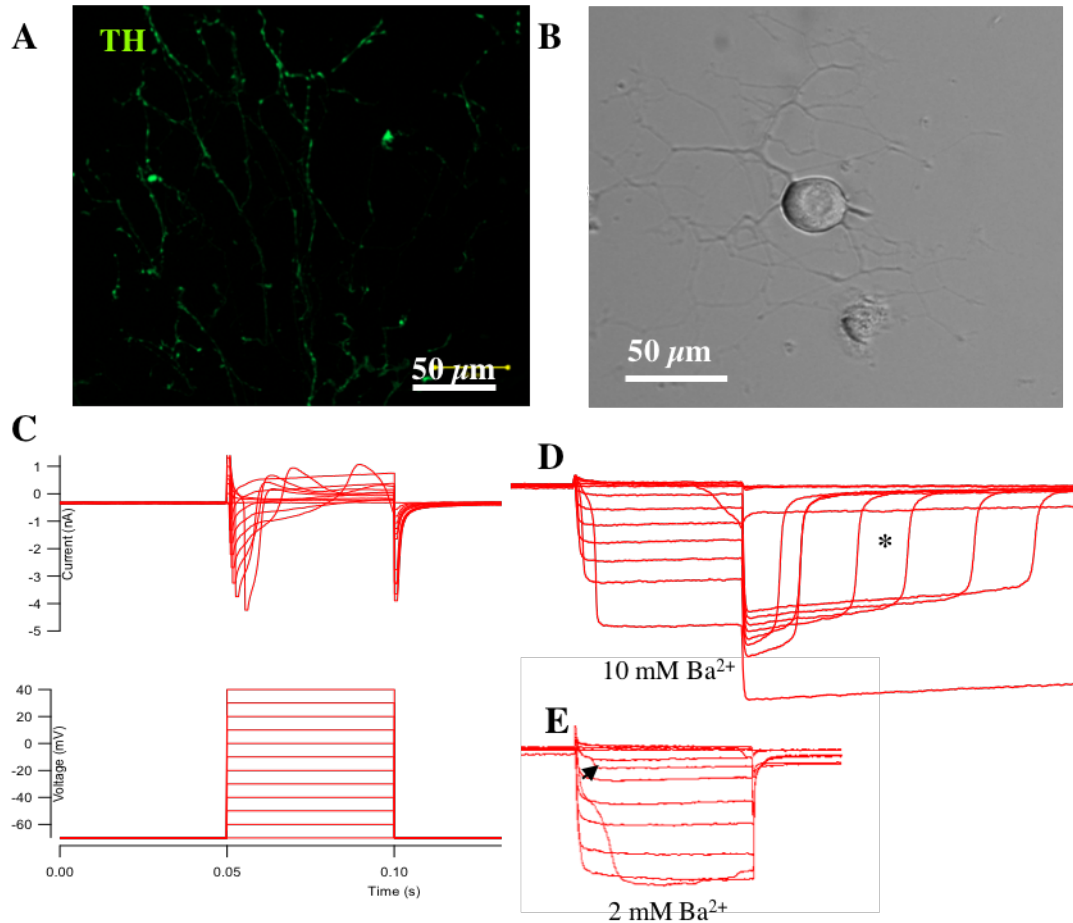


Figure 3.6: The space clamp problem. Sympathetic stellate neurons portray extensive axonal branching after short term in culture leading to significant space clamp errors. Tyrosine hydroxylase staining of the neuronal branching (A) and phase contrast of a second cell after 3 hours in culture (B). Space clamp errors can be difficult to detect as they are often hidden. Most evident on the Na⁺ currents where the peak current occurs at different time points (C). When the Ba²⁺ current is isolated (D-E), the errors are evident through delayed activations of the currents with respect to the start of the voltage pulse (arrowheads), and variable closing (asterix). The degree of space clamp errors are linked to the size of the current and thus the concentration of the charge carrier. A reduction from 10 mM Ba²⁺ (D) to 2 mM Ba²⁺ (E) significantly reduced the errors.

A low and stable R_{series} is also needed to accurately compare the currents between runs when doing paired recordings. Despite these changes and further efforts to reduce R_{series} (see section 3.2.6), it did not stay constant. This forced me to conduct unpaired recordings. The R_{series} was measured before and after each voltage recording, and cells

with any deviation were discarded. Cells with any clear evidence of space clamp errors were also discarded from this study.

3.3.4 Current isolation

The method described above, allowed for, at the time, the first reported successful voltage clamp of PGSNs (See figure 3.7). Current isolation was successfully obtained following the appropriate design of the intra and extracellular solutions. Subjecting the cell to the voltage steps in normal tyrode solution resulted in the appearance of Na^+ and Ca^{2+} inward currents, and outward K^+ currents (figure 3.7C), whilst the current isolating solution resulted in clean inward Ba^{2+} currents as a proxy of the whole cell Ca^{2+} currents (figure 3.7D).

3.3.5 Rundown of the current

Significant run down of the Ba^{2+} current was evidenced in all recordings (see figure 3.9). Five recordings at 4, 9, 14, 19 and 21 minutes post breakthrough of three identical voltage steps around the peak current (-20, -10 and 0 mV, figure 3.9A) resulted in significant reduction of the current between each set of recordings (3.9B-E). The grouped peak (-10mV) currents (3.9F) show the extent of the run down in pA. In this cell, the peak current was reduced by 27 % in 4 minutes, with a total reduction of 72% after only 21 minutes. To minimise the occurrence of this rundown, all recordings in Chapter 4 were made at 4 minutes post breakthrough. 4 minutes was the minimum time required for the bath solution to change (measured using dyes).

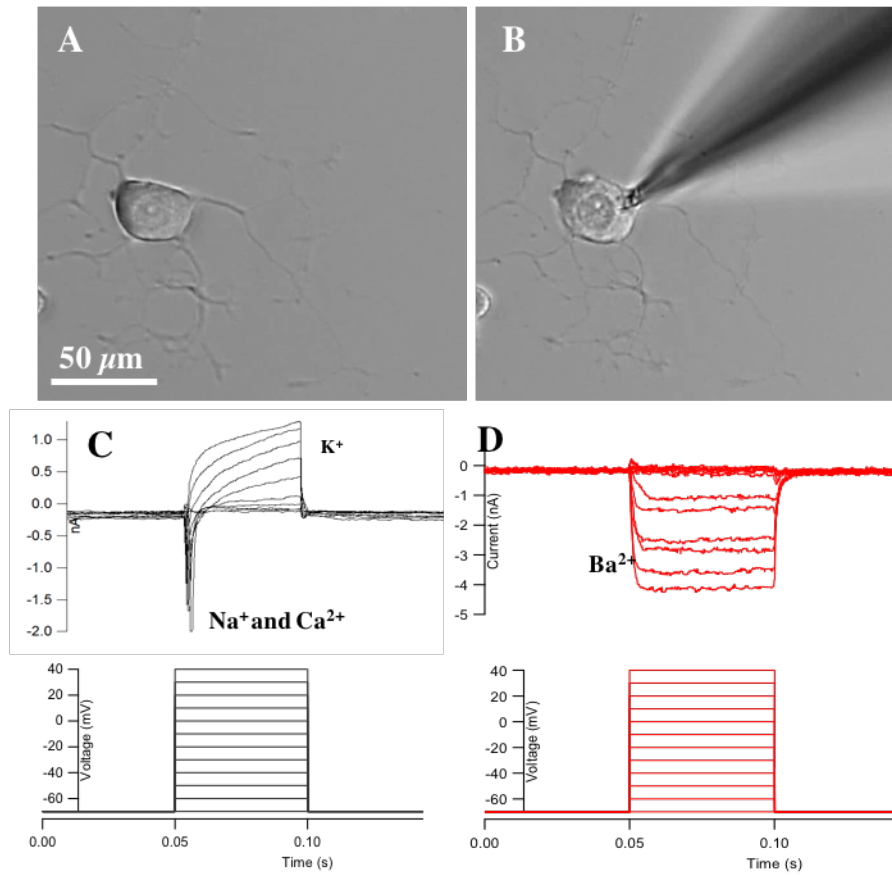


Figure 3.7: Voltage clamp experiment and current isolation. A cell before and at the end of a voltage clamp experiment (A-B). Voltage step protocol in normal Tyrode solution (C) showing whole cell current responses. Inward Na^+ and Ca^{2+} currents and outward K^+ currents can be clearly seen. Identical voltage steps in the current isolating solution (D), results in pure inward Ba^{2+} currents, as a measure of the native Ca^{2+} channel currents

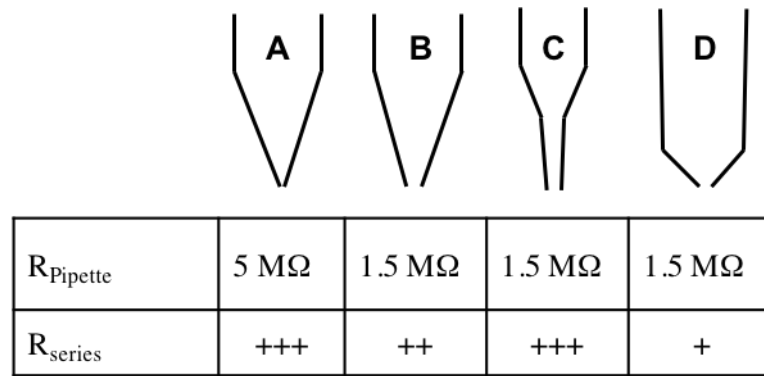


Figure 3.8: Pipette shapes to minimise R_{series} and space clamp issues. Pipettes can have the same size pipette opening (R_{pipette}) but significantly different shape of the shank, leading to different R_{series} . Care must be taken to optimise both the shape and size of the pipette for each cell type being studied, In this thesis, pipette shape D was used.

Efforts were made to investigate whether the rundown was time- and/or activity dependent and to which extent it could be compensated for. To test for activity and time dependence, 10 identical -10mV steps (3.10C) were applied with the intervals between the steps varying from 5 to 1 seconds. The cell was allowed to sit for two minutes between each of these steps to investigate whether there was also a passive time dependent run down. Figure (3.10A and B show the raw traces of the first two identical step protocols with 5 seconds between each voltage step. The results showed that the rundown is linear, but with unpredictable and varying slopes between cells (data not shown). In all cells, the steps in quicker succession resulted in faster run down (figure 3.10D), as measured by the slope of the fitted line. These results, suggest that intracellular factors in the cell is being used up. The slope of the traces with 1 second between each step had a considerably steeper slope when compared to the 5 second interval recording, indicating a faster run down (23.07 vs 9.69).

In order to further test the activity dependence of the run down, only the three steps around the peak (see figure 3.9) were run. However, this did not significantly reduce the rundown, and additionally leads to a loss of information about the voltage dependency of the channels due to the lack of voltage steps needed to generate a current-voltage relationship (an IV curve). There was a slight time dependency as can be seen from the two-minute break times between each as well as what can be seen from figure 3.8, but this dependency was not reproducible amongst different cells, making effective compensation impossible. Taken together it was not possible to accurately predict and compensate for the rundown.

3.3.6 Efforts to stabilise the rundown

Efforts were then turn to ways to reduce the run down. A common way to reduce run down is to prevent the dialysis of the cell using a perforated patch approach-an experiment where antibiotics are used to punch holes in the membranes under the pipette that allow the movement of charged ions, but not larger intracellular proteins²⁵⁶. This was not attempted in this study as an inherent side effect of this is an increased R_{series} (due to the patch not being fully broken) which would significantly worsen the space clamp issues. Many studies have been published, discussing the nature and reason for the run down, often seen only with Ca^{2+} currents, but there are no clear conclusions. A common theory is that the cell is using up a limited factor (often thought to be ATP) as a result of the voltage protocol and the dialysis of the cell. A common way to prevent this is to include in your intracellular solution, the building blocks and enzymes needed for the cell to recycle AMP and ADP back into ATP - an ATP regenerative internal solution (see section 3.2.5).

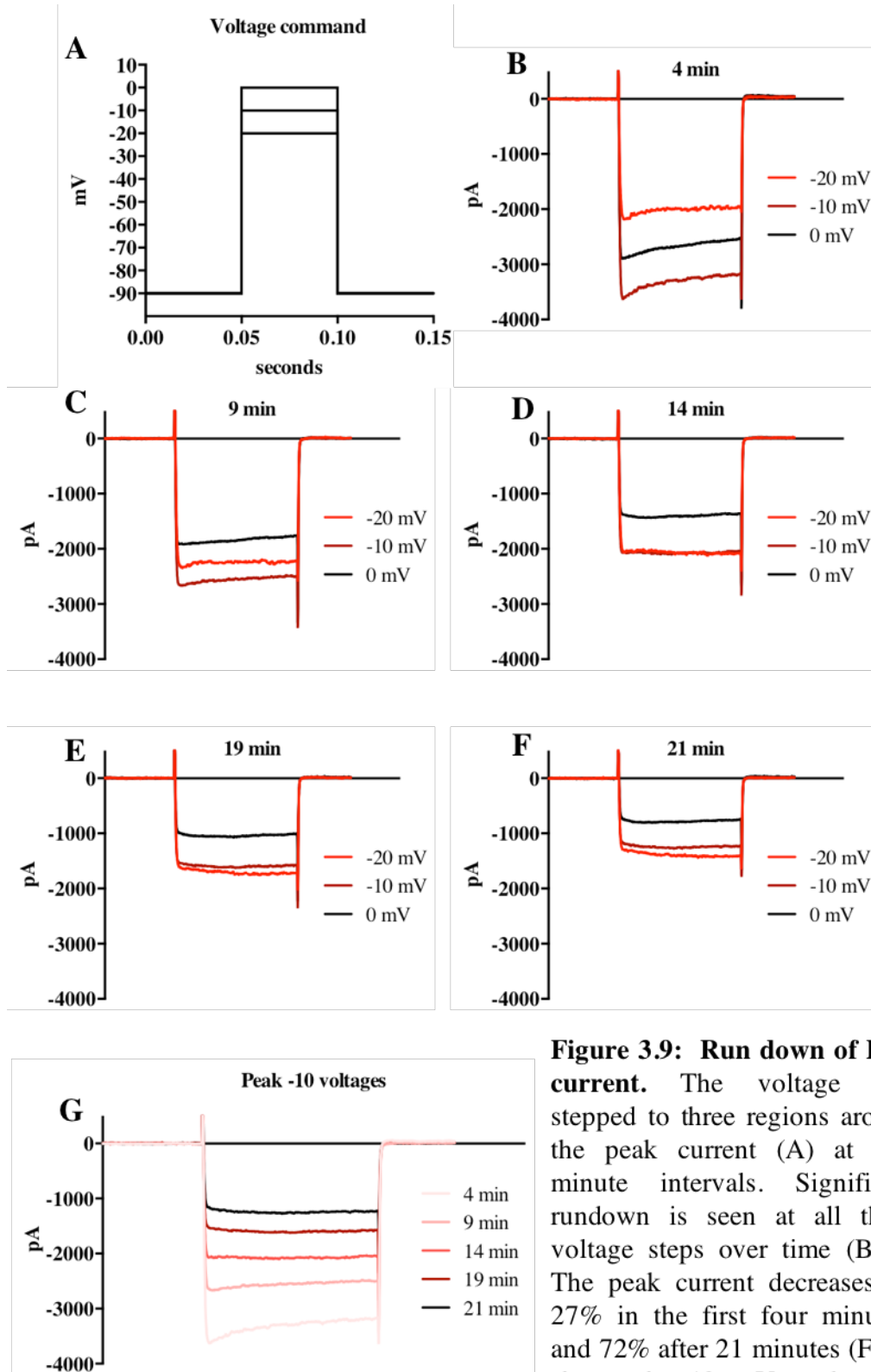


Figure 3.9: Run down of Ba^{2+} current. The voltage was stepped to three regions around the peak current (A) at five minute intervals. Significant rundown is seen at all three voltage steps over time (B-E). The peak current decreases by 27% in the first four minutes, and 72% after 21 minutes (F). G shows the 10 mV peak steps overlaid.

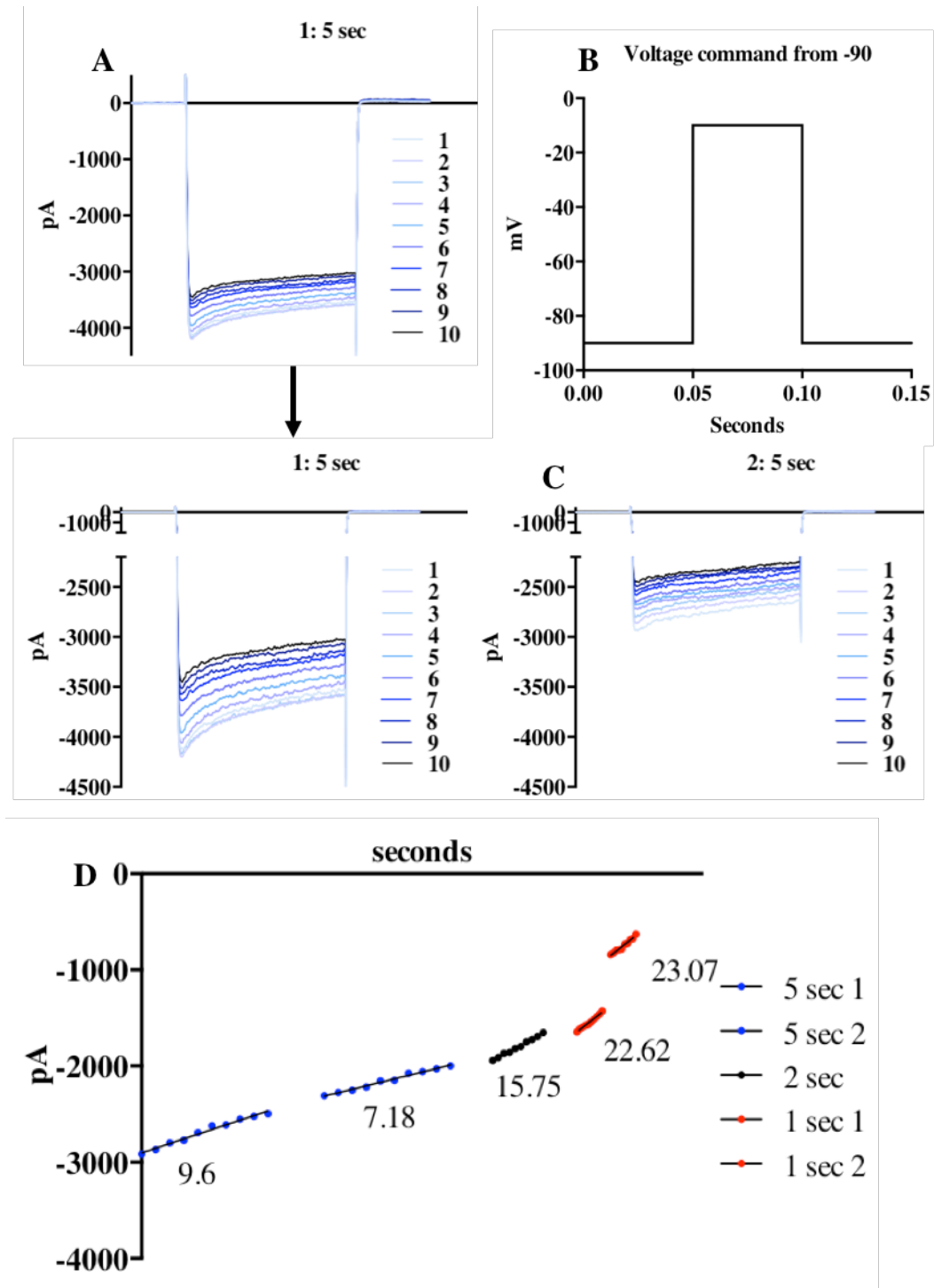


Figure 3.10: Time and activity dependency of current rundown. 10 voltage steps at the peak current (-10 mV B) were repeated with varying intervals (5, 2 and 1 seconds between steps) to examine the time and activity dependence of the current rundown. Example voltage traces of the two 5 second sweep time runs show significant rundown (as measured by the slope of the fitted line) (A and C). The rundown shows activity and time dependence with the shorter and faster step protocols causing a more dramatic rundown (D).

3.3.6.1 ATP regenerative internal solution

The experiment to test the time and voltage dependency of the rundown was repeated with the ATP regenerative solution. The results showed that the ATP regenerative solution, not only halts the rundown, but actually resulted in an activity dependent run-up of the current (see figure 3.11 A-D). This was evident through a negative slope of the 10 step protocol. Interestingly there was still a significant time-dependent rundown between each step. However, it was significantly more difficult to successfully obtain seals with the ATP regenerative solution when compared to the normal one (1 in every 20 vs 1 in every 2 cells). Pre-loading of the pipette with a normal internal solution did not significantly increase the success rate. As the processes by which the run-up occurs is unclear, the presence of time dependent run down, and the ethical implications associated with the need to use many animals to compensate for the low success rate, it was decided to use the internal solution as described in table 3.2, and to conduct unpaired experiments in a tightly time controlled manner.

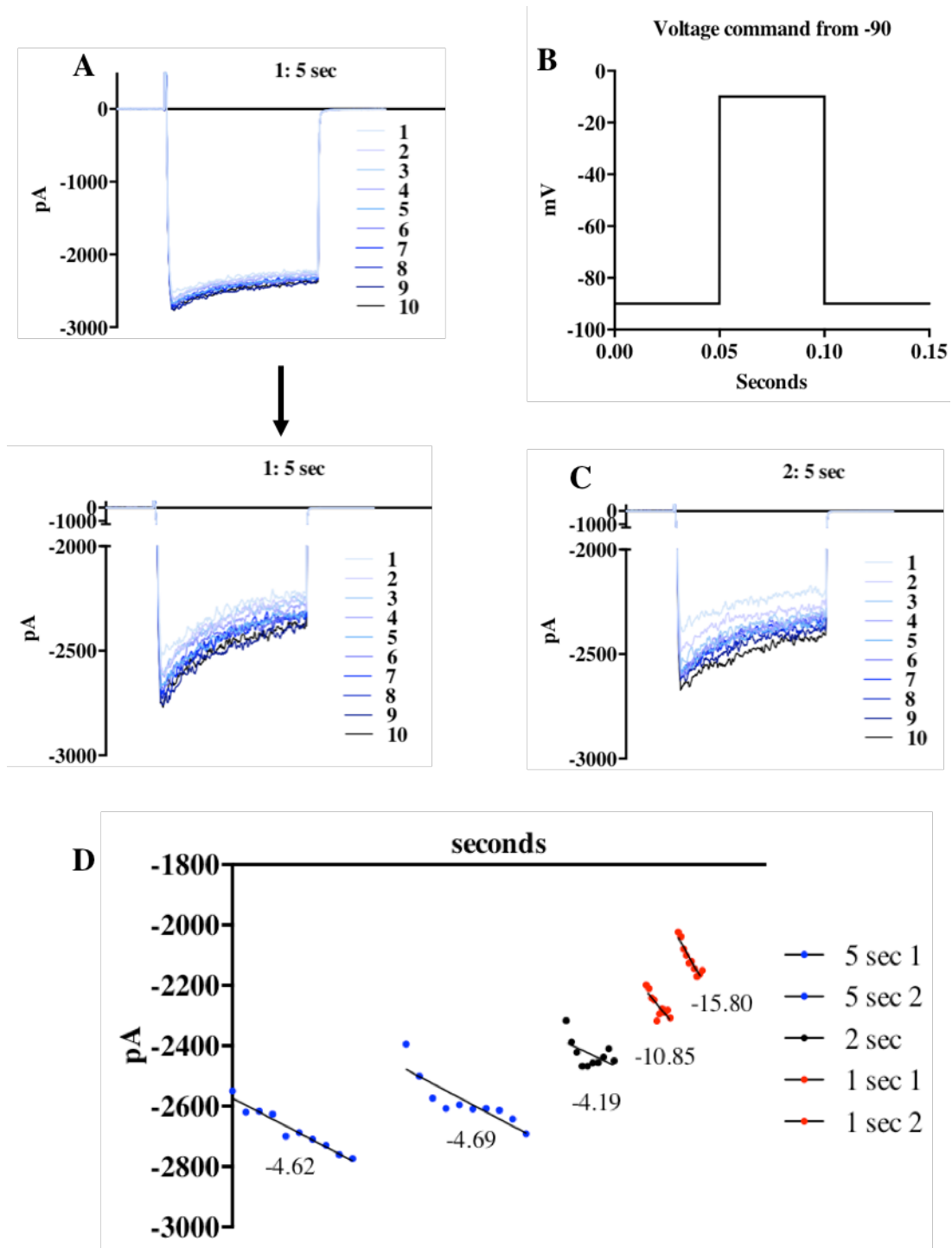


Figure 3.11: ATP regenerative intracellular solution. Allowing the cell to regenerate used ATP significantly reduced the activity dependent rundown when repeating the same -10 mV voltage step (B) and actually induced a seemingly activity dependent run-up (as evidenced by a negative slope) of the current (A and C). The time dependent rundown was still present, though slightly less than for the normal internal solution(D).

3.3.7 Optimal configuration of the experimental setup

Table 3.4 illustrates the final experimental setup for a successful voltage clamp of sympathetic PGSNs.

Table 3.4. Final experimental patch setup	
Rig setup	Microscope on an air table with a temperature controlled recording chamber with an agar bridge ground electrode.
Cell isolation	Acute isolation of neurons with voltage clamp experiments performed between 2-6 hours post culturing to avoid extensive axonal branching and the resulting space clamp risks.
Extra and Intracellular solutions	See tables 1 and 2
Holding potential and voltage steps	• Create seal in tyrode and break through. • Hold cell at -90 mV. • Change external solution to current isolating solution (with drugs when needed) and wait 4 minutes. • Run a voltage step from -90 to -80 mV to get a trace of the passive capacitance to be used for leak subtraction. • Generate an IV curve by performing 10, 10 mV voltage steps from -50 to +50 mV with 5 second sweep times.
Cell phenotype	Obtain pictures of the cells pre and post recording to record the cell type the recording is from (see figure 3.7), and to monitor cell health during the experiment.
Data analysis	• Express currents as pA/pF where pF is the cell capacitance, to account for differences in cell size. • Perform leak subtraction using the 10 mV step from -90 to -80 mV using the pClamp software.
Inclusion criteria	• No apparent delay in the start-peak of the current in any of the IV steps • Peak current occurring at -10 mV • Resting membrane potential of <-40 mV • Holding current >-300 pA • R_{series} must remain constant throughout the experiment

3.4 Discussion

3.4.1 Cellular phenotype

Upon investigation of the cultured cells, four distinct cell types were noted (see figure 3.4), each of which had a different current profile. These cell types have been identified independently by other researchers²⁵⁷⁻²⁵⁹. Many studies have demonstrated a link between the cellular morphology, axonal branching and the cellular activity, resulting in a particular cellular phenotype²⁵⁷⁻²⁶¹. In fact research has shown that the dendritic complexity of neurons is directly related to the size of the organs they innervate²⁶². An aim of the electrophysiological work in this thesis was to study the cells that innervate the heart both alone and in combination with myocytes. For this reason, the cells with marked axonal branching (as expected from peripheral postganglionic neurons) were therefore selected for experimental study (Figure 3.4D). Cells are known to get damaged during culturing processes which will greatly affect the recordings. Great care was therefore taken to ensure cell health both before and after recordings. Figure 3.5 illustrates cells of different health that can be found on a coverslip two hours post isolation. In order to obtain effective seals, it is of utmost importance that the cell membranes are healthy. Any damage to the integrity of the cell membrane would make seal formation extremely challenging and could cause the cell to explode upon impact with the patch electrode.

3.4.2 Space clamp irregularities

PGSNs portray extensive axonal branching, making an effective voltage clamp exceptionally difficult to perform due primarily to space clamp errors (see section 3.1.8). Space clamp errors at best lead to increased variability of the data, but at worst lead to erroneous results. Priority was therefore given to reduce the space clamp errors. In order to get detailed and sensitive information on the effect of drugs on channel function, paired recordings should be performed. However, paired recordings rely on a

low and stable R_{series} and absence of run down in the system. Unfortunately for this study, the methods that most effectively reduce the R_{series} and rundown problems, worsen the space clamp errors. As limiting the space clamp errors take priority, forced to collect strictly time controlled unpaired data. This effectively doubles the time and cells needed to collect sufficient amount of experimental data, but perhaps for the first time allowed recordings from these neurons in culture.

It is worth mentioning that another electrophysiological method that could be used to study Ca^{2+} channels is to employ the current clamp (I_{clamp}). This technique is not as sensitive to the above mentioned limitations. Pilot I_{clamp} experiments were conducted with the hope that the Ca^{2+} current differences could be isolated from the plateau phase of an action potential. Unfortunately, the nature of the action potential shape did not allow for such discrimination. As the primary aim of the work in this thesis was to investigate the channel properties of the neuronal Ca^{2+} channels, the V_{clamp} was the preferred method.

Recent research has employed dual soma/dendritic V_{clamp} experiments with the view to both counteract, but also model the degree of space clamp issues seen in neurons^{218,253}. The space clamp issue is of huge importance in neuroscience as V_{clamp} remains one of the most popular electrophysiological methods. Recently a series of important studies were conducted to fully describe the space clamp issue and to raise awareness. Developing ways to reduce, remove or compensate for these issues could revolutionise the field²⁵³.

3.4.3 Limitations of the study

3.4.3.1 Space clamp errors and protocol design

The primary limitation of this study is the presence of space clamp errors, forcing the experimenter to employ strict exclusion criteria to make sure the data accurately represents physiology. In this study roughly 60% of all data collected had to be excluded based on the parameters in section 3.2.8. Furthermore, the rundown and R_{series} issue prevented ideal protocol design, forcing the experimenter to conduct unpaired experiments. This in combination with the possibility of undetected space clamp errors in some cells is expected to significantly increase the variability in the data, potentially resulting in false negative results. The prohibitive expense and ethical concerns associated with the resulting increased need of cells (and thus animals), may leave this study at risk of being underpowered.

3.4.3.2 Acute cell isolation

The need to conduct experiments on acutely isolated cells, limits the future experimental procedures to those involving the acute applications of drug. Viral based experimental methods to modulate intracellular pathways would not be possible with this preparation. Furthermore, it is reasonable to think that some cell damage could have occurred as a result of the isolation process, that may be invisible to the experimenter. This could account for some of the cell exclusions. Finally, the need to use acutely isolated cells, may limit the validity of comparisons of this work with previous work often done on cultured cells.

3.4.3.3 Current separation of Ca^{2+} channels

Ca^{2+} ion entry may activate and/or alter the voltage dependency of other ion channels in the preparation. The substitution of Ca^{2+} with Ba^{2+} ions may therefore significantly alter the health and behaviour of the cell and also potentially the currents under investigation.

3.4.4 Concluding remarks

V_{clamp} is a powerful experimental technique to investigate the properties of membrane bound voltage gated ion channels. Unfortunately, the presence of space clamp errors has limited its use in cells that portray extensive axonal branching such as the sympathetic neurons innervating the heart. It has long been postulated that these neurons have a critical role in the initiation and perhaps maintenance of cardiac disorders. For over a decade it has been clear that these neurons have significant intracellular Ca^{2+} dysregulation, yet relatively few studies have thus far focused on the characterisation of the voltage gated Ca^{2+} channels that are likely to play a major role. See Chapter 4 for complete details.

This study is one of the first, that has characterised not only the problem of space clamp errors in these cells, but also details how one should go about solving them. In this study a detailed protocol for whole cell voltage clamp of sympathetic neurons was described, with particular details on the specific isolation of Ca^{2+} currents.

CHAPTER FOUR

4 Voltage clamp to characterise autonomic dysfunction in sympathetic neurons from the SHR

4.1 Introduction

4.1.1 Autonomic dysfunction in hypertension

Hypertension – often referred to as a silent killer - is a major predisposing factor for cardiac conditions such as heart failure, arrhythmias and sudden cardiac death ^{1,84,263-265}. Increased activity of the sympathetic nervous system is a well-established contributing factor to the pathophysiology of human hypertension ^{9,48-50,77,266,267} and that observed in the spontaneously hypertensive rat (SHR) ^{51,52,63,82}. Interestingly dysautonomia occurs in young pro-hypertensive animals, suggesting it is an early hallmark of the disease itself, although the exact mechanisms that underpin the phenotype remain poorly understood. When comparing age matched PGSNs from normotensive rats (WKY), with those from pro-hypertensive SHRs, the latter have increased intracellular Ca^{2+} transients ($[\text{Ca}^{2+}]_i$) ⁶³, and enhanced neurotransmission ^{54,74,174}. This translates into increased post synaptic excitability of the sinoatrial node due to a greater conductance of ICa^{2+}_L ⁸⁰. The observed differences in $[\text{Ca}^{2+}]_i$ may be partially explained by altered mitochondrial Ca^{2+} handling ⁶³, but it is unclear whether there are additional changes at the membrane level to account for the greater calcium transients.

4.1.2 The role of the voltage gated Ca^{2+} channels in the sympathetic neurons.

Influx of Ca^{2+} through the N-type and possibly the L and T-type channels is important for the release of noradrenaline (see figure 4.1) from sympathetic neurons in humans, rats and rabbits ^{25,26,103,104,107-109}. The importance of neuronal Ca^{2+} channel function in hypertension was first reported in the early 90s when it was observed that N-type Ca^{2+} channel ($\text{Ca}_v2.2$) blockers led to hypotension ²⁶⁸. Later, $\text{Ca}_v2.2$ knock out mice revealed

inherent problems with blood pressure control, manifested in reduced baroreflex responses and heart rate responses after electric stimulation of the sympathetic neurons^{269,270}. More recently Tu et al demonstrated an enhanced activity of the N-type voltage gated Ca^{2+} channels in heart failure²⁶⁵. Similarly we also observed increased whole cell neuronal Ca^{2+} currents in the PGSNs of pro-hypertensive SHR animals⁶⁴ suggesting that channel dysregulation is highly conserved over several developmental stages from the pre-diseased phenotype to advanced heart failure.

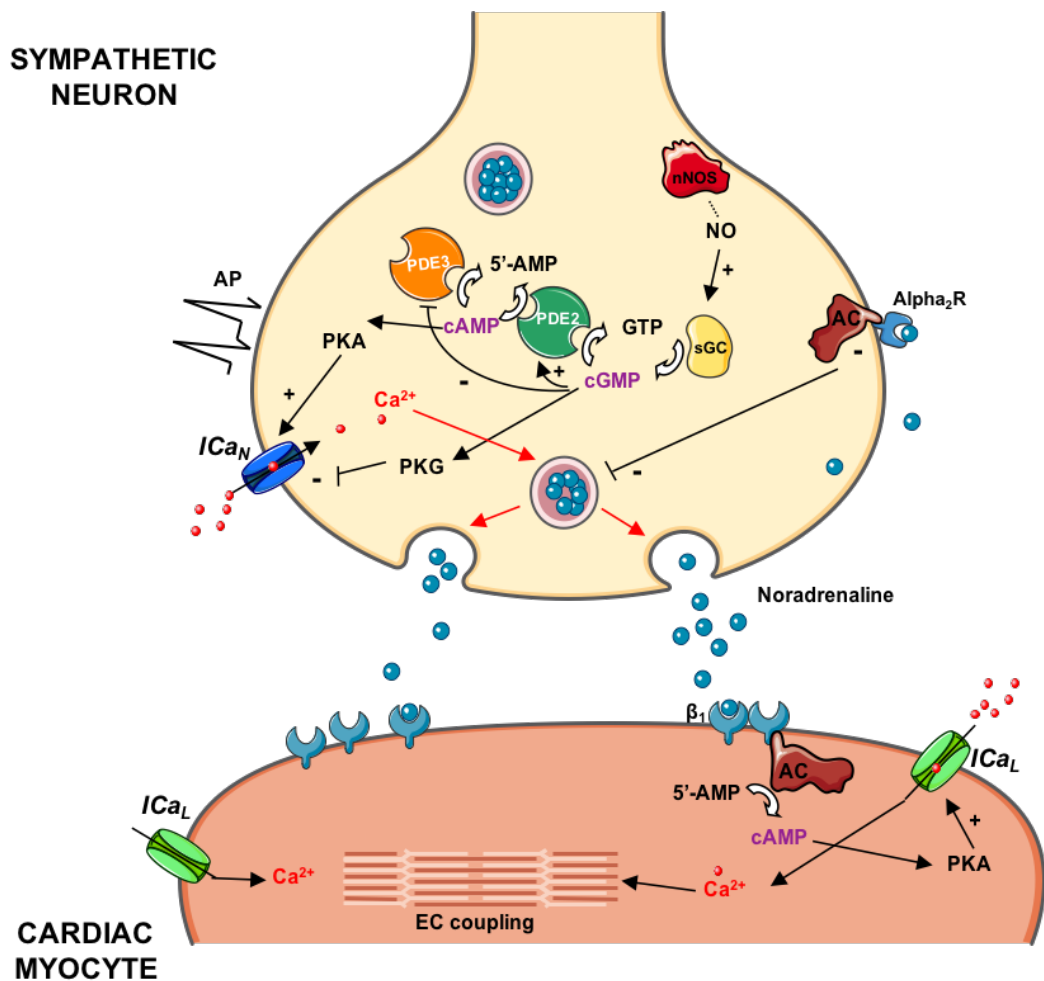


Figure 4.1: Noradrenaline release in the neuro-cardiac junction. The arrival of an action potential in the synaptic nerve terminal, leads to Ca^{2+} entry via voltage gated Ca^{2+} channels. This triggers exocytosis of noradrenaline into the synaptic clefts which acts on the β_1 adrenoceptor, altering myocyte behaviour.

4.1.3 Cyclic nucleotide dysfunction

What links ion channel dysregulation to sympathetic hyper-responsiveness? One suggestion is that early metabolic-oxidative stress could be coupled to abnormal cyclic nucleotide regulation ^{127,128,136}. Voltage gated Ca^{2+} channels are known to be strongly regulated by cyclic nucleotide signalling ^{114,271} – a signalling cascade that is dysfunctional in hypertension ^{54,62,65,82,134}. Here PGSNs have reduced neuronal nitric oxide synthase (nNOS) activity, low level expression of the β_1 subunit of soluble guanylyl cyclase ⁶⁵ and high PDE2A activity ⁷⁰, leading to a reduction in cGMP levels ^{54,108}.

4.1.4 Aims of the chapter

This chapter tests the hypothesis that impaired cyclic nucleotide regulation provides a direct link to abnormal signalling of neuronal calcium channels in pro-hypertensive rats, and that targeting cGMP signalling pathways can restore normal channel phenotype in the diseased pro-hypertensive SHR PGSNs. Could the alterations in the membrane Ca^{2+} currents provide the trigger for the intracellular Ca^{2+} dysfunction and the autonomic dysfunction we see in hypertension?

4.2 Methods

4.2.1 Neuronal isolation

PGSNs innervating the heart were isolated as described in Chapter 2. Briefly, the stellate neurons from four-week old pro-hypertensive SHR and normotensive WKY were dissected out, desheathed and digested with collagenase (1 mg/ml) and trypsin (2 mg/ml) to create a single cell suspension. These were grown on 6 mm Poly-D-Lysine/Laminin coated coverslips (37°C 5% CO₂) for 3 hours before all voltage clamp recordings were made. All animal work was done in accordance with the Home Office Animals (Scientific Procedures) Act 1986 (UK).

4.2.2 Voltage clamp

V_{clamp} experiments were performed as described in Chapter 2 and 3. Briefly, the neurons were held at -90 mV before a set of 50 ms voltage steps from -50 to +50 mV were applied with 10 mV steps, 5 sec intervals. Cells were superfused with Tyrode or Ca²⁺ isolating solution using a custom built perfusion system, into a heated microscope stage chamber maintained at 37°C. Access to the cell was obtained in normal Tyrodes solution before the solution was changed to the Ca²⁺ isolating solution containing no Na⁺, and K⁺ channel blockers (TEA and Cs⁺) with Ba²⁺ as the charge carrier in order to isolate the Ca²⁺ currents either in the presence or absence of drugs (1 μ M ω -Conotoxin GVIA, 100 μ M 8 bromo-cGMP, 1 μ M BAY 60-7550). 75-90% series resistance compensation was applied to all cells. To minimize space clamp errors²¹⁸, the cells were held at -90 mV, low concentrations of the charge carrier was used, cells were patched acutely and large bore, blunt electrodes (1.5-2 M Ω) were used in addition to strict exclusion criteria. Only cells that has a resting membrane potential of <-40 mV, a series resistance of <10 M Ω throughout the recording, a holding potential of >-300 pA and

cells where no visible contusion of the currents were visible, were included in this study.

4.2.2.1 Protocol

The following protocols were used for all experiments: A successful seal was obtained in tyrode solution. Immediately following rupture of the patch, the solution was changed to the charge carrying solution containing relevant drugs. Exactly four minutes was allowed for the drugs to have their effects before the voltage step protocol described above was triggered. The four minute time window allows for the chambers content to be entirely replaced by the new drug containing solution in one minute. The remaining three minutes allowed the membrane permeable drugs to cross the cell membranes and act. This time period was determined as follows: a bright dye was added to the charge carrying solution and perfused into the chamber at a set rate.

A three-part voltage protocol was used; first a 10 mV step from -90 to -80 mV was applied in order to generate pure capacitive currents to be used in off line leak subtraction; second, a step was made to -10 mV to isolate the immediate peak current that is not affected by activity dependent rundown; thirdly, a 10 step voltage protocol was applied to generate the IV curve (see 4.2.2).

4.2.2.2 Data analysis

Current traces were normalised to cell capacitance to eliminate differences due to cell size. The conductance (G) was calculated as follows: $G = I / (V_m - V_e)$ where I is the membrane current, V_m is the membrane voltage step command and V_e is the reversal potential as determined by the IV curve. For relative conductance and generation of the activation curve, G/G_{max} was plotted against the voltage command. A Boltzmann curve was fitted to the data points using the Prism software.

4.2.3 Immunofluorescence

Cells were fixed with 4% formaldehyde for 10 minutes before being blocked and permeabilised using a solution containing 10% goat serum, 0.3% bovine serum albumin and 0.1% Triton X in PBS for one hour at room temperature. The cells were washed in PBS before primary antibodies against TH (Tyrosine Hydroxylase 1:250, Abcam), ChAT (Choline-Acetyltransferase 1:500, Abcam) and Cav2.2 (1:250, Alomone Labs) were added and incubated overnight at 4°C. Appropriate secondary antibodies (Alexafluor 1:1000) were added for two hours before the coverslips were washed in PBS and mounted using DAPI containing mounting media (Vectashield, Vector labs). Cells were imaged using a Live Cell Olympus confocal microscope.

4.2.4 Statistical analysis

Data passed three separate normality tests (D'Agostino & Pearson omnibus normality test, Shapiro-Wilk normality test and Kolmogorov–Smirnov). Unpaired T tests were used to compare peak currents and conductances. All data are expressed as the mean $\pm$ SEM. Statistical significance was accepted at P values <0.05 .

4.3 Results

4.3.1 Sympathetic phenotype of the acutely isolated stellate neurons.

Immunofluorescence analysis of the cardiac stellate neurons confirms their sympathetic phenotype. All cells showed TH positivity - a sympathetic neuron specific marker. No cells were positive for ChAT - a common parasympathetic neuronal marker. Figure 4.2 depicts a representative stained neuron from a WKY rat.

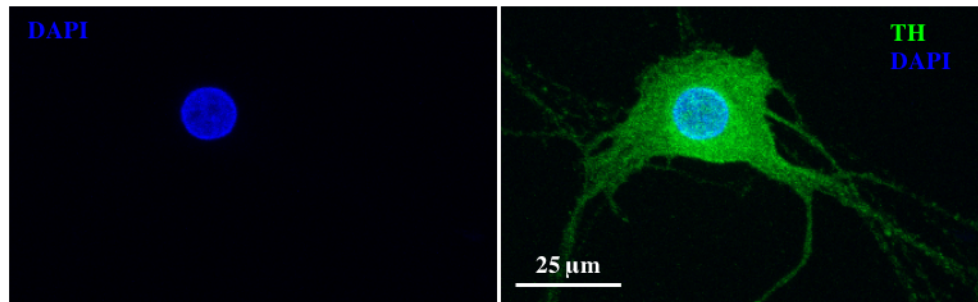


Figure 4.2: Confirmation of sympathetic phenotype. Representative image of a WKY sympathetic stellate neuron stained for Tyrosine Hydroxylase (TH) a sympathetic neuronal marker.

4.3.2 Cellular characteristics of the WKY and SHR stellate neurons

As described in Chapter 3, four distinct cell types were identified in the stellate ganglions from both the WKY and SHR. Only cells that portrayed significant dendritic arborisation were used in this chapter (Figure 4.3A). No significant differences were found in the resting membrane potential (4.3B) or the cell capacitance (an estimate of cell size) (4.3C) between the WKY and SHR (-46.8 ± 0.95 mV vs -45.4 ± 0.87 mV and 21.4 ± 1.03 vs 21.3 ± 1.17 pF, $n=32$ and 30). The data were found to be normally distributed (4.3D).

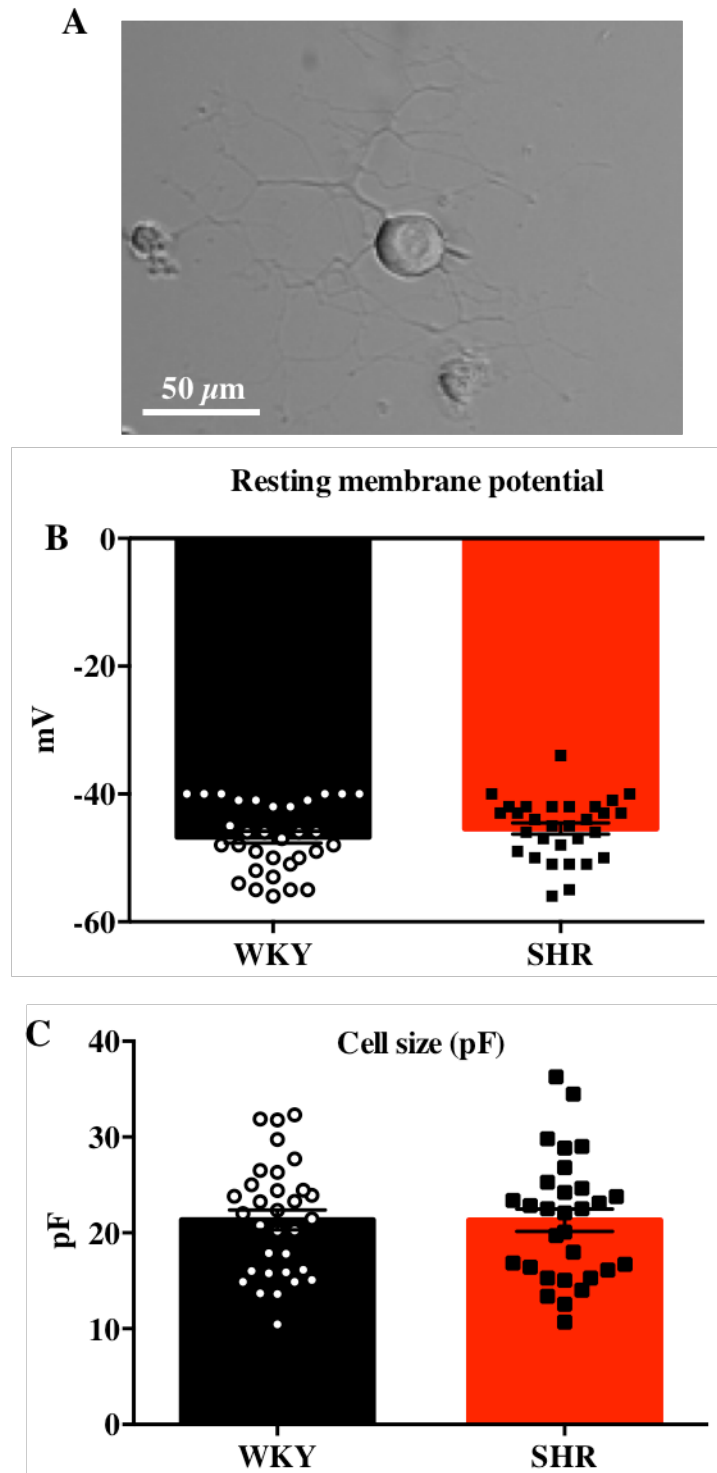


Figure 4.3: Cellular phenotypes of the WKY and SHR. All cells used in this thesis were acutely isolated branched sympathetic neurons (A). No significant changes were found in the resting membrane potential (B) or the cell size (C) between the WKY and SHR neurons (n=32 and 30).

4.3.3 Neuronal Ca^{2+} currents of the pre-hypertensive SHR are larger than that of the normotensive control.

The peak whole cell voltage gated Ca^{2+} currents of the pro-hypertensive SHR were significantly larger than that of the normotensive WKY animals at multiple voltages (Figure 4.4D). The peak current (-10 mV) of the SHR was -127.5 ± 5.94 pA/pF (n=10) whereas in the WKY it was -108.0 ± 6.80 pA/pF (n=10, $p < 0.05$). The specific conductance of the channels was significantly larger in the SHR when compared to the WKY (2.38 ± 0.11 vs 2.01 ± 0.13 , n=10, $p = 0.041$). The activation curve (figure 4.5) showed no significant change in voltage dependency of the channels between the SHR and WKY (V50: -22.04 ± 0.31 vs -22.50 ± 0.25 , slope: 4.56 ± 0.29 vs 4.68 ± 0.22 , $R^2 = 0.99$) (Figure 4.5) suggesting that the biophysical properties of the channels were the same.

4.3.4 The N-type Ca^{2+} channel ($\text{Ca}_v2.2$) is a major player

1 μM ω -Conotoxin GVIA reduced the whole cell currents by 75% and 83% respectively, down to similar levels in both animals (WKY -26.88 ± 1.70 , n=6; SHR -22.04 ± 1.60 pA/pF, n=5), suggesting that a major player is the neuronal N-Type Ca^{2+} channel^{272,273} (figure 4.6). The specific conductance was reduced by similar amounts. The resulting conductances were not significantly different between the WKY and SHR (0.3746 ± 0.029 vs 0.4439 ± 0.038 , $p = 0.20$ n=5-6). Immunofluorescence analysis confirmed the expression of these channels on the membrane of the TH positive cells (figure 4.7).

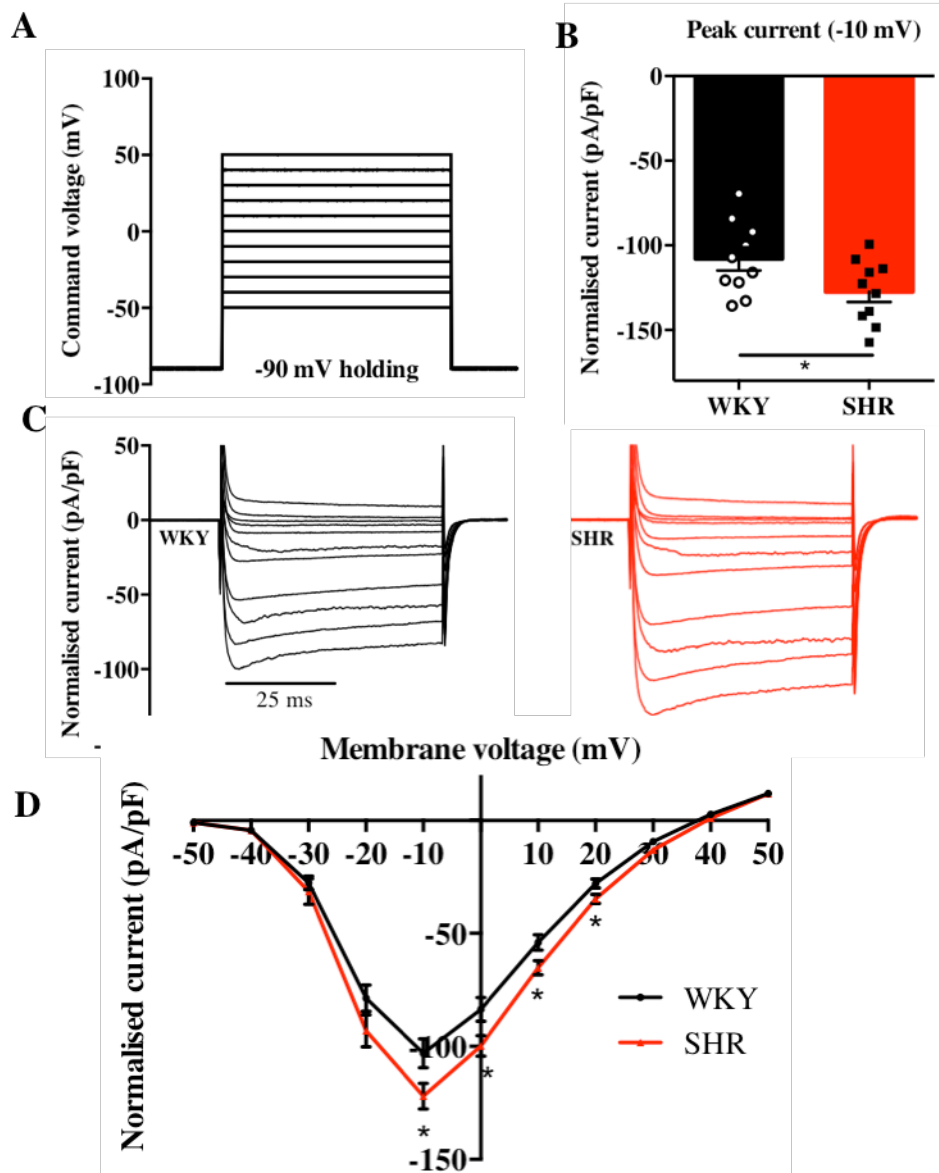


Figure 4.4: The whole cell Ca^{2+} current and specific conductance is larger in the PGSNs of the pro-hypertensive SHR. Whole cell voltage clamp was performed on the PGSNs innervating the heart to investigate the whole cell Ca^{2+} properties of four-week old pro-hypertensive SHR and normotensive WKY rats. 50 ms, 10 mV voltage steps from -50 to +50 mV were applied to the cell (A). The peak current (-10 mV) was significantly larger in the SHR (-127.5 ± 5.94 pA/pF, $n=10$) when compared to the WKY (-108.0 ± 6.80 pA/pF, $n=10$, $p<0.05$) (B). Example voltage clamp traces of WKY and SHR neurons showing significantly larger currents in the SHR neurons (C). Current voltage relationship (IV curve) showing significantly larger currents in the SHR when compared to the WKY at multiple voltages (D). Data are represented as the mean $\pm$ SEM. $*=p<0.05$

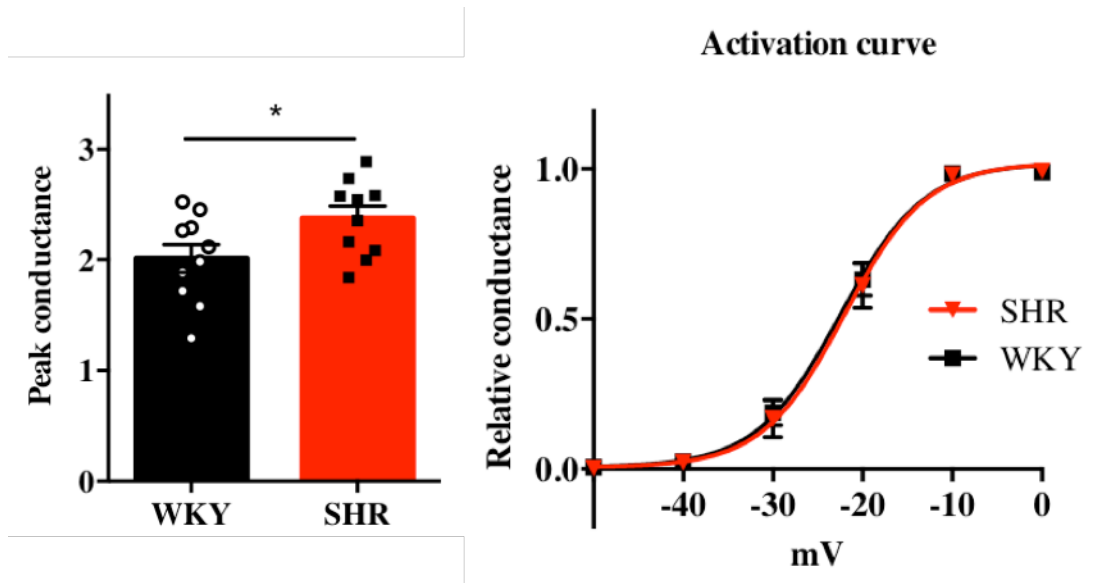


Figure 4.5: The biophysical property and voltage dependency of the channels is not changed in hypertension. The peak relative conductance was significantly larger in the SHR when compared to the WKY (2.377 ± 0.1088 vs 2.011 ± 0.1254 , $p=0.0407$, $n=10$). The activation curve shows no change in the voltage dependency of the channels, suggesting that the biophysical properties of the channels were identical between WKY and SHR V_{50} : -22.04 ± 0.31 vs -22.50 ± 0.25 , slope: 4.56 ± 0.29 vs 4.68 ± 0.22 , $R^2 = 0.99$. All data are represented as the mean $\pm$ SEM.

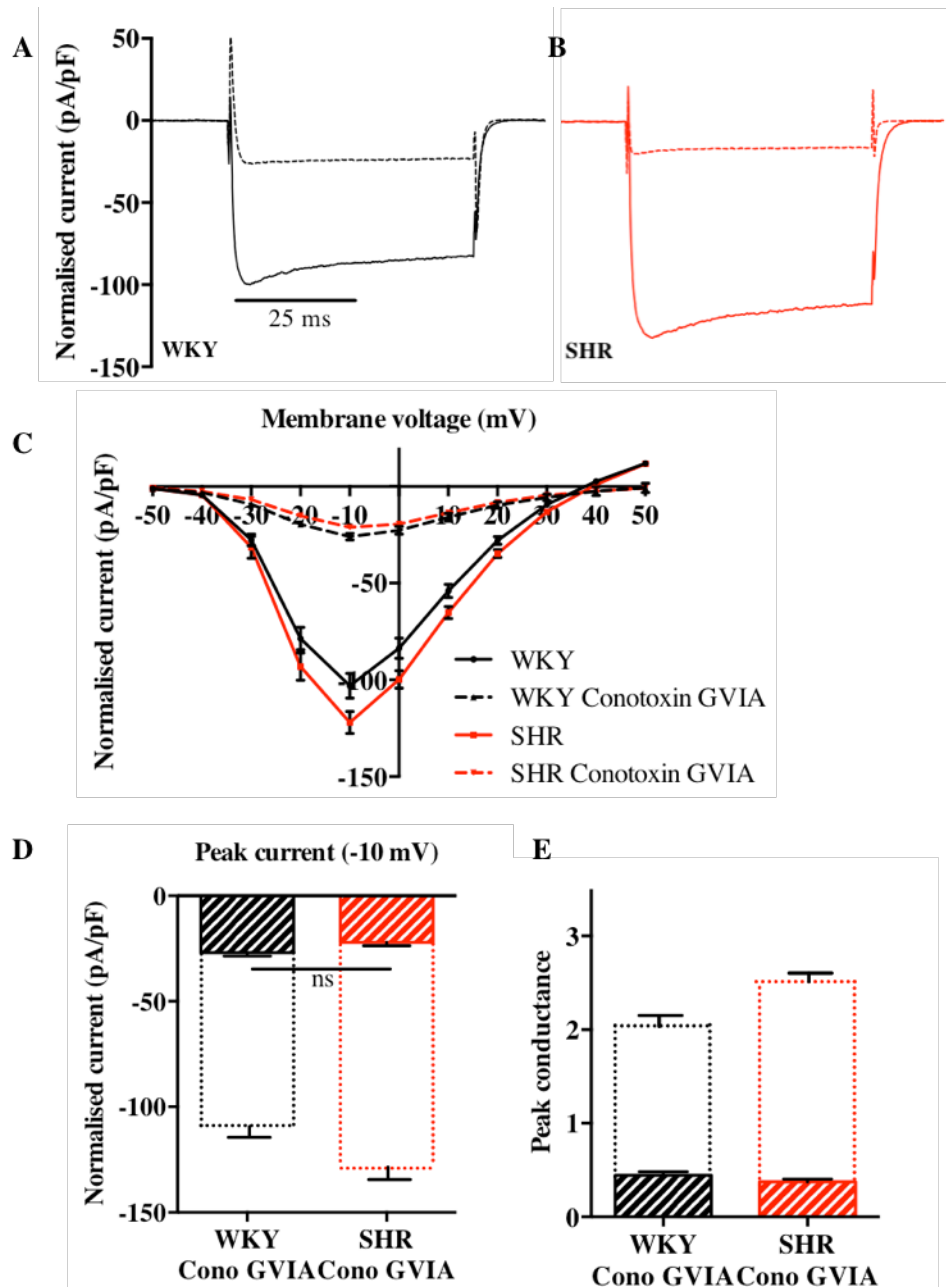


Figure 4.6: 1 μ M ω -Conotoxin GVIA blocks 75 and 83% of the current, suggesting the N-Type Ca^{2+} channel is the major player. Representative peak (-10 mV) voltage clamp traces of WKY (A) and SHR (B) in normal conditions (solid lines) and after treatment with 1 μ M ω -Conotoxin GVIA (dotted lines). Current voltage relationship showing uniform reduction in both the SHR and WKY neurons at multiple voltages. The peak current remains at -10 mV and is not significantly different between the WKY and SHR (C). Bar chart of the peak currents (-10 mV) showing a 75% (WKY) and 83% (SHR) reduction of the N-type Ca^{2+} current down to levels that were not significantly different between the strains (-26.88 ± 1.7 pA/pF, $n=6$ and -22.04 ± 1.60 pA/pF, $n=5$). Dashed lines represent the mean of the WKY (black) and SHR (red) control data (D). The specific conductance was also significantly reduced resulting in conductances that are not significantly different between the WKY and SHR (0.375 ± 0.029 vs 0.444 ± 0.038 , $p=0.20$ $n=5-6$). Data are represented as the mean $\pm$ SEM.

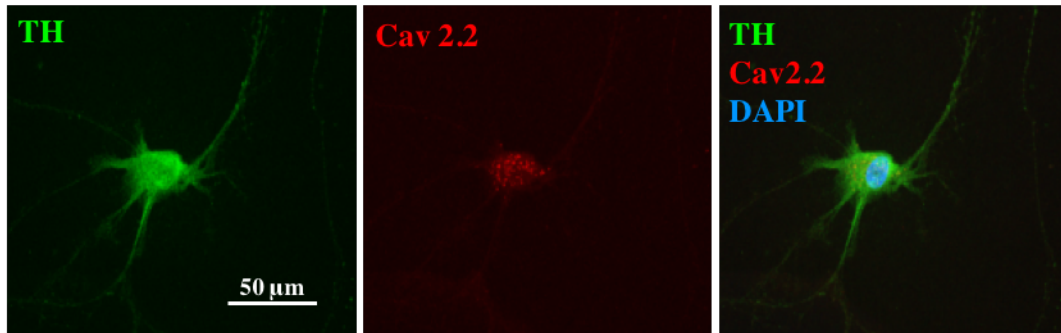


Figure 4.7: The cardiac sympathetic neurons express the N-Type Ca^{2+} channel ($\text{Ca}_v2.2$) on their membranes. The cells were stained with antibodies against Tyrosine Hydroxylase and $\text{Ca}_v2.2$ confirming the cell surface expression of the N-Type Ca^{2+} channel on both the WKY and SHR neurons. Figure depicts a representative stain of a SHR neuron. The nuclear stain DAPI is included on the overlay image.

4.3.5 Increasing the intracellular cGMP concentration significantly lowers the currents and reverses the channel phenotype

To test the involvement of cyclic nucleotides in the regulation of the N-type channel, a membrane soluble cGMP analogue (100 μM 8-bromo-cGMP) was perfused before the voltage clamp experiment was repeated. Elevating cGMP did not alter the Ca^{2+} currents in the normotensive WKY animals (-108.0 ± 6.80 pA/pF, n=10 to -103.1 ± 9.40 pA/pF, n=7), but significantly reduced the SHR currents (-127.5 ± 5.94 pA/pF, n=10 to -105.2 ± 7.79 pA/pF, n=7) down to levels seen in the WKY animals (-108.0 ± 6.80 pA/pF, n=10) (Figure 4.8). The peak conductance of the SHR was reduced to levels not significantly different from the WKY (2.125 ± 0.1834 vs 2.190 ± 0.1429 , n=7 p=0.78). No changes were observed in the activation curves for either the WKY or the SHR neurons (Figure 4.9).

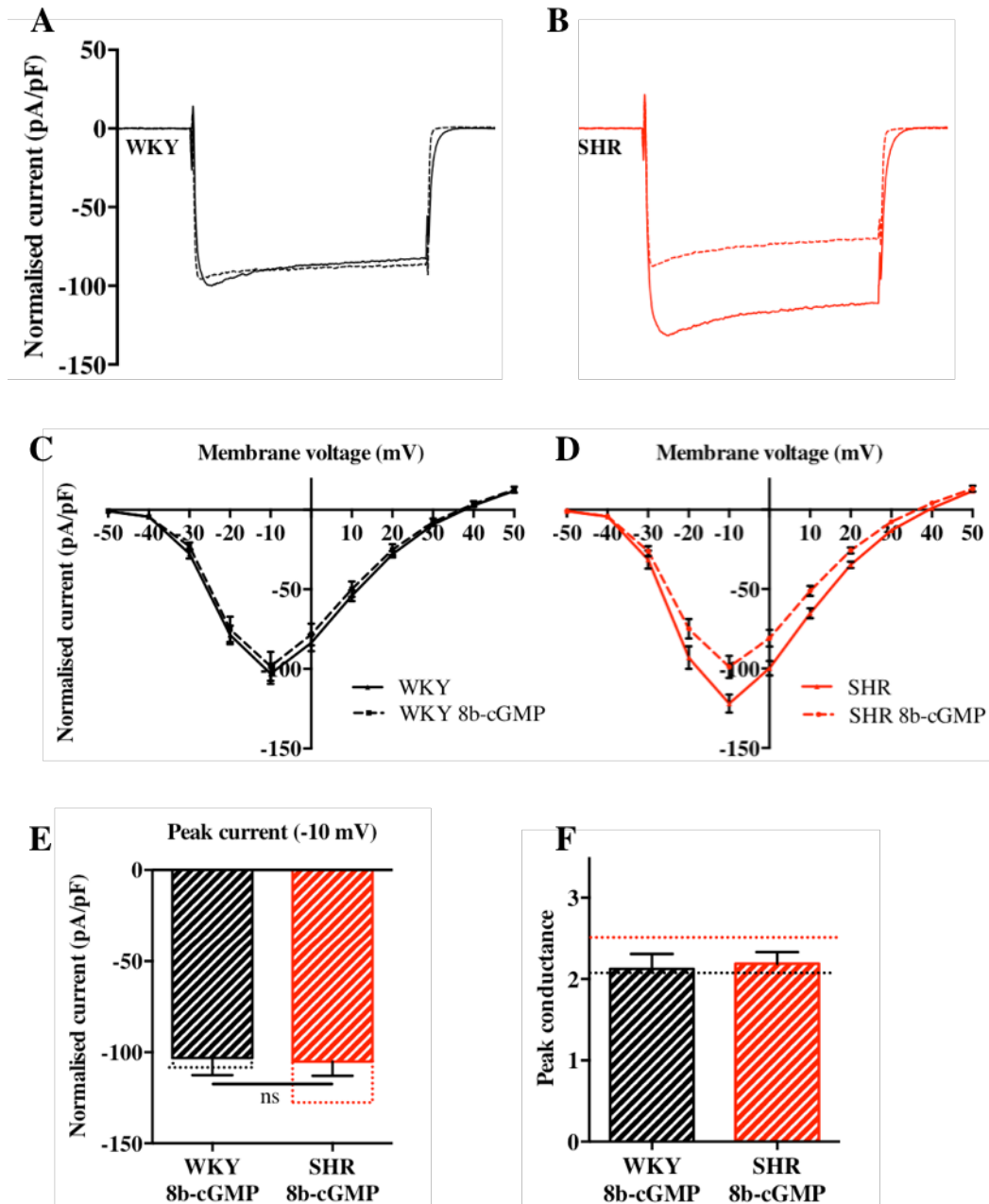


Figure 4.8: Restoring the cGMP levels rescues the channel phenotype seen in the SHR. Example peak voltage clamp traces at -10 mV of WKY (A) and SHR (B) neurons showing a decrease in the SHR current following application of 100 μ M of a membrane permeable cGMP analogue; 8 bromo cGMP. Current voltage relationship of WKY (C) and SHR (D) Bar chart of the peak currents (-10 mV - E) showing a significant reduction ($p < 0.05$) and normalisation of the SHR current (-127.5 ± 5.94 pA/pF, $n=10$ to -105.2 ± 7.79 pA/pF, $n=7$) down to WKY levels (-108.0 ± 6.80 pA/pF, $n=10$). The drug does not significantly alter the WKY currents. The peak conductance (F) was also reduced to levels not significantly different from the WKY (2.125 ± 0.1834 vs 2.190 ± 0.1429 , $n=7$ $p=0.78$) Dashed lines represent the mean of the WKY (black) and SHR (red) control data. All data are represented as the mean $\pm$ SEM.

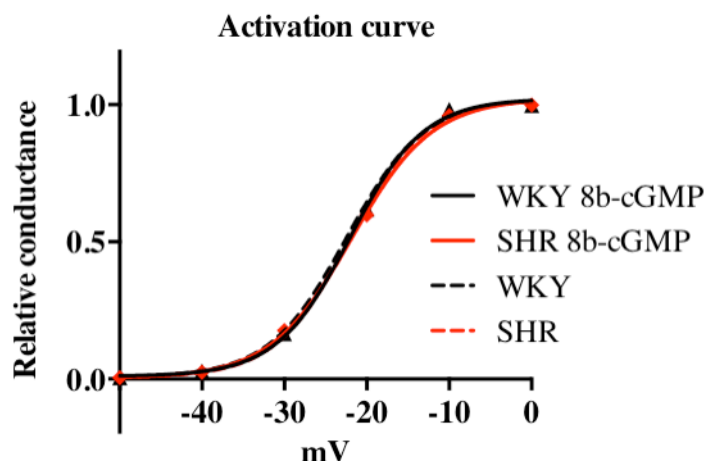


Figure 4.9: The activation plot showed no significant difference between the SHR and WKY or the drug groups, suggesting that the biophysical properties of the channels are unchanged.

4.3.6 Differential response to PDE2 blockade of the WKY and SHR neurons

The application of 1 μ M BAY 60-7550, a PDE2 selective inhibitor, significantly increased the WKY channel currents (-108.0 ± 6.80 to -138.7 ± 9.61 pA/pF $p=0.0169$ $n=9$ and 10). Interestingly in the SHR, 1 μ M BAY 60-7550 showed no significant change in currents (-127.5 ± 5.937 to -118.0 ± 6.673 pA/pF, $p=0.30$ $n=10$ and 9) (figure 4.10 A-C) Following PDE2 inhibition, the control currents were trending towards being larger than the SHR but this was not quite significant (138.7 ± 9.610 pA/pF to -118.0 ± 6.673 pA/pF, $p=0.052$). The conductance data showed a similar pattern with an increase in the WKY (2.01 ± 0.125 to 2.61 ± 0.214 $p=0.0169$ $n=10$ and 9) and no significant change in the SHR (2.38 ± 0.109 to 2.19 ± 0.126 $p=0.28$ $n=10$ and 8) (figure 4.10D). Following PDE2 inhibition the WKY conductance was higher than that of the SHR neurons, but this was not significant ($p=0.062$). No changes were observed in the activation curves (figure 4.10E).

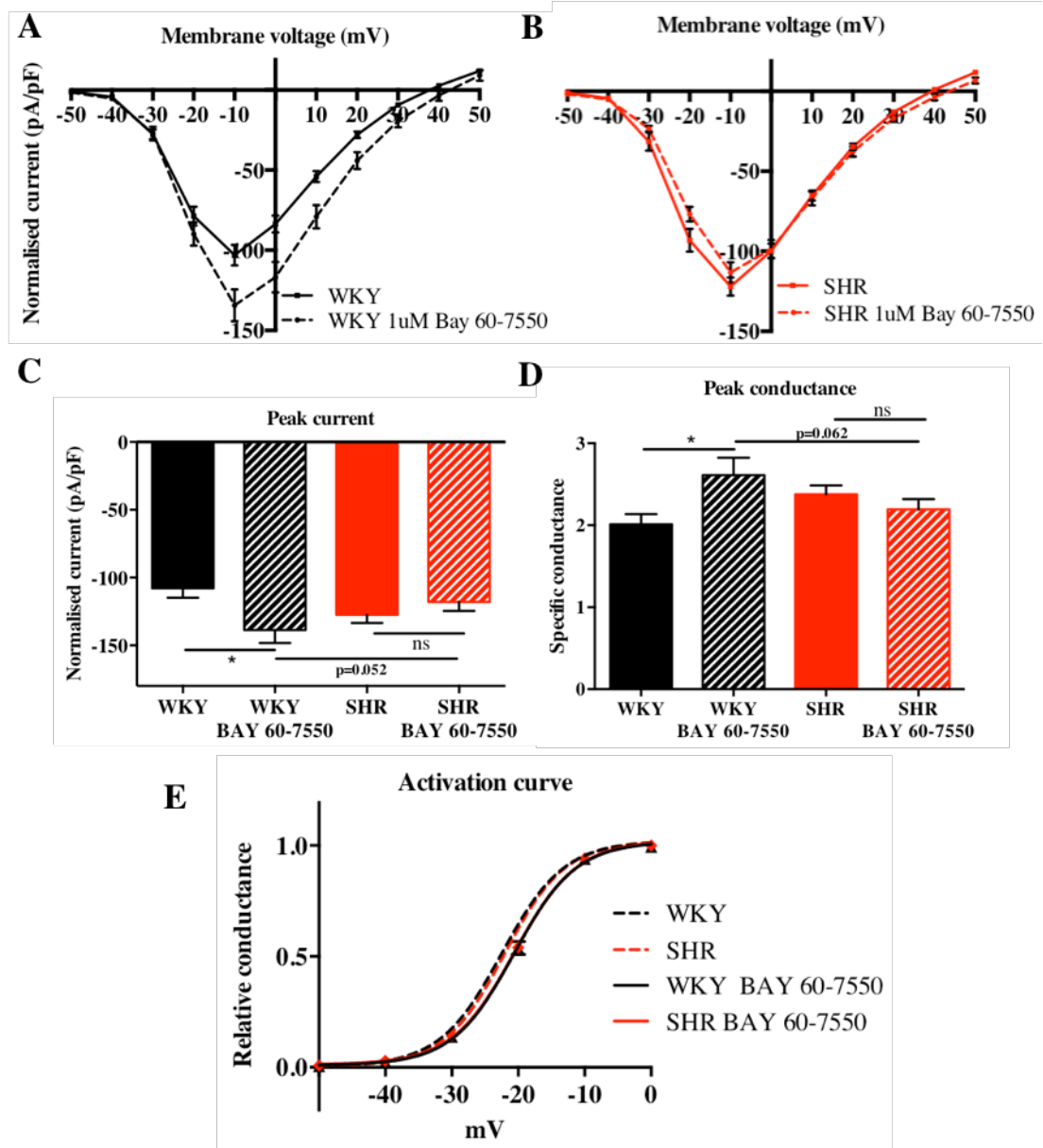


Figure 4.10: PDE2 blockade suggests differential involvement of PDEs in the WKY and SHR. IV relationships of the WKY (A) and SHR (B) neurons following the application of 1 μ M BAY 60-7550, a PDE2 selective inhibitor. BAY 60-7550 significantly increased the peak WKY channel currents (-108.0 ± 6.80 pA/pF to -138.7 ± 9.61 pA/pF, $p=0.0169$ $n=9$ and 10), but showed a non significant decrease in the SHR currents (-127.5 ± 5.937 pA/pF to -118.0 ± 6.67 pA/pF, $p=0.30$ $n=10$ and 9). Following PDE2 inhibition, the control currents were trending towards being larger than the SHR but this was not quite significant (138.7 ± 9.61 pA/pF to -118.0 ± 6.67 pA/pF, $p=0.052$) (C). The conductance data showed similar patterns with an increase in the WKY (2.01 ± 0.125 to 2.61 ± 0.214 $p=0.0169$ $n=10$ and 9) and no significant change in the SHR (2.38 ± 0.109 to 2.19 ± 0.126 $p=0.28$ $n=10$ and 8). Following PDE2 inhibition the WKY conductance was higher than that of the SHR neurons, but this was not significant ($p=0.062$) (D). No changes were observed in the activation curve (E).

4.4 Discussion

4.4.1 Cell phenotypes and neuronal switching

Synaptic plasticity with respect to neurotransmitter release is a commonly known phenomena in neuroscience. For review see ^{177,274}. Many groups have demonstrated that sympathetic neurons are able to co-release both noradrenaline and acetylcholine in vivo and in vitro ^{183,275,276}. The switching was initially thought to be a developmental stage of neonatal neurons as they form their first connections with their targets ²⁷⁷ however, it is now clear that it occurs in adult cells as well, and could even be a stable condition of some neurons ²⁷⁸. It was therefore important to conduct regular immunohistochemistry experiments to confirm that this switching was not present in our cultures. All cells tested throughout this thesis were found to be TH positive (see figure 4.2).

No changes were found in the cell capacitance or the resting membrane potential of the WKY and SHR PGSNs (Figure 4.3) suggesting the experimental conditions affected the two cell types in similar ways, indicated that the overall health of the two cell types was the same. This is in contrast to a study showing significant hypertrophy of the stellate ganglion of the SHR ²⁷⁹, but the disparity is likely due to the relatively inaccurate estimation of the cell volume in the axonal processes that was performed in this study. It is worth noting that both cells were relatively depolarised, however this is consistent with observations made by others ²⁸⁰. Whilst it is possible this has some physiological relevance, it could also be due to two factors: the membrane stress occurring when using large tipped electrodes; and changing of the intracellular environment (which sets the membrane potential) due to immediate dialysis of the cell. Interestingly when using the ATP regenerative solution, the few cells obtained were significantly more polarised (~ -70 mV) suggesting that this could indeed be an artefact of the experimental conditions rather than inherent properties of the cells. Please refer to Chapter 3 section

3.3.6.1 for details on why the ATP regenerative solution was not employed in this study.

For accurate recordings of membrane potential, the following methods could be employed: intracellular, sharp electrode recordings; perforated voltage clamp; voltage dyes or other potentiometric probes.

4.4.2 The role of the autonomic nervous system in hypertension

Previously thought to be a passive relay of information, it is now becoming clear that the autonomic nervous system has a major active role to play in both the development and progression of most cardiac diseases. Abnormalities of cardiac ion channels are well-established contributing factors; however relatively little work has been done to characterize ion channels in the peripheral nerves innervating the heart. More than a decade's worth of work has shown that the peripheral neurons innervating the heart have significant intracellular⁶²⁻⁶⁸ and membrane level^{69,70} molecular changes that contribute to the cardiac phenotype. This sympathetic dysfunction is in many cases seen prior to the development of clinical symptoms, suggesting that its role may be causative and not merely a contributor to the disease. These changes are all acutely linked to dysregulation of intracellular calcium, which in neurons, is critically responsible for cellular functions such as neurotransmitter release.

4.4.3 Ca²⁺ channels in disease

The importance of the N-type channel function in sympathetic neurons has long been known^{26,104}, but its relevance to hypertension came from the evidence that N-Type Ca²⁺ blockade resulted in hypotension²⁶⁸, and more recently from a study using Ca_v2.2 knockout mice²⁷⁰. These mice show a relatively mild phenotype where the lack of Cav2.2 expression is compensated for by other Ca²⁺ channels, allowing for continued synaptic transmission^{114,281}. However, the mice show significant problems with blood pressure control linked to reduced baroreflex responses and heart rate responses after

electrical sympathetic neuronal stimulation. They report no changes in the parasympathetic system ^{269,270}. More recently it was shown that heart failure is associated with a marked increase of the N-type channel currents ²⁶⁵. Interestingly this is not linked to a lower mRNA or protein expression of the channel, suggesting the channel phenotype could be due to altered channel regulation.

Though the N-type channel is a major player in sympathetic neurotransmission in normal and hypertensive animals, it is possible that some of the other Ca²⁺ channel types are implicated. Most of the studies of the N-type channels employ ω -conotoxin GVIA to specifically isolate the N-type channel, however reports have shown that in rat sympathetic neurons the N-type specific blocker ω -Conotoxin GVIA also block the L-type channels ^{111,282}. It is therefore possible that some of the effects attributed to the N-type channel could also be partly L-type mediated. In fact, inhibition of the L-type channel on the mesenteric artery of the SHR lead to significantly reduced NA release in response to neuronal stimulation ²⁸³ suggesting this channel is indeed involved in the modulation of NA release from sympathetic neurons. We also know this channel is dysregulated at the myocyte level ⁸⁰. Interestingly a novel and clinically used dual vascular L-type and sympathetic N-type blocker cilnidipine ²⁸⁴ significantly reduces blood pressure in anesthetized dogs without affecting heart rate or maximum upstroke velocity ²⁸⁵ and also resets the cardiac autonomic imbalance in heart failure, preventing lethal arrhythmias in mice ²⁸⁶. Whether the drug also acts on the L-type channels in the nerves is unclear.

Together, the multiple reports of channel dysfunction suggests that channel dysregulation is highly conserved over several developmental stages from the pre-

diseased phenotype ⁶⁴ to advanced heart failure ²⁶⁵. What links the ion channel dysregulation to sympathetic hyper-responsiveness? One suggestion is that early metabolic-oxidative stress could be coupled to abnormal CN regulation ^{127,128,136}.

4.4.4 Why are the currents larger in the pro-hypertensive SHRs?

4.4.4.1 Differential channel regulation

Ca²⁺ channels are regulated by a variety of intracellular mediators including kinases and G proteins, as well as by Ca²⁺ itself. Much attention has been given to the cyclic nucleotide mediated protein kinase regulation of the channels. CNs act via three main mechanisms; they can directly modulate CN-gated ion channels; activate PKG or PKA resulting in phosphorylation of channels; and activate PDEs and protein phosphatases (PPs) resulting in de-phosphorylation of cellular targets and the breakdown of cAMP/cGMP. PKA and PKG mediated phosphorylation leads to stimulation and/or inhibition of Ca²⁺ channel currents ^{98,113,114}. Whilst well characterised in the myocyte ²⁸⁷, these pathways have to my knowledge not been fully investigated in PGSNs. In fact our group are among the first to have reported changes in intracellular signalling pathways linked to Ca²⁺ handling and exocytosis in the PGSNs of the SHR. Therefore the larger Ca²⁺ currents observed in the SHR neurons could be due to reduced PKG mediated inhibition and/or increased PKA mediated activation of the channel currents. That ω -conotoxin GVIA reduced the currents down to similar levels could suggest that the differences seen are due to the N-type Ca²⁺ channel. However, as discussed above, due to the reported non-selectivity of ω -conotoxin GVIA in sympathetic neurons, the changes could also be due to the L-type channels.

What is more, a recent study demonstrated that in HEK cells, NO significantly attenuates the voltage gated Ca²⁺ current via S-nitrosylation of the channel. This

predominantly affected the N-type channel, but also marginally reduced P/Q and L-type currents. A significant shift of the voltage dependency of the N-type channel was identified²⁸⁸. We know NO generation in the sympathetic neurons is reduced and so this could help explain the base differences in the Ca^{2+} currents in this study.

4.4.4.2 Cell surface expression

Different cell surface expression of the Ca^{2+} channels could also account for the difference in channel currents. However no changes were found in the expression of the Ca^{2+} channels in a recent comprehensive study of PGSNs in heart failure²⁶⁵. Nor did any changes in membrane expression result from increased S-nitrosylation mediated inhibition of the channel²⁸⁸. Western blot analysis was not possible due to the small tissue size. It would be interesting to perform single cell PCR to investigate the mRNA levels but this technique was not available in the lab.

4.4.4.3 Differential expression/association of ancillary subunits

Differential expression of the different channel subtypes and ancillary subunits in the SHR and WKY could also explain the phenotype. The different Ca^{2+} channel types have different channel properties and also associate with different ancillary subunits. As described in the introduction, the association of the Ca^{2+} channels with ancillary subunits significantly affects the channel behaviour and localisation and thus cell function. It is known that the β subunit significantly modulate the size of the currents, and so a difference in expression and/or association of β subunits could account for the smaller currents we see in the WKY. Interestingly when the $\text{Ca}_v\beta 3$ subunits are knocked out in the superior cervical ganglions (the sympathetic neurons upstream of the stellate ganglia used in this study), the result is a significant reduction in the N and P/Q type currents²⁸⁹. Overexpression of $\text{Ca}_v\beta 3$ leads to enhanced N-type channel currents and increased basal plasma NA levels, suggesting a link with sympathetic over activity²⁹⁰. More recently,

Ca_vβ3 knock out has been linked to hypertension ²⁹¹. However, though data from knockout models is exciting, it often relays little information about the molecular pathways involved, nor whether the resulting phenotype is a product of compensatory mechanisms and not the gene in question. Whilst changes in gene expression could account for the differences seen between the WKY and SHR, it is unlikely that if present, this is the only story. This is evident from the fact that pharmacological manipulation (8 bromo-cGMP) of the currents in the SHR, significantly reduces the current in 4 minutes. Most likely, a different or combination of mechanisms are likely at play.

4.4.5 Importance of NO-cGMP pathway in hypertension

Previous work has demonstrated a key involvement of the nitric oxide (NO)-cGMP pathway in the pathophysiology of hypertension, where it is acutely linked to intracellular Ca²⁺ homeostasis ^{63,174} and neurotransmission ⁵⁴. NO gets synthesized by a family of enzymes called nitric oxide synthases (NOS) ¹³⁶ and acts as a neurotransmitter or a paracrine signal. NO activates guanylyl cyclase via its binding to the β subunit (soluble guanylyl cyclase) ^{292,293} leading to an elevation of intracellular cGMP ^{294,295}. cGMP is integral to many cellular pathways and can go on to activate PKG resulting in the phosphorylation of downstream targets often resulting in altered cellular function. cGMP also exert its effects via cyclic nucleotide gated ion channels and via PDEs ¹³⁰. The NO/cGMP/PKG signalling pathway has been linked to cardiac protection, neuronal plasticity and gene transcription ¹³⁰. Previously thought to be restricted to particular tissues, it is now realised that this signalling pathway is present in a diverse range of tissues – even in places where the concentrations of signalling components are very low ²⁹⁶. Altered levels of NO or the downstream cGMP, PKG or PDEs have been implicated in diseases such as hypertension, hypercholesterolemia and diabetes ¹⁷³. In neurons

nNOS modulates autonomic balance ¹²⁷, and disruption of the NO/cGMP pathway leads to increased sympathetic drive which is a well-established factor of heart diseases such as hypertension and heart failure ^{127,128}.

4.4.6 cGMP mediated cyclic nucleotide dysfunction in hypertension

In hypertension, we know from previous studies that PGSNs from the SHR have a lower expression of the β_1 subunit of soluble guanylyl cyclase and as a result cGMP when compared to the WKY neurons ⁵⁴. Gene transfer of nNOS increases neuronal cGMP levels ^{54,134}, rescues the high intracellular Ca^{2+} transients ^{64,65} resulting in reduced neurotransmission ^{54,134,141,142}. Furthermore, re-expression of CAPON – an important modulator of nNOS – reverses the cyclic nucleotide phenotype and indeed normalises the Ca^{2+} current seen in the SHR down to levels seen in the WKY ⁶⁴.

To investigate whether the lower expression of cGMP in the SHR PGSNs could account for the larger Ca^{2+} currents I observed, I bath applied a membrane permeable cGMP analogue and repeated the voltage clamp experiments. Interestingly 8-bromo cGMP application significantly reduced the whole cell Ca^{2+} currents in the SHR neuron down to levels seen in the WKY, whilst having no effects on the WKY currents. This suggests altered cyclic nucleotide regulation of the channel could account for the differences seen between the SHR and WKY. Cyclic nucleotide signalling in peripheral autonomic neurons is still a relatively new field of study. However much can be learnt from studies in other cell types. Previous studies have shown that the NO-cGMP pathway is critical for the regulation of Ca^{2+} channels via PKG activation, but also through cross talk with PDEs ^{132,135}. In the myocyte and pinealocytes 8-bromo cGMP application leads to a direct phosphorylation of Ca^{2+} channels via PKG ^{117,297} resulting in smaller currents, with no changes in the voltage dependency ¹¹⁸ of the channel, which is analogous to what I

describe in this study. This may be surprising as the modulation of ion channels by intracellular mediators often involve a change in the voltage dependency of the channel²⁴⁶. It is possible that small changes in this study are masked by the need to conduct unpaired experiments, leading to more variability in the data. However as similar results have been reported before, it is possible that cGMP acts through mechanisms that do not involve such a shift in the activation curve.

Furthermore, as described in section 4.4.4.1, NO donors significantly reduce N-type channel current in HEK cells²⁸⁸ and dorsal root ganglion neurons²⁹⁸. The channel inhibition was independent of the activity of guanylyl cyclase²⁹⁸. However these experiments were conducted in cells of spinal origin and HEK cells, with NO being generated by S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) - known to be a relatively unspecific drug. Further as PKG inhibition of the current was not attempted, a cGMP/PKG dependent pathway could still be involved. What is more, the evidence that the elevation of cGMP rescues the current phenotype, provides strong evidence for the involvement of a cGMP mediated pathway in cardiac sympathetic neurons.

4.4.7 Differential involvement of PDEs in the WKY and SHR neurons

A signal as powerful and diverse as cGMP must be terminated. This is done both by protein phosphatases (PPs) that dephosphorylate the downstream proteins, and phosphodiesterases (PDEs) that break down cGMP and cAMP. As the role of PDE2A and its heightened activity in hypertension is coming to light^{70,173}, I sought to inhibit PDE2A using 1 μ M BAY 60-7550. Blockage of PDE2A could lead to an increase of cAMP, but also possibly cGMP leading to increased/decreased channel activity. It is likely that both of the pathways are affected, perhaps to different degrees in the two cell types. A further complication comes from the cyclic nucleotides' ability to cross talk

with each other via the PDEs¹²², resulting in their own (or indeed the other's) breakdown. In the WKY, PDE2A inhibition led to significantly increased channel currents, likely due to a direct increase in the intracellular cAMP as a result of inhibition of cAMP hydrolysis. It is also possible that an indirect increase in cAMP occurred, mediated via a potential increased cGMP inhibiting PDE3, resulting in further inhibition of cAMP hydrolysis. Together the increased cAMP levels would facilitate PKA dependent activation of the current. Interestingly in the SHR, BAY 60-7550 resulted in a slight but not significant decrease in channel currents. This could come as a result of PDE2 mediated increases of cGMP leading to channel inhibition. However I cannot rule out that the changes may also be due to effects on the cAMP side. This dysfunction could lie at any point in the downstream signalling pathway, and could suggest changes in levels, activity or subtype expression of PP and PDEs between the SHR and WKY neurons. Furthermore, it is now well-established that cyclic nucleotide signalling occurs in distinct subcellular locations within a cell^{176,299}. The voltage clamp and the drug treatments used in this study detect global changes within a cell and as such may not be able to resolve the full extent of the CN dynamics in these neurons.

4.4.8 Limitations of the study

The space clamp issue (described in detail in chapter three), remains a significant limitation of the study. These issues led to the exclusion of large amounts of data leaving the study at danger of being underpowered. Furthermore, due to R_{series} instability it was necessary to conduct unpaired experiments. This increased the variability of the data, and could have masked subtle differences between the WKY and SHR neurons. Being able to make longer term, paired recordings would allow for pharmacological manipulation of the intracellular pathways in a more creative way.

This study uses acutely isolated neurons, thought to recapitulate normal physiology better when compared to cultured neurons, however one cannot rule out that membrane damage occurred during the isolation process. Furthermore, comparisons are made with previous work done in cultured cells, which are at risk of changing their phenotype due to their culture environment.

4.4.9 Concluding remarks

This study is the first to describe the involvement of the voltage gated Ca^{2+} channels in the intracellular Ca^{2+} dysfunction seen in a model of pro-hypertension. PGSNs from the pro-hypertensive SHR show significantly larger whole cell Ca^{2+} currents with the N-type channel being responsible for 70-80% of the current. It was subsequently demonstrated that the channel dysfunction is linked to the previously reported dysfunction of the CN signalling pathway in the neurons possibly via changes in PKA/PKG mediated regulation. Restoring the intracellular levels of cGMP normalised the SHR currents down to levels seen in the WKY. Finally, preliminary data are provided suggesting a differential involvement of PDEs in the WKY and SHR sympathetic neurons. The evidence provided in this thesis demonstrate that the WKY and SHR neurons have differential responses to the PDE2A blockade. This points towards a potential role for these enzymes in the pathophysiology of hypertension. Interestingly the molecular changes illustrated in this thesis are present prior to the development of clinical symptoms of hypertension, and thus could potentially form the trigger for the autonomic dysfunction seen in these neurons.

4.4.10 Future work

To my knowledge, this is the first studies linking the intracellular calcium phenotype to changes at the membrane level in PGSNs from pro-hypertensive SHRs. It has answered many questions but also posed many more that need to be investigated if we are to fully

understand the Ca^{2+} dysfunction seen in the hypertensive neurons, and how this is linked to the disease phenotype. Given more time it would be informative to perform the following experiments:

- PDE2A blockade in the presence of 8b-cGMP
- Repeat the experiments in the presence of PKA or PKG blockers to dissect out whether the pathway dominance varies between the WKY and SHR neurons.
- Employ different PDE blockers to test for the differential involvement of signal terminators in the WKY and SHR.
- Current clamp experiments to investigate the basal excitability of the WKY and SHR neurons.
- Analysis of cell surface expression of the Ca^{2+} channels and their subunits. It would be of interest to investigate cellular location and clustering.

CHAPTER FIVE

5 Differential cGMP/cAMP cross-talk in pro-hypertension

5.1 Introduction

Increased activity of the sympathetic nervous system and cardiac hyper-responsiveness are well-established hallmarks of human hypertension^{47,264} and that observed in the spontaneously hypertensive rat (SHR)^{13,80,300}. When comparing the sympathetic neurons innervating the heart (PGSNs) from the pro-hypertensive SHR rats to those of their normotensive controls, it is clear that the hypertensive state is associated with significant Ca^{2+} dysregulation resulting in increased neurotransmission⁵³. This Ca^{2+} dysfunction manifests itself in significantly greater membrane Ca^{2+} currents⁶⁴ (see Chapter 4 for details) and intracellular Ca^{2+} transients⁶³ in response to membrane depolarisation. This elevation in intracellular Ca^{2+} , coupled with reduced function of the noradrenaline reuptake transporter (NET)⁶⁹ results in significantly increased noradrenaline (NA) remaining in the synaptic cleft and increased neurotransmission^{53,54}.

5.1.1 Neuronal cyclic nucleotide signalling

The exact molecular mechanisms that underpin the sympathetic hyperactivity remain poorly understood, but it is now clear that the dysregulation of Ca^{2+} forms one of the central players. What links the Ca^{2+} phenotype to sympathetic hyper-responsiveness? One suggestion is that early metabolic-oxidative stress could be coupled to abnormal CN regulation of neurotransmission^{127,128,136}.

The CNs cAMP and cGMP are important intracellular second messenger molecules that function as central points of signal amplification within a cell. In neurons cGMP is produced by guanylyl cyclase (GC) as a result of its activation by nitric oxide (NO). In central and peripheral neurons this pathways functions as an important regulator of

neurotransmitter release^{130,134,166}. Similarly, cAMP is produced by adenylate cyclase (AC) in response to receptor activation. The mechanism by which the CNs modulate neurotransmission, is largely related to their protein kinase dependent effects on cellular proteins critical to Ca^{2+} homeostasis. The CNs activate the cGMP activated protein kinase (PKG) and the cAMP activated protein kinase (PKA) leading to the phosphorylation of downstream proteins¹⁵⁰. In neurons, PKG and PKA directly phosphorylate Ca^{2+} channels resulting in the inhibition and activation of the channel respectively^{114,119,301}. The balance of these two governs the net level of Ca^{2+} entry into the cell and thus modulates the level of neurotransmission in a cell^{25,26,104,108}.

In addition to this direct effect on PKG and PKA, cGMP and cAMP can affect and control each others signalling through the activation and/or inhibition of cellular phosphodiesterases (PDEs)^{122-125,130}. This functions as a direct signalling mechanism but also as a positive/negative feedback control system to maintain signal fidelity. This process has been identified as critically important in cardiac myocyte function^{122,164} and also plays a significant role in central neurotransmission¹²⁴. However, its role in the autonomic nervous system remains entirely unexplored.

5.1.2 Cyclic nucleotide dysfunction in the hypertensive state

It is now clear that the PGSNs of the pro-hypertensive SHR have significantly altered CN signalling when compared to the normotensive controls. In these cells there is a significant down-regulation of the β_1 subunit of sGC⁶⁵, which is coupled to a low activity of nNOS (the enzyme responsible for the generation of NO)⁶⁵. Together this results in significantly lower intracellular levels of cGMP in the SHR neurons⁵⁴. What is more, it was recently identified that whilst PDE2A expression was the same between the SHR and its controls, the activity of PDE2A was significantly higher in the SHR

PGSNs. This has been shown to facilitate larger Ca^{2+} transients and enhanced neurotransmission⁷⁰. Increasingly PDEs are gaining attention for the crucial role they play in orchestrating intracellular CN signalling by constraining them to specific subcellular compartments^{160,165,170}. Whilst historically relatively unexplored in PGSNs, it is now becoming clear that they have a dominant role to play. Interestingly, in the last year both PDE2A and PDE3 have been implicated in the pathogenesis of hypertension^{70,302}, suggesting a larger role for PDEs than has previously been appreciated.

The relative importance of this cGMP-centred dysfunction is evident from a multitude of experiments demonstrating that gene transfer with nNOS and its adaptor protein CAPON restores the Ca^{2+} dysfunction seen and rescues the heightened neurotransmission^{54,64,65,141} – both in ways that centre around increases in neuronal cGMP levels^{54,64,134}. It is clear that cGMP signals directly via PKG to modulate cellular Ca^{2+} homeostasis. However it remains unclear whether it could also elicit effects via cross-talk with cAMP/PKA pathway.

5.1.3 Aims of the Chapter

This chapter tests the hypothesis that the cGMP centred cyclic nucleotide dysfunction linked to aberrant Ca^{2+} handling could be affecting the cAMP pathway to elicit changes in Ca^{2+} handling. Could this be mediated by the differential expression of PDEs in the PGSNs of the WKY and pro-hypertensive SHR? To test this, I employed novel, highly sensitive FRET sensors to monitor cAMP levels and PKA activity. Finally, I used the FRET cAMP sensor to test whether the activation of the presynaptic neuron via the native nicotinic acetylcholine receptors could be different in the pro-hypertensive SHR and its normotensive control neurons.

5.2 Methods

5.2.1 Cell isolation

Please see Chapter 2 for details on the cell isolation. Briefly, the PGSNs from four-week old SHR and control rats were isolated, de-sheathed and minced into $\sim 1 \text{ mm}^2$ pieces. The ganglions were exposed to enzymatic digestions using collagenase (1 mg/ml) and trypsin (2 mg/ml) and mechanically dissociated before being plated on 6 mm coverslips. In this chapter both the Wistar and Wistar Kyoto rat was used. Hereafter, these are referred to as normotensive control neurons.

5.2.2 Immunofluorescence

After three to four days in culture the cells were fixed in 4% paraformaldehyde, blocked and permeabilised. Primary antibodies to tyrosine hydroxylase (TH) and the nicotinic acetylcholine receptor (nAChR) were added and visualised via the aid of fluorescently labelled secondary antibodies. Cells were imaged on an Olympus confocal microscope.

5.2.3 FRET sensors used in this study

The two main sensors used in this study were the cAMP sensor H187²²⁴ and the PKA sensor AKAR4¹⁷². These are both adenoviral sensors driven by a CMV promoter. However in the experiment assessing cAMP levels in response to forskolin, the H30 sensor was used²²⁶. These experiments were performed before the H187 sensor was made available to our lab. All subsequent cAMP experiments employ the newer and more sensitive H187 sensor.

5.2.4 Viral infection of the FRET sensors

The neurons were infected with cAMP and PKA sensors for 24 hours. The following particle forming units (PFUs) were used: H187; 1.4×10^7 and AKAR4; 4×10^9 . The cells were allowed to recover in an incubator kept at 37°C 5% CO₂ for three days prior to experimentation. FRET sensor-expressing neurons were imaged three to four days post-

isolation on an inverted Nikon microscope connected to an OptoLED fluorescence imaging system.

5.2.5 FRET imaging of sympathetic stellate neurons

FRET imaging was performed on the sympathetic stellate neurons of the pro-hypertensive SHR and its normotensive controls to investigate both basal and stimulated cAMP levels and PKA activity in response to pharmacological drug treatment (see Chapter 2, section 2.7-8). Briefly, the cells were excited at 420 nm and CFP and YFP emissions were measured as a change in the ratio between 480/535 nm fluorescent emission intensities following a 100 ms excitation at 430 nm, every 15 seconds. Background fluorescence was subtracted from emission intensities and intensity ratios were plotted against time. Mean FRET responses were expressed as the percentage change from baseline ($\Delta R/R_0$ where $\Delta R = R - R_0$. R_0 is the ratio of intensity at time = 0 seconds and R is the ratio at time = t seconds).

In all experiments, the maximal FRET change of each cell was recorded by exposing the cells to saturating concentrations of an AC activator and a PDE inhibitor (25 μ M forskolin and 100 μ M IBMX respectively) to ensure the cells responded similarly to the sensor. All data are reported as the mean raw FRET ratio. Due to small variability in the rate of superfusion (2.5-3 mL/min) and image acquisition every 15 seconds, the peak occurred over 3 image points (time span of 30 seconds). For this reason, the absolute peak cAMP levels are reported in bar charts. Statistical analysis was performed on the absolute peak values. As a consequence of the variable flow rates, it was not possible to draw any conclusions about the rate of cAMP increase. Imaging at 15 second intervals was critically important to avoid photo toxicity of the neurons which could alter their behaviour.

5.2.6 FRET protocols

The cells were perfused continuously with tyrode solution and flow rate was controlled at 2-3 ml/min. Pharmacological agents were diluted in tyrode and perfused at the following concentrations: forskolin (0-25 μ M); 3-isobutyl-1-methylxanthine (IBMX: 1-100 μ M); the cGMP analogue 8b-cGMP (100 μ M); PDE3 inhibitors cilostamide (10 μ M) or milrinone (10 μ M); PDE2 inhibitor BAY-60-7550 (1 μ M).

Specifically, I looked at:

- The cells ability to generate cAMP and resulting PKA activity by administering the adenylyl cyclase (AC) activator forskolin to both cells types in the presence of the AKAR4 or H187 FRET sensors.
- I assessed the cells ability to hydrolyse cAMP by applying a global pharmacological PDE inhibitor (IBMX) which inhibits PDEs 1-7 and 10-11.
- To test the involvement of the NO-cGMP pathways in the regulation of basal cAMP levels and PKA activity I applied the membrane permeable cGMP analogue 8-bromo-cGMP (8b-cGMP) and measured cAMP levels and PKA activity.
- To test the relative involvement of PDE2A and PDE3 in the cells, the cells were incubated with selective PDE2 (BAY 60-7550) and PDE3 (Cilostamide or Milrinone) inhibitors whilst the cAMP levels were monitored.
- I chose native neuronal stimulation (nicotine) to depolarise the cells and investigated the resulting changes in cAMP levels.
- Finally, I tested whether IBMX insensitive PDEs could be expressed in these cells, and indeed be involved in the nicotine evoked responses. I pre-incubated the cells with 25 μ M IBMX before repeating the nicotine stimulation.

5.3 Results

5.3.1 The cAMP-PKA signalling pathway remains unchanged in the pro-hypertensive animals

To determine the cells ability to generate cAMP and activate PKA the cells were challenged with increasing doses of the adenylyl cyclase (AC) activator forskolin (0.1-25 μ M) and measured the FRET responses of the two sensors. There was no significant difference between the generation of cytosolic cAMP (n=15-18/21-28), and PKA activity (n=20-23/16-17) between normotensive control and pro-hypertensive SHR (see figure 5.1). This indicated that the cAMP-PKA signalling pathway remains intact in the pro-hypertensive animals.

5.3.2 Nicotinic acetylcholine receptor activation resulted in similar increases in cAMP levels in the control and SHR neurons.

To test whether the physiological stimulation of the stellate neurons via the nicotinic acetylcholine receptor resulted in different cAMP responses I measured neuronal FRET responses to nicotine. The application of nicotine (1, 10 and 100 μ M) led to large increases in cAMP levels in both neurons (see figure 5.2). The absolute peak control responses were found to be not statistically different between the SHR and control cells at all nicotine concentrations (1 μ M: 35.57 ± 13.26 , n=6 vs 34.19 ± 6.10 , n=5 p=0.93; 10 μ M: 27.09 ± 6.14 , n=6 vs 17.94 ± 3.11 , n=7, p=0.18; 100 μ M: 34.19 ± 6.10 , n=5 vs 37.96 ± 2.54 , n=8, p= 0.52, unpaired t-tests).

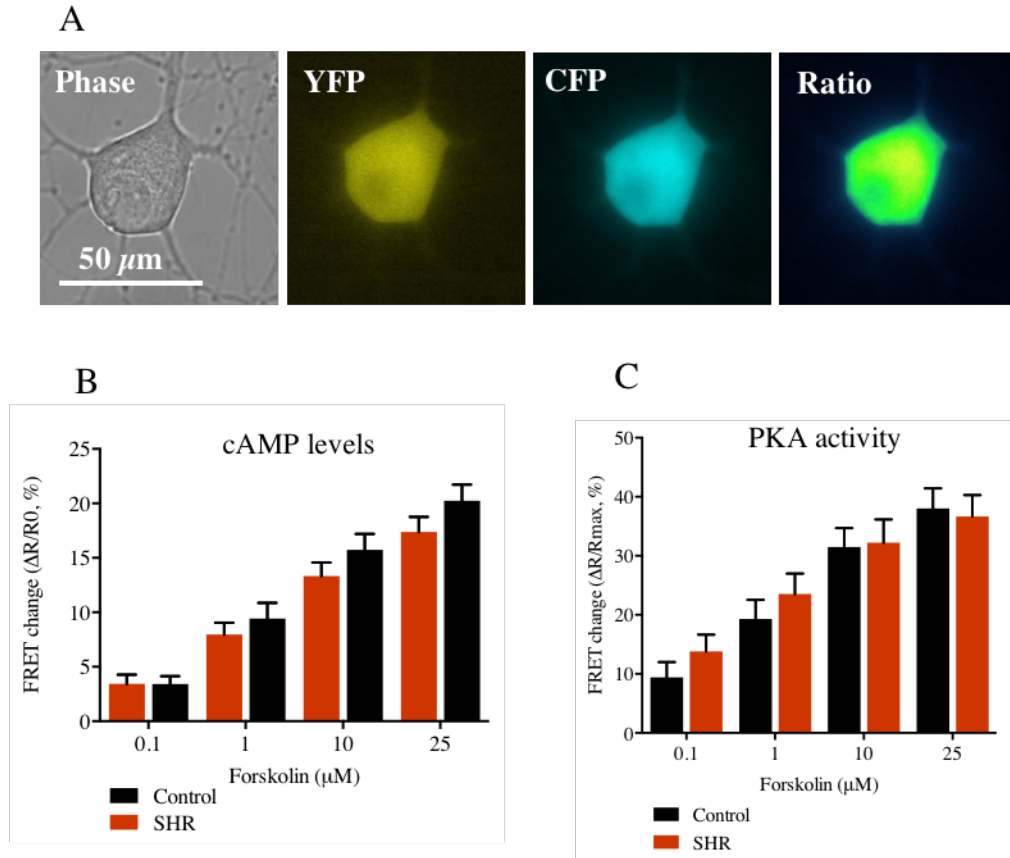


Figure 5.1: cAMP-PKA signalling pathway is functional in the SHR neurons.

To investigate the ability of the PGSNs to generate cAMP and PKA activity was compared between the SHR and control neurons using FRET imaging. Cells were infected with the H30 EPAC and AKAR4 sensors and the changes in fluorescence emission intensity of CFP and YPF was monitored (A). No significant difference were found in forskolin induced cAMP generation (B, $n=15-18/21-28$) or PKA activity (C, $n=20-23/16-17$) in the sympathetic neurons from normotensive controls and SHR neurons. Comparisons were made using the Mann-Whitney test and were found to be non-significant.

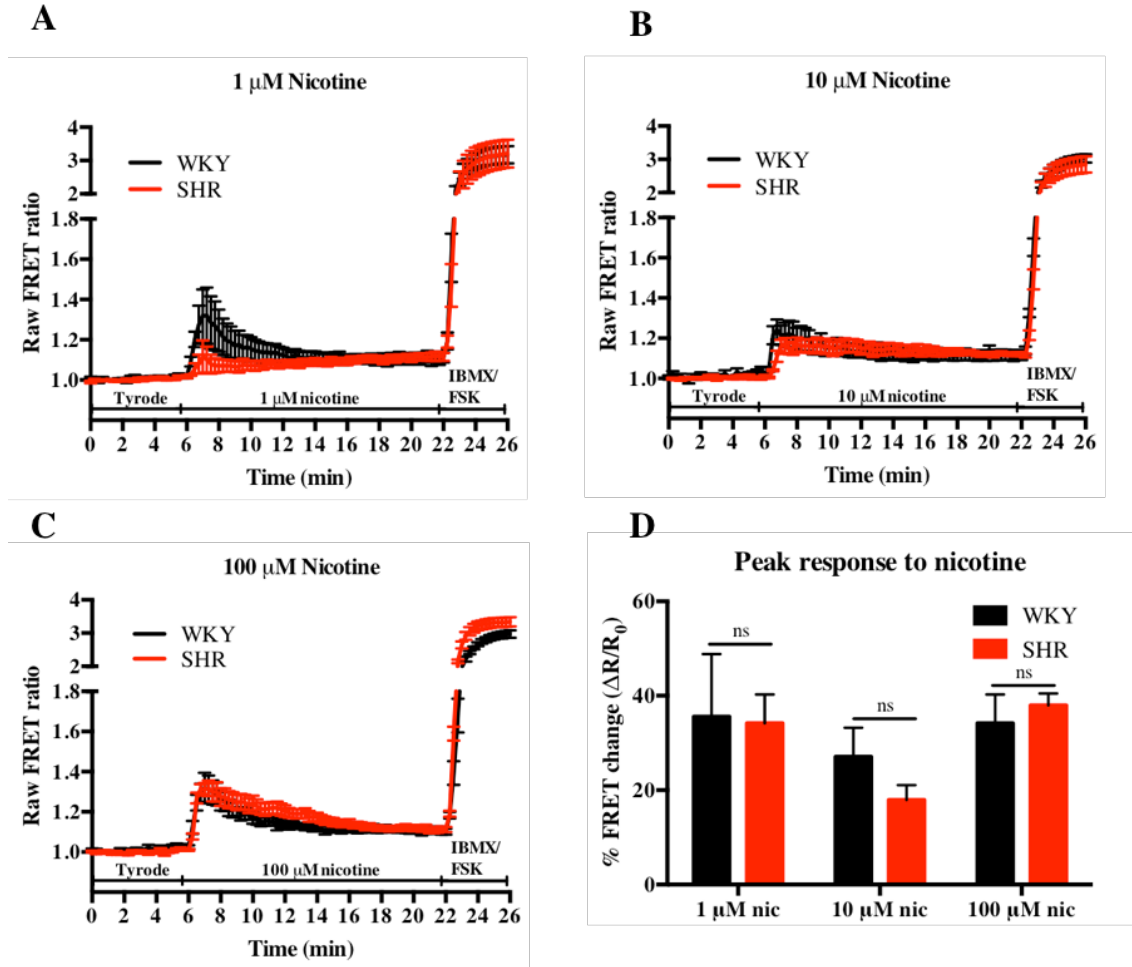


Figure 5.2: Nicotine equally elevates cAMP levels in both the WKY and SHR sympathetic neurons. Sympathetic stellate neurons from the pro-hypertensive SHR and normotensive WKY were infected with the H187 cAMP FRET sensor and imaged following the application of nicotine (1, 10 and 100 μM : A-C). The application of nicotine led to immediate increases in cAMP levels in the neurons. The absolute peak WKY responses were found to be not statistically different from the SHR responses at all nicotine concentration (unpaired t-tests). The basal levels of cAMP are dictated by the equilibrium between the generation of cAMP by adenylyl cyclase and its breakdown by the phosphodiesterases (PDEs). The application of nicotine results in a peak-plateau response which is explained by the increases in cAMP activating PDEs, resulting in its breakdown. A plateau is reached when a new equilibrium has formed.

5.3.3 Altered sensitivity to global PDE block in SHR and control neurons.

Next, I tested the cells ability to hydrolyse cAMP using the global PDE inhibitor IBMX.

Compared to the control neurons, the PGSNs of the pro-hypertensive SHRs showed significantly reduced cAMP hydrolysis at 1 μ M ($0.96 \pm 1.23\%$, n=15 vs $32.96 \pm 9.67\%$, n=6, p=0.006) and 10 μ M ($34.77 \pm 3.34\%$, n=15 vs $75.48 \pm 13.74\%$, n=6, p=0.014) IBMX. At 100 μ M IBMX ($105.4 \pm 7.9\%$, n=15 vs 124.7 ± 9.48 , n=6, p=0.23) the response of the SHR neurons were not statistically significant to those of the controls (Mann Whitney tests, n=6-15. See figure 5.3). At 100 μ M half of the control cells reached the maximum activation of the FRET sensors. This resulted in the under-representation of the control response, which is likely to have been higher, and could explain the lack of significant difference. Together these data suggest that there could be a difference in the PDE expression profile between the control and SHR neurons.

5.3.4 The sympathetic stellate neurons from the pro-hypertensive SHR express IBMX insensitive PDEs

The peak-plateau appearance of the nicotine-evoked cAMP response provided a good tool to test the presence of IBMX insensitive PDEs. If the cells expressed only IBMX-sensitive PDEs, IBMX pre-treatment would eliminate the peak-plateau caused by nicotine. The IBMX pre-treatment did not eliminate the peak-plateau (see figure 5.4), suggesting the presence of IBMX-insensitive PDEs in rat sympathetic neurons. Pilot data showing two example traces of an SHR neuron (n=2).

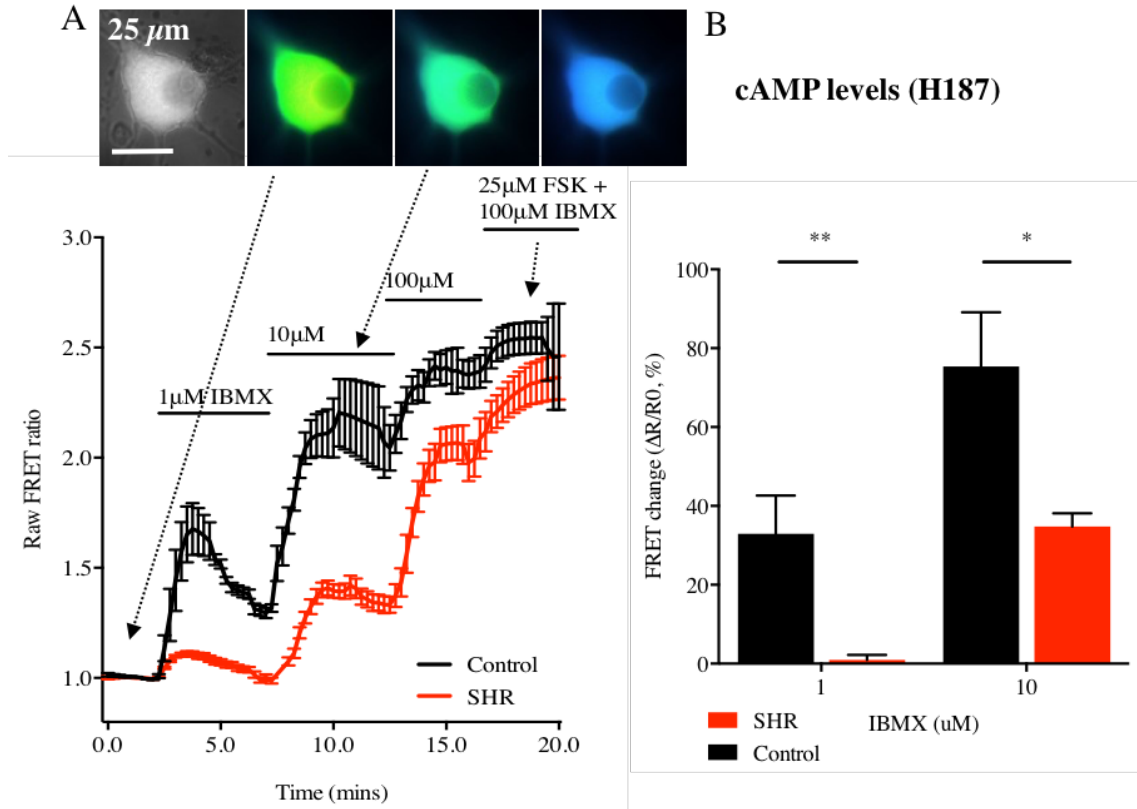


Figure 5.3: cAMP responses to global PDE inhibition. To investigate the PGSNs ability to hydrolyse cAMP, the cells responses to global pharmacological PDE inhibition using 1, 10 and 100 μM IBMX was investigated. Panel A depicts the real time changes in fluorescent intensities (trace) and FRET change (image panel) following drug application. The grouped data (B) revealed that the cells ability to hydrolyse cAMP was significantly different between the SHR and control neurons. The SHR neurons have significantly reduced cAMP responses as a result of PDE inhibition when compared to the control neurons at 1 μM ($0.96 \pm 1.23\%$, $n=15$ vs $32.96 \pm 9.67\%$, $n=6$, $p=0.006$) and 10 μM ($34.77 \pm 3.34\%$, $n=15$ vs $75.48 \pm 13.74\%$, $n=6$, $p=0.014$). At 100 μM ($105.4 \pm 7.9\%$, $n=15$ vs 124.7 ± 9.48 , $n=6$, $p=0.23$) the response of the SHR cells was not significantly different from the control ($n=6-15$, Mann Whitney tests). At 100 μM half of the control cells reached the maximum activation of the FRET sensor (last drug step in panel A). This resulted in the under-representation of the control response, which is likely to be higher, and could explain the lack of significant difference. Together these results suggests a different expression/activity profile of PDEs may be present in the control vs. SHR neurons.

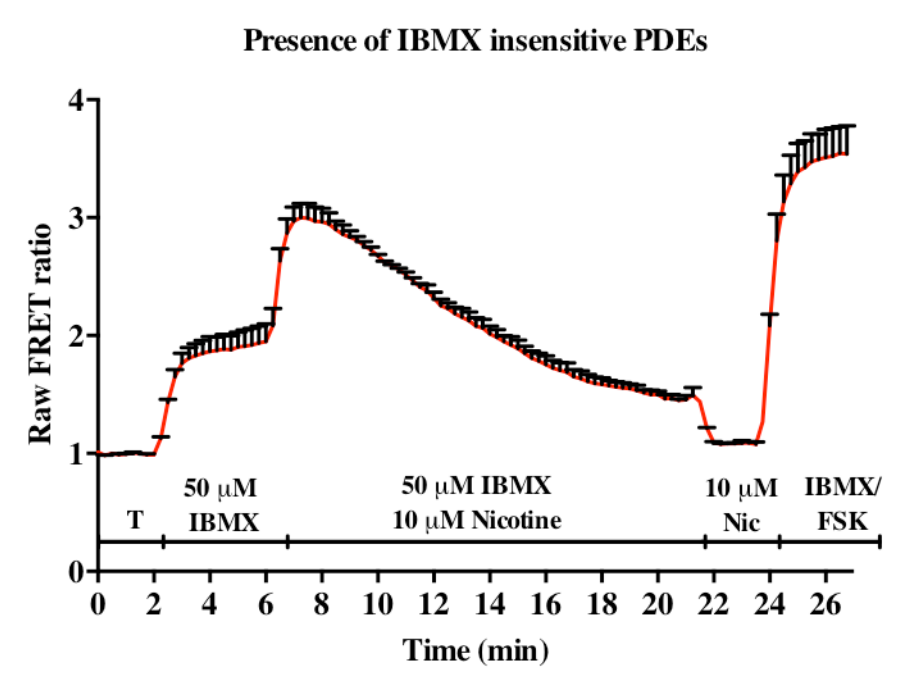


Figure 5.4: Pre-treatment with IBMX suggests the presence of IBMX insensitive PDEs in the sympathetic neurons. The peak plateau of the nicotine response in the neurons is due to cAMP hydrolysis by PDEs. Currently, only IBMX sensitive PDEs have been described in sympathetic neurons (PDE2A and 3). To test whether these cells could be expressing IBMX insensitive PDEs we pre-incubated the cells with 50 μ M IBMX. If the cells only express IBMX sensitive PDEs, the IBMX pre treatment would eliminate the peak-plateau response. The IBMX pre-treatment does not eliminate the peak-plateau, suggesting the presence of IBMX insensitive PDEs in rat sympathetic neurons. Pilot data showing two example traces of an SHR neuron (n=2).

5.3.5 Differential cGMP-cAMP crosstalk in the SHR

To investigate the ability of the cGMP pathway to cross-talk with the cAMP pathway, I applied 8b-cGMP (membrane permeable cGMP analogue) and measured changes in cAMP levels and PKA activity. The application of 8b-cGMP led to significant increases in cAMP levels in the control neurons ($11.68 \pm 1.54\%$, $n=14$). In contrast, in the pro-hypertensive SHR neurons, this increase was attenuated. In fact, a slight decrease of cAMP levels was observed ($-4.82 \pm 2.02\%$, $n=16$). The differences between the control and SHR responses were found to be highly significant ($p<0.0001$, unpaired t test). See figure 5.5 A-B.

5.3.6 The cAMP responses to cGMP elevation translate into functional PKA activation

I determined the functional significance of the cAMP changes by assessing the resulting changes in the phosphorylation of PKA. Following 8b-cGMP application, the PKA activity in the normotensive neurons was significantly increased ($19.15 \pm 3.51\%$, $n=6$). No significant increase from basal levels was observed in the SHR neurons ($1.09 \pm 0.57\%$, $n=8$). The differences between the control and SHR neurons were found to be highly significant ($p<0.0001$, Mann Whitney test). See figure 5.5 C-D.

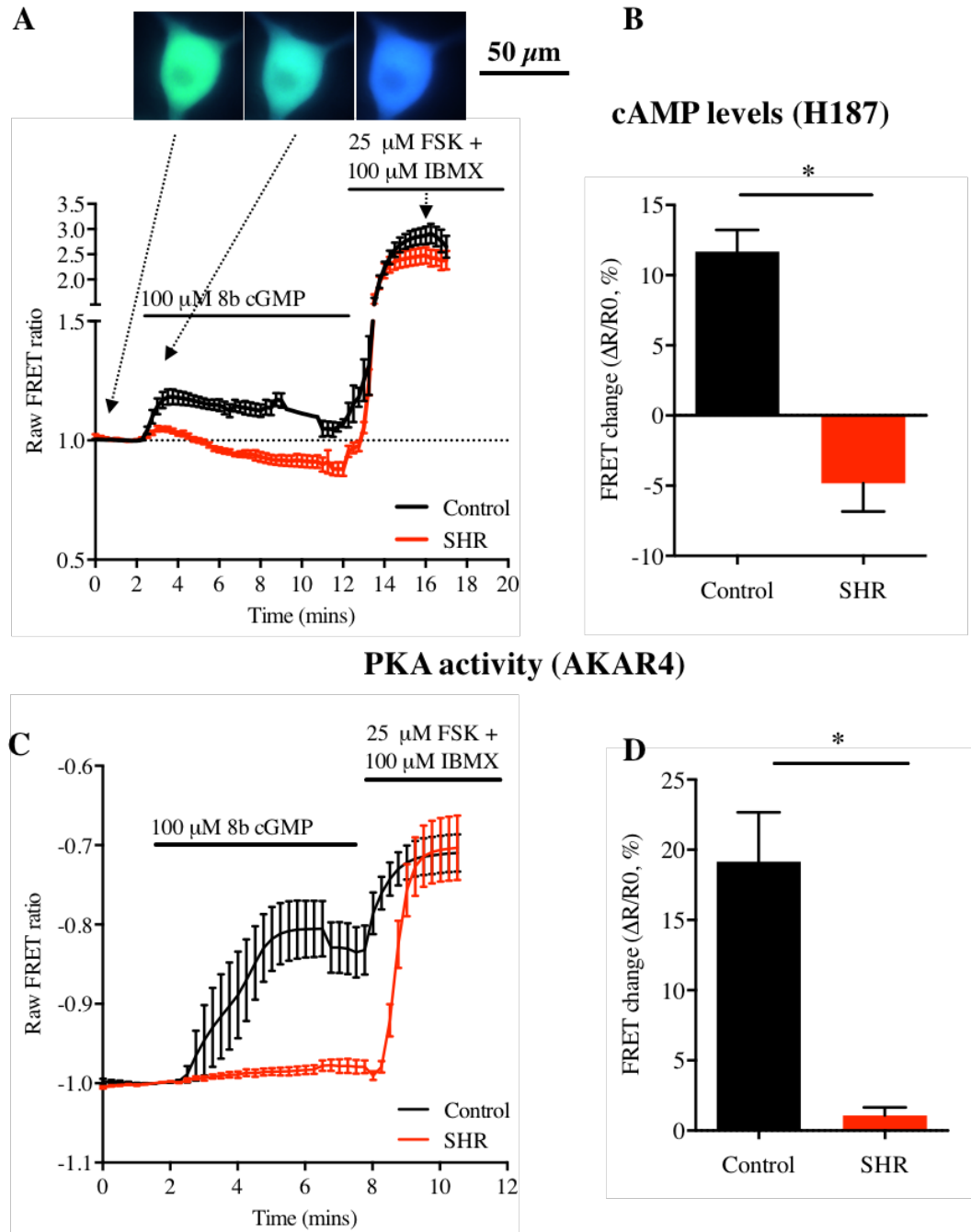


Figure 5.5: cAMP levels and PKA activation following the application of 8b-cGMP. In the control cells 8b-cGMP application resulted in an increase in cAMP levels but a reduction in the SHR neurons ($11.68 \pm 1.54\%$, $n=14$ vs $-4.82 \pm 2.02\%$, $n=16$, $p<0.0001$, unpaired t-test) (A-B). As a result, the PKA activity in the control neurons was found to be significantly higher than that of the SHR ($19.15 \pm 3.51\%$, $n=6$ vs $1.09 \pm 0.57\%$, $n=8$, $p<0.0001$, Mann Whitney test) (B-C). These results point towards PDE2 dominance in the SHR but a greater regulation by a cGMP inhibited PDE in the normotensive control.

5.3.7 Evidence for the functional expression of PDE2 in the sympathetic neurons of control and SHR neurons

Both the SHR and control neurons were found to functionally express PDE2. The application of a specific PDE2 inhibitor (1 μ M BAY 60-7550) increased the cAMP levels in both the control and SHR neurons ($19.69 \pm 5.16\%$, $n=10$ vs $36.55 \pm 21.47\%$, $n=8$, $p=0.94$, Mann Whitney). This was found to be non-significant between the two strains (see figure 5.6).

5.3.8 Sympathetic neurons functionally express PDE3

Both the SHR and control neurons were found to functionally express PDE3. The application of two clinically used specific PDE3 inhibitors (10 μ M milrinone and 10 μ M cilostamide) significantly increased cAMP levels in both the control and SHR neurons (see figure 5.7). Milrinone: $22.98 \pm 6.81\%$, $n=10$ vs $31.14 \pm 6.25\%$ $n=16$, $p=0.41$, Mann Whitney. Cilostamide: $28.15 \pm 15.45\%$, $n=5$ vs $33.23 \pm 8.18\%$ $n=8$, $p=0.58$, Mann Whitney.

5.3.9 Single or dual PDE2 and PDE3 inhibition did not rescue the sympathetic phenotype observed following cGMP elevation

Pharmacological inhibition of PDE2 and PDE3 in combination or alone did not rescue the pro-hypertensive phenotype seen following cGMP elevation using 8b-cGMP. The data were found to be incredibly variable and non significantly different between any of the treatment groups using the Mann Whitney test (see figure 5.9).

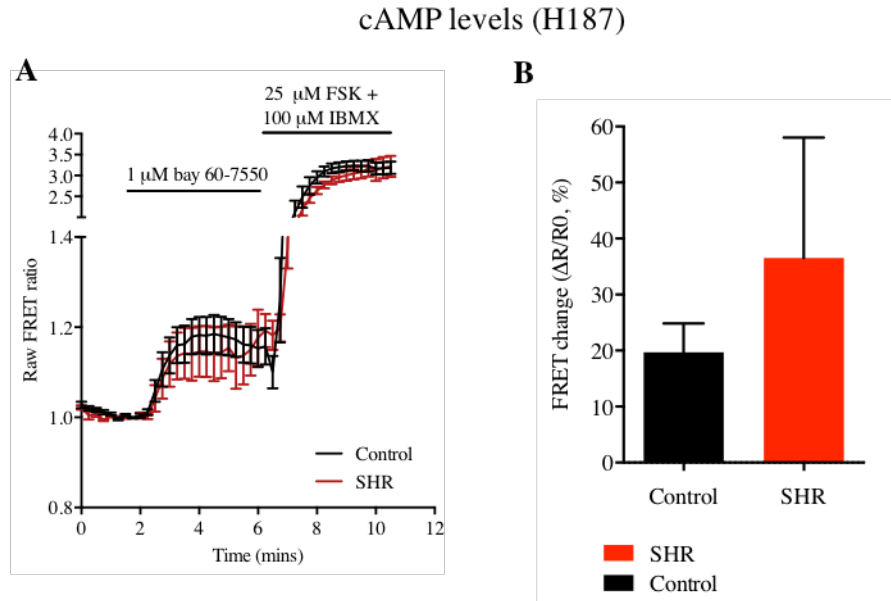


Figure 5.6: Evidence for the functional expression of PDE2 in the sympathetic neurons of the SHR and control. To assess the functional expression of PDE2, FRET imaging of cAMP levels (H187) in response to the well characterised selective PDE blocker BAY 60-7550 was performed. Pharmacological inhibition of PDE2 (1 μ M Bay 60-7550) increased the cAMP levels in both the control and SHR neurons ($19.69 \pm 5.16\%$, $n=10$ vs $36.55 \pm 21.47\%$, $n=8$, $p=0.94$). This was found to be non-significant between the two strains (Mann Whitney).

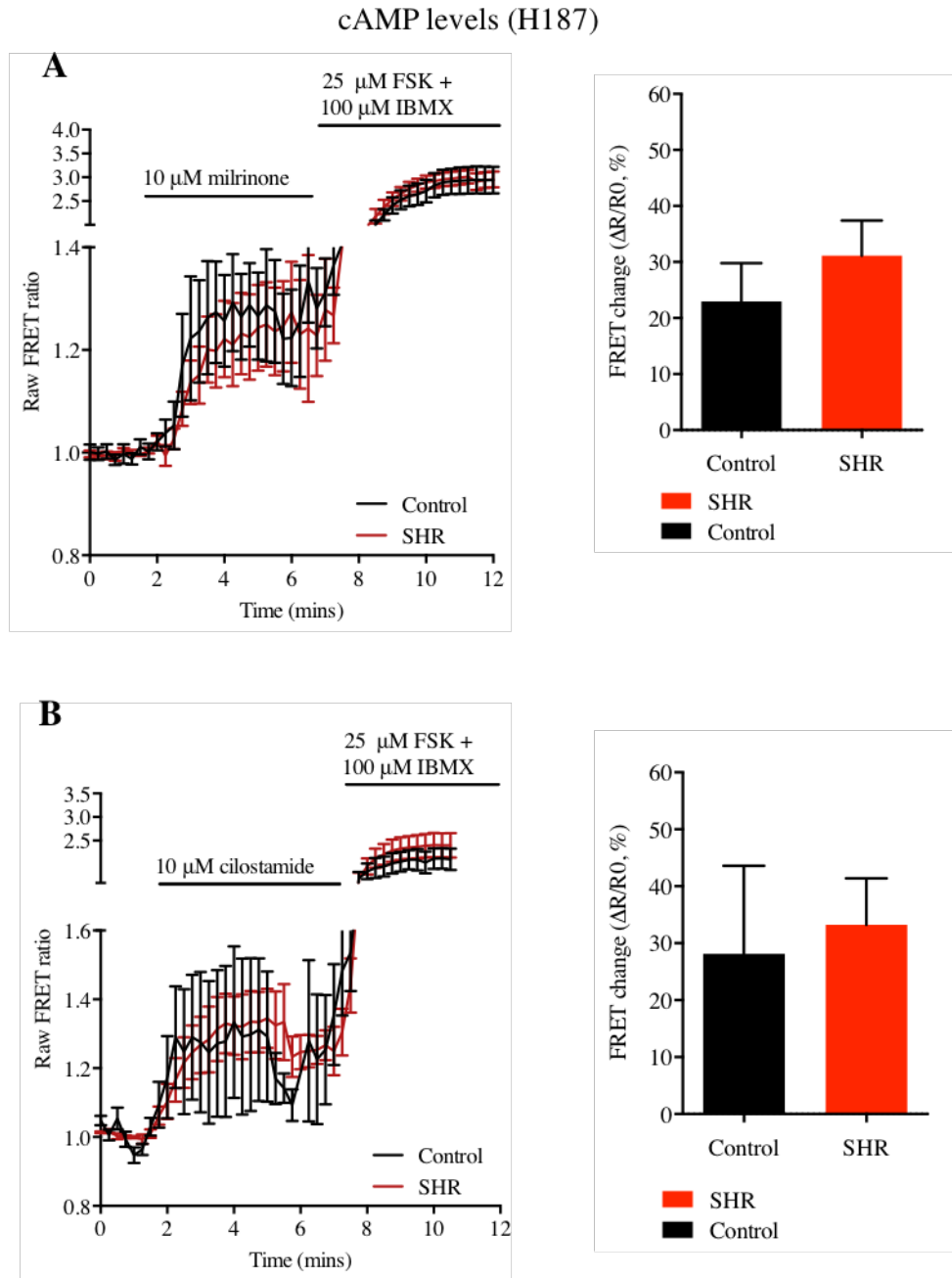


Figure 5.7: Confirming the functional presence of PDE3 using two clinically used PDE3 inhibitors. To test for the functional presence of PDE3s I sought to pharmacologically inhibit the enzyme using two clinically used PDE3 inhibitors (10 μ M milrinone – A, and 10 μ M cilostamide - B). Both inhibitors increased the cAMP levels in both the control and SHR neurons (milrinone: $22.98 \pm 6.81\%$, $n=10$ vs $31.14 \pm 6.25\%$ $n=16$, $p=0.41$. Cilostamide: $28.15 \pm 15.45\%$, $n=5$ vs $33.23 \pm 8.18\%$ $n=8$, $p=0.58$, Mann Whitney). This was found to be non significant between the two treatments and the two strains ($n=8-16$). PDE3 has previously been identified in the superior cervical ganglions (Nunes *et al.*, 2012), but this is the first report of its functional presence in the cardiac sympathetic stellate ganglions.

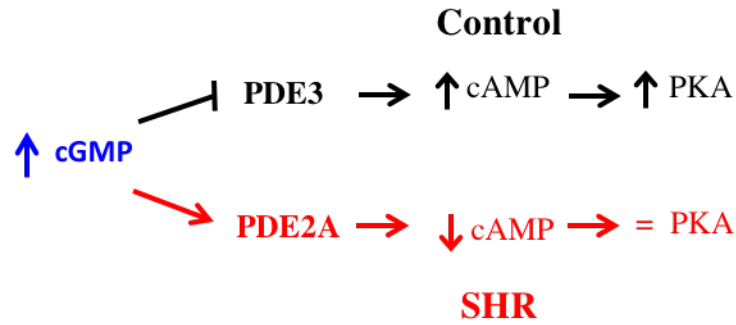


Figure 5.8: Differential activation of PDEs facilitate cyclic nucleotide cross talk in hypertension. cGMP is able to cross talk with the cAMP pathway via its activation and/or inhibition of PDEs that hydrolyse both nucleotides. Based on the evidence gained in this study, we propose the differential involvement of PDEs in the sympathetic stellate neurons of the pro-hypertensive SHR and its normotensive control. In the control neurons, cGMP inhibits PDE3 resulting in increased cAMP levels and PKA activity. Conversely, in the neurons from the pro-hypertensive SHRs, cGMP activates PDE2A resulting in increased cAMP levels and PKA activity. This is consistent with previous work suggesting PDE2A dominance in hypertension.

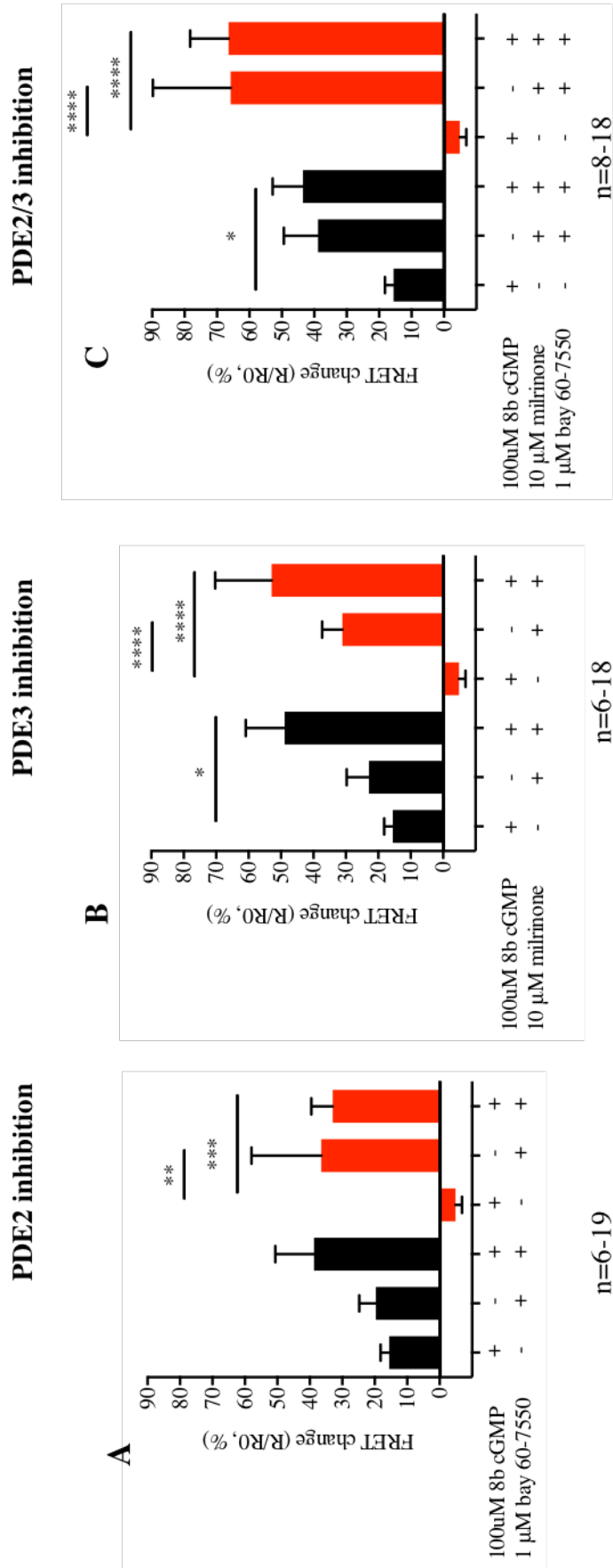


Figure 5.9: Individual PDE2/PDE3 and dual PDE2/3 inhibition does not change the cells response to 8b-cGMP.

I hypothesised that if the 8b-cGMP responses in the control and SHR is a result of action of PDE3 and PDE2A respectively, the individual or dual inhibition of these PDEs in the presence of 8b-cGMP would rescue the responses. The inhibition of PDE2 (A), PDE3 (B) and PDE2/3 in combination (C) did not significantly (Mann Whitney test) change the responses of the cells to 8b-cGMP. Cyclic nucleotide signalling is known to occur in distinct subcellular locations. My results could be explained by the lack of compartmentalised sensitivity of the experimental technique. In addition, the rate of PDE hydrolysis is critically dependent on the relative concentrations of both cAMP and cGMP, making it challenging to isolate the direct action of one drug.

5.4 Discussion

Cyclic nucleotide regulation of neuronal Ca^{2+} levels and their modulation of neurotransmission is critical for appropriate neuronal function^{70,136,137}. It is now clear that in the hypertensive state, sympathetic stellate neurons are associated with significant cGMP centred cyclic nucleotide dysfunction resulting in aberrant neurotransmission^{53,54,70}.

5.4.1 cGMP centered CN dysfunction in pro-hypertension

With the important role of NO in the modulation of neurotransmission and its clear involvement in the sympathetic dysfunction associated with the hypertensive state, most of the current work has focussed on cGMP dysfunction. cAMP is critically important for neurotransmission via presynaptic inhibition, yet little work has thus far been done to investigate whether the CN dysfunction could also be present on the cAMP side. To address this, I first used FRET to investigate the ability of the PGSNs to generate cAMP and its ability to increase PKA activity. As the principle membrane receptor responsible for raising neuronal intracellular cAMP is not clear, I measured the responses to the known AC activator forskolin. There were no significant differences between the SHR and control neurons ability to generate cAMP. Nor was there any difference in cAMPs ability to activate PKA. This suggests that the signal-generating machinery is fully functioning in both cells and provides indirect evidence that the cGMP impairment seen in the SHR neurons is not affecting the cells ability to generate cAMP. Second, I investigated the PGSNs ability to hydrolyse cAMP by measuring cAMP changes in response to global PDE inhibition using IBMX. The SHR and control neurons displayed significant differences in their ability to hydrolyse cAMP. The control cells portraying significantly larger cAMP responses at 1 and 10 μM IBMX. This suggests that there are differences in the PDE expression and/or activity profiles between the SHR and control

neurons (see figure 5.3). At 100 μ M IBMX the responses of the control cells, were larger but deemed not significantly different (Mann Whitney). At this concentration, roughly half of the control cells reached the maximum activation of the FRET sensors, resulting in masking of the true cAMP elevation. Thus the lack of significance in these data is likely due to under representation of the true effect. IBMX is generally thought to be a global PDE inhibitor, but it does not inhibit PDE 8 and 9 (and any unknown PDEs). Thus, the results observed could also point towards the potential expression of IBMX insensitive PDEs. Taken together there was no apparent impairment in the cells ability to generate global cAMP, however their ability to hydrolyse it could be different in the two states.

5.4.2 Differential cross talk between cGMP and cAMP in the SHR and control PGSNs

From Chapter 4 we know that the PGSNs from the pro-hypertensive SHR have significantly larger Ca^{2+} currents associated with CN dysfunction. Rescuing the CN impairment by pharmacologically elevating cGMP levels reduced the membrane currents of the pro-hypertensive SHR neurons down to levels seen in the normotensive control. Previous studies have demonstrated that the NO-cGMP pathway is acutely involved in directly regulating these Ca^{2+} currents via PKG activation, but also through cross talk with PDEs ¹³⁵. This cross talk occurs via the CNs causing the activation/inhibition of cellular PDEs ^{122-125,130} thereby allowing the CNs to control their own levels. Often this cross talk forms only a part of a CNs actions, but it has been shown to be dominant in some tissues, such as in atrial tissues ³⁰³.

cGMP and cAMP compete with each other for the catalytic sites on the PDEs with varying affinities resulting in inhibition and/or activation of these enzymes ^{130,163}. cGMP activates PDE2A leading to the breakdown of cAMP (and thus reduction in PKA

mediated phosphorylation of downstream targets) whilst it inhibits PDE3A with the opposite result ¹³⁰. Could the cGMP mediated rescue of the CN and Ca²⁺ dysfunction we see also signal via affecting the cAMP-PKA pathway? This has been indirectly postulated to occur in the post-ganglionic vagal nerves ¹⁶¹ and also in the heart ³⁰⁴ of hypertensive animals, however no direct experimental evidence has yet confirmed it. To address this possibility, I used FRET cAMP sensors to detect changes in cAMP levels following elevation of cGMP using the membrane permeable 8-bromo cGMP. I went one step further and investigated the functional relevance of this signalling by assessing resulting PKA activity. The application of 8b-cGMP led to larger increases in cAMP levels in the control neurons that translated into significant activation of PKA (see figure 5.5). Conversely in the SHR neurons I observed a significant reduction of cAMP with no activation of PKA. This is the first direct evidence confirming the functional presence of cGMP/cAMP cross talk in sympathetic stellate neurons. The difference in these responses can be explained by the differential involvement of PDEs, adding a further level of complexity to the CN dysfunction observed in these neurons.

PDE2A and PDE3A are dual-specificity PDEs that get activated by, and can hydrolyse both cGMP and cAMP ^{130,163}. In the SHR neurons the elevation of cGMP results in the activation of PDE2 leading to increased hydrolysis of cAMP ¹³⁰. This might provide a link to the reduction in Ca²⁺ channel current I see following 8b-cGMP and further supports the notion of PDE2A dominance in the PGSNs associated with hypertension ⁷⁰. In the control cells, the increased cAMP levels and PKA activity suggests the involvement of a second PDE – one that is inhibited by cGMP. With the recent evidence suggesting the involvement of PDE3 in sympathetic neurons ¹⁶¹ and its well characterised role as the major facilitator of cGMP/cAMP cross-talk in cardiac

myocytes^{122,123,130,165}, it is conceivable that this could be PDE3. Whilst there is no direct evidence PDE3 is expressed sympathetic stellate neurons, a recent study has functionally identified its presence in superior cervical ganglion cells¹⁶².

5.4.3 PDE3 and PDE2 are functionally expressed in PGSNs.

To test whether PDE3 was functionally expressed in the sympathetic stellate neurons of control and SHR, I examined the cAMP responses to two clinically used selective PDE3 inhibitors – milrinone and cilostamide. Both inhibitors resulted in increased cAMP levels in both the control and SHR neurons confirming its presence in sympathetic neurons. The role of PDE3 in the sympathetic neurons remains unclear, but it has previously been implicated in vagal nerves where it facilitates acetylcholine release¹⁶¹ and more recently in the blunting of sympathetic hyper-innervation observed following myocardial infarctions in rats³⁰⁵.

Previous work has confirmed the presence of PDE2A in the PGSNs of the SHR and control rats using western blotting⁷⁰. In order to demonstrate the functional expression of PDE2A I applied the selective PDE2A inhibitor BAY 60-7550 and observed the changes in cAMP levels. 1 μ M BAY 60-7550 resulted in increased cAMP levels in both the SHR and control neurons. This response was slightly, but not significantly increased in the SHR neurons which is consistent with the notion of higher PDE2A activity in the SHR neurons⁷⁰. Over expression of PDE2A in PGSNs significantly increases intracellular Ca²⁺ levels and enhances neurotransmission⁷⁰, suggesting PDE2A could play a major role in the pathophysiology of hypertension and other conditions of aberrant sympathetic activity.

5.4.4 Evidence of altered PDE2A/PDE3A balance in sympathetic neurons

With the functional expression of PDE3 and PDE2A in the PGSNs established, I proposed that cGMP elevation would result in differential activation of PDEs in the SHR and control neurons (see figure 5.8). Though both enzymes are likely involved in both animal models, I proposed the existence of PDE3 dominance in the control cells with cGMP inhibition of PDE3 resulting in increased cAMP levels and PKA activation in the cell. Conversely in the pro-hypertensive SHR neurons, cGMP activation of PDE2A, resulting in a reduction in cAMP levels and PKA activity is the major effector (see figure 5.10). It is likely that the CN cross talk also significantly interferes with cellular cGMP levels and PKG activity levels through altered PDE dependent hydrolysis of cGMP itself, and that this forms an important feedback pathway in the neurons. However, this was not measured in this study, due to the unavailability of well characterised cGMP sensors.

The notion of differential PDE2/3 involvement in the PGSNs of control and SHR neurons helps clarify the effects PDE2 blockade has on the Ca^{2+} currents (see Chapter 4, figure 4.10). In the control neurons this leads to significantly increased currents, likely due to the direct elevation of cAMP due to inhibition of its hydrolysis. But also via the indirect elevation of cAMP mediated by cGMP inhibition of PDE3, resulting in increased PKA mediated phosphorylation of Ca^{2+} channel currents. Conversely in the SHR the net result of PDE inhibition is a slight (though not significant – $p=0.052$) decrease in channel currents. This is likely mediated through cGMP mediated channel inhibition. This is consistent with the evidence that PDE2A has a higher affinity for cGMP than cAMP^{70,306}.

5.4.5 Functional relevance of cGMP-cAMP cross-talk in sympathetic neurons

In ventricular myocytes, the cross-talk between cAMP and cGMP via the PDEs, functionally connects two crucial and opposing signalling pathways in the human heart; the β -adrenergic-cAMP pathway that increases cardiac function, and the NO-cGMP pathway that reduces it ^{122,164}. The evidence provided here suggesting this cross-talk plays an important role in PGSNs both in normal and disease conditions. Further it opens up the question of whether the well described and critical NO-cGMP pathway that critically involved in the modulation of neurotransmission, could interact with an equally critical and perhaps opposing cAMP pathway? What is more, the dysregulation of each of these pathways has been implicated in disease, which opens the possibility that the dysfunction of a single protein in either of the pathways could have more far reaching effects than previously thought, contributing to the complex disease picture.

This is hugely relevant to the work described in this thesis as I observed: 1: the functional presence of these PDEs in sympathetic neurons, 2: that there is significant cross-talk taking place and 3: that this cross-talk is perturbed in disease. There is mounting evidence suggesting cAMP and cGMP interact via Ca^{2+} to control the spatiotemporal dimension of CN signalling ¹²⁴. Ca^{2+} is able to activate and regulate CN generators (ACs) and terminators (PDEs/PPs) ^{124,163}. Could the increased intracellular Ca^{2+} associated with sympathetic hyperactivity ⁶³ indeed be working through these mechanisms?

5.4.6 Compartmentalisation of cyclic nucleotide signalling

To confirm that PDE2A and PDE3 are the two PDEs that facilitate the cGMP/cAMP cross-talk in the PGSNs, I hypothesised that the application of PDE2 and PDE3 blockers alone and in combination, would abolish the cells responses to 8b-cGMP.

However, no differences were found between the SHR and control neurons in response to any of the combinations tested. This most likely reflects the lack of sensitivity when using global FRET sensors to detect compartmentalised signalling. This is supported by recent work in the lab (unpublished data) demonstrating significant differences in forskolin induced cAMP generation between the SHR and control neurons at the outer mitochondrial membrane, but not in the cytosol (see figure 5.1).

It now is clear that CNs^{123,170,307-309} and PDEs reside in distinct subcellular compartments⁴⁴. Specifically in the rat, there are three splice variants of PDE2A that are localised in the cytosol (PDE2A1) or membrane (PDE2A2 and 3)³¹⁰, although their exact roles remain to be established. Similarly PKA/PKG regulation of the Ca²⁺ channels also occurs in distinct signalosomes near the channels themselves, thus conferring site specific regulation of Ca²⁺ entry coupled to neurotransmission^{166,311}. Furthermore the rate of PDE hydrolysis is critically dependent on the concentration of both cAMP and cGMP^{130,165}, the latter of which we know to be different between the pro-SHR neurons and its controls⁵⁴. Thus global CN sensors used in this study are unlikely to allow sufficient resolution to detect any localised changes in CN signalling, perhaps in particular when looking at PDE mediated effects. This could explain why I see no significant changes between any of the experiments using PDE2A and PDE3 blockers. Although these global cAMP responses following PDE2A and PDE3 inhibition were similar between control and diseased neurons, this does not in itself establish that localised control is normal in the diseased neuron.

Indeed, the NO-cGMP pathway has been shown to be highly localised in neurons¹⁶⁶. This pathway is known to be highly dysregulated in hypertension. Could parts of the

dysregulation come as a result of altered compartmentalisation and localisation of critical components of the signal transduction pathway? The answers to this depends on our ability to design novel intracellular sensors that are specifically localised to the cellular compartments we wish to study ²³³. Some progress has been made with the recent development of sensors targeted to the outer and inner mitochondrial membrane ¹⁷², AC ³¹² and SERCA ³¹³. However, in order to clarify the CN regulation of the Ca^{2+} channels, a membrane- or channel-linked sensors is needed. Further the use of cGMP and PKG sensors in combination with the arsenal of cAMP/PKA sensors could help shed light on localised signalling dynamics in neurons. cGMP and PKG sensors do exist but they are less well characterised ³¹⁴⁻³¹⁶.

5.4.7 Sympathetic hyperactivity is not caused by changes in the activation of pre-synaptic nAChRs

In a further attempt to elucidate whether a cAMP dysfunction could exist in these neurons, I used the nAChR agonist nicotine to achieve physiologically stimulation of the PGSNs. It was realized early that nicotinic drugs activating the nAChR caused an “explosive release of NA” from sympathetic neurons ³⁶, however the link with cAMP levels remained unclear. It is now appreciated that cAMP has a critical role in the modulation of NA release from PGSNs by its activation of PKA ^{34,317}. NA mediated activation of presynaptic α_2 receptors facilitates a reduction in cAMP levels and reduced neurotransmission ^{28,34,35}. The exact molecular mechanisms remain unclear, but it has been postulated that Ca^{2+} plays a key role ²⁸. We know that PKA dependent phosphorylation of the Ca^{2+} channels lead to increased Ca^{2+} entry and neurotransmitter release, but it is likely that PKA also has direct effects on the exocytotic machinery. PKA directly phosphorylates many components of this machinery including the SNARE proteins, resulting in their enhanced Ca^{2+} sensitivity (see ²⁸ for extensive review).

It is now clear that there is significant interplay between Ca^{2+} and cAMP both in myocytes³¹⁸ and neurons¹²⁴. We know that PGSNs from the SHR have significantly larger intracellular Ca^{2+} transients when compared to normotensive controls, but to this date there has been no direct evidence suggesting the cAMP levels are dysregulated. It is therefore perhaps not unsurprising that the nicotine-induced changes in cAMP remain unchanged between the pro-hypertensive SHR and control neurons at all concentrations tested (1, 10 and 100 μM).

Despite there being no difference between the control and SHR neurons, these data do provide us with clues as to what level the sympathetic dysfunction lies in the cell. It suggests that the nAChRs remain unaffected by the hypertensive state – a notion that is further supported by previous findings demonstrating that the acetylcholine-induced currents possess similar properties in the sympathetic neurons from the SHR and its normotensive controls⁷³. The autonomic dysfunction associated with the hypertensive state is therefore unlikely to be caused by changes in the presynaptic nAChRs. Rather, a part of the intracellular pathway directly coupled to NA release could be affected. One theory is that the activation of a postganglionic sympathetic neuron with a given stimulus could elicit more NA release in the pro-hypertensive SHR neurons than in the normotensive cells, however this remains to be firmly established.

5.4.8 The presence of IBMX insensitive PDEs in the sympathetic stellate neurons of the pro-SHR

The cAMP responses to nicotine had a marked peak – plateau appearance. The peak is caused by the rapid cAMP increases in the cell. As the cAMP levels rise, PDEs get activated causing the hydrolysis of cAMP^{44,222}. Once a balance between cAMP generation and hydrolysis is reached, a plateau becomes apparent on the FRET ratio trace²³³. This phenomenon offers us a unique way of assessing the presence of IBMX-

insensitive PDEs in these cells. If all the PDEs in the cells were sensitive to IBMX, pre-incubation with IBMX before exposing the cell to nicotine would result in the elimination of the peak-plateau as the cAMP would remain elevated due to lack of PDE action. As is evident from figure 5.4, the peak – plateau shape was still present after IBMX pre-treatment, although it occurred on a somewhat delayed timescale. This could suggest the presence of IBMX-insensitive PDEs in the SHR neurons. The traces reported here represent two pilot data traces and so further work needs to be done to characterise this in both the SHR and control neurons before any conclusions are made. Once this response has been characterised in both cell models, the addition of PDE8 and PDE9 inhibitors in combination with IBMX could shed light on which IBMX-insensitive PDE is involved and potentially identify further subspecies of the enzyme family.

5.4.9 Limitations of the study

Perhaps the biggest limitation of this study is the use of globally acting drugs and global FRET sensors to detect molecular signalling events that are known to be highly organised into subcellular compartments. This lack of cellular resolution could mask any true effect as is described in the sections above. However due to the large area of the cell, any changes that are seen are likely to be correct, if perhaps under represented. Further the FRET sensors only respond to dynamic changes in the levels of cAMP and PKA – they are not able to accurately detect the basal levels of cAMP or PKA activation. As such, despite there being no changes in the stimulated levels of cAMP and PKA, there could be significant change in the basal levels that is predicted to have significant effects on the interpretation of the data presented here. Efforts have been made to use PKA and AC inhibitors to determine basal levels ²²⁶, though we were not able to reproduce these results.

A further limitation associated with the use of FRET sensors, is that they only accurately represent the changes in cAMP levels if these occur in the dynamic range of the sensors^{226,237}. It is therefore possible that the results I see in the SHR following 8b-cGMP application are underrepresented. As discussed in Chapter 2, the cAMP FRET sensor Epac H30 is significantly pH sensitive, whilst having a limited dynamic range. It could therefore be argued that the lack of difference between the SHR and controls ability to generate cAMP in response to forskolin could come as a result of the lack of sensitivity. To ensure this was not the case, the experiments were repeated by Dr Dan Li in the lab using the H187 sensor, and the results were found to be the same (data not shown). Finally, the functional evidence of the presence of PDE3 in sympathetic neurons provided in this chapter would have been supported by the presence of a western blot. This could also provide hints as to whether there is a difference in expression between these two enzymes. However, due to the small amounts of tissue generated from the sympathetic stellates of young rats, this was not possible. Similar problems have been encountered by other groups that instead employed immunofluorescence²⁶⁵. The lack of specific PDE antibodies prevented us from using such techniques.

5.4.10 Concluding remarks

The data reported in this chapter are first to provide evidence of a functional CN cross-talk between cGMP and cAMP in the PGSNs of the rat. I provide evidence that this cross-talk is facilitated by different PDEs in the SHR and control neurons, suggesting it could form a critical player in the well established CN dysfunction associated with the pro-hypertensive state. I provide some evidence that suggests the differential expression of PDEs in the sympathetic neurons of the SHR and control neurons. I also provide

functional evidence supporting the presence of PDE3 in the PGSNs of the pro-hypertensive and normotensive neurons. Finally, I provide further evidence suggesting the sympathetic dysfunction is not present at the level of nAChR activation – the first step in the activation of the sympathetic neuron.

CHAPTER SIX

6 Dysregulation of cardiac cAMP in nicotine stimulated sympathetic neuronal-myocyte co-cultures from hypertensive rats.

Are sympathetic PGSNs the primary driver of the cardiac autonomic phenotype in hypertension?

6.1 Introduction

6.1.1 Autonomic dysfunction in hypertension

Hypertension is associated with autonomic dysfunction marked by significant sympathetic hyper-responsiveness and vagal impairment¹⁹. This heightened activity of the sympathetic nerves is a well-established contributing factor to the development of hypertension in humans^{12,13,24,47,77} and that observed in animal models such as the spontaneously hypertensive rat (SHR)^{51-53,82,300}. In the pro-hypertensive SHR, the sympathetic PGSNs that innervate the heart⁶¹ show increased membrane Ca^{2+} currents⁶⁴, increased intracellular Ca^{2+} transients and altered mitochondrial Ca^{2+} handling⁶³ – all contributing to enhanced noradrenaline (NA) release and neurotransmission^{53,54,74,79}. These neurons also have significant impairment of the noradrenaline reuptake transporter (NET)⁶⁹, when compared to the normotensive Wistar Kyoto (WKY), resulting in NA remaining in the synapse for longer.

This heightened sympathetic activity results in over-excitability of postganglionic neurons at the end organ, and β -adrenergic hyper-responsiveness of the myocyte^{80,81,319}. The SHRs display increased basal heart rate⁵³, and increased heart rate responses to electrical stimulation of the stellate ganglion^{53,79}. This is linked to significantly greater basal and NA stimulated L-type Ca^{2+} currents associated with elevated cAMP levels in both sinoatrial cells⁸⁰ and ventricular myocytes⁸¹.

It is becoming apparent that the heightened sympathetic responsiveness is present on both the PGSNs and the myocytes they innervate, however the relative contribution each makes to the autonomic phenotype is unclear.

6.1.2 The importance of modelling the neuro-cardiac axis

The (patho-) physiology of the individual ion channels and signalling molecules on the single neuron and myocytes as separate systems are well described, but relatively little is known about the cell-to-cell interaction that takes place. It is now becoming clear that the sympathetic neurons play a large role in modulating the behaviour of myocytes both via antero- and retrograde signalling, but also through eliciting changes in myocyte receptor expression. As a result, the study of these cells in conjunction becomes paramount if we are to fully understand the molecular mechanisms governing sympathetic neurotransmission, and how it might be altered in disease states.

In co-cultures of sympathetic neurons and ventricular myocytes, the myocyte membrane is directly in touch with the neuron^{184,197}. These points of contact are enriched in NA containing granular vesicles^{320,321}. The tight postsynaptic junctions are facilitated in part by the interaction of neuronal integrins binding to myocyte Vascular Cell Adhesion Molecule-1 (VCAM1)³²² – molecules that are known to play important roles in the formation cellular junctions. The neuro-cardiac junction is also enriched with synaptic scaffolding proteins known to be associated with adrenergic signalling. In these junctions there is an increased association of A kinase anchoring proteins (AKAP 79/150)¹⁸⁴, known to be important facilitators of compartmentalisation of cAMP signalling via the molecular anchoring of downstream signalling components^{167,323}. There is also an accumulation of synapse associated protein 97 (SAP97) – an important molecule associated with membrane receptor trafficking and synaptic plasticity¹⁸⁴. In

addition to the proteins involved in synapse architecture, the presence of sympathetic neurons has been shown to directly alter the expression of important myocyte ion channels³²⁴; L-type Ca^{2+} ¹⁸⁷, K^{+} ³²⁵, and I_f ³²⁶. The direct molecular mechanisms underpinning this remain largely unknown but it is thought to involve the neuropeptide system and adrenergic receptors³²⁴, and has been suggested to be activity dependent¹⁸⁴.

Thus it becomes clear that the communication between sympathetic neurons and myocytes is critically important both to maintain healthy homeostasis, but also to elicit changes in behaviour through cellular signalling. However, the degree to which this signalling is altered in diseases such as hypertension remains largely unknown. It is unlikely the sympathetic phenotype resides in one spatial domain, thus it becomes critical to study the entire neuro-cardiac axis and not just cells in isolation. Tao et al mathematically modelled this neuro-cardiac interaction and its dysfunction in hypertension, incorporating biological data taken from single cell experiments¹⁷⁵. Interestingly, based on the current biological data, the model quantitatively suggested the presence of hitherto unknown molecular impairments on both sides of the synaptic cleft, highlighting the need to use such dual cell models if we are to fully understand the pathophysiology of hypertension and indeed other cardiovascular diseases. The ability of the neuron to elicit changes in the myocytes they innervate points towards a pivotal role for the sympathetic neurons in potentiating neurotransmission both in health and disease.

6.1.3 Aims of the chapter

This chapter tests the hypothesis that the sympathetic PGSNs are the dominant drivers behind the sympathetic hyperactivity observed in hypertension. It aims to:

- Develop a novel co-culture of physiologically coupled sympathetic stellate neurons and ventricular myocytes that will allow the investigation of cell-cell communication.
- Test whether the myocytes from the pro-SHR are hyper responsive to β adrenergic stimulation.
- Test whether the sympathetic hyperactivity observed in the single cells of the pro-hypertensive and normotensive rats translate into a dual cell model of PGSNs and ventricular myocytes.
- Develop a cross-culture to assay whether the PGSNs are the dominant driver behind the sympathetic hyperactivity observed in the pro-hypertensive state.

6.2 Methods

6.2.1 Cell isolation

6.2.1.1 Myocyte isolation

Neonatal ventricular myocytes (P3) from SHR and WKY littermates were isolated as described in Chapter 2 (section 2.3.3). Briefly, the hearts were swiftly removed from the animal and placed into cold Hanks Balanced Salt Solution (HBSS). The atria were discarded before the ventricles were finely chopped and placed into an enzyme solution containing trypsin (1 mg/ml) and incubated for three hours at 4°C. A series of collagenase (1 mg/ml) digestions followed, resulting in a single cell suspension that was plated on 6 mm coated coverslips. Roughly 78 000 cells were plated per four-well plate, each containing four 6 mm coverslips. This left room for the neurons to sit down in-between the myocytes and achieved a 75% confluent cell layer at day five. The myocyte-only cultures were plated in myocyte media, whilst the myocyte cultures awaiting neurons were plated in co-culture media (see table 2.1).

6.2.1.2 Neuronal isolation

Immediately following the myocyte culture, the right and left stellate ganglia from two four-week SHR or WKY animals were removed and placed into HBSS. Briefly, the tissue was desheathed and finely chopped before being subjected to a series of trypsin (2 mg/ml) and collagenase (1 mg/ml) digestions and triturated to achieve a single cell suspension of PGSNs (see section 2.2.3).

6.2.1.3 Co-culture formation

The PGSNs were immediately plated on top of the myocytes to form the co-cultures. The cells were allowed to settle for 24 hours before media was changed and 1 μ M Ara-C was added to reduce fibroblast growth. 24 hours later, fresh co-culture media was

added and the cells were kept at 5% CO₂, 37°C for five days to allow the synaptic connections to form between the two cell types^{179,184,214}.

6.2.1.4 Cross-culture formation

To investigate whether the PGSNs are the dominant driver behind the sympathetic hyperactivity observed in the pro-hypertensive state, two cross-cultures (see figure 6.7) were developed. These cultures were exposed to the same experimental protocols as the normotensive and pro-hypertensive co-cultures.

6.2.2 Immunofluorescence

Cellular phenotypes and expression of the acetylcholine receptor was determined using immunofluorescence as described in Chapter 2. From each myocyte culture, immunofluorescence using TH and CHaT was performed to confirm the absence of potentially contaminating intra cardiac neurons.

6.2.3 Native stimulation of the co-cultures

The use of physiologically coupled cells, maintains the integrity of the synapse and enable the stimulation of one cell and recording from the other. In order to mimic physiological stimulation, the co-cultures were activated by nicotine (a nAChR agonist) to allow native release of noradrenaline. The resulting cAMP changes in the myocytes were recorded using the cAMP FRET sensor H187²²⁴. Various concentrations of nicotine (1-500 µM) were added to simulate increasing sympathetic drive.

6.2.4 FRET imaging of the co-cultures

6.2.4.1 Infection of FRET sensor

24 hours prior to recording, the cells were infected with 3.6x10⁵ particle forming units (PFUs) of the adenoviral H187 FRET sensor for three hours in co-culture media at 5% CO₂, 37°C. The cells were washed twice to remove any residual viral particles before

media was replaced and the cells placed back in the incubator. 20 hours was allowed for the cells to express appropriate levels of the FRET sensor^{233,236}.

6.2.4.2 FRET imaging protocols

Cells were imaged as described in chapter 2 section 2.8. Briefly, the cells were perfused with tyrode solution (containing drugs when appropriate) and imaged using an inverted Nikon microscope connected to an OptoLED fluorescence imaging system. The cells were excited at 420 nm and CFP and YFP emissions were measured as a change in the ratio between 480/535 nm fluorescent emission intensities following a 100 ms excitation at 430 nm, every 15 seconds. Background fluorescence was subtracted from emission intensities and intensity ratios were plotted against time. Mean FRET responses were expressed as the percentage change from baseline ($\Delta R/R_0$ where $\Delta R = R - R_0$. R_0 is the ratio of intensity at time = 0 seconds and R is the ratio at time = t seconds).

6.2.4.3 FRET protocols

The myocytes were superfused with tyrode solution for two minutes to establish a stable baseline. To test the difference in cAMP responses to bath applied isoprenaline, each cell was exposed to either 0.1, 3 and 30 or 1, 10 and 100 nM isoprenaline. The myocytes were also exposed to 1-500 μ M nicotine to ensure no non specific actions of nicotine on the myocytes, and to confirm the absence of any contaminating intra cardiac neurons.

The co-cultures were superfused with tyrode solution for six minutes to ensure a stable background before 1, 10, 100, 300 and 500 μ M nicotine was applied. Each cell was only exposed to one concentration of nicotine, as the nAChR is prone to rapid inactivation²⁷.

To ensure that the responses seen were adrenoreceptor mediated, the baseline was

allowed to develop for two minutes, before the cells were pre-incubated with the selective β_1 receptor antagonist metoprolol (10 μ M) for four minutes. Metoprolol in combination with nicotine (at all concentrations) was then applied at six minutes.

The mean ratio traces of all cells recorded are reported in the figures. Due to small variability in the rate of perfusion (2.5-3 ml/min) and image acquisition every 15 seconds, the peak occurred over three image points (time span of 30 seconds). For this reason, the absolute peak cAMP levels are reported in bar charts, and it was these data that the statistics were performed on. It was therefore not possible to draw any conclusions about the rate of cAMP increase. It is possible that the absolute peak occurred in the interval between image acquisition, however such errors are mitigated by the large n number. Imaging at 15 second intervals was critically important to avoid photo toxicity of both the neurons and myocytes which could alter their behaviour.

6.3 Results

6.3.1 Co-culture phenotype and cross culture formation

The co-culture of PGSNs and ventricular myocytes was successfully created (see figure 6.1). The functional connection of the two cells was initially observed as increased beat rate and coordination between myocytes that were innervated by the same neurons (unquantified observations made by H Larsen, R Burton and G Bub, article in preparation). This was subsequently proved experimentally as is described below where I observed β_1 adrenergic increases in myocyte cAMP levels following nicotinic stimulation. Figure 6.1A illustrates the signalling pathway through which nicotine causes these effects. Immunofluorescence confirms the phenotype of the cells in culture with the sarcomeric alpha-actinin denoting the myocytes, and tyrosine hydroxylase (TH) denoting the sympathetic neurons. Visual innervation was confirmed in all cultures.

6.3.2 The myocytes from the pro-hypertensive SHR are over-responsive to beta adrenergic stimulation.

To test the β -adrenergic responses of the myocytes, I bath applied the β_1 agonist isoprenaline and measured changes in myocyte cAMP levels using FRET. Isoprenaline treatment of the culture revealed that the SHR myocytes (n=27-33) were significantly hyper-responsive when compared to the WKY myocytes (n=50-55) at isoprenaline concentrations ≥ 3 nM (see figure 6.2 and 6.3). The response was increasing over the six concentrations tested but plateaued between 30 nM and 100 nM.

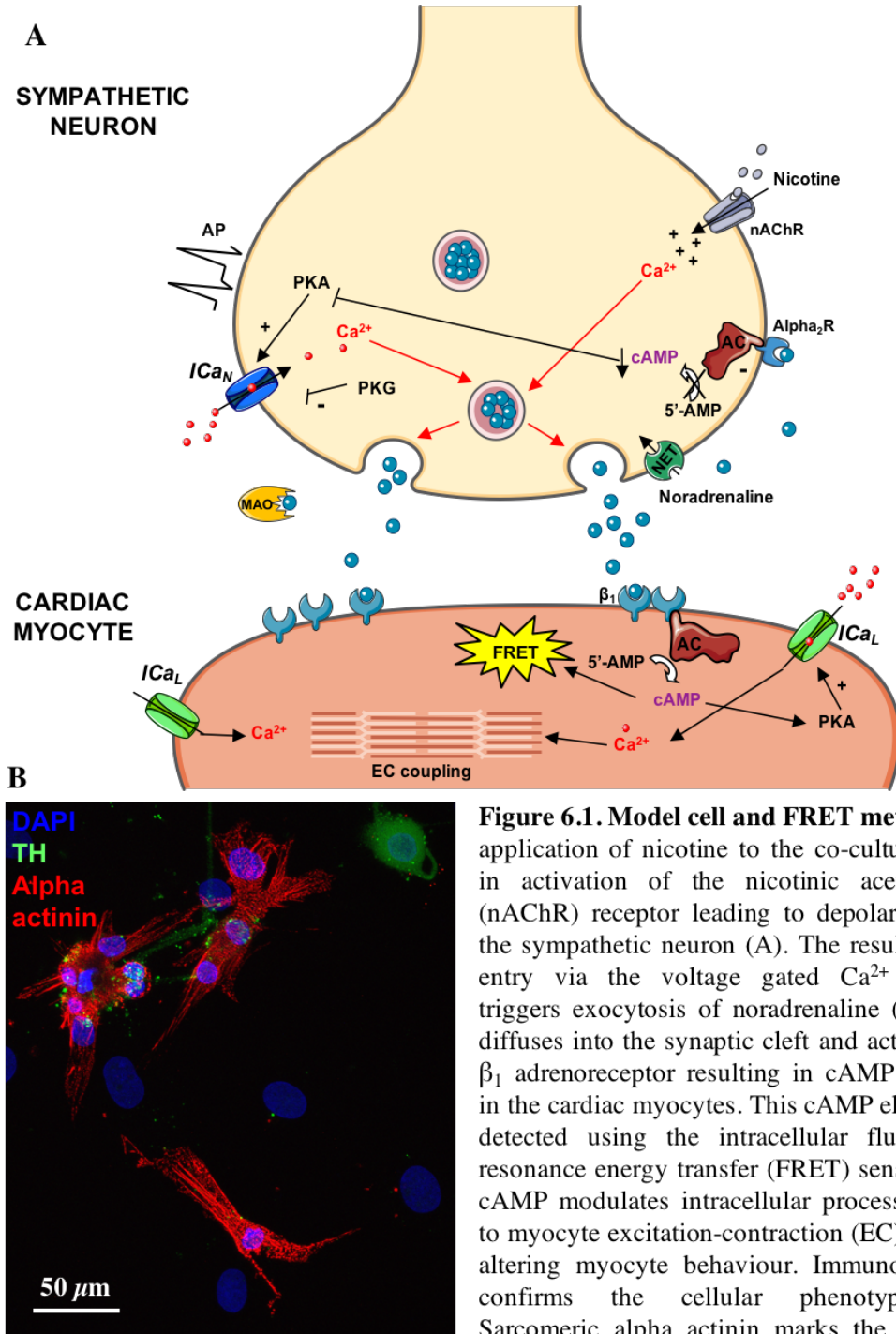


Figure 6.1. Model cell and FRET method. The application of nicotine to the co-culture results in activation of the nicotinic acetylcholine (nAChR) receptor leading to depolarisation of the sympathetic neuron (A). The resulting Ca^{2+} entry via the voltage gated Ca^{2+} channels triggers exocytosis of noradrenaline (NA). NA diffuses into the synaptic cleft and activates the β_1 adrenoreceptor resulting in cAMP elevation in the cardiac myocytes. This cAMP elevation is detected using the intracellular fluorescence resonance energy transfer (FRET) sensor H187. cAMP modulates intracellular processes linked to myocyte excitation-contraction (EC) coupling, altering myocyte behaviour. Immuno staining confirms the cellular phenotypes (B). Sarcomeric alpha actinin marks the myocytes (red), Tyrosine hydroxylase (TH – green) marks the sympathetic neurons. Note the punctate staining there the neurons innervate the myocyte.

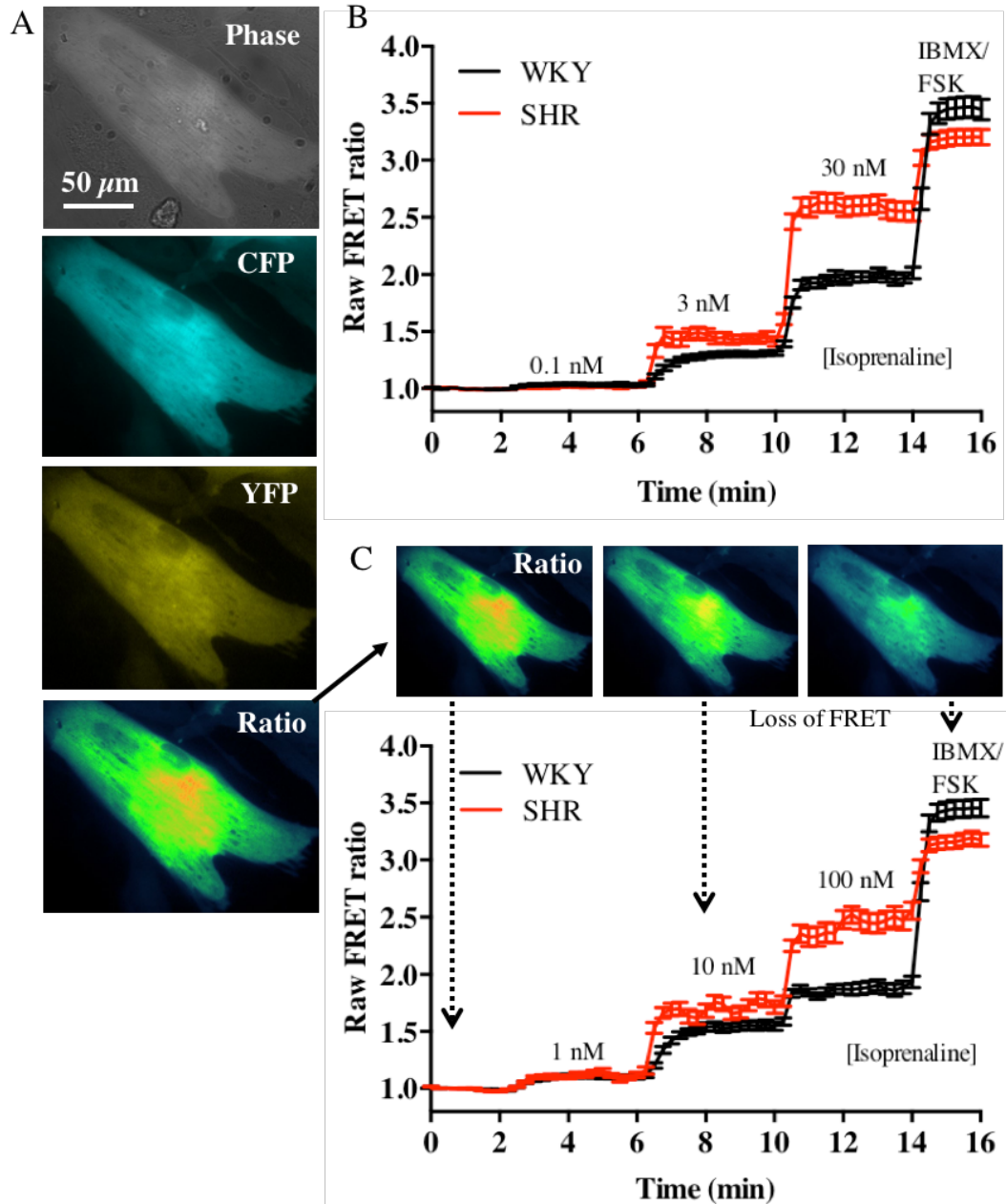
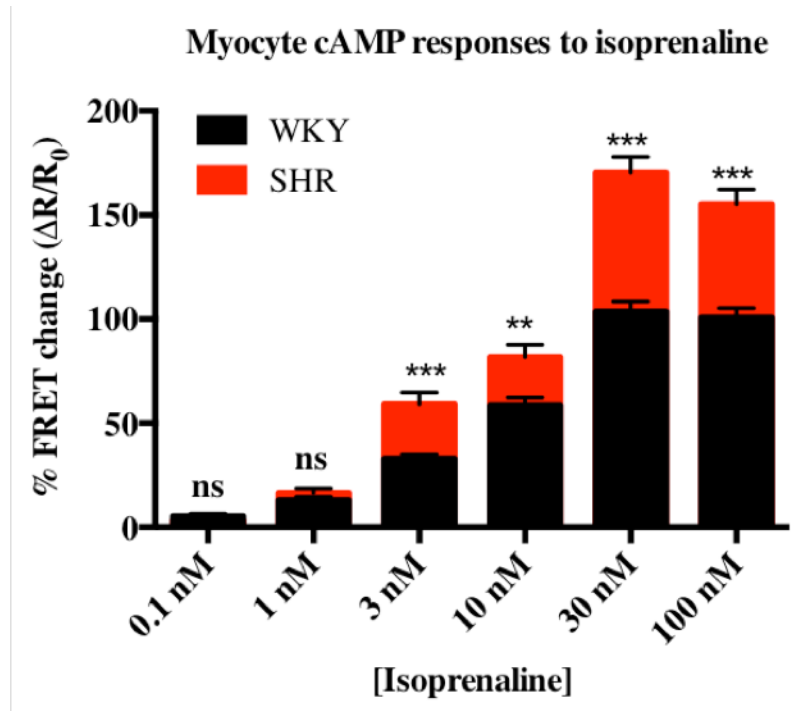


Figure 6.2: The ventricular myocytes from the pro-hypertensive SHR are hyper-responsive to β adrenergic stimulation. To investigate the β adrenergic responses of the myocytes, I perfused the cells with isoprenaline and used the fluorescence resonance energy transfer (FRET) sensor H187 to detect changes in cAMP levels following stimulation. CFP and YFP fluorescence intensities (A) were measured and the ratio plotted (B). The binding of cAMP to EpacH187 causes a conformational change in the sensor that moves the fluorophores apart, resulting in loss of FRET signal (C). When compared to normotensive controls, the myocytes from the SHRs were found to have significantly larger cAMP responses to isoprenaline at concentrations ≥ 3 nM. Concentrations and n numbers (WKY/SHR) as follows: 0.1 nM (55/33), 1 nM (50/27), 3 nM (55/33), 10 nM (50/27), 30 nM (55/33), 100 nM (50/27).



	SHR				WKY		
[Iso]	Mean	SEM	N	P value	Mean	SEM	N
0.1 nM	3.275	0.766	33	= 0.2203	5.266	1.160	55
1 nM	16.380	2.172	27	= 0.2156	12.919	1.665	50
3 nM	59.037	5.757	33	< 0.0001	32.934	1.941	55
10 nM	81.762	5.775	27	= 0.0007	58.588	3.709	50
30 nM	170.394	7.550	33	< 0.0001	103.580	4.831	55
100 nM	155.313	6.889	27	< 0.0001	100.784	4.522	50

Figure 6.3: Summary of myocyte hyper responsiveness to β adrenergic stimulation. The myocytes from the SHR were found to be significantly over responsive to β adrenergic stimulation when compared to control WKY myocytes at concentrations ≥ 3 nM (A). The mean increase in cAMP from baseline ($\Delta R/R_0$ %) and the SEM for all six isoprenaline concentrations tested are depicted (B). ** $p < 0.0007$, *** $p < 0.0001$

6.3.3 The co-cultures from the pro-hypertensive SHR are hyper responsive to neuronal activation

To test the β adrenergic response of the myocyte to neuronal activation, I applied nicotine and measured cAMP level in the myocytes. The nicotinic stimulation of the sympathetic neurons in the co-cultures led to rapid increases in myocyte cAMP levels that returned to baseline. The myocytes from the pro-hypertensive SHR co-cultures showed significantly larger nicotine evoked cAMP responses when compared to the WKY at 1 μ M ($55.89 \pm 7.292\%$, $n=28$ vs $5.952 \pm 1.623\%$, $n=24$, $p<0.0001$) and 10 μ M ($44.02 \pm 5.310\%$, $n=36$ vs $17.05 \pm 3.715\%$, $n=29$, $p<0.0002$) nicotine (see figure 6.4). At 100 μ M the responses of the two cultures were the same ($38.25 \pm 6.105\%$, $n=20$ vs $39.35 \pm 3.283\%$, $n=27$ $p=0.87$). At the higher concentrations of nicotine (300 and 500 μ M) significantly reduced responses were found in both cultures that were not significantly different from each other (results not shown). Both co-cultures responded identically to maximal activation of the sensor with 100 μ M IBMX and 25 μ M forskolin (WKY: $241.2 \pm 5.94\%$ $n=76$. SHR: $239.0 \pm 5.74\%$ $n=87$, $p=0.79$. See chapter 2, figure 2.12B).

Furthermore, roughly half of the SHR cultures portrayed double peaks (see arrow on figure 6.4C and D) of cAMP elevation. This double peak behaviour was not observed in any of the WKY cells. This contributed to the the area under the curve (a rough measure of total cAMP change over time) being consistently larger in the SHR cultures when compared to the WKY. However due to limitations of the experimental method, precise quantification of this was not possible.

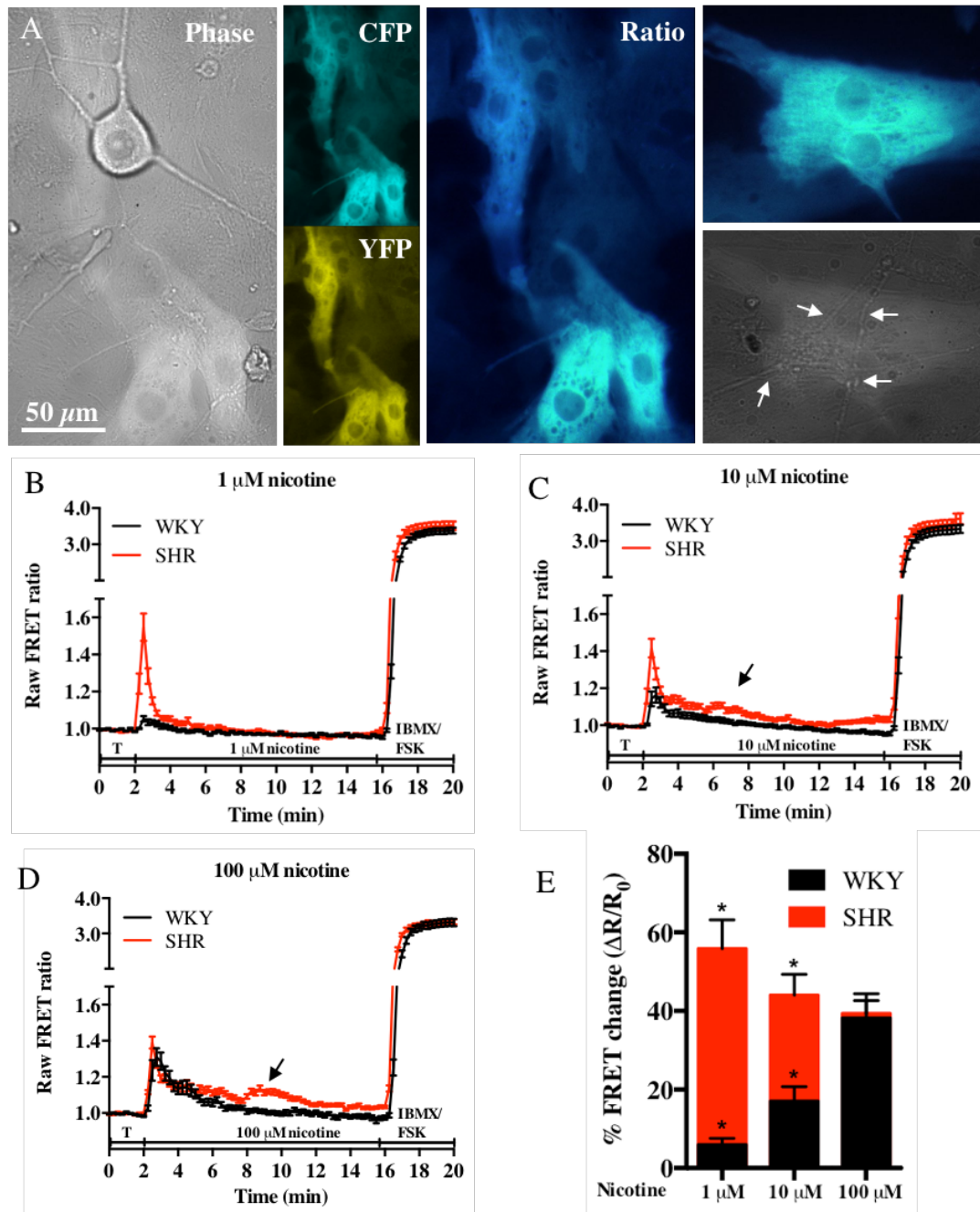


Figure 6.4: The co-cultures from the pro-hypertensive SHR animals are hyper responsive to nicotinic stimulation when compared to the normotensive WKY. The myocytes of the pro-hypertensive SHR and WKY co-cultures were selectively infected with the H187 cAMP FRET sensor (A). The neurons in the co-culture were stimulated using increasing concentrations of nicotine to stimulate sympathetic drive. The resulting effect of the released noradrenaline on the β adrenergic receptors was detected as changes in myocyte cAMP levels. The myocytes from the SHR co-cultures had significantly higher cAMP responses to nicotine stimulation at 1 μ M (55.89 ± 7.292 , $n=28$ vs 5.952 ± 1.623 , $n=24$, $p<0.0001$: B) and 10 μ M (44.02 ± 5.310 , $n=36$ vs 17.05 ± 3.715 , $n=29$, $p<0.0002$: C) nicotine. At 100 μ M nicotine (D) the responses of the two cultures were the same (38.25 ± 6.105 , $n=20$ vs 39.35 ± 3.283 , $n=27$ $p=0.87$). B-D represent the raw traces of all recordings, whilst the absolute peak data that statistic were performed on is shown in E.

6.3.4 cAMP elevation in response to nicotine activation of the sympathetic neurons in the co-culture was mediated via the β_1 adrenergic receptor

The application of the β_1 selective antagonist metoprolol completely blocked the nicotine evoked cAMP responses (see figure 6.5) in the SHR and WKY co-cultures at all nicotine concentration in all cells tested (n=60).

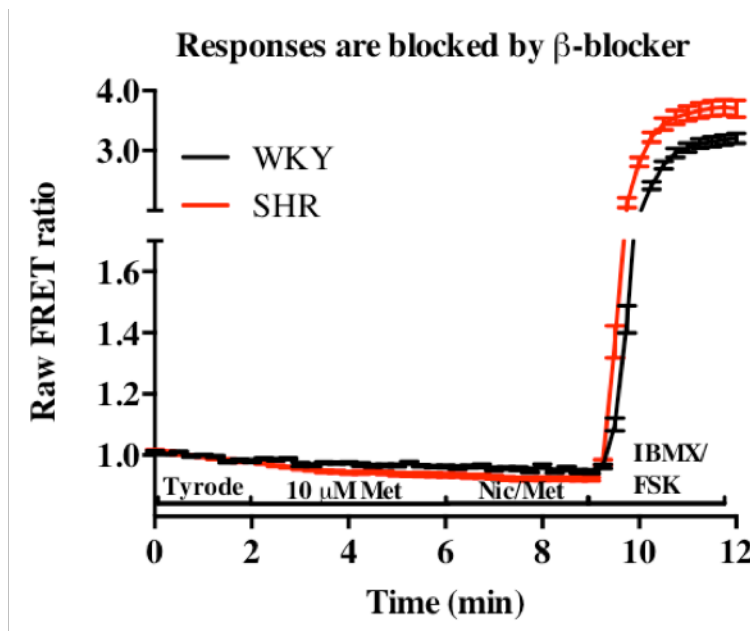


Figure 6.5: cAMP elevation in response to nicotine activation of the sympathetic neurons in the co-culture is mediated via the β_1 adrenergic receptor. To confirm that the nicotine (1-500 μ M) evoked cAMP responses I saw were mediated by activation of the β_1 adrenergic receptors, I added bath applied nicotine in the presence 10 μ M metoprolol. In the presence of metoprolol the nicotine induced cAMP responses were entirely absent in all cells tested (n=60) in both the WKY and SHR neurons.

6.3.5 Absence of contaminating intra cardiac neurons and non specific nicotine effects.

To confirm that there were no contaminating neurons in the myocyte cultures, I tested the myocytes cAMP response to nicotine, and performed immunofluorescence. Nicotine application (1-500 μ M) to myocytes cultured alone led to no changes in cAMP levels in all cells tested (WKY n=9, SHR n=20) (figure 6.6A). Immunofluorescence (6.6B) confirmed negative tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) staining, establishing that no contaminating neurons were present in the myocyte only cultures. This indirectly confirmed that the co-culture responses seen were solely due to PGSNs and not contaminating intra cardiac neurons. The data also confirmed that nicotine has no effect on cAMP levels in myocytes.

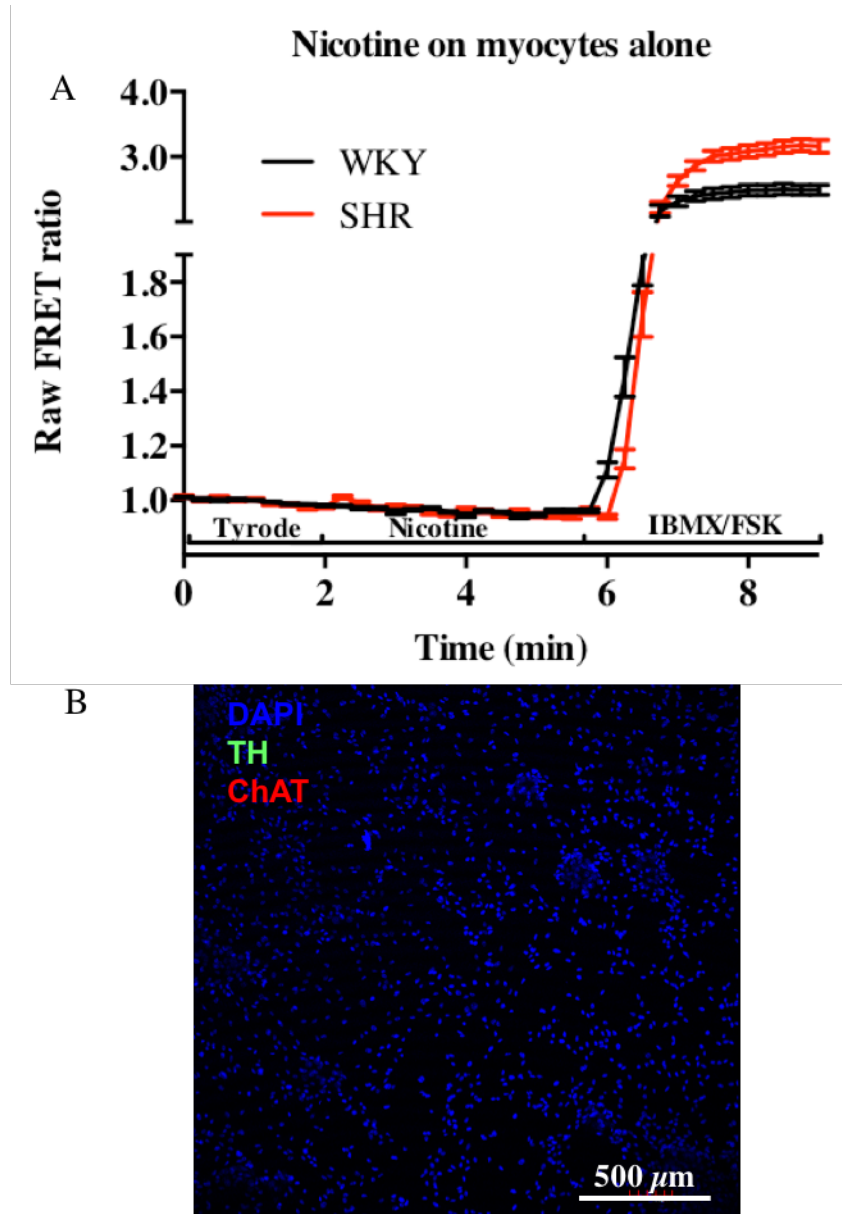


Figure 6.6: Nicotine has no effect on the cAMP levels of cultured myocytes.

To ensure there were no contaminating intra-cardiac neurons present in the myocyte cultures and that nicotine had no non-specific effects on the myocytes, I exposed the myocytes to nicotine and monitored their cAMP levels. Nicotine application (1-500 μ M) had no effect on myocyte cAMP levels in all cells tested (WKY n=9, SHR n=20) (A). Immunofluorescence (B) was performed on each culture and showed negative tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) stain confirming no contaminating neurons were present in the myocyte only cultures. This indirectly confirms that the co-culture responses seen are solely due to sympathetic stellate neurons and not contaminating intra cardiac neurons. The data also confirm that nicotine has no effect on cAMP levels in myocytes.

6.3.6 The sympathetic PGSNs are the drivers behind cardiac-autonomic dysfunction.

I employed a series of cross-cultures to elucidate which of the cells play the dominant role in neuro cardiac excitation both in the healthy and diseases states. Figure 6.7 details the cross-culture setup.

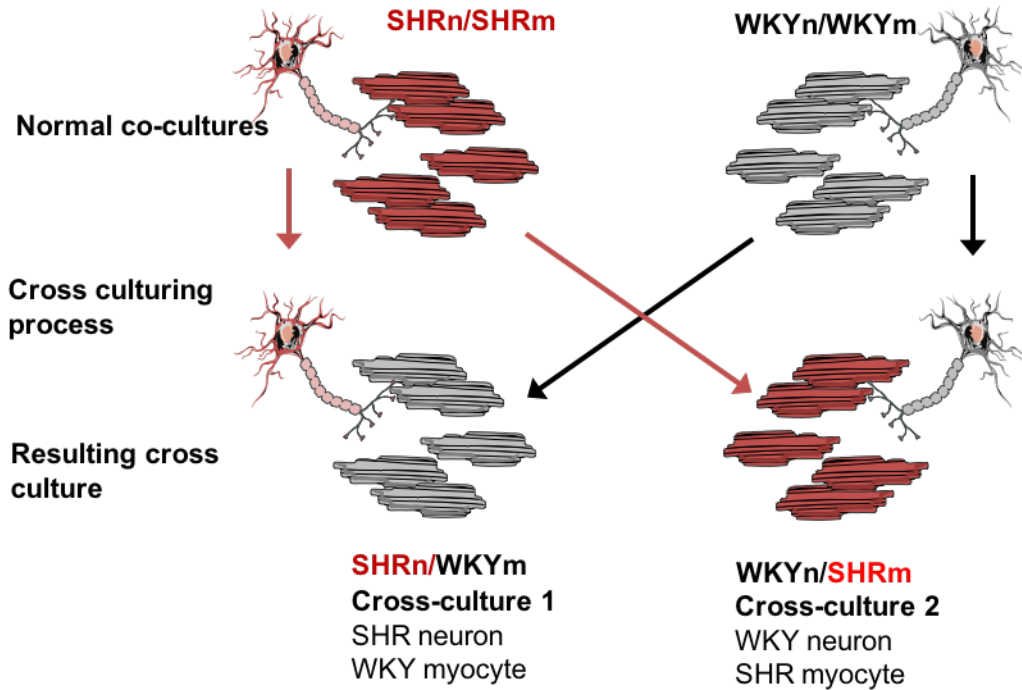


Figure 6.7: The cross-cultures used to test whether the PGSNs are the dominant driver of the sympathetic hyperactivity observed in the pro-hypertensive state. The pro-hypertensive SHRn/SHRm cultures were compared with the normotensive WKYn/WKYm cultures to establish whether the sympathetic hyperactivity translates into a dual cell model. The cross-cultures were developed to test whether the PGSNs are the dominant drivers behind this phenotype. Specifically, could a diseased neuron drive an otherwise healthy myocyte into a diseased state (SHRn/WKYm), or conversely attenuate the hypertensive phenotype (WKYn/SHRm)? To test this, the four co-culture combinations were exposed to the same time controlled experimental protocols.

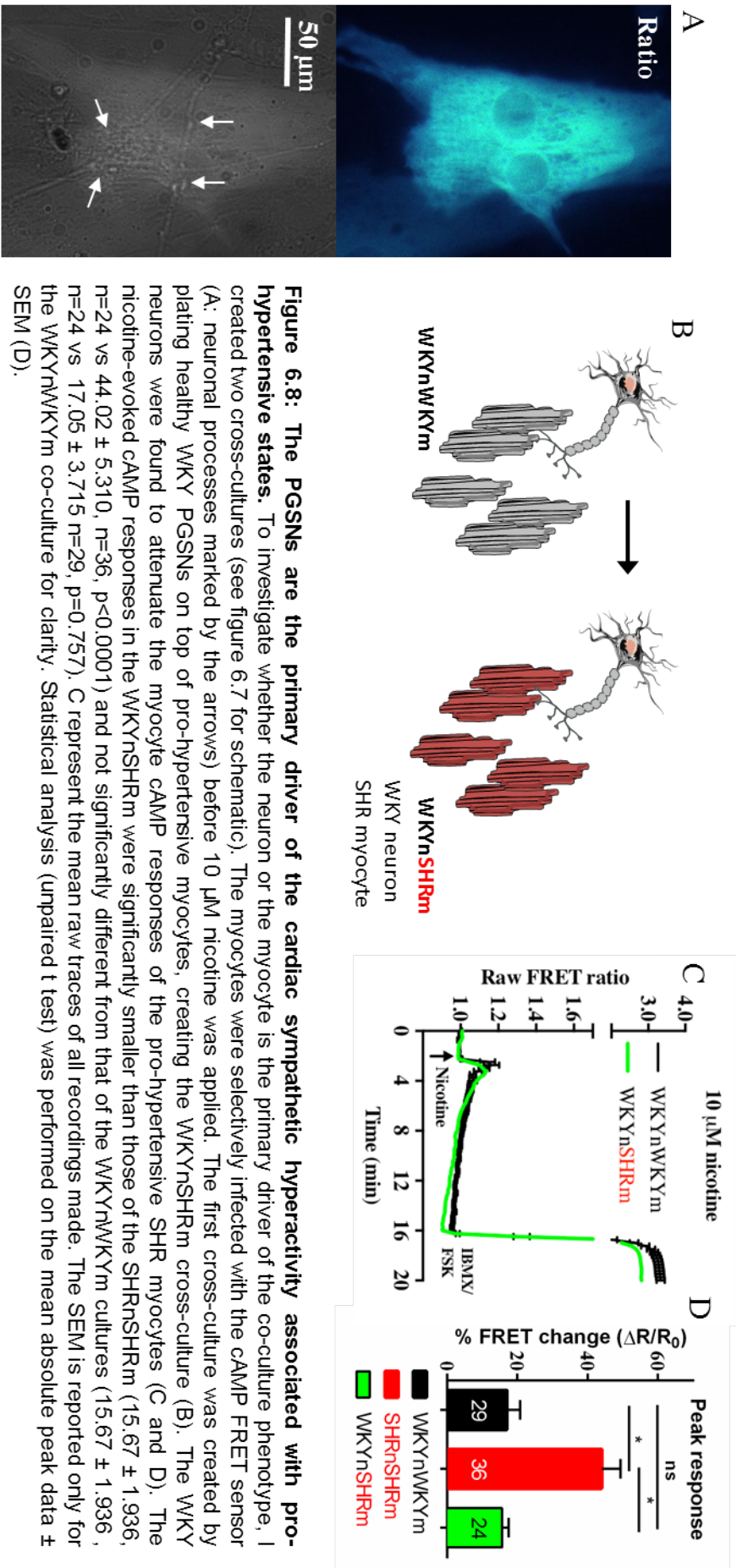
6.3.6.1 Healthy WKY neurons attenuated the myocyte hyper responsiveness seen in the SHR

To test whether a healthy WKY neuron could reduce the cAMP responses of a diseased SHR myocyte, I created the WKYnSHRm cross-culture (figure 6.8B). The nicotine-evoked cAMP responses of the myocytes in the WKYnSHRm were significantly smaller than those from the SHRnSHRm ($15.67 \pm 1.936\%$, $n=24$ vs $44.02 \pm 5.310\%$,

n=36, $p<0.0001$) and not significantly different from that of the WKYnWKYm cultures ($15.67 \pm 1.936\%$, n=24 vs $17.05 \pm 3.715\%$, n=29, $p=0.757$. Figure 6.8 C and D). This suggests the normotensive WKY neurons were found to attenuate the myocyte cAMP responses of the pro-hypertensive SHR myocytes and normalise it to levels seen in the WKY myocytes.

6.3.6.2 Diseased SHR neurons induced myocyte hyper-responsiveness in otherwise healthy WKY myocytes

To test whether a diseased SHR neuron could affect the cAMP responses of a healthy WKY myocyte, I created the SHRnWKY cross-culture (see figure 6.9A). The nicotine-evoked cAMP responses of the myocytes from the SHRnWKYm culture was significantly larger than that of the WKYnWKYm ($31.37 \pm 5.194\%$, n=42 vs $17.05 \pm 3.715\%$, n=29, $p=0.0441$) and not significantly different from those of the SHRnSHRm culture ($31.37 \pm 5.194\%$, n=42 vs $44.02 \pm 5.310\%$, n=36, $p=0.094$). This suggest the pro-hypertensive SHR neurons were able to induce a diseased myocyte cAMP phenotype on the otherwise healthy WKY myocytes (see figure 6.9 B and C).



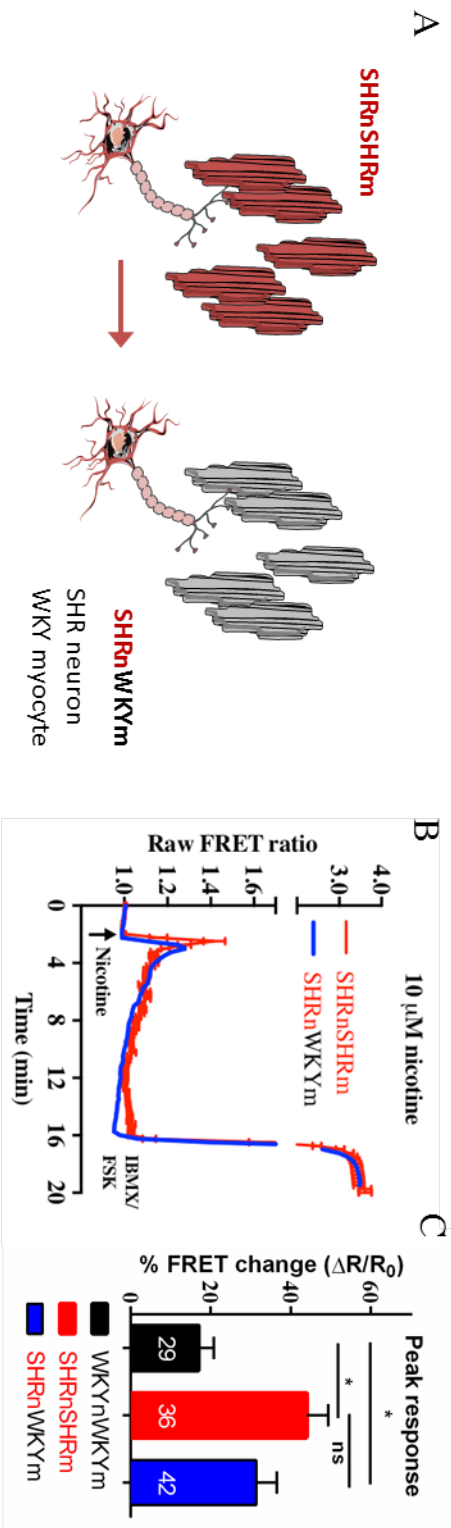


Figure 6.9: The PGSNs are the primary driver of the cardiac sympathetic hyper-activity associated with pro-hypertensive states. The second cross-culture was created by plating pro-hypertensive PGSNs from the SHR on top of healthy WKY myocytes, creating the SHRn WKYm cross-culture (A). The pro-hypertensive SHR neurons were able to induce a diseased myocyte cAMP phenotype on the otherwise healthy WKY myocytes (B and C). The cAMP levels of the SHRnWKYm were significantly larger than those of the WKYnWKYm (31.37 ± 5.194 , $n=42$ vs 17.05 ± 3.715 , $n=29$, $p=0.0441$) and not significantly different from the SHRnSHRm culture (31.37 ± 5.194 , $n=42$ vs 44.02 ± 5.310 , $n=36$, $p=0.094$). The SEM is reported only for the SHRnSHRm co-culture for clarity. Statistical analysis (unpaired t test) was performed on the mean absolute peak data $\pm$ SEM (D).

6.4 Discussion

The novel findings presented here are: (i) The myocytes of the pro-hypertensive SHRs are hyper responsive to β_1 adrenergic stimulation; (ii) the cardiac autonomic dysfunction identified on the single neuron and myocyte, is present in the physiologically coupled co-cultures; (iii) the PGSNs are the principle drivers behind the sympathetic phenotype in pro-hypertensive states as revealed in the *in vitro* cross-cultures.

Sympathetic nervous system hyperactivity is increasingly being recognized as a causal factor for hypertension^{7,13,14,47}, however to this date, no direct experimental evidence exists to support this notion in the case of the neuro-cardiac junction. This study attempted to provide experimental evidence for this hypothesis by employing a physiologically viable preparation of ventricular myocytes and sympathetic stellate ganglions from the normotensive WKY and pro-hypertensive SHR. Successful innervation of the myocytes was observed both by their cAMP response to nicotinic stimulation and immunofluorescence showing positive sarcomeric α -actinin and tyrosine hydroxylase stain (6.1B). The sympathetic neurons displayed the typical ‘beads on a string’ appearance with regular swellings (varicosities) indicating sites of synapse formation^{27,42}. Analogous to that seen in the human heart, the myocytes in the co-cultures were richly innervated, with a neural process being identified next to almost every myocyte^{327,328}.

The myocytes in the co-cultures responded to nicotine-induced neuronal activation with rapid cAMP elevation. All responses were blocked by pre-incubation with 10 μ M metoprolol (see figure 6.5), confirming the nicotine-induced elevation of cAMP I observed was due to NA released from the neuron, activating the β_1 -adrenoreceptor.

Further, the application of nicotine had no effects on the cAMP levels of myocytes cultured alone (figure 6.6B), confirming that nicotine had no effect on the cAMP levels of myocytes cultured alone, indirectly establishing the absence of contaminated intra-cardiac neurons. Taken together, the co-culture model presented in this chapter forms a viable and physiologically relevant model to study cell-to-cell signalling in the neuro-cardiac axis.

6.4.1 Beta adrenergic hyper responsiveness of myocytes in the pro-hypertensive state

The action of NA on cardiomyocytes is facilitated via its action on the β -adrenergic receptors. This results in rapid cAMP elevation and PKA mediated phosphorylation of proteins linked to cardiac function ⁴². When exposed to increasing concentrations of the β -adrenergic agonist isoprenaline (0.1-100 nM), the SHR myocytes generated significantly larger cAMP responses when compared to the WKY controls at concentrations ≥ 3 nM (figure 6.2 and 6.3). This is consistent with previous reports demonstrating β -adrenergic hyperactivity associated with the hypertensive state ^{79-81,329-331}. Early work demonstrated that young adults with resting tachycardia were more likely to develop hypertension ³³². This was later supported by the notion that the β -blocker propranolol caused a greater reduction of heart rate in hypertensive patients than in normotensive controls ³³³. More recently it was demonstrated that SHRs have higher basal heart rates and increased tachycardic responses to isoprenaline ^{79,80} and right stellate stimulation ^{53,79} when compared to normotensive controls. This was linked to larger basal- and β -adrenoreceptor stimulated L-type Ca^{2+} currents in pacemaking cells ⁸⁰ and ventricular myocytes ⁸¹. The latter may contribute to the increased arrhythmogenic action of isoprenaline observed in the SHR ³¹⁹.

A study assessing myocardial adrenoreceptors and adenylate cyclase (AC) activity in the developing pro-hypertensive SHRs (0-125 days post birth) demonstrated that whilst β -adrenergic receptor levels receptor expression was unchanged between the pro-SHR and WKY, a significant increase in the isoprenaline- and forskolin-induced activity of adenylate cyclase (AC) was identified in the SHR myocytes³²⁹. Consistent with the work of Blumenthal et al³²⁹, a more recent study confirmed these findings and identified significantly increased chronotropic responses of neonatal SHR myocytes to isoprenaline³³⁰. This could explain the increased isoprenaline-induced cAMP responses of the neonatal SHR myocytes seen in this study.

Interestingly, the increased heart rate responses are only present in adult animals with established hypertension^{79,80}, and not in the pro-hypertensive four-week old animals⁵³. Intriguingly, this is correlated with the development of increased sympathetic drive^{14,63} which could suggest that the β -adrenergic hyperactivity comes about as a result of the neuronal dysfunction¹³.

What none of these studies address, is the true synaptic NA concentration experienced by the WKY and SHR myocytes, which could yield significantly different results than when globally applied to the cell. In vitro this can only be obtained in a co-culture, using carbon fibre amperometry techniques³³⁴. Work in our lab has attempted this (Dr. Julia Shanks and Dr. Guoliang Hao), but it remains technically challenging. Plasma NA levels form an important predictor of the development of hypertension^{13,47,335,336} and an indicator of mortality in cardiovascular disease¹³. The measurement of plasma NA levels has therefore been attempted in the clinic, however, accurate measurements remain challenging to obtain^{47,76,78,337,338}.

6.4.2 Hyper-responsiveness of the SHR co-cultures to nicotinic stimulation

The results described here provide the first evidence that the dysfunction of the individual neurons and myocytes seen in hypertension, functionally translate into co-cultures. Hypertension is associated with increased sympathetic drive that is associated with increasing severity of the condition. To simulate this, I exposed the co-cultures to increasing concentrations of nicotine (1, 10 and 100 μ M). When compared to the WKY, the nicotine-evoked cAMP responses of the SHR myocytes were consistently and significantly elevated at 1 and 10 μ M nicotine. In contrast, the WKY responses were graded - increasing with rising concentrations of nicotine. At 100 μ M nicotine the responses of the WKY was identical to that of the SHR (see figure 6.4). This could suggest that the SHR PGSNs have a heightened sensitivity to nicotinic stimulation, until a maximum is reached. We know that the myocytes are hyper-responsive (see figure 6.2 and 6.3). However, if this is the only answer then, given the persistent differences of the myocytes at the top (un-physiological) concentrations of isoprenaline, one would expect the nicotine-evoked responses to stay different and not reach a common maximum. Given that the responses to 100 μ M nicotine was identical in the two co-cultures, it could be possible that a more dominant role is played by the PGSN, and that the neuronal physiological response to autonomic stimulation takes precedence over any myocyte phenotype.

To further test the notion of neuronal dominance, I created two cross-cultures (see figure 6.7) where I coupled healthy PGSNs to pro-hypertensive myocytes (termed WKYnSHRm) and vice versa (termed SHRnWKYm). These cross-cultures were exposed to identical experimental conditions as the SHR and WKY cultures (these will hereafter be referred to as SHRnSHRm and WKYnWKYm for clarity). The true acetylcholine concentration experienced by the PGSNs remains unknown, with

literature reporting the use of nicotine concentrations ranging from 1–500 μM ^{27,184}. To prevent the risk of false negatives due to under-stimulation of the WKY co-cultures, and to maximise the chance of resolving the cross-culture phenotypes, 10 μM nicotine was chosen for the cross-culture studies. At this concentration there were clear nicotine evoked cAMP responses from all co-cultures within the dynamic range of the FRET sensor and a significant pro-hypertensive phenotype present in the SHR co-cultures.

6.4.3 The PGSNs are the dominant drivers behind cardiac autonomic hypertension

Interestingly my data found that the PGSNs of the pro-hypertensive SHR were able to induce a disease phenotype on otherwise healthy WKY myocytes (see figure 6.9). What is more, the healthy WKY neurons were able to attenuate the cAMP responses of the hyper-responsive SHR myocytes (see figure 6.8). This provides the very first experimental evidence suggesting the dysfunction in the peripheral neuro cardiac axis seen in the pro-hypertensive state is driven by the PGSNs. It suggests that the autonomic dysfunction of the neuron occurs prior to the development of end organ hyper-responsiveness and could indeed be causal.

Interestingly, the first (and to my knowledge, only) study employing SHR and WKY co-cultures also sought to determine the dominant cell behind the hypertensive phenotype ¹⁸⁶. In contrast to the results obtained here, they identified decreased β -adrenergic responses of the SHR myocytes. Further by employing a cross-culture, analogous to the one described in this thesis, they identified the myocytes as the primary driver behind autonomic hypertension. This study is however significantly weakened by substantial methodological limitations. The study used a crude preparation of cultured ventricular myocytes with un-cultured thoracolumbar explants. These neurons do not solely innervate the heart and could be contaminated by preganglionic, acetylcholine

releasing cells. Further, the study provided no evidence for the correct phenotyping of their cells and their method of assessing contractility was crude and lacked support by any molecular evidence.

Thus the data presented here, using PGSNs, supported by significant molecular control provide a more compelling platform for the investigation of the mechanisms behind cardiac autonomic hyper-responsiveness. The dominant role of the neuron described here is consistent with the mounting evidence in both human and animal studies suggesting the sympathetic neurons are indeed the principal drivers behind autonomic hypertension^{2,12,13,47}.

6.4.4 Molecular mechanism behind the sympathetic hyperactivity

What is the molecular cause of this neuronal hyper-responsiveness? Early work demonstrated that acetylcholine induced currents in postganglionic sympathetic neurons had similar properties between the SHR and WKY rats⁷³, suggesting that the autonomic phenotype is unlikely to be present at the level of the nAChRs. This is consistent with my work in chapter 5, demonstrating no difference in nicotine-evoked cAMP increases between the WKY and SHR PGSNs at any concentration tested (1-100 μ M - see figure 5.2 in chapter 5). The neuronal dysfunction is likely to reside inside the PGSN, which is consistent with work by us and others, demonstrating the presence of a significant Ca^{2+} dysfunction in the pro-hypertensive neurons. These neurons are associated with significantly increased intracellular Ca^{2+} transients⁶³ linked to enhanced neurotransmission⁵³. How the Ca^{2+} dysregulation affects the exocytotic machinery remains unclear.

6.4.4.1 Enhanced NA release

One explanation for the result observed in this study could be that the PGSNs of the SHR have an increased sensitivity to autonomic stimulation, resulting in increased NA release per unit of nicotine. This is supported by the graded appearance of the response (discussed in section 6.4.2). The apparent maximum reached at 100 μ M nicotine could be explained by the depletion of the neurotransmitter from intracellular stores. This notion is supported by numerous studies both in human and animal models of hypertension. It was established early, that the human hypertensive condition was associated with significantly higher circulating levels of NA ^{47,76,78}. Consistent with this, the SHR display significantly higher tissue levels of noradrenaline at multiple developmental stages ³³⁹. What is more, the PGSNs of the SHR portray enhanced NA release following electrical stimulation before and after the development of hypertensive symptoms ^{53,64,74}. Further, the SHR neurons display reduced reuptake of NA ⁶⁹, and impaired α_2 mediated auto inhibition of NA release ⁷⁴, further supporting the notion of enhanced NA release. However, until now, no study has employed graded pharmacological autonomic stimulation which would allow the detection of a potential sensitivity difference between the SHR and WKY.

Interestingly, when exposed to identical electrical stimuli, the SHR neurons portrayed multiple action potentials, where as the WKY cells only elicit one ³⁴⁰. This spontaneous multiple spiking behaviour could provide an explanation to the appearance of double cAMP peaks in the SHRnSHRm cultures. No studies have looked at the vesicular content of NA in the SHR and WKY neurons, but the SHRs have been shown to express higher levels of tyrosine hydroxylase ³⁴¹ which could point towards enhanced NA synthesis in these neurons.

6.4.4.2 Innervation

Another explanation could be that the myocytes from the SHR are hyper-innervated. It is clear that both the vascular bed and the heart of SHR animals have significantly higher expression of nerve growth factor (NGF)^{342,343}. NGF is a neurotropic factor important for neuronal development and survival⁴². It is conceivable that the increased NGF expression could result in enhanced neuronal growth and facilitation of increased innervation. Quantification of innervation of the co-cultures was not attempted in this study as it was difficult to guarantee equal neuronal distribution between different cultures. However hyper-innervation has indeed been demonstrated in the stroke-prone SHRs at multiple developmental stages prior to the development of hypertension³⁴⁴. This was postulated to be caused by hyperactivity of the stellate ganglion and could help explain the increased incidence of arrhythmias associated with hypertension^{1,67,263}.

6.4.4.3 Differential neurotransmitter release

It is now clear that in addition to catecholamines, sympathetic neurons also release other molecules such as ATP (adenosine triphosphate) and various neuropeptides (NPs) such as NPY¹⁹. These are released with different kinetics, allowing the neuron to elicit short- and long term changes in myocyte behaviour⁴². Interestingly, many of the structural changes the sympathetic neurons impose on the myocytes, occur independently of NA. The sympathetic neuron-mediated modulation of myocyte L-type Ca²⁺ channels was shown to be independent by NA^{187,345}, and later determined to be caused by NPY³⁴⁶. Interestingly, NA and NPY had no effect on I_f when applied alone, however when applied together they caused a significant negative shift in its activation curve³²⁶. Mounting evidence suggests that the co-release of neurotransmitters is activity-dependent^{19,177,275}. This poses the question of whether the heightened sympathetic stimulation seen in the hypertensive states could result in the release of a different arsenal of neurotransmitters. This remains to be tested but the co-culture described here

will prove an invaluable tool to shed light on transmitter co-release and its potential role in disease^{19,278}.

6.4.4.4 Structural changes in the synapse?

The notion that sympathetic neurons effect changes in myocyte membrane receptor expression^{184,324}, highlights the apparent importance of synapse structure. The functional relevance of synaptic integrity in hypertension was described recently. Four-week old pro-hypertensive animals demonstrated significantly increased heart rate responses to right stellate ganglion stimulation when compared to normotensive controls⁵³. However, no changes in heart rate were reported following norepinephrine application at any of the nine concentrations tested (0.025 – 5 μ M)⁵³. What is more, it was recently demonstrated that the neuro-cardiac junction is structurally sensitive to the activity of the neurons¹⁸⁴. The reorganisation of structural synaptic proteins, and indeed the β -adrenoreceptors on the myocyte membranes were dependent on nicotine activation¹⁸⁴. The activity-dependence of such processes could have significant relevance to conditions such as hypertension where there is significant sympathetic hyperactivity and vagal impairment. Could these diseases be associated with molecular remodelling of the synaptic space? This remains to be tested.

6.4.5 Limitations of the study

Perhaps the biggest limitation of this study is the assumption that fully functional synapse formation had occurred within a relatively short amount of time in culture (five days). However, studies from others demonstrated the presence of functional synapse formation after three days in culture^{208,214}. Evidence of sympathetic innervation was determined visually (and recorded using a CCD camera), with care being taken to ensure the myocytes were only innervated by one sympathetic neuron. However, it is possible that some cells in this study were not innervated, which could lead to

misrepresentation of the data. However this error was expected to be similar between the four co-cultures. An original aim of this study was to investigate the cAMP responses to neighbouring innervated and non-innervated myocyte. It was thought this could provide information of the differential effects global vs. synapse mediated NA action could have on myocyte cAMP signalling. This would provide information about the relative importance of targeting the whole synapse. However this proved impossible due to the extensive branching of the neurons, the limited field of view of the microscope and the difficulty in guaranteeing the lack of innervation using a conventional low powered FRET microscope. Further, isoprenaline has a slightly different affinity for the adrenoreceptors than NA. As such care must be taken when comparing the effects of NA release in the synapse with global isoprenaline application.

6.4.6 Concluding remarks

The sympathetic hyperactivity associated with the hypertensive state does not reside in one cell alone, but in both the PGSNs and the myocytes they innervate. However, the work presented here demonstrated that the sympathetic neurons are the primary drivers behind cardiac autonomic hypertension and could be the causal factor. This is aligned with the marked autonomic phenotype observed in the cell, organ, whole animal and human models of hypertension. The notion that a healthy sympathetic neuron can attenuate the hyper-responsiveness of a diseased neuron, could significantly alter how this hypertension is viewed and could provide an alternative rationale for the pharmacotherapy of hypertension and indeed other conditions associated with dysautonomia. It could provide an avenue for developing cell therapies targeted at the postganglionic, presynaptic neuron to turn down the gain of neurotransmission, akin to a smart pre-synaptic β -blocker.

CHAPTER SEVEN

7 Concluding discussion

7.1 *The main findings of the thesis*

1. The sympathetic dysfunction seen in pro-hypertensive rats is underpinned by significant dysregulation of the voltage gated Ca^{2+} channels ($\text{Cav}_{2.2}$), providing the trigger for the Ca^{2+} impairment seen in these neurons.
2. This neuronal Ca^{2+} dysfunction is linked to significant impairment of cGMP signalling that could be facilitated through the differential involvement of PDE2 and PDE3 in the pro-hypertensive PGSNs.
3. There is significant β -adrenergic hyper-responsiveness of the myocytes in pro-hypertension.
4. Cross-culture studies suggest the PGSNs are the dominant drivers behind the neuro-cardiac hyper-responsiveness associated with the pro-hypertensive state.

7.2 *Sympathetic neurons as the principal drivers behind autonomic hypertension*

It has long been clear that neuronal sympathetic dysfunction plays a major role in the development and maintenance of hypertension^{10,12,13}. However, until now it has remained unclear whether this sympathetic impairment is causal, or whether it appears as a result of the hypertensive state itself. Whilst the significant hyper-excitability of the myocytes is well described, research is now pointing towards a more dominant involvement of both the central and peripheral nervous system^{7,12,13,47}. However, this has remained challenging to establish experimentally. This thesis provides the first direct evidence that the neurons function as the principle drivers behind the cardiac sympathetic hyperactivity, and could be causal in the early onset of the pathology. This was done by first establishing the presence of the well-established hyperactivity on both the PGSNs and the myocytes of pro-hypertensive SHR. Second, I for the first time

confirmed that these molecular changes were present in a physiologically coupled co-culture from the normotensive and pro-hypertensive animals. Finally, I employed a cross-culture that physiologically coupled healthy sympathetic neurons with hypertensive myocytes, and vice versa. Through these experiments I established that the healthy WKY PGSNs were able to attenuate the myocyte hyper responsiveness seen in the SHR and that the diseased SHR PGSNs were able to induce a disease phenotype in the otherwise healthy WKY myocytes.

The clinical relevance of these findings may be significant. Cardiovascular diseases are associated with significant pathology on the cardiac myocytes that can precipitate sudden cardiac death. In these patients their hearts form ‘ticking bombs’, waiting to go off; incidents of sudden cardiac death are often associated by a bout of high sympathetic activity⁶⁷ that in essence lights the fuse. With the dominant role of the neurons in driving the myocytes, suggested in this thesis, it is likely that the neurons could provide the trigger and light the fuse. It could indeed be the case that sympathetic neurons form the substrate for arrhythmias. This has huge implications for hypertensive patients with known sympathetic hyperactivity, but could also help explain the often puzzling causes of sudden cardiac death in otherwise “healthy” subjects.

Further, our results could help explain why β -blockers – previously used as a first line treatment for hypertension - do not have the same positive effects on hypertensive endpoints as other antihypertensive agents^{347,348}. The β -blockers specifically target the hyper-responsiveness of the cardiac myocytes. Here I provide intracellular molecular evidence for this β -adrenergic hyper responsiveness, but I show that it can be attenuated by the presence of healthy sympathetic neurons. This could suggest that the β -

adrenergic hyper-responsiveness may not be causal, but rather a compensatory mechanism that comes about as a result of the sympathetic hyperactivity. This has huge implications for the pharmacotherapy of sympathetic dysautonomia as in order to reduce its incidence and mortality rate, it is vital to treat the cause of the disease and not merely the symptoms. Further, recent evidence has suggested that β -blockage could increase cardiac sympathetic innervation³⁴⁹ which could contribute to the sympathetic hyperactivity seen in hypertension. Today, β -blockers are no longer used as a first line treatment and are generally only prescribed when others medications are less effective, or in distinct subgroups of the population. Interestingly one such subgroup of patients are those with evidence of increased sympathetic drive (NICE guidelines CG127 1.6.11, 2011).

7.3 Importance of targeting the autonomic dysfunction early

The prolonged elevation of blood pressure causes significant organ damage and directly contribute to the high level of cardiovascular diseases associated with hypertension^{13,18}. To reduce the mortality rate it therefore becomes critical to catch the disease early³. The sympathetic dysfunction seen in hypertension increases along with the progression of the disease, both in humans¹⁴ and animal models^{53,63}. Further, it occurs irrespective of other heterogeneous clinical aspects that accompany the state (such as obesity and diabetes)¹³. As such the autonomic dysfunction could be seen to precede these comorbidities. Indeed, the work in this thesis and that of others^{53,63} has demonstrated that the sympathetic dysfunction seen in the PGSNs is seen prior to the development of clinical symptoms. This has great significance for the potential identification of a pre-disease diagnostic marker that would allow early pharmacological intervention and the prevention of cardiovascular complications.

7.4 *Novel treatment avenues for hypertension*

A multitude of clinically used drugs exist to treat hypertension, however relatively few of these are aimed at the sympathetic neurons ⁴⁷. Protecting people from the organ damage occurring as a complication hypertension is predicted to significantly lower mortality rates ³. What is more, the current four-tiered treatment scheme (NICE guidelines, 2011) consisting of multiple different drug groups to cater for resistant patients, highlights the need for novel anti-hypertensive treatment strategies ¹³.

7.4.1 Sympathetic denervation

It was realised early that cardiac stellate sympathetctomy had significant blood pressure reducing effects, and was antiarrhythmic ^{350,351}. However, the significant risk of complications following such intrusive surgery, prevented its widespread use ⁸. Today, cardiac sympathetic denervation is relatively well-established for the treatment of cardiac arrhythmias ³⁵¹, however my results could provide rationale its potential use to treat the dysautonomia associated with hypertension. Targeted sympathetic denervation of other organs associated with sympathetic hyperactivity have also been explored as a treatment of hypertension ^{10,352}. Both renal denervation and more recently carotid body resection has been shown to improve blood pressure control ^{6,353,354}, however clinical trials have yielded conflicting results ³⁵²⁻³⁵⁵.

It is clear the the sympathetic dysfunction is present at multiple autonomic nuclei. But with the lack of direct success of stand alone sympathetic denervation in all patients, the role played by the synergy between these systems should be explored ³⁵².

7.4.2 Cell therapy

Our results demonstrating that the normotensive neurons are able to attenuate the myocyte β -adrenergic hyper responsiveness of the pro-hypertensive SHRs, could

provide the rationale for the use of cell therapy in the treatment of ‘sympathetic-myocyte’ phenotypes. Interestingly, the transplant of both foetal and adult SHR hypothalami into normotensive rats induces hypertension³⁵⁶⁻³⁵⁸. Whilst these studies employ relatively primitive experimental design and the potential transplant of peripheral cells is expected to be technically challenging, it has proven to be successful in more complex settings such as in the brain³⁵⁹.

7.4.3 Nicotinic drugs for the treatment of autonomic dysfunction

The nicotinic acetylcholine receptor (nAChR) antagonists mecamylamine and hexamethonium were some of the earliest pharmacological agents used in the treatment of hypertension^{360,361}. However, due to significant peripheral side effects they have both been withdrawn and are today only used in a laboratory setting³⁶². The only ganglion blocker in use today is trimethaphan, which causes a rapid drop in blood pressure¹⁴⁷. However its use is restricted to the treatment of malignant hypertension and the hypertensive crisis in a hospital setting³⁶³. The peripheral side effects of these drugs are widely attributed to their non-discriminative block of multiple nAChRs. The nAChR is a pentamer composed of $\alpha 1$, $\beta 1$ and δ , subunits, each of which presenting multiple splice variants. The tissue-specific expression of some of these subunits convey different agonist profiles for each receptor⁴³, allowing the cell to fine-tune its response to acetylcholine. It should therefore be possible to design sympathetic neuron specific nicotinic antagonist that could be used as anti-hypertensives. The work presented in this thesis provides compelling evidence that the neurons are the drivers behind autonomic hypertension, and supports the rationale for the search for such drugs. Interestingly it has been found that different types of nAChR can be present in different locations on the same sympathetic neuron³⁶⁴. Differentially targeting one or multiple of these receptors on the same neurons could allow discrete pharmacological control

neurotransmission. What is more the absence of net changes in nAChR activation (see Chapter 5 and ⁷³) between the hypertensive and normotensive neurons could provide a solid platform for stable and predictable drug action. However, before this can be done, more research needs to be conducted into the relative expression profiles of the nAChR subunits in different tissues of the body.

7.4.4 Targeting the PDEs in autonomic dysfunction

PDEs facilitate the cross-talk between the cGMP and cAMP pathways. These cyclic nucleotides have been shown to be strongly dysregulated in the SHR both at the neuron ⁷⁰ and myocyte level ⁸⁰, with emerging roles suggested for the PDEs ⁷⁰. The evidence provided in this thesis suggests that the cGMP dysfunction could be facilitated by the differential expression and/or activity of PDEs in the sympathetic neurons from the pro-hypertensive SHR. Whether this apparent change in cGMP mediated PDE signalling, plays a role in the initiation of the disease, or if it comes as a result of the cyclic nucleotide dysfunction, remains to be elucidated. Nevertheless, these findings have implications for the understanding of the role of the CNs in sympathetic neurotransmission. The ability of cGMP/cAMP cross-talk to simultaneously effect changes in a multitude of signalling events, resulting in a finely tuned response, could enable the design of very specific molecular therapies against a multitude of conditions ranging from cardiovascular disease to erectile dysfunction ¹⁵⁸. PDE inhibitors are relatively well tolerated in the clinic. The large PDE subfamily, with their many splice variants (>100 PDE proteins, each with a unique tissue expression ^{160,310}) makes them attractive targets for drug therapy ¹⁵⁸. Combined, these data suggests that they could open up a new avenue for the design of novel anti-hypertensives.

7.5 A novel cell model to study the initiation and development of hypertension

The presence and physiological relevance of a co-culture of sympathetic neurons and cardiac myocytes is predicted to allow unprecedented detail and fine control in the study of the molecular mechanisms behind the initiation and maintenance of hypertension. It will allow the investigation of the nature of the cell-to-cell communication in the neuro-cardiac junction and how this might be altered in disease states. This is of particular interest to our group, as it could help the understanding of how sympathetic NO/CN signalling occurs; such insight might help us understand how peripheral neural circuits operate in hypertension. What is more, the co-culture will allow us to provide biological data that can be used to verify the mathematical predictions made by Tao *et al.* in 2011¹⁷⁵. This in turn could lead to novel avenues of pharmacological treatment for hypertension and indeed other cardiovascular conditions associated with heightened sympathetic activity.

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