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STIM1 mediates multiple signalling pathways in neuronal growth cones

Camilla B Mitchell

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University of Tasmania

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The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Name and School = **Camilla Bessie Mitchell**, Menzies Research Institute Tasmania

Name and institution = **Robert J Gasperini**, Menzies Research Institute Tasmania

Name and institution = **David H Small**, Menzies Research Institute Tasmania

Name and institution = **Lisa Foa**, School of Medicine and Menzies Research Institute Tasmania

Author details and their roles:

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Percentage contribution of each author is outline in the table below:

Author	Research Performed	Data analysis	Paper writing	Designed project
CM	85	70	30	5
RJ	15	20	25	10
DS	0	5	5	5
LF	0	5	40	80

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: 

Lisa Foa
Primary supervisor
Menzies Research Institute Tasmania
School Of Medicine
University of Tasmania



Head of School
Menzies Research Institute Tasmania
University of Tasmania

Date: 3/9/13

Statement of ethical conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Abstract

Calcium is an intracellular second messenger that is vital for normal neuronal function. The maintenance of calcium homeostasis is critical for healthy neuronal function, and disruption in calcium homeostasis has been implicated in diseases such as epilepsy and Alzheimer's disease. In developing neurons, calcium signalling regulates the precise wiring of neurons, in a process known as axon guidance. Axon guidance is extremely important in the normal healthy development of the nervous system. Aberrant axon guidance is highly associated with several neurodevelopmental disorders including autism and mental retardation syndromes such as fragile-X syndrome. Axons navigate the environment by a dynamic navigational structure located at the distal tip of an extending axon, known as a growth cone. Cytosolic calcium is crucial in mediating growth cone navigation. Correct understanding of the signalling mechanisms that regulate cytosolic calcium is key to understanding normal growth cone function.

This thesis focuses on the molecular mechanisms that regulate a vital source of calcium within growth cones, the endoplasmic reticulum (ER). Little is known about the function of the ER within growth cones. Stromal Interaction Molecule 1 (STIM1) is a calcium sensing protein in the ER membrane, which interacts with Orai proteins in the plasma membrane to initiate store-operated calcium entry (SOCE) and refill depleted intracellular calcium stores. The central hypothesis of this thesis is that STIM1 is necessary for SOCE in neuronal growth cones, and is required for axon guidance.

The results presented within this thesis demonstrate the presence and function of STIM1-mediated processes within the developing nervous system. This thesis has utilised primary cell culture of embryonic dorsal root ganglia neurons and immunocytochemistry to investigate the presence and localisation of STIM1 within developing growth cones. STIM1, along with its binding partners Orai1 and Orai2 reside in two different localisation patterns within growth cones; active (punctate) and inactive (diffuse). Depletion of calcium stores resulted in the activation of STIM1 within growth cones, increasing the number of growth cones displaying punctate STIM1 protein distribution. Calcium depletion also increased colocalisation between STIM1 and Orai1. Furthermore, STIM1 localisation appeared to be biased towards the turning side of the growth cone, in response to a calcium-dependent guidance cue. These data suggest that STIM1 and the Orai proteins are dynamic proteins that function in the regulation of calcium within growth cones.

While immunocytochemistry data suggested that STIM1 was functional within growth cones, a target morpholino approach was used to determine if STIM1 was necessary for growth cone function. A reduction of endogenous STIM1 reversed turning towards BDNF and netrin-1, and demonstrated that STIM1-mediated SOCE was necessary for BDNF signalling in growth cones. Unexpectedly, a reduction in STIM1 abolished turning away from Sema-3a in a manner independent of SOCE. In a growth cone collapse assay, STIM1 was also found to be necessary for Sema-3a-induced collapse, suggesting that STIM1 is implicated in multiple Sema-3a signalling pathways. This knockdown approach clearly demonstrates the necessity of STIM1 function for normal growth cone turning.

While the main function of STIM1 is thought to be the activation of Orai proteins, and subsequent activation of SOCE, STIM1 has been shown to interact with other signalling proteins, including the second messenger cAMP, in a process termed store-operated cAMP signalling. This study utilised cAMP analogues to determine if store-operated cAMP signalling was functional within growth cones. Upon the activation of cAMP, repulsive turning away from Sema-3a was restored in growth cones with reduced levels of STIM1. Sema-3a collapse was also prevented upon addition of cAMP agonists in control growth cones, but not restored in STIM1 morphants. Similar results were achieved with cGMP agonists. These data suggest that STIM1 mediates cyclic nucleotide signalling within growth cones. Furthermore, STIM1 has also recently been implicated in the reciprocal control of L-type voltage-gated calcium channels (VGCCs) and Orai proteins. While L-type VGCCs are important in mature neurons, there is conflicting data in the literature as to their role in axon guidance. This study investigated whether there was a potential interaction between STIM1 and L-type VGCCs in growth cones, and found that if there is an interaction, it is not essential for growth cone turning, but may be required for axon extension.

These results indicate a number of novel findings: Firstly, that STIM1 mediates growth cone navigation in response to both calcium-dependent and -independent guidance cues. Secondly, that STIM1 is required for Sema-3a signalling. Thirdly, that STIM1 mediates cyclic nucleotide signalling pathways within growth cones, and likely does not interact with

L-type VGCCs for growth cone navigation. In conclusion, this thesis has significantly added to the understanding of the regulation of the calcium signalling pathways that are crucial for normal growth cone guidance, enhancing our understanding of growth cone navigation, and in particular the regulation of the calcium signalling pathways that are crucial for normal growth cone guidance. These findings add to the pool of knowledge of how growth cones function and regulate calcium, which is crucial for normal neuronal health within development.

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Abbreviations List

[Ca ²⁺] _i	Intracellular calcium concentration
6-Phe-cAMPS	Sp-6-Phe-cAMPS
8-Me-cAMPS	8-pCPT-2'-O-Me-cAMPS
AC	Adenylate cyclase
Ach	Acetylcholine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BDNF	Brain derived neurotrophic factor
CAD	CRAC activation domain
CaMKII	Calcium/calmodulin kinase II
cAMP	Cyclic adenosine monophosphate
CaN	Calcineurin
cGMP	Cyclic guanosine monophosphate
CIF	Calcium Influx Factor
CNS	Central nervous system
CRAC	Calcium-release activated calcium
CICR	Calcium-induced calcium release
DCC	Deleted in Colon Cancer
DRG	Dorsal root ganglia
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol tetraacetic acid
Epac	Exchange protein directly activated by cAMP
ER	Endoplasmic reticulum
ER-PM	Endoplasmic reticulum-plasma membrane
ERM	ezrin/radixin/moesin
f-actin	Filamentous actin
FLIP	Focal laser-induced photolysis
GTP	Guanosine triphosphate
HBSS	Hank's Buffered Salt Solution
I_{CRAC}	CRAC current
IP ₃	Inositol trisphosphate
IP ₃ R	IP3 receptor
IR	Immunoreactivity
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
mRNA	messenger RNA
NaCl	Sodium Chloride
NFAT	Nuclear Factor of Activated T cells
NGF	Nerve Growth Factor
Npn	Neuropilin
NT-3	Neurotrophin 3

NT-4/5	Neurotrophin 4/5
P75 ^{ntr}	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PDE	Phosphodiesterase isozyme
PI3-K	Phosphoinositide 3-Kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKG	Protein Kinase G
PLC	Phospholipase C
PTEN	Phosphatase and tensin homolog
PVDF	polyvinylidene difluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation assay buffer
RNAi	RNA interference
ROI	Region of interest
RyR	Ryanodine Receptor
SAM	Sterile Alpha Motif
SCID	Severe Combined Immunodeficiency
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean

Sema-3a	Semaphorin-3a
SERCA	Sarco/endoplasmic reticulum
SNM	Sensory Neuron Media
SOC	Store- operated calcium
SOCC	Store- operated calcium channel
SOCE	Store-operated calcium entry
STIM	Stromal Interaction Molecule
Tg	Thapsigargin
TrK	Tyrosine Kinase
TRP	Transient receptor potential
TRPC	Transient receptor potential canonical
VGCC	Voltage-gated calcium channel
w/w	weight/weight
<i>Xenopus</i>	<i>Xenopus laevis</i>

Chapter 1:

Introduction and literature review

Chapter 1. Introduction and literature review

1.1 Axon pathfinding is vital for nervous system formation

The nervous system is comprised of millions of neurons that create specific synapses to form a highly ordered neuronal network. The intricacy and precision of neuronal connectivity is the hallmark of neuronal development. One vital process that is required in the formation of this network is axon pathfinding. Axon pathfinding is the process whereby developing neurons send out their axons to navigate through the embryonic environment to find and make connections with their correct targets (reviewed in Tessier-Lavigne and Goodman, 1996). Correct axon pathfinding is crucial to the correct formation of the nervous system. If the process of axon pathfinding goes wrong, aberrant neuronal connections can form. These aberrant connections can lead to neurodevelopmental disorders such as autism spectrum disorder (Geschwind and Levitt, 2007), epilepsy (Yaron and Zheng, 2007), schizophrenia (Bunney et al., 1995, Eastwood et al., 2003, Chen et al., 2011) and mental retardation syndromes including fragile-X syndrome (Waage-Baudet et al., 2005, Bassell and Warren, 2008, Degano et al., 2009). It is extremely important that the process of axon pathfinding is well understood throughout normal development, in order to understand the cellular basis of neuronal disorders and diseases.

1.1.1 Pioneer neurons establish a scaffold for axon guidance

The first neurons to extend axons into a guidance cue filled environment are known as pioneer neurons. The extracellular environment is rich in cell surface, extracellular matrix

and soluble molecules, acting as guidance cues (Araújo and Tear, 2003). Pioneer axons navigate through this environment establishing a scaffold of axon pathways, or tracks (Bate, 1976, Bentley and Keshishian, 1982, Bastiani et al., 1984, Jacobs and Goodman, 1989, Boyan et al., 1995, Araújo and Tear, 2003, Bak and Fraser, 2003). These early laid axon tracks are important for the guidance of later-developing neuronal axons, referred to as follower axons. Follower axons track along the existing axonal scaffold, fasciculating with individual tracks to reach their final targets (Whittington, 1993, Araújo and Tear, 2003). If pioneer axon pathways are ablated or removed, the guidance of follower axons is perturbed, either by being delayed or misrouted (Raper et al., 1984, Bastiani and Goodman, 1986, Gan and Macagno, 1995, Hidalgo and Brand, 1997). This can result in adverse effects in the development of neuronal connections, however it does not necessarily mean that follower axons will not extend or find their correct targets. Pioneer axons contribute to guidance by providing a suitable substrate for follower axons to extend along. It is rare for pioneer neurons to be essential for follower axons to reach their targets (Pittman et al., 2008)

1.1.2 Growth cones lead the developing axon

At the tip of an extending axon is a specialised structure known as a growth cone, which initiates axon pathfinding, and therefore neuronal connectivity. Growth cones are extremely motile, sensitive organelles, which navigate and lead the axon through the surrounding environment (reviewed in Tessier-Lavigne and Goodman, 1996). Different axons (pioneer or follower) have differing growth cone morphologies. Pioneer axon growth cones are more

complex, larger, but shorter and wider than those of follower axons, which are more elongated in morphology (Bak and Fraser, 2003). The more complex morphology of pioneer growth cones is consistent with their motility as they pause, spread and sample the environment in order to interpret guidance cues to make decisions at choice points such as the optic chiasm or floor plate, as to where to generate axonal scaffolding (Tosney and Landmesser, 1985). Follower axon growth cones have less to interpret, because they are following the pioneer growth cones when they are in the tracks. Therefore, follower growth cones have simpler, narrow, more streamlined growth cone morphologies that grow at a much faster rate (Bovolenta and Dodd, 1990, Wilson and Easter Jr, 1991, Mason and Wang, 1997, Bak and Fraser, 2003, Raper and Mason, 2010), however these can become more complex when follower growth cones reach decision points, such as at the plexus region.

Growth cones have a myriad of behaviours that result from the extension and retraction of their membranes. Growth cone behaviours are elicited in response to the external environment and guidance cues. These behaviours include outgrowth, retraction, stalling/arrests (permanent or transient pauses in motility), branching, turning and fasciculation. All of these behaviours are required for axon pathfinding to occur correctly (Kater and Mills, 1991, Kalil et al., 2000, Suter and Forscher, 2000).

1.1.2.1 Growth cone structure

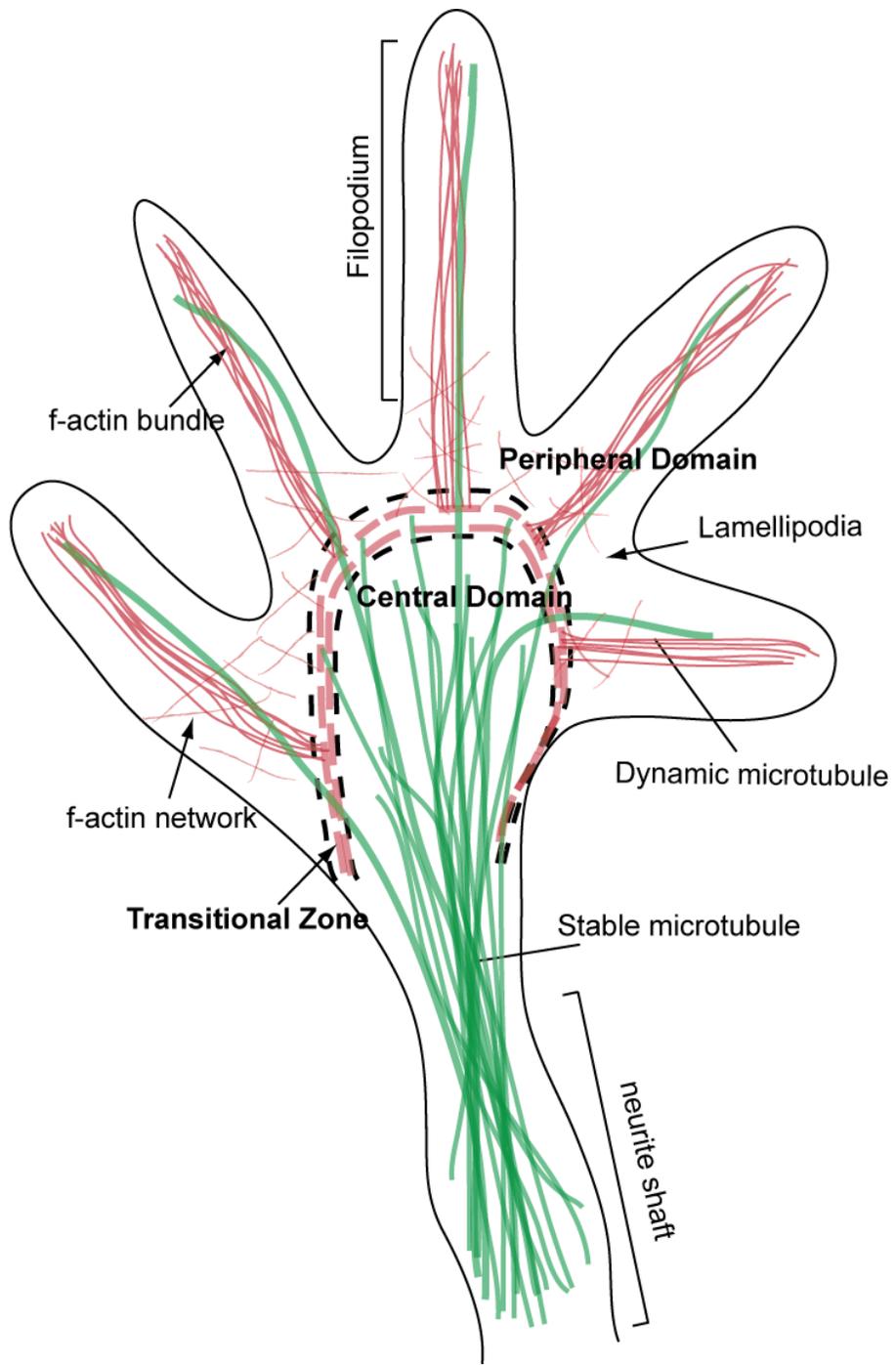
Neuronal growth cones consist of three domains; the peripheral domain, the central domain and the transitional zone (Fig. 1.1; Goldberg and Burmeister, 1986, Forscher et al., 1987, Forscher and Smith, 1988, Jay, 2000). Each domain is structurally different. The peripheral domain is mostly composed of actin-based structures known as filopodia and lamellipodia, and is thought to be largely devoid of cytoplasmic organelles (Sheetz et al., 1992, Henley and Poo, 2004). The central domain makes up the palm of the growth cone and consists of microtubules, vesicles and organelles. The transitional zone is located between the peripheral and central domains and is characterised by actin-based ruffling activity. Actin filaments and microtubules are domain specific. Actin filaments are found more predominantly in the peripheral domain, while the cytoplasmic central domain contains mostly stable microtubules (Gordon-Weeks, 1987, Forscher and Smith, 1988, Bridgman and Dailey, 1989), with the distal tips of microtubules residing in the transition zone. Dynamic microtubules are also able to extend into the peripheral domain, through the transition zone (Suter and Forscher, 2000).

Growth cones are made up of lamellipodia and filopodia, which are highly dynamic components that mediate the direction of axon growth to be determined (O'Connor et al., 1990, Fan et al., 1993, Zheng et al., 1994, Jay, 2000). Filopodia are long, thin, finger-like processes that can change their length rapidly (Fig. 1.1). Lamellipodia are more veil-like membranous structures, that expand between the filopodia in the direction of growth of the growth cone (Fig. 1.1) (Bray, 1970). Both filopodia and lamellipodia project in multiple

directions, and continuously extend and retract, sensing the environment around them (Bray and Chapman, 1985, Goldberg and Burmeister, 1986). Filopodia and lamellipodia extension and retraction occurs many times in comparison to the actual rate of growth of the axon, and is able to occur due to the extensive adhesive contacts that are made with substrates and the surrounding environment (Sheetz et al., 1992). The direction in which filopodia and lamellipodia become stabilised, is the direction the growth cone will continue to develop, allowing for neurite extension as the filopodia and lamellipodia become filled with actin and cytoplasm (Fan et al., 1993, Bentley and O'Connor, 1994, Zheng et al., 1994). As filopodia are stabilised, the lamellipodia expand and extend to fill the spaces in between them, in a veil-like manner. Neurite extension involves not only the protrusion and stabilisation of filopodia, but the initial peripheral domain also becomes engorged with vesicles and organelles from the central domain, resulting in the advancement of the central domain (Harris et al., 1987, Aletta and Greene, 1988, Goldberg, 1988, Mitchison and Kirschner, 1988, Tanaka and Sabry, 1995, Suter and Forscher, 2000, Henley and Poo, 2004).

Figure 1.1. The growth cone structure and cytoskeleton

A typical growth cone, demonstrating its two membranous structures, filopodia (finger-like projections) and lamellipodia (veil-like structures interspersed between filopodia). The growth cone is divided into three domains: peripheral domain, central domain and the transitional zone. F-actin (pink/red) resides in two populations: a mesh-like network primarily in lamellipodia, and bundles within each filopodia (Ishikawa and Kohama, 2007). Stable microtubules (green) extend out of the axon shaft and splay out in the central zone. Dynamic microtubules extend into the lamellipodia and filopodia. (Figure adapted from Lowery and Van Vactor, 2009).



1.1.2.2 Growth cone cytoskeleton

Growth cone motility is dictated by the motility of filopodia and lamellipodia. Filopodial and lamellipodial motility is based on the dynamics of the underlying cytoskeleton, the actin filaments and microtubules (Bentley and O'Connor, 1994, Letourneau, 1996, Jay, 2000, Suter and Forscher, 2000, Dent et al., 2011).

Filamentous actin (f-actin) exists in growth cones in two major populations (Lewis and Bridgman, 1992). The first is bundles of longer filaments, with their plus ends toward the leading edge of the growth cone. These bundles of longer filaments are linear in orientation, and contain up to a dozen filaments (Fig. 1.1). This population of f-actin is found primarily within filopodia, but they are also found in lamellipodia and extend across the full width of the lamellipodia (Lewis and Bridgman, 1992, Bentley and O'Connor, 1994, Suter and Forscher, 2000). These f-actin bundles often extend to the edge of the central domain (Bridgman and Dailey, 1989). The second population of f-actin is the shorter, randomly cross-linked network. These shorter filaments are located between the bundles of longer filaments (Fig. 1.1) (Lewis and Bridgman, 1992, Korey and Van Vactor, 2000, Suter and Forscher, 2000). This population is known as the volume filling f-actin, and gives structure to the lamellipodia (Letourneau, 1983, Bridgman and Dailey, 1989, Bentley and O'Connor, 1994).

F-actin operates within the growth cone via several kinetic processes, including: actin filament assembly from G-actin monomers at the leading edge of the growth cone; constant retrograde transport of f-actin networks powered by myosin motors; and proximal recycling of f-actin in the transition zone of the growth cone (Forscher and Smith, 1988, Lin et al., 1996a, Suter and Forscher, 2000, Bard et al., 2008, Chan and Odde, 2008). The extension and retraction of a filopodium reflects the balance between actin polymerization at the barbed ends, and the retrograde flow of entire filaments (Okabe and Hirokawa, 1991, Lin et al., 1996b, Mallavarapu and Mitchison, 1999).

Microtubules are the dominant cytoskeletal component within neurites (Suter and Forscher, 2000). Microtubules are arranged as stable, parallel bundles within the neurite shaft (Fig. 1.1) (Dickson, 2002), but as they enter the central domain of the growth cone, they splay apart, spread out, and turn, as they probe the actin-rich periphery of the growth cone (Bridgman and Dailey, 1989, Suter and Forscher, 2000, Dent and Gertler, 2003, Gordon-Weeks, 2004). Microtubules are also able to interact with f-actin within the peripheral domain (Fig. 1.1) (Zhou et al., 2002, Dent and Gertler, 2003, Gordon-Weeks, 2004). The microtubules that emerge and enter into the growth cone display classic properties of dynamic instability, enabling them to explore both the lamellipodia and filopodia of growth cones, as they extend and retract (Tanaka and Sabry, 1995, Dent et al., 1999, Dent and Kalil, 2001, Schaefer et al., 2002, Zhou et al., 2002). These dynamic microtubules preferentially grow along actin filaments within the filopodia (Tanaka and Sabry, 1995, Schaefer et al., 2002), and local stabilisation or destabilisation within the filopodia may be

sufficient to induce an attractive or a repulsive growth cone turning response (Buck and Zheng, 2002, Dickson, 2002).

1.1.2.3 Directed growth cone motility

Growth cones move as they extend and retract their filopodia and lamellipodia in response to guidance cues within the external environment. Any signal that can mediate growth cone turning responses must regulate the assembly of both f-actin and microtubules (Zheng et al., 1996, Zhou et al., 2002, Dent and Gertler, 2003, Henley and Poo, 2004, Kalil and Dent, 2005, Wen and Zheng, 2006). The interactions that occur between actin and microtubules are essential for growth cone motility and navigation (Rodriguez et al., 2003) 'Positive' external guidance cues promote actin filament polymerization, while 'negative' external guidance cues cause actin depolymerisation and reorganisation. The local balance of actin filament dynamics and organisation within a growth cone determines the direction of axon growth (Fan and Raper, 1995, Gallo and Letourneau, 2002). While much has been learnt about the identity of extracellular guidance cues and the corresponding receptors on the growth cone membrane, it is still unclear how activation of receptors regulates the cytoskeleton and subsequent growth cone motility.

1.2 Guidance cues mediate axon guidance

Axon outgrowth is controlled by an orchestrated effect on growth cones via attractive and repulsive guidance cues (Chilton, 2006). Guidance cues are proteins that bind to receptors on growth cone membranes and cause the active attraction or repulsion of developing axons (Chilton, 2006, O'Donnell et al., 2009). There are different types of guidance cues which can be categorised into at least four mechanistic groups: contact-mediated attractants, contact-mediated repellents, diffusible attractants (chemoattraction), and diffusible repellents (chemorepulsion) (Goodman, 1996, Flanagan, 2006, Kennedy et al., 2006). The focus of this thesis will be on diffusible guidance cues, as they were used for the majority of the experiments performed. Guidance cues are interpreted by growth cones, first by binding to their appropriate receptors on lamellipodial and filopodial membranes (Goodman, 1996, Gordon-Weeks, 2004, Gupton and Gertler, 2007, Tojima et al., 2011). When a growth cone moves through a guidance cue gradient, the side of the growth cone which faces a higher concentration of the cue displays higher receptor occupancy (Bouzigues et al., 2007, Tojima et al., 2011). The binding of receptors at the edges of growth cones will have more contribution to a growth cones turning response, than those that are located in the centre of the growth cone, due to the localised cytoskeletal events that determine the direction the growth cone becomes stabilised (Mortimer et al., 2009).

The formation of ligand-receptor complexes on the growth cone membrane triggers intracellular changes that initiate intracellular signalling cascades, ultimately affecting cytoskeletal dynamics that regulate growth cone motility, resulting in either growth cone attraction or repulsion. Guidance cue receptor activation initiates intracellular signalling through the generation of second messengers, in particular calcium and cyclic nucleotides (Hong et al., 2000, Henley and Poo, 2004, Li et al., 2005, Wang and Poo, 2005, Togashi et al., 2008b, Akiyama et al., 2009). Asymmetrically produced second messengers are thought to coordinate multiple cellular mechanisms, which include membrane trafficking, adhesion dynamics and cytoskeletal reorganisation, all with the purpose of executing bidirectional growth cone turning (Tojima et al., 2011).

Guidance cues have been described previously as belonging to one of two groups: calcium-dependent or calcium-independent guidance cues. Calcium dependent guidance cues include netrin-1, brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), acetylcholine (Ach), ephrins, and Myelin-associated glycoprotein (MAG). Calcium-independent guidance cues include semaphorin-3a (Sema-3a) and neurotrophin-3 (NT-3) (Song and Poo, 1999). Calcium-dependent guidance cues are classified such that the normal turning response is abolished by depletion of extracellular calcium. Conversely, calcium-independent guidance cues are classified by their turning response remaining unchanged after the depletion of extracellular calcium, that is, they signal independently of extracellular calcium. Calcium-dependency is determined by the reliance of extracellular calcium on a guidance cues downstream function. While calcium-independent guidance cues do not rely on extracellular calcium, they may utilise intracellular calcium to carry out

their function. Each member of these groups also share distinct cytoplasmic signalling pathways. Calcium-dependent guidance cues also co-activate phosphoinositide 3-kinase (PI3-K) and phospholipase C (PLC)- γ pathways (Ming et al., 1999), and the levels of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activity play a major role in determining the turning response (Tigyi et al., 1996, Cai et al., 1999, Song and Poo, 1999). If PKA is inhibited, the attractive turning responses of netrin-1, BDNF, NGF and Ach are converted to repulsion. If PKA is activated, the repulsive response of MAG is converted to attraction (reviewed in Song and Poo, 1999). In a similar manner, calcium-independent guidance cues are regulated by cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG). If PKG is activated, repulsion in response to Sema-3a is converted to attraction. Inhibition of PKG converts NT3 attraction into repulsion (Song et al., 1997, Ming et al., 1999).

While several families of diffusible guidance cues have been described, this review will focus on three examples from three different guidance cue families: netrin-1 (a netrin), BDNF (a neurotrophin) and Sema-3a (a semaphorin). These three guidance cues are utilised extensively in the experiments described throughout this thesis, and represent guidance cues from both the calcium-dependent and calcium-independent families of guidance cues.

1.2.1 Netrins

Netrins are a small family of proteins with conserved functions in axon guidance (Culotti and Merz, 1998). Netrins are secreted molecules that are related to laminin, which is associated with the extracellular matrix. Netrins can act as both chemoattractants and chemorepellents depending on receptor expression (Hedgecock et al., 1990, Hamelin et al., 1993). Netrin genes are highly conserved homologues of *Caenorhabditis elegans* Unc6 (Hedgecock et al., 1990, Ishii et al., 1992). Netrin receptors were originally discovered in *Caenorhabditis elegans* mutants with defects in circumferential guidance (Hedgecock et al., 1990). Unc5 is necessary for dorsal cell guidance, while Unc40 is necessary for ventral cell guidance (Hedgecock et al., 1990, Chan et al., 1996). The vertebrate homologue of Unc40, Deleted in Colorectal Cancer (DCC), functions as a netrin receptor (Keino-Masu et al., 1996). DCC (Unc40) is necessary for the mediation of attractive effects of netrin-1 (Keino-Masu et al., 1996). Unc5 (and its three mammalian homologues UNCH1, UNCH2 and UNCH3) are required for netrin-1 repulsion (Hamelin et al., 1993, Ackerman et al., 1997, Leonardo et al., 1997). In *Drosophila melanogaster* motor neurons, Unc5 alone mediates netrin-1 repulsion (Keleman and Dickson, 2001), but in *Xenopus laevis* (*Xenopus*) spinal neurons Unc5 and DCC associate in response to netrin-1 binding, to mediate repulsion (Hong et al., 2000). Bidirectional chemotactic responses of netrin-1 also depend on intracellular cAMP levels within the cells of interest (Ming et al., 1997). Netrin-1 attraction requires an optimal level of PKA activation, with low cAMP levels. A change in cAMP levels results in a switch from attraction to repulsion in *Xenopus* spinal neurons *in*

vitro (Ming et al., 1997, Wu et al., 2006). Downstream targets of netrin-1 signalling include Rho-GTPases (Causeret et al., 2004, Chang et al., 2004) and Enabled (Gitai et al., 2003) which play roles in cytoskeletal rearrangements within growth cones. Netrin-1 mediated attraction is calcium dependent, and relies upon the downstream signalling of calcium/calmodulin-dependent protein kinase II (CaMKII), mitogen-activated protein kinases (MAPK), calcineurin (CaN), as well as nuclear factor of activated T-cells (NFAT) for gene transcription, which is important for growth cone function (Hong et al., 2000, Graef et al., 2003, Tang and Kalil, 2005).

1.2.2 Neurotrophins

Neurotrophins are a family of proteins containing BDNF, NGF, NT-3 and neurotrophin-4/5 (NT-4/5). Neurotrophins are well characterised in developing neurons (Campenot and MacInnis, 2004). Neurotrophins signal through two receptor types: tyrosine kinase (TrK) receptors and the low-affinity p75 neurotrophin receptor (p75^{ntr}) (Keil et al.). The p75^{ntr} is common to all four neurotrophins (Rodriguez-Tebar and Barde, 1988), however different neurotrophins utilise different TrK receptors (Huang and Reichardt, 2001). NGF binds TrKA (Kaplan et al., 1991), BDNF and NT-4 bind TrKB (Chao, 2003) and NT-3 binds TrKC (Lamballe et al., 1991). TrK receptors act as a scaffold for the recruitment of signalling molecules, such as PLC (Vetter et al., 1991), PI3-K (Ming et al., 1999) and RhoA (Yamashita et al., 1999). TrK receptors are also required for the activation of many signalling pathways, including PI-3K and MAPK for neuronal survival (Vaillant et al., 1999) and RAS, for normal neuronal differentiation (Huang and Reichardt, 2001). BDNF

messenger RNA (mRNA) is found predominantly in the central nervous system (CNS) (Leibrock et al., 1989), and its expression is mediated by CREB and CaMKII. BDNF has multiple functions within the nervous system including neuronal survival, morphogenesis, synaptogenesis, and synaptic plasticity, but it also functions as a calcium-dependent chemoattractive guidance cue (Ming et al., 1997, Song et al., 1997). The binding of BDNF to TrKB results in the influx of extracellular calcium into the cytoplasm (Shieh and Ghosh, 1999, Jia et al., 2007), which can activate PLC- γ (Berninger et al., 1993). BDNF has growth promoting actions on filopodia, through signalling via RhoA (Gehler et al., 2004). BDNF can also be chemorepulsive when cAMP levels are low (Song et al., 1998).

1.2.3 Semaphorins

Semaphorins are a large family of both membrane-bound and secreted guidance molecules. Semaphorins are defined by the presence of a conserved ~420 amino acid sema domain at the N-terminus. Semaphorins are divided into eight classes based on their structure. Classes 1-2 are found in invertebrates, classes 3-7 reside in vertebrates, and class V are encoded by viruses (Raper, 2000). Semaphorins are required for the development of the nervous system, and act primarily as short-range inhibitory or repulsive guidance cues (Raper, 2000). Their role is to deflect axons away from inappropriate regions or targets, and to guide them through repulsive pathways (Raper, 2000, Cheng et al., 2001). There is evidence that some semaphorins may act as attractive guidance cues for specific types of axons (Wong et al., 1999, Raper, 2000). Class 3 semaphorins (3A-3G) are the most extensively studied in axon guidance. Class 3 semaphorins were originally identified as

molecules that caused growth cone collapse and were termed ‘collapsins’ (Luo et al., 1993, Luo et al., 1995). It was further found that localised application of these collapsing factors could also result in the turning of a growth cone away from the source of the collapsing factor (Fan and Raper, 1995). Since then, class 3 semaphorins have been shown to elicit repulsive activity on a myriad of neurons, including motor, sensory, olfactory and hippocampal neurons (Luo et al., 1995, Messersmith et al., 1995, Kobayashi et al., 1997, Koppel et al., 1997, Varela-Echavarría et al., 1997, Chedotal et al., 1998)

Semaphorins signal through multimeric receptor complexes. The composition of these receptor complexes is not fully known or understood. However, many semaphorin receptor complexes contain a plexin protein. Plexins are a large family of transmembrane proteins, divided into four groups (A-D) determined by sequence similarity (Tamagnone et al., 1999). Plexins act as semaphorin receptors (Comeau et al., 1998, Winberg et al., 1998). The vertebrate plexin-As are functional receptors for secreted class three semaphorins (Tamagnone et al., 1999). The multimeric receptor complexes for class 3 semaphorins also contain neuropilins (Npn) (Raper, 2000). The neuropilin family contains Npn1 and Npn2. Npn1 forms a homodimeric receptor complex for the binding and signalling of Sema-3a (He and Tessier-Lavigne, 1997, Kolodkin et al., 1997). To confirm the link between Sema-3a and Npn1, studies have been done on knockout mice, where those that lack Npn1 have similar axonal guidance defects as those mice that lack Sema-3a. These axonal guidance defects include defasciculation of nerve bundles, and aberrant projections from both sensory nerves and cranial motor nerves (Kitsukawa et al., 1997, Taniguchi et al., 1997). In addition, dorsal root ganglia growth cones from Npn1-deficient mice do not collapse in the

presence of Sema-3a (Kitsukawa et al., 1997). However, Npn1 does not have any signalling function. Npn1 plays a role in coupling Sema-3a and plexinA to contribute to ligand specificity and enhance the affinity of Sema3a for the receptor complex (Takahashi et al., 1999).

1.3 Two major intracellular second messengers mediate growth cone navigation

Guidance cues elicit their effects on growth cone navigation by activating intracellular second messengers. Two of the most important intracellular second messengers within growth cones are cyclic nucleotides and calcium. While there are other second messengers that are active in growth cones, this thesis will only focus on these two major pathways.

1.3.1 Cyclic nucleotides as second messengers

Cyclic nucleotides are second messengers that are known to be key regulators of growth cone motility, and therefore axon guidance (Song and Poo, 1999). Cyclic nucleotide levels are controlled by the enzymatic conversion of adenosine triphosphate (ATP) and guanosine triphosphate (GTP), to cAMP and cGMP, respectively (Chilton, 2006). Cyclic nucleotide signalling requires an optimal concentration range of nucleotides for normal function within growth cones (Kim and Wu, 1996, Song and Poo, 1999). Membrane permeable cAMP analogues cause attraction when applied in a gradient manner on growth cones (Lohof et al., 1992). As noted earlier, some guidance cues can be both attractive and

repulsive, depending on intracellular cAMP levels. Activation of intracellular cAMP on just one side of the growth cone triggers attraction in response to pituitary adenylate cyclase-activating polypeptide, MAG and netrin-1 (Guirland et al., 2003, Murray et al., 2009). Inhibition of PKA, a downstream effector of cAMP, converts both BDNF and netrin-1 turning responses from attraction to repulsion (Ming et al., 1997, Song et al., 1997). One way in which cAMP triggers growth cone attraction is through inhibition of CaN, which is an effector in the growth cone repulsion pathway (Wen et al., 2004). By inhibiting CaN, cAMP is preventing the activation of growth cone repulsion, shifting the growth cone turning response towards attraction.

There is a critical range in which cyclic nucleotides operate to effect growth cone motility. Calcium-dependent guidance cues act on cAMP levels. Higher cAMP levels are associated with attraction, while lower levels of cAMP are associated with repulsion (Ming et al., 1997, Song et al., 1998, Song and Poo, 1999, Shewan et al., 2002). Calcium-independent guidance cues act on cGMP levels, with higher cGMP levels resulting in growth cone attraction, while lower cGMP levels are associated with growth cone repulsion (Song et al., 1998, Song and Poo, 1999). Sema-3a signals via the downstream production of cGMP and is normally repulsive (Togashi et al., 2008b). However, when cGMP levels are artificially elevated Sema-3a repulsion is converted into growth cone attraction (Song et al., 1998). While this early work might seem conflicting, it suggested that there are optimum ranges for cyclic nucleotides to operate in. Although there appears to be a critical range for cyclic nucleotide signalling, it is thought that the absolute level of cyclic nucleotides is not what modulates growth cone turning behaviours, but rather the relative ratio of cyclic

nucleotides. It is the ratio of cAMP to cGMP that determines growth cone turning (Song et al., 1998, Song and Poo, 1999, McFarlane, 2000, Nishiyama et al., 2003). When the ratio of cAMP:cGMP was systematically varied, with the total concentration remaining at 10 μ M, turning responses to netrin-1 altered. High ratios of cAMP:cGMP were found to favour growth cone attraction, while low ratios of cAMP:cGMP favoured repulsion (Nishiyama et al., 2003).

The ratio of cAMP:cGMP is driven by reciprocal inhibition. Elevations in cAMP levels result in the reduction of cGMP, through cGMP-specific phosphodiesterase isozyme 5 (PDE5). In contrast, elevations in cGMP cause subsequent reductions in the levels of cAMP through cAMP-specific PDE4 (Shelly et al., 2010). The reciprocal inhibition of cAMP and cGMP operate within growth cones so that attractive guidance signals may reduce cGMP levels, and repulsive guidance cues may reduce cAMP levels. This reciprocal inhibition of cyclic nucleotides may contribute to amplification of guidance signals, due to an inhibition of the counteractive regulation of endoplasmic reticulum (ER) membrane calcium channels that are gated by cyclic nucleotides (Shelly et al., 2010). In addition, cyclic nucleotides have been shown to self-suppress through PDEs in cardiac cells (Zaccolo and Movsesian, 2007). This may be a way in which the amplification of guidance signals is limited.

1.3.2 Calcium as a second messenger

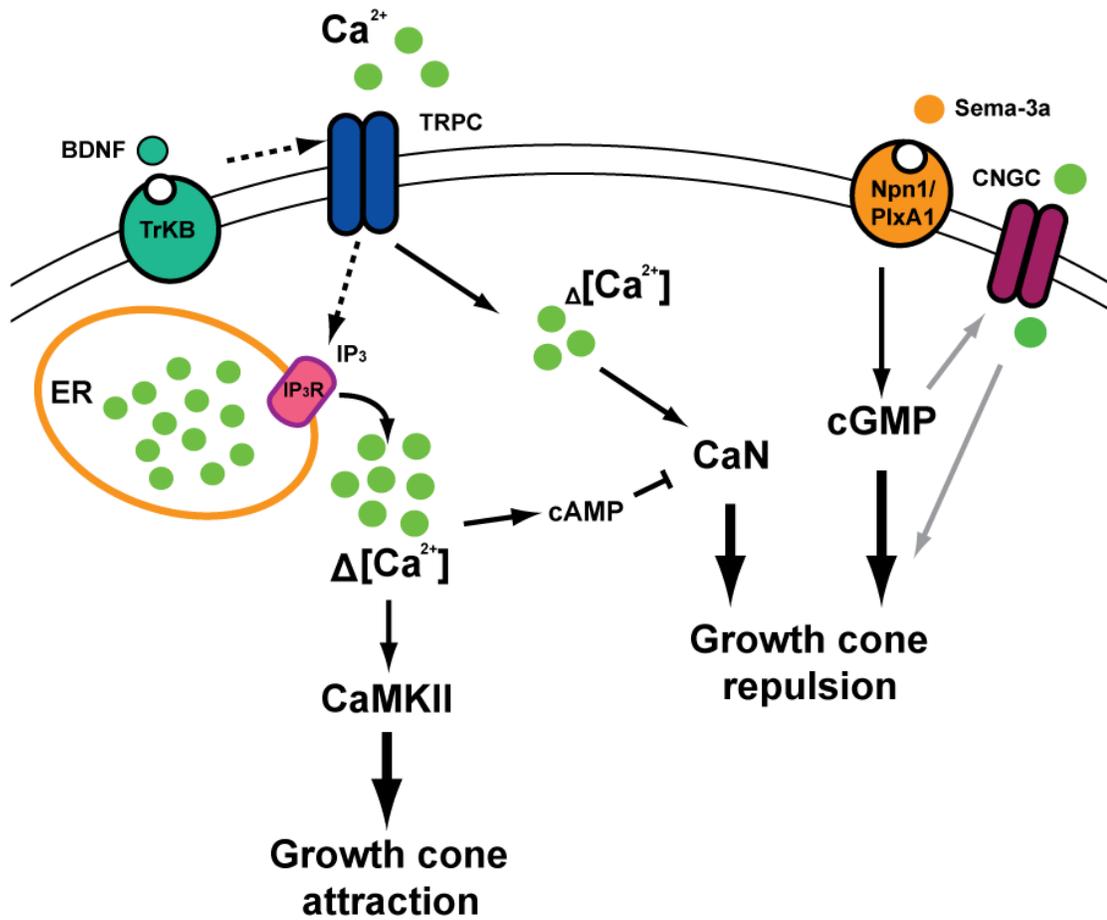
Calcium signalling does not only occur in combination or cross talk with cyclic nucleotide signalling. Calcium is a major intracellular second messenger in downstream signalling cascades within many cell types (Chilton, 2006). In developing neurons, calcium is particularly important in the transduction of guidance cue signals (Hong et al., 2000, Zheng, 2000), and in the regulation of growth cone motility and turning (Kater and Mills, 1991, Kater et al., 1994, Gomez and Spitzer, 1999, Song and Poo, 1999). Calcium signalling can activate both attractive and repulsive guidance (Fig. 1.2). Higher concentrations of calcium are observed on the side of the growth cone exposed to a source of soluble guidance cue, regardless of whether it is an attractant or a repellent (Zheng, 2000, Tojima et al., 2011). There are two major sources of calcium that are utilised for growth cone function. The first is the extracellular environment, which requires calcium influx across the plasma membrane (Fig. 1.2). The second are internal stores, in particular the ER (Fig. 1.2). Both extracellular and intracellular calcium stores are necessary for the turning of growth cones in response to most guidance cues.

Cytosolic calcium levels can be altered by either source of calcium; by the influx of calcium through membrane channels, or by the release of calcium from internal stores (Berridge, 1998). Extracellular calcium is required to initiate and sustain rises in intracellular calcium. If extracellular calcium is removed, the turning responses of neurons to ACh, BDNF, MAG and netrin-1 are abolished (Zheng et al., 1994, Ming et al., 1997, Song et al., 1997, Song et al., 1998).

Changes in the concentration of calcium can change the growth cone response to a guidance cue from attraction to repulsion. Netrin-1, which is normally an attractive cue, can induce a repulsive response when calcium influx is blocked, resulting in a reduction in cytosolic calcium signals (Hong et al., 2000). Fluctuations in basal resting calcium concentration in the growth cone can direct the growth cone turning response. In a landmark study in calcium signalling, focal laser-induced photolysis (FLIP) was utilised to uncage calcium from spatially restricted areas of *Xenopus* growth cones. Direct focal elevation of calcium induced growth cone attraction, when released in a locally defined area, even though no guidance cues were present. When the resting level of cytosolic calcium was decreased by removing calcium from the extracellular environment, local calcium release caused focal calcium elevations resulting in growth cone repulsion, instead

Figure 1.2 Intracellular second messenger signalling within growth cones

A simplified schematic of intracellular second messenger signalling within a growth cone. An example of a calcium-dependent guidance cue: BDNF binds to the TrKB receptor on the plasma membrane. This activates TRPC channels to open, allowing calcium (green circles) to influx. Calcium can activate the production of IP₃. IP₃ binds to the IP₃R on the ER membrane, to release calcium from the ER (calcium-induced calcium release). Released calcium results in a relatively large change in cytoplasmic calcium concentration, activating CaMKII, resulting in growth cone attraction. The initial rise of calcium can be small, resulting in a small change in cytoplasmic calcium concentration, activating CaN, resulting in growth cone repulsion. An example of a calcium-independent guidance cue: Sema-3a binds to Npn1/PlxA1 to activate the downstream production of cGMP. cGMP activates cyclic nucleotide gated channels (CNGC) to allow a very small influx of calcium into the cytoplasm. Both this calcium influx, and cGMP production result in growth cone repulsion.



of attraction (Zheng, 2000). This work clearly demonstrates that the resting concentration of intracellular calcium, and not only the absolute change in the focal elevation of calcium, is a determining factor in the nature of the growth cone turning response. Although it has been known for a long time that calcium is important in growth cone motility and turning responses (Cohan et al., 1987, Kater and Mills, 1991, Gomez et al., 1995), this study revealed that spatial and temporal characteristics of calcium, in addition to the amount of calcium present, are critical for bidirectional growth cone turning responses.

Intracellular calcium fluctuations can occur in a temporal manner within growth cones. They can be spontaneous or agonist-induced (Gu et al., 1994, Gomez et al., 1995, Gu and Spitzer, 1995, Williams et al., 1995, Tang et al., 2003), and can be either global or highly localised signals within growth cone filopodia (Gomez et al., 2001, Lohmann et al., 2005). Global elevations of calcium concentration can regulate neurite growth by causing growth cones to slow their rate of growth, stop, or retract (Lankford and Letourneau, 1989, Bandtlow et al., 1993), while decreased levels of intracellular calcium resulted in increases in neurite extension (Mattson and Kater, 1987). The extracellular application of neurotransmitters causes an increase in intracellular calcium concentration in growth cones in a global manner, inhibiting neurite growth (Haydon et al., 1984, Mattson et al., 1988). However, if a gradient of neurotransmitter such as glutamate (Zheng et al., 1996) or Ach (Zheng et al., 1994) is applied locally to a growth cone, the subsequent localised rise in calcium specifically induces growth cone attraction. Local elevations of calcium on one side of the growth cone can promote extension of filopodia (Zheng et al., 1994, Zheng et al., 1996), lamellipodia (Zheng et al., 1994, Zheng, 2000) and axon outgrowth (Goldberg,

1988), demonstrating highly spatial, localised calcium elevations are important for growth cone navigation.

Distinct calcium transients and localised signals are activated by specific calcium channels, which are located in both the plasma membrane, and the ER membrane, to gate both major sources of calcium (Bandtlow et al., 1993, Gomez et al., 1995, Tang et al., 2003). Bidirectional growth cone turning most likely relies on the gating of different sets of calcium channels located on the plasma membrane, such as the Transient Receptor Potential Canonical (TRPC) channels, L-type voltage-gated calcium channels (VGCC), and the calcium release-activated calcium (CRAC) channels (Orai proteins). Each of these calcium channels has the ability to trigger select spatial and temporal patterns of calcium signals, and hence growth cone turning (Berridge et al., 2003, Nishiyama et al., 2003, Li et al., 2005, Shim et al., 2005, Wang and Poo, 2005, Tojima et al., 2011, Mitchell et al., 2012).

Calcium signals can cause a switch from attraction to repulsion, depending on which downstream effectors are activated. Calcium has a limited ability to diffuse throughout the cytoplasm (Gabso et al., 1997). Due to this, it is assumed that calcium elevations are confined to small spatial vicinities, in close proximity to the calcium channels that the calcium entered through. However, the influx of calcium can be both amplified and extended from its location of entry (Tojima et al., 2011). These amplifications and extensions of calcium signals occur via a secondary rise in cytoplasmic calcium levels, by

release of calcium from the ER through IP₃Rs and RyRs (reviewed in Tojima et al., 2011). This process is termed calcium-induced calcium release (CICR), and it produces large elevations in intracellular calcium concentration (Hong et al., 2000, Jin et al., 2005, Akiyama et al., 2009). These increases in cytosolic calcium result in the activation of growth cone attraction. Conversely, when CICR is not activated, there is no secondary release of calcium, and relatively smaller elevations in calcium concentration occur (Henley and Poo, 2004, Ooashi et al., 2005). These smaller elevations in calcium concentration result in growth cone repulsion (Hong et al., 2000, McFarlane, 2000, Zheng, 2000). Higher or lower calcium concentrations result in differing growth cone behavioural responses, due to downstream calcium effectors that detect the different amplitudes of calcium (Fig. 1.2) (Wen et al., 2004, Gomez and Zheng, 2006). The two main calcium effectors are CaMKII and the calcium/calmodulin-dependent protein phosphatase, CaN (Song and Poo, 1999, Wen et al., 2004, Tojima et al., 2011). Calmodulin is a calcium binding protein that senses intracellular calcium (Clapham, 1995, Ghosh and Greenberg, 1995). When calcium is bound to calmodulin, the complex associates with kinases and phosphatases to act as downstream calcium effectors (Wen et al., 2004, Gomez and Zheng, 2006). CaMKII and CaN have differing affinities for calcium. CaMKII has a lower affinity for calcium, while CaN has a higher affinity for calcium (Rusnak and Mertz, 2000, Hudmon and Schulman, 2002, Wen et al., 2004). This means when there are relatively smaller elevations in calcium, CaN is preferentially bound by calcium, and when there are higher elevations in calcium, CaMKII is bound (Fig. 1.2). These two calcium effectors have very different growth cone motility responses. When CaMKII is bound and activated, it activates growth cone attraction (Wen et al., 2004). When CaN is bound and activated repulsion ensues (Fig.

1.2). The higher amplitude of calcium is sufficient to cause attractive turning on the side of the growth cone with these calcium signals, such as when calcium is released focally on one side of the growth cone (Zheng, 2000). It has also been hypothesised that the calcium effectors are localised to different compartments of the cell. CaMKII is thought to be localised within close proximity of the ER calcium channels, RyRs and IP₃Rs (Tojima et al., 2011), which is consistent with the idea that calcium influx across the plasma membrane can be further amplified both spatially and temporally by a secondary release of calcium from the internal stores, resulting in attraction (Takei et al., 1998, Song and Poo, 1999, Tojima et al., 2011). Growth cone repulsion occurs when there are lower amplitude calcium signals, caused only by calcium influx through plasma membrane bound channels, with no additional calcium release from the ER (Henley and Poo, 2004, Ooashi et al., 2005, Tojima et al., 2011). CaN has been hypothesised to exist in a diffuse manner throughout the cytosol, in areas where it is more likely to bind to calcium as it has just entered the cytosol via membrane bound channels (Tojima et al., 2011). These initial and secondary increases in cytoplasmic calcium concentrations are crucial for bidirectional growth cone turning, in response to guidance cues.

1.3.3 Second messenger cross talk

While cyclic nucleotides and calcium act as second messengers within growth cones, they do not do this independently of each other. There is a lot of cross talk between cyclic nucleotides and calcium. Cyclic nucleotides act as a switch for growth cone turning responses, by switching attraction into repulsion, through the modulation of calcium

channel activities (Nishiyama et al., 2003). Sema-3a signalling stimulates the production of cGMP on the side of the growth cone facing the Sema-3a source. This production of cGMP leads to a very small influx of calcium via cyclic nucleotide gated calcium channels on the plasma membrane, stimulating the calcium-related pattern of growth cone repulsion to occur (Togashi et al., 2008b). Cyclic nucleotides and their direct effectors PKA and PKG counteractively regulate calcium release from the ER via ER membrane bound calcium channels (Ooashi et al., 2005, Akiyama et al., 2009, Tojima et al., 2009). The gating of ER-bound calcium channels is modulated by the phosphorylation of PKA and PKG (Zalk et al., 2007, Foskett, 2010, Tojima et al., 2011). cAMP is associated with the activation of both inositol trisphosphate (IP₃) receptors (IP₃R) and ryanodine receptors (RyR) on the ER membrane, and facilitates calcium mobilisation, resulting in growth cone attraction. cGMP inactivates RyRs, blocking calcium release from the ER (Foskett et al., 2007, Zalk et al., 2007). While cAMP is able to cause mobilisation and release of calcium from the ER, calcium can also stimulate the production of cAMP, in a positive feedback loop, amplifying the attractive signal and turning response (Willoughby and Cooper, 2007, Tojima et al., 2011). Calcium produces cAMP via its downstream target, adenylate cyclases (AC) (Xia and Storm, 1997, Song and Poo, 1999). AC1 and AC8 are both calcium-dependent ACs that are required for cAMP production (Willoughby and Cooper, 2007, Tojima et al., 2011).

1.4 Two major calcium sources in growth cones: extracellular and intracellular

There are many different calcium channels present within neurons. Some of these calcium channels are important for synaptic transmission, such as multiple types of VGCCs, and the glutamate, NMDA and AMPA receptors. However, this review will only focus on the main calcium channels that have been implicated within growth cones, and in particular growth cone motility and guidance.

Extracellular calcium enters the cell cytoplasm through a variety of calcium channels located on the plasma membrane (Bootman et al., 2001). These channels are grouped based on their activation mechanisms and include VGCCs, TRP channels and store-operated calcium channels (SOCCs) (Berridge et al., 2000, Bootman et al., 2001, Berridge et al., 2003). VGCCs are activated by the depolarisation of the plasma membrane and generate rapid calcium fluxes that can control fast cellular processes (Bootman et al., 2001). The main group of VGCCs that have been implicated in growth cone turning are the L-type VGCCs (Berridge et al., 2000). TRP channels are transiently activated in response to many stimuli, including temperature, mechanical stress and osmolality (Clapham 2003). TRPC channels are the most extensively examined within growth cone navigation. SOC channels on the plasma membrane are activated in response to depletion of intracellular calcium stores (Bootman et al., 2001). For many years, the channels which operated as SOC channels, were unknown, with the number one candidate being the TRP channels. Orai proteins are now thought to be the main SOC channels. Calcium can also enter the cytoplasm when calcium-dependent guidance cues bind to their G protein coupled receptor

on the plasma membrane, activating associated ion channels. How intracellular calcium levels change in growth cones in response to guidance cues is an important question. Guidance cue-induced calcium entry into growth cones can occur via the VGCC, TRP channels and SOC channels. The following section will examine these major calcium sources in more detail.

1.4.1 Extracellular calcium source: voltage-gated calcium channels

Voltage-gated calcium channels are located on the plasma membrane and open and close due to changes in the membrane potential. In vertebrate sensory neurons, multiple calcium conductances were predicted based on voltage recordings (Llinas and Sugimori, 1980). These calcium conductances have since been assigned as multiple calcium channels, known as L-, T-, N- and P-type voltage-gated calcium channels (Fox et al., 1987, Bean, 1989, Llinas et al., 1989). There is evidence to show that these VGCCs co-exist throughout cell bodies, neurites, dendrites and growth cones of sensory neurons (Bolsover and Spector, 1986, Ross et al., 1986, Ross and Werman, 1987, Thayer et al., 1987, Lipscombe et al., 1988, Bean, 1989). VGCCs have been shown to function in neurite outgrowth, as well as the projection of growth cone processes (Goldberg, 1988, Silver et al., 1990). VGCCs also facilitate calcium entry into growth cones during axon guidance, in response to guidance cues (Nishiyama et al., 2003). In particular, L-type and N-type VGCCs have been shown to mediate a role in axon and growth cone motility. Calcium influx through N-type VGCCs is vital for the rate of migration of mouse cerebellar granule cells (Komuro and Rakic, 1992, 1996). Netrin-1-induced growth cone turning responses are thought to be dependent

upon both calcium influx through the L-type VGCCs on the plasma membrane (Hong et al., 2000). When L-type VGCCs are blocked with nimodipine, netrin-1 induced attraction is converted to repulsion in *Xenopus* spinal neurons (Hong et al., 2000). Netrin-1 has also been shown to induce an increase in the amplitude of L-type VGCC currents across the plasma membrane in turning growth cones (Nishiyama et al., 2003). Guidance cue receptor activation also stimulates L-type VGCCs on growth cones indirectly, causing calcium influx into the cytoplasm. Netrin-1 activates TRPC channels on growth cones, through binding of the DCC receptor. Activation of TRPC channels causes calcium influx into the cytoplasm, which in turn depolarises the growth cone membrane. Subsequently, L-type VGCCs are activated which results in a further calcium influx (Shim et al., 2009).

While these studies provide evidence that VGCCs, in particular L-type VGCCs, are located on growth cone membranes, and are functional in growth cone motility and axon guidance, there is still some conflict within the field. L-type VGCCs have only been implicated in the turning response of *Xenopus* spinal neurons (Hong et al., 2000, Nishiyama et al., 2003). When mammalian neuronal growth cones were treated with the L-type VGCC inhibitor nifedipine, there was no effect on growth cone turning, suggesting that L-type VGCCs are not necessary for mammalian growth cone turning (Li et al., 2005, Gasperini et al., 2009). However, both of these studies used a different calcium-dependent guidance cue, BDNF. This raises two questions: is the requirement of L-type VGCCs for guided growth cone turning a cell type or species specific effect, and is the requirement of L-type VGCCs dependent on the guidance cue? These questions will be examined in this thesis.

1.4.2 Extracellular calcium source: Transient Receptor Potential Channels

TRP channels are ion channels that are transiently activated in response to temperature, osmolality, mechanical stress and taste (Montell, 2001, Clapham, 2003). Generally, TRP channels are not selective to one ion. Mammalian TRP channel proteins form six-transmembrane cation permeable channels that are further divided into seven sub families. These are TRPC, TRPV, TRPM, TRPA, TRPP, TRPML, and TRPN and they are categorised based on their ligands and modes of activation, and their tissue distribution (Ramsey et al., 2006). TRP channels have been found to be present in neuronal growth cones, with the TRPC family (TRPC1-7) the most extensively studied in axon guidance. In *Xenopus* spinal neurons, TRPC1 functions in netrin-1, BDNF, and MAG mediated growth cone turning responses, but not Sema-3a responses (Shim et al., 2005, Wang and Poo, 2005). TRPC1, TRPC3 and TRPC6 are found in cerebellar granule neurons (Ramsey et al., 2006), and TRPC3 and TRPC6 are both involved in BDNF-mediated intracellular calcium elevations and chemoattraction in these neurons (Li et al., 2005). TRPC1 is also found in embryonic dorsal root ganglia (DRG) sensory neuronal growth cones (Gasperini et al., 2009). TRPC3 is temporally and spatially distributed in parallel to TrKB receptors in the brain (Li et al., 1999), and BDNF activation of TrKB generates a PLC-dependent calcium influx through TRPC3 channels (Li et al., 1999). TRPC4 is upregulated in DRG neurons after sciatic nerve transection injuries and is thought to contribute to axonal regeneration after nerve injury (Wu et al., 2008). TRPC5 is translocated to hippocampal growth cones, and modulates growth cone morphology, filopodial length and neurite extension, suggesting that TRPC channels may regulate some aspects of neurite outgrowth, as well as axonal pathfinding (Greka et al., 2003, Moran et al., 2004). These studies provide evidence

that members of the TRPC channel family are able to function as potential key mediators for calcium influx and axon guidance regulation during neuronal development. Different TRP channels may serve different distinct regulatory functions within growth cones, potentially by creating different spatio-temporal patterns of calcium influx (Ramsey et al., 2006).

The binding of guidance cues to their receptors on growth cone membranes are thought to activate TRPC channels indirectly, potentially via phosphatidylinositol 4,5-bisphosphate (PIP₂). Activation of G-protein coupled receptors and receptor tyrosine kinases causes the hydrolysis of PIP₂, via PLC, to generate diacylglycerol and IP₃. For example, when BDNF binds to its TrKB receptor, it activates PLC- γ , which can start the cascade of IP₃ signalling to result in the activation of TRPC3 and TRPC6 channels (Li et al., 2005). Diacylglycerol remains in the plasma membrane, and can activate TRPC3 and TRPC6 indirectly (Hofmann et al., 1999). When IP₃ is generated it binds to the IP₃R on the ER membrane. When bound, IP₃ can also interact with, and activate TRPC3 (Kiselyov et al., 1998). TRPC1 channels have also been shown to be coupled to IP₃R through direct interactions with the calcium signalling scaffold protein Homer (Tu et al., 1998, Yuan et al., 2003). The activation mechanisms of TRPC channels results in a short, sharp influx of calcium into the cytoplasm. This influx of calcium can cause both subsequent calcium release from stores, via the IP₃R, and membrane depolarisation, activating VGCCs, increasing calcium influx further (Hong et al., 2000, Li et al., 2005, Wang and Poo, 2005).

1.4.3 Intracellular calcium source: Calcium-Induced Calcium Release via IP₃ receptors and ryanodine receptors

Neuronal cytoplasmic calcium concentrations can increase from two main stores of calcium: the extracellular store and the intracellular store. The intracellular store of calcium resides within the ER lumen.

The neuronal ER is an elaborate continuous membrane system, which extends throughout the whole cell, including neuronal soma, axons and dendrites (Terasaki et al., 1994). The ER membrane forms subsurface cisternae that allow the ER membrane to reside 20-80nm away from the plasma membrane (Rosenbluth, 1962). Throughout the axon, the ER forms connecting tubules that run parallel to the axon, allowing for endoplasmic reticulum-plasma membrane (ER-PM) connections, and extension into neuronal synapses (Droz et al., 1975, Broadwell and Cataldo, 1984). The ER is also found to extend throughout the neuron, into dendrites, terminating in dendritic spines (Berridge, 1998). The neuronal ER acts as an internal calcium source, integrating calcium-related information from within the ER lumen, and the neuronal cytoplasm (reviewed in Berridge, 1998). IP₃Rs and RyRs are distributed along the entire length of the neuronal ER membrane. These receptors act as calcium channels to control the release of calcium from the ER (Henzi and MacDermott, 1992, Kostyuk and Verkhatsky, 1994). These receptors are activated either by IP₃, or calcium itself. This process of channel activation and calcium release is known as calcium-induced calcium release (CICR), and it allows the ER to play an active role in neuronal calcium signalling (Berridge, 1998).

The process of CICR is initiated by external cues binding to receptors on the neuronal membrane. The activation of two membrane receptor types, G-protein coupled receptors and tyrosine kinase receptors results in the production of IP₃ (Berridge, 1993, Berridge, 1998, Berridge et al., 2000). IP₃ is highly mobile within the cytoplasm, and diffuses quickly towards the IP₃R (Allbritton et al., 1992). IP₃ binds to the IP₃R on the ER membrane to activate calcium release. When IP₃ binds to the IP₃R, a conformational change occurs opening an integral calcium channel on the ER membrane (Bootman et al., 2001). This channel opening releases calcium from the high concentration in the ER, to the lower concentration in the cytoplasm (Bootman et al., 2001). The IP₃R is not only activated by IP₃. There are a variety of factors that the IP₃R is sensitive to. One of these is calcium itself. The most effective combination of IP₃R activation occurs when both IP₃ and calcium act together (Berridge, 1998). The binding of IP₃ increases receptor sensitivity to calcium. The receptor has a biphasic action where it activates at lower concentrations of calcium (0.5-1 μM), but it inhibits at higher concentrations of calcium (>1 μM), particularly after calcium releases from the ER, acting as a negative control mechanism (Bootman et al., 2001, Berridge et al., 2003).

The process of CICR from the ER store of calcium is critical for growth cone turning responses. There is substantial evidence implicating the activation of CICR within growth cones. The high amplitude change in calcium concentrations caused by CICR is necessary for neurite outgrowth (Takei et al., 1998). CICR through IP₃R and RyRs is also required

for calcium-dependent growth cone attraction (Hong et al., 2000, Jin et al., 2005, Li et al., 2005, Akiyama et al., 2009). Netrin-1-induced growth cone attraction was switched to repulsion upon inhibition of RyRs, and CICR in *Xenopus* spinal neurons (Hong et al., 2000). A gradient of NGF has been shown to elicit an asymmetric production of IP₃ across growth cones, with a higher concentration of IP₃ present on the side of the growth cone closest to the NGF source. This asymmetric production of IP₃ triggers localised CICR and growth cone attraction in chick DRG neurons (Akiyama et al., 2009). Low concentrations of ryanodine can also increase cytoplasmic calcium concentration through CICR to cause growth cone attraction in *Xenopus* spinal neurons (Jin et al., 2005). Furthermore, blocking IP₃Rs abolishes BDNF-induced attraction in rat cerebellar granule growth cones (Li et al., 2005). In repulsive guidance, RyRs are inhibited by the production of cGMP in response to repulsive guidance cues such as Sema-3a and netrin-1 (Nishiyama et al., 2003, Tojima et al., 2009). Taken together these data demonstrate that CICR is required for the augmentation and amplification of calcium signals in growth cone attraction, but it is not required for repulsion.

Calcium release from the ER is transient. It is deactivated within a few seconds due to the quick buffering of calcium within the cytoplasm. Calcium buffering is necessary in order to maintain a low resting level of calcium within the cytoplasm to improve signal:noise ratio. Many calcium-dependent processes within cells require a sustained rise in intracellular calcium concentration, making a secondary influx of calcium into the cell a crucial mechanism. Furthermore, the ER store of calcium is a finite store. Upon calcium release, the ER becomes depleted of calcium. However, the ER requires a full calcium store for

future calcium-related events to occur within the cell. ER stores of calcium are replenished by the activity of the calcium adenosine triphosphatase (ATPase) or sarco/endoplasmic reticulum (SERCA) pump, which refills emptied ER stores (Putney Jr, 2007, Smyth et al., 2008, Várnai et al., 2009). However, for the stores to be replenished, there needs to be a mechanism in place for calcium to re-enter the cytoplasm first. In addition, cells require a mechanism to be in place for the augmentation of the initial release of calcium from the ER. Electrophysiologists have been aware of such a mechanism since the 1980's, where depletion of intracellular calcium stores through IP₃Rs and RyRs causes an influx of extracellular calcium into cells (Putney Jr, 1986). The existence of this process, and the channels that allow for this to occur have been confirmed, however, the actual mechanism of action, and the proteins and channels involved have remained a mystery until the last decade.

1.5 Store-operated calcium entry

1.5.1 Discovery of store-operated calcium entry

The process by which cells are able to sustain calcium signals, and refill depleted calcium stores, is known as store-operated calcium entry (SOCE). Depletion of intracellular calcium stores induces the activation of a sustained inward calcium current (Hoth and Penner, 1992). It was previously thought that IP₃ was required for the activation of this calcium influx to replenish stores, until it was shown that it was the actual event of store depletion that triggered calcium influx (Putney Jr, 1986). This calcium influx was termed the calcium release-activated calcium (CRAC) current (I_{CRAC}), and was shown to be activated

independently of changes in membrane voltage (Hoth and Penner, 1992). It was postulated that this may be the mechanism by which cells can maintain raised intracellular calcium concentrations as well as replenish empty internal calcium stores (Hoth and Penner, 1992).

There were several theories proposed for the molecular mechanisms that controlled the activation of I_{CRAC} . The two main theories were: a diffusible second messenger released from the stores, referred to as Calcium Influx Factor (CIF), which signalled to unknown calcium channels on the membrane (Randriamampita and Tsien, 1993, Bolotina and Csutora, 2005); or, a conformational or direct coupling between proteins in the two membranes, the ER and plasma membrane (Franzini-Armstrong and Protasi, 1997, Patterson et al., 1999). No real evidence existed for the support of a diffusible second messenger as the mechanism for signalling from the stores to the plasma membrane. However, evidence began to build to generally support the theory of a physical coupling, a direct interaction, between the ER and plasma membrane, to activate store-operated channels (Venkatachalam et al., 2002). Centrifugation was employed to redistribute the organelles inside intact *Xenopus* oocytes into specific layers (Jaconi et al., 1997). Laser scanning confocal microscopy was used to uncage IP_3 to activate CICR. Calcium imaging revealed that calcium release was localised to the stratified ER layer of the oocytes. In addition, calcium entry was restricted to regions of the plasma membrane directly adjacent to the ER layer (Jaconi et al., 1997). This study concluded that calcium depletion from the ER stores, and calcium entry through the plasma membrane colocalised, suggesting that calcium entry only occurred in regions where the plasma membrane and the ER were closely apposed (Jaconi et al., 1997). In combination with studies that disrupted the

communication between calcium stores and the plasma membrane SOC channels, with the use of drugs that promote peripheral actin polymerisation (Patterson et al., 1999, Bakowski et al., 2001), these results led to the idea that a close membrane relationship must exist, potentially through protein-protein interaction. This led to a search for the proteins involved in the interaction that causes SOCE.

While most of the early work on SOCE was conducted in immune cells, eventually most cell types were shown to exhibit SOC influx through CRAC channels on their plasma membranes (Putney Jr, 2007), although it was thought that excitable cells, such as neurons, relied more on VGCCs rather than SOCCs (Park et al., 2010b, Wang et al., 2010a). *Drosophila* S2 cells, which lack contaminating currents from other cell types, display a SOC-selective current which shares many properties with the CRAC current in mammalian immune cells (Yeromin et al., 2004). In a screen of *Drosophila* S2 cells searching for the molecular components of CRAC channels, 170 candidate genes were identified as potentially functioning in SOCE, based on channel-like domains, transmembrane regions, calcium-binding domains and putative function in SOC influx. Using RNA interference (RNAi) one gene, *stim*, demonstrated a major reduction of SOCE when expression was suppressed in *Drosophila* S2 cells (Roos et al., 2005). Reduction in Stromal Interaction Molecule (STIM) 1, the mammalian homologue of *Drosophila stim*, in Jurkat T cells, HEK293 cells and SH-SY5Y cells also resulted in a significant decrease in SOCE (Roos et al., 2005). These studies revealed that STIM1 is required for SOC influx and CRAC channel activity. STIM1 is a key mediator of the influx of calcium in response to calcium store depletion.

STIM1 is a novel gene that was first cloned from human chromosome region 11p15.5 (Parker et al., 1996). STIM1 was previously designated GOK, and it was originally identified as a growth suppressor gene thought to be associated with tumour development (Parker et al., 1996). Chromosome region 11p15.5 contains genes that have been implicated in Beckwith-Wiedemann syndrome, Wilms tumour (nephroblastoma), rhabdomyosarcoma, rhabdoid tumour development, adrenal carcinoma, hepatoblastoma, bladder, breast, lung, ovarian and testicular cancers (Parker et al., 1996, Sabbioni et al., 1997, Manji et al., 2000). STIM1 protein originally appeared to have no obvious function, but it was found to be highly conserved in mouse and human, suggesting that it may have an important cellular function (Parker et al., 1996). Early studies showed that STIM1 expression was absent from rhabdomyosarcoma, even though it was present in high levels in normal skeletal muscle, where rhabdomyosarcoma originates. This downregulation of STIM1 led to the hypothesis that STIM1 was involved in tumour development (Sabbioni et al., 1997).

1.5.2 STIM1 is the key mediator of store-operated calcium entry

There are three well studied STIM proteins; STIM1, STIM2 and the *Drosophila melanogaster* D-Stim (Williams et al., 2001). The focus of this thesis is on STIM1. As such, the structure and function of mammalian STIM1 will be discussed in detail. STIM1 is a 90 kDa protein comprising 685 amino acids, which is widely expressed in many cell types including the nervous system of human and mouse (Manji et al., 2000, Dziadek and Johnstone, 2007). The protein structure of STIM1 provided important clues regarding its function.

STIM1 is a type 1 transmembrane protein that resides in the ER membrane (Putney, 2005, Zhang et al., 2005, Wu et al., 2006, Liao et al., 2007). STIM1 is also found in the plasma membrane, although its function there is unclear. STIM1 contains a single transmembrane segment that separates the N terminal (ER luminal) region from the C terminal cytoplasmic region (Williams et al., 2001, Dziadek and Johnstone, 2007). The N-terminus of STIM1 contains a signal peptide, cysteine residues and a single helix-loop-helix region, which conform to an EF-hand motif (Williams et al., 2001). This EF-hand motif binds calcium, allowing STIM1 to function as an ER luminal calcium sensor (Mercer et al., 2006, Stathopoulos et al., 2006, Dziadek and Johnstone, 2007). The EF hand has a low affinity for calcium, as well as a 1:1 binding stoichiometry (Stathopoulos et al., 2006). STIM1 binds calcium within the ER, when ER stores are full. The ER has a high resting calcium concentration, which is sensed by STIM1 due to its low affinity for calcium. STIM1-triggered calcium release from the ER results in calcium mobilisation. Mutations in the STIM1 EF-hand prevent the binding of calcium, effectively leaving STIM1 constantly active. STIM1 mutants behave as if the ER stores are constantly depleted of calcium (Liou et al., 2005, Spassova et al., 2006, Zhang et al., 2006, Dziadek and Johnstone, 2007). This demonstrates that the binding of calcium to the EF hand in STIM1 is sufficient to prevent the entire sequence of events that lead to SOCE.

STIM1 has been shown to exist in two states: monomers and oligomers. When ER calcium stores are full, STIM1 binds calcium through its EF-hand, and resides in a relatively uniform distribution (Liou et al., 2005), referred to as a diffuse distribution (Wu et al.,

2006) (Fig. 1.3). Within this state, STIM1 resides in the ER membrane as a monomer (Ji et al., 2008). The binding of calcium to the EF-hand motif is sufficient to keep CRAC channels in the plasma membrane closed (Zhang et al., 2005, Huang et al., 2006). Depletion of calcium stores within the ER, either through CICR or experimentally by thapsigargin treatment (a pharmacological agent known to deplete calcium stores), causes STIM1 to dimerise and translocate (Fig. 1.3). Redistributed STIM1 oligomerises within seconds into puncta that accumulate within areas of the ER membrane located close to the plasma membrane (Zhang et al., 2005, Baba et al., 2006, Luik et al., 2006, Wu et al., 2006, Liou et al., 2007, Muik et al., 2008). STIM1 oligomerisation is facilitated by the coiled-coil domains, ezrin/radixin/moesin (ERM) domain and sterile alpha motif (SAM) domain located in STIM1 (Williams et al., 2002, Baba et al., 2006, Huang et al., 2006, Luik et al., 2008). The coiled-coil/ERM domain mediates the aggregation of STIM1 dimers into puncta (Williams et al., 2002), and deletion of the SAM domain makes STIM1 incapable of forming puncta in response to ER store depletion (Baba et al., 2006). The coiled-coil domains reside within the ERM domain, which allows for the mediation of protein-protein interactions (Mangeat et al., 1999). STIM1 oligomerisation precedes and triggers the translocation of STIM1 near the plasma membrane in order to activate the CRAC channel (Fig. 1.3) (Liou et al., 2007, Luik et al., 2008, Muik et al., 2008).

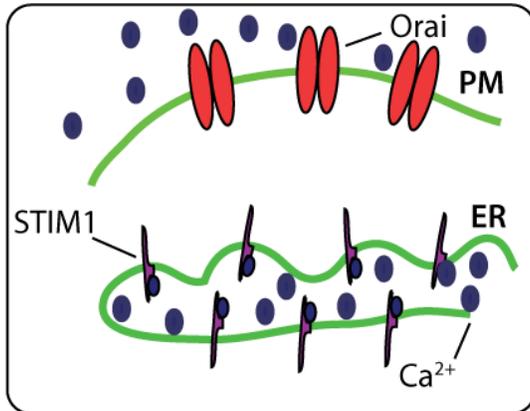
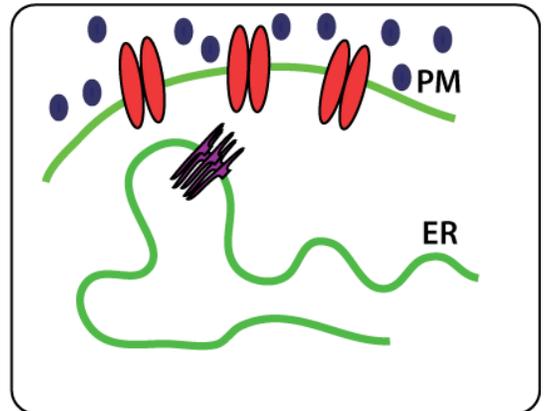
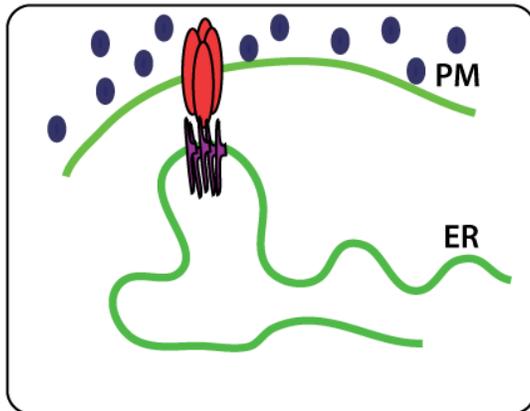
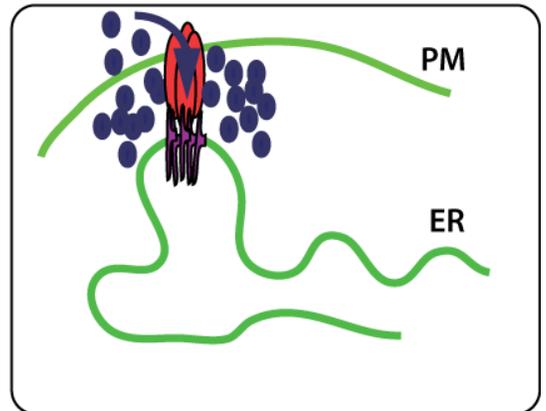
The STIM1 puncta that form after store depletion are translocated throughout the ER. STIM1 undergoes rapid, organised movement within the ER, due to the ability STIM1 has to bind to, and interact with a microtubule plus-end protein, EB1 (Baba et al., 2006, Smyth et al., 2007, Grigoriev et al., 2008). After translocation, STIM1 puncta are located

intracellularly near the plasma membrane (Wu et al., 2006). There was some suggestion that STIM1 puncta translocate and insert into the plasma membrane (Zhang et al., 2005). However, it has been extensively shown that STIM1 puncta do not insert into the plasma membrane (Mercer et al., 2006, Wu et al., 2006). STIM1 plasma membrane insertion is not a required step in the activation of the CRAC channel and SOCE, instead STIM1 puncta translocate close to the plasma membrane, but remain within the ER membrane (Spassova et al., 2006, Wu et al., 2006). STIM1 puncta form in areas of the ER that are located close to the plasma membrane, known as ER-PM junctions, or contact sites. It was found that upon store-depletion, junctional ER (ER tubules within 50 nm of the plasma membrane) increased in both length, and the number of contact sites with the plasma membrane (Wu et al., 2006), suggesting that the ER-PM junctions that STIM1 translocates to may be pre-existing or newly formed. In addition, there is evidence that STIM1 accumulation near the plasma membrane does not involve bulk movement of the ER (Wu et al., 2006). The distance between the ER and plasma membrane at these junction sites is 10-25 nm (Wu et al., 2006), which is close enough for a direct interaction to form between ER bound STIM1 puncta and plasma membrane CRAC channels. For years, evidence has existed that SOCE sites in the plasma membrane occur in areas where the ER is close by to the plasma membrane (Putney, 1990) and that SOC influx occurs in areas that have a high density of ER (Jaconi et al., 1997, Golovina, 2005). SOC influx via CRAC channels is highly concentrated in areas where STIM1 puncta and the ER closely appose the plasma membrane. SOCE does not occur globally throughout a cell, but is instead a localised cellular response, with calcium influx only occurring in areas where ER bound STIM1 puncta closely appose the plasma membrane (Luik et al., 2006).

STIM1 functions as the missing link between internal calcium store depletion, and SOCE (Zhang et al., 2005). Overexpression of STIM1 results in significant increases in the activity of CRAC channels (Spassova et al., 2006). Furthermore, CRAC channels open in direct proportion to the concentration of STIM1 within ER-PM junctions (Spassova et al., 2006, Luik et al., 2008). STIM1 contains a CRAC activation domain (CAD) on its cytoplasmic region. The CAD resides within the ERM domain. This CAD in STIM1 binds directly to CRAC channels, for the activation of SOCE (Park et al., 2009). STIM1 mutants missing the CAD reveal that this domain is required for the clustering and activation of CRAC channels (Park et al., 2009). This suggests that STIM1 acts as both an ER calcium sensor, as well as the CRAC channel activator. STIM1 oligomerisation is a cause of, rather than a consequence of calcium influx (Liou et al., 2005). STIM1 oligomerisation slightly precedes STIM1 redistribution (Luik et al., 2008), and STIM1 redistribution always precedes CRAC channel activation (Wu et al., 2006).

Figure 1.3. STIM1 and Orai form the CRAC channel

A. STIM1 (purple) is located predominantly in the ER membrane. STIM1 binds calcium (blue) and is located in a monomeric uniform distribution on the ER membrane. Orai (red) is expressed as dimers on the plasma membrane. **B.** Upon calcium depletion STIM1 is expressed as dimers on the plasma membrane. **C.** Upon calcium depletion STIM1 clusters on the ER membrane close to the plasma membrane. **D.** STIM1 clustering signals Orai dimers to form tetramers. **E.** STIM1 clusters bind to Orai tetramers to activate the CRAC channel. SOCE is activated and calcium enters into the cytoplasm. (Figure adapted from Soboloff et al., 2006a, Spassova et al., 2006).

A**B****C****D**

STIM1 oligomerisation is the primary event in the activation of the CRAC channel and subsequent SOCE. Once STIM1 oligomerisation occurs, all subsequent steps in the SOCE pathway occur (Luik et al., 2008). Although STIM1 activation triggers the SOCE pathway to occur, and it activates the CRAC channel, it does not form the CRAC channel itself. Following the success of RNA interference studies to discover STIM1 and its role in SOCE, similar studies were carried out to discover the molecular makeup of the CRAC channel. Members of two potential protein families have been proposed to interact with STIM1 and play a role in SOCE.

1.5.3 STIM2

STIM2 is the second *Stim* homologue, and it has high sequence homology with STIM1 (Williams et al., 2001). STIM2 also contains an N-terminal SAM domain and an EF hand, as well as a C-terminal ERM domain. STIM1 and STIM2 differ at both the C and N termini (Williams et al., 2001). The ER lumen N-terminus exhibits structural diversity to STIM1, which corresponds to a difference in function, that is, STIM2 is more sensitive to calcium than STIM1 (Zhou et al., 2009). STIM2 has the ability to detect very small changes within ER calcium concentration, while a larger change in calcium concentration is required for STIM1 activation. These observations led to the suggestion that STIM2 may function as a regulator of basal cytosolic and ER calcium (Brandman et al., 2007). However, the role of STIM2 remains controversial, with STIM2 being proposed as a powerful inhibitor of calcium entry through CRAC channels (Soboloff et al., 2006a). The localisation of STIM2 is also controversial, with STIM2 being reported within neurons, and even proposed as

being the major STIM homologue within neurons (Berna-Erro et al., 2009). However, recent data from our laboratory disputes this, revealing that although STIM2 is present within the nervous system, it does not localise to neurons (Hadrill, in preparation). Given this, STIM2 will not be discussed in detail within this review.

1.5.4 TRPC channels and their role in store-operated calcium entry

TRPC channels have long been thought to play a role in SOCE. TRPC channels appeared likely candidates to form SOC channels, as they are plasma membrane bound, allow for the influx of calcium, and interact with STIM1. Co-immunoprecipitation experiments in HEK293 cells suggested that the ERM domain of STIM1 binds to TRPC1, 2, and 4 (Huang et al., 2006). The role of TRPC channels in SOCE is a controversial topic. There is much evidence that supports the role of TRPC channels in SOCE (Zhu et al., 1996, Zitt et al., 1996, Kiselyov et al., 1998, Mori et al., 2002, Philipp et al., 2003, López et al., 2006). There have been reports of diminished SOCE following knockdown of TRPC (Mori et al., 2002, Parekh and Putney, 2005). However, many early results came from overexpression studies, and were often found to be unreproducible (Zhu et al., 1998, Trebak et al., 2003). Generally, calcium imaging studies have supported the role of TRPC channels in SOCE (Zhu et al., 1996, Mori et al., 2002), however, electrophysiological patch clamp experiments showed that TRPC channels do not recapitulate the biophysical properties of the CRAC current (Friel, 1996, Zitt et al., 1996, Schaefer et al., 2000). CRAC channels are known for their high calcium selectivity (Hoth and Penner, 1992), but TRPC channels form non-selective cation channels, which only have modest calcium selectivity (Zitt et al., 1997, Hurst et al., 1998). It is likely that TRPC channels can be activated in response to store-

depletion and STIM1 activation, playing some role in SOCE. However, most of the evidence would suggest that TRPC channels are not the classical plasma membrane component that forms the CRAC current.

1.5.5 Orai proteins are essential components of the calcium-release activated calcium channel

Orai1 was discovered in the search for the mutation in two patients with hereditary severe combined immunodeficiency (SCID) syndrome (Feske et al., 2006). SCID is a disorder of impaired SOCE and CRAC channel function in T lymphocytes (Feske et al., 2005). The CRAC current and SOCE are highly important for lymphocyte activation and immune defence. When defective, the cellular and humoral arm of the adaptive immune system is impaired. SCID patients have a marked propensity for viral, bacterial and fungal infections from the early months of age (Bortin and Rimm, 1977, Gelfand and Dosch, 1982). Orai1 was identified as being crucial for SOCE and CRAC channel function with the use of a combination of unbiased genetic approaches. A modified linkage analysis was carried out in the SCID patients to identify the gene mutation responsible for defective SOCE. A point mutation in human Orai1 was revealed to be responsible for the defect in calcium influx (Feske et al., 2006). Furthermore, a genome-wide RNAi screen was carried out in *Drosophila melanogaster* to identify regulators of SOCE. RNAi-mediated reduction in *Drosophila* Orai expression significantly reduced SOCE (Feske et al., 2006). In addition, overexpression of Orai1 restored SOCE in T cells derived from the SCID patients, however restoration did not occur with mutant Orai1 (Feske et al., 2006).

There are three mammalian homologues of *Drosophila* Orai; Orai1, Orai2 and Orai3 (Feske et al., 2006). Orai1 is the most extensively studied member of the Orai family (Peinelt et al., 2006, Prakriya et al., 2006, Vig et al., 2006, Yeromin et al., 2006, Zhang et al., 2006, Muik et al., 2008, Vig and Kinet, 2009). Orai1 is a small, four transmembrane domain protein, localised to the plasma membrane, with both termini in the cytosol (Feske et al., 2006, Vig et al., 2006, Zhang et al., 2006). Overexpression studies show that Orai1 alone does not increase the magnitude of the CRAC current (Peinelt et al., 2006, Vig et al., 2006, Zhang et al., 2006). However, when Orai1 is overexpressed in combination with STIM1, CRAC currents and SOCE are amplified up to 100-fold above baseline (Mercer et al., 2006, Peinelt et al., 2006, Soboloff et al., 2006b, Vig et al., 2006, Zhang et al., 2006, Hewavitharana et al., 2007), suggesting that Orai1 and STIM1 work in combination with each other to mediate CRAC currents. Interactions between STIM1 and Orai1 were established by co-immunoprecipitation (Soboloff et al., 2006b, Vig et al., 2006, Yeromin et al., 2006, Ong et al., 2007), and FRET studies revealed dynamic coupling of the two proteins before activation of calcium entry (Muik et al., 2008). Similar to the oligomerisation and redistribution of STIM1, Orai also translocates throughout the plasma membrane. When ER calcium stores are full and cells are resting, Orai1 is evenly distributed in a diffuse manner in the plasma membrane. In this resting state, Orai1 proteins form dimers (Fig. 1.3) (Gwack et al., 2007, Penna et al., 2008). Upon calcium store depletion, Orai1 is recruited to ER-PM junctions in areas where STIM1 clusters and forms puncta (Luik et al., 2006, Park et al., 2009, Yuan et al., 2009). Upon recruitment with STIM1, Orai proteins form the active pore of the CRAC channel in the plasma membrane, which consists of 4 Orai molecules forming a tetramer (Fig. 1.3) (Ji et al., 2008, Mignen et

al., 2008). Interactions between STIM1 and Orai1 are mediated by the CAD located on the cytosolic C terminus of STIM1, which directly interacts with both the N- and C-termini of Orai1 (Muik et al., 2008, Park et al., 2009). The STIM1 and Orai1 interaction can be reversed upon store-refilling, suggesting that this interaction only occurs after store-depletion, in order to activate CRAC channels and the CRAC current (Muik et al., 2008).

Orai2 and Orai3 also contain C-terminal coiled-coil domains, and it has been confirmed that both of these proteins also interact with STIM1, and function as SOCE channels (Mercer et al., 2006). Orai2 and Orai3 produce much smaller CRAC currents than Orai1 does and have a less powerful role in SOCE (Mercer et al., 2006, Frischauf et al., 2011). Orai2 and Orai3 overexpression studies reveal a much lower fold increase in SOCE compared to Orai1 overexpression. Although Orai2 and Orai3 are capable of SOCE, they are not the main Orai proteins involved, although they can compensate for Orai1 in SOCE if required (Gwack et al., 2007). These data suggest that Orai proteins, in particular Orai1, are an essential component of the CRAC channel, and are involved in the activation of the CRAC current. Orai1 has been shown to be the pore forming component of the CRAC channel (Prakriya et al., 2006, Vig et al., 2006, Yeromin et al., 2006), and its presence and function are crucial for SOCE.

1.6 Store-operated calcium entry in neurons

The regulation of intracellular calcium concentration is crucial to normal neuronal function. The concentration of intracellular calcium is the dominant regulator of a myriad of neuronal functions, which include differentiation, excitation, synaptic transmission, apoptosis, and gene expression (Ghosh and Greenberg, 1995, Paschen, 2001, Clapham, 2007). Neurons require a low basal concentration of calcium for a strong signal to noise ratio. Neurons also need to be able to sustain an increase in cytoplasmic calcium for the augmentation of calcium signals. It is thought that neurons harness SOCE in order to perform many of these critical calcium-related processes described above (Zufall et al., 2000, Emptage et al., 2001, Venkiteswaran and Hasan, 2009, Gemes et al., 2011).

Neuronal calcium levels fluctuate within an optimal range for normal growth and function. Calcium is very efficiently buffered within neurons, to keep the basal level of calcium low (Berridge et al., 2000, Berridge et al., 2003). Tightly controlled calcium homeostasis is required to keep neuronal calcium within an optimal range for normal growth and function. This is particularly important within the developing growth cones. If there is too much calcium, neuronal growth cones undergo collapse, where they withdraw their lamellipodia and filopodia. In contrast, if there is not enough calcium within growth cones, neurites fail to extend and develop (Kater and Mills, 1991). There is an optimal range of calcium required for normal growth cone function, and this optimal range is maintained by tightly controlled calcium homeostasis. The concentration of intracellular calcium is critical in axonal outgrowth and correct growth cone turning in response to calcium-dependent

guidance cues (Gomez and Zheng, 2006). In order to sustain this intracellular calcium level, growth cone stores are emptied. However, these stores are finite and it has been suggested that developing neurons and growth cones utilise the process of SOCE in order to direct and regulate growth cone motility during development (Zufall et al., 2000, Emptage et al., 2001, Li et al., 2005, Wang and Poo, 2005).

A voltage-independent calcium influx was previously recorded in neuroblastoma cell lines (Mathes and Thompson, 1994, Williams et al., 2001), NG108-15 cells (Takemura et al., 1991) and neurosecretory PC12 cells (Clementi et al., 1992). This voltage-independent calcium influx was activated upon intracellular calcium store depletion, and was unable to be blocked by VGCC inhibitors. This calcium influx has a strong similarity to the voltage-independent CRAC current described in non-excitable cells (Hoth and Penner, 1992). This early work in neuronal cell lines prompted the study of the CRAC current within primary cultures of neuronal cells. Multiple studies in different neuronal cell types, including hippocampal neurons, cortical neurons, olfactory receptor neurons and bag cell neurons, demonstrated the existence of this voltage-independent calcium entry from the extracellular space, activated by intracellular calcium store depletion (Arakawa et al., 2000, Bouron, 2000, Zufall et al., 2000, Emptage et al., 2001, Kachoei et al., 2006). Taken together these studies provide the physiological evidence for functional store-operated calcium entry in neurons; however, the molecules, pathways and mechanisms involved remained unknown.

Following the discovery of STIM1 and Orai1 within non-excitabile cells, and the demonstration of their role in SOCE (Roos et al., 2005, Feske et al., 2006), the presence of the STIM and Orai proteins was assessed throughout the nervous system. STIM1 and Orai1 expression was demonstrated in cell bodies and dendrites of pyramidal neurons, Purkinje neurons and granule cells within the cortex and hippocampus of the mouse brain (Klejman et al., 2009). These proteins were also shown to operate in a similar mechanism to non-excitabile cells, by forming co-localised puncta upon calcium store depletion with thapsigargin treatment, suggesting a functional role of STIM1 and Orai1 in neurons (Klejman et al., 2009). STIM1 expression was also demonstrated within both the adult and developing CNS and peripheral nervous system, within the cerebellum and dorsal root ganglia, particularly within axonal projections (Dziadek and Johnstone, 2007). Expression of STIM1, Orai1 and functional SOCE was confirmed in sensory neurons in a study of adult rat DRG neurons by immunocytochemistry, western blotting, calcium imaging, and electrophysiological recordings (Gemes et al., 2011). *Stim1* and *Stim2* mRNA was demonstrated to be present in cortical neurons (Gruszczynska-Biegala et al., 2011). In a study of the mouse brain, prominent protein expression of both STIM1 and STIM2 was found in the cerebrum. Both STIM1 and STIM2 were also present in the cerebellum and spinal cord, but to a lesser extent (Steinbeck et al., 2011). Within human tissue, STIM1 was demonstrated to be most prominently expressed within excitatory tissues of the central nervous system, with the highest expression within the cortex and the hippocampus (Steinbeck et al., 2011). STIM1 and STIM2 expression was confirmed within all sections of the brain, with STIM2 expression highest in the hippocampus (Skibinska-Kijek et al., 2009). STIM1 expression was also demonstrated to be present in neuronal dendrites, as

well as in a subset of spine synapses (Ng et al., 2011). STIM1 expression has also been shown to increase throughout development, with a stable expression within mature neurons (Keil et al., 2010).

Taken together, the data described here conclusively demonstrates the presence of the STIM and Orai proteins within the nervous system. Furthermore, STIM1 has not only been shown to be present in, but also functional within the nervous system. Blocking SOCE with Lanthanum attenuated spontaneous calcium transients within synaptic boutons, suggesting a role of SOCE in short-term plasticity (Emptage et al., 2001). Blocking SOCE with 2-APB (inhibitor of IP₃R (Maruyama et al., 1997), TRP channels (Xu et al., 2005, Togashi et al., 2008a) and SOC channels (Bootman et al., 2002)) and SKF-96365 (inhibitor of TRPC, SOCE, STIM1 and VGCCs) within hippocampal slices accelerated the decay of NMDA-induced calcium transients, and attenuated tetanus-induced dendritic calcium accumulation and long term potentiation (Baba et al., 2003). It is of interest to mention that 2-APB can both potentiate, as well as inhibit SOCE in a dose-response manner, where lower concentrations of 2-APB potentiate calcium entry, while higher concentrations inhibit calcium entry (Prakriya and Lewis, 2001). In addition, SOCE has also been reported to increase the frequency of miniature inhibitory postsynaptic potentials (Savic and Sciancalepore, 1998) and miniature excitatory postsynaptic potential (Emptage et al., 2001). These data all suggest a role of SOCE in the regulation of neuronal plasticity, although at present the role of SOCE in plasticity is unclear (Baba et al., 2003). The inhibition of SOCE within DRG sensory neurons increases neuronal excitability (Gemes et al., 2011), potentially through the 2-APB activation of TRPV channels (Chung et al., 2004),

while SOCE has been implicated in the modulation of the activity of neuronal networks, as inhibition of SOCE causes a profound reduction in activity (Steinbeck et al., 2011). SOCE is also present within flight neurons of *Drosophila melanogaster* (Agrawal et al., 2010). When Orai1 and STIM1 expression are reduced in *Drosophila melanogaster*, normal flight and flight-associated patterns of rhythmic firing of flight motor neurons is abolished (Venkiteswaran and Hasan, 2009). SOCE also functions in bag cell neurons of *Aplysia californica*, which are involved in reproductive behaviour (Kachoei et al., 2006). While these studies demonstrate that STIM1, and indeed SOCE, are functional within the nervous system, they do not provide any evidence of the function of SOCE within neuronal growth cones, where the regulation of calcium is crucial for normal motility and guidance throughout development.

Furthermore STIM1 has been shown to interact with other proteins and signalling pathways that could profoundly affect neuronal function. STIM1 has been shown to regulate a process known as store-operated cAMP signalling, within colonic epithelial cells (Lefkimmatis et al., 2009). While this study was not performed in neuronal cells, cyclic nucleotides play a vital role as neuronal second messengers, affecting neuronal function, and growth cone motility and guidance. STIM1 has also been shown to interact with, and inhibit Cav1.2 L-type VGCCs, in order to regulate SOCE (Park et al., 2010b, Wang et al., 2010b). L-type VGCCs are important in the regulation of extracellular calcium influx into neurons. If STIM1 were to mediate L-type VGCCs or cAMP signalling within neurons, and particularly within growth cones, this could have profound implications for neuronal function, and growth cone guidance.

1.6.1 Store-operated calcium entry in growth cones

Calcium-dependent guidance cues elicit rises in intracellular calcium within the growth cone (Song et al., 1997, Ming et al., 1999, Hong et al., 2000, Gasperini et al., 2009). Both growth cone turning responses, as well as increases in intracellular calcium continue as long as the guidance cue is present (Gasperini et al., 2009, Mitchell et al., 2012). These results suggest that there is a mechanism within growth cones that refills internal calcium stores to help sustain this rise in intracellular calcium concentration. While SOCE has been proposed to be functional in neuronal growth cones (Li et al., 2005, Wang and Poo, 2005), very little research has been done on the presence and function of SOCE within growth cones. Recently, endogenous STIM1 was demonstrated in DRG neuronal growth cones. STIM1 was shown to be located throughout the central domain of the growth cone, but to also extend in to the periphery and extending filopodial processes (Gasperini et al., 2009). Furthermore, overexpressed STIM1-GFP has also been found to localise and extend into growth cone filopodial extensions. STIM1 puncta also form at the tips of these growth cone extensions (Keil et al., 2010). These studies hint at the presence and localisation of STIM1 within neuronal growth cones.

1.6.2 Endoplasmic reticulum in growth cones

If STIM1 and Orai proteins are to function in SOCE within the growth cone, then the ER store of calcium would need to be present within growth cones. Calcium dynamics have been observed within active filopodia, but it is not known if these are mediated by the ER (Gomez et al., 2001). If calcium signalling, and indeed SOCE, is necessary for correct

growth cone functioning, it stands to reason that the ER must be a prominent member of the growth cone organelle family. The presence of ER within growth cones has been demonstrated using electron microscopy in fixed cells (Tennyson, 1970, Yamada et al., 1971, Bunge, 1973, Pouwels, 1978). The ER is orientated along the axis of axons, and continues in to the centre of the growth cone (Yamada et al., 1971), but also extends into the periphery of the growth cone (Deitch and Banker, 1993). Growth cone ER often forms interconnecting channels throughout the centre of the growth cone, and at the base of filopodia (Yamada et al., 1971, Landis, 1983). Filopodia often protrude from segments of the growth cone rich in ER (Kawana et al., 1971), and possess occasional elements of ER (Landis, 1983). ER dynamics were demonstrated in live cells with the use of a fluorescent dye, DiOC₆(3), which revealed that ER is located mostly at the base and central domain of the growth cone (Dailey and Bridgman, 1989). Several thin ER processes also extend within the peripheral domain, which sometimes extend into the filopodia (Dailey and Bridgman, 1989, Deitch and Banker, 1993). Peripheral ER processes undergo extension and retraction, along with the growth cone (Dailey and Bridgman, 1989). More recently, ER bound protein PTPB1 has been demonstrated to localise prominently in the central domain of growth cones, but it also extends dynamically into the peripheral region, including the filopodia (Fuentes and Arregui, 2009).

1.7 Hypothesis

While it has been shown that SOCE is necessary for the regulation of calcium within neurons, and indeed throughout the nervous system, there has been little work to examine the presence of STIM and Orai proteins within developing neuronal growth cones. Furthermore, the function of these proteins within growth cone navigation has never been examined. Given that growth cones are highly dynamic structures that rely heavily on temporal and spatially localised calcium signals, it follows that growth cones may utilise the process of SOCE for the regulation of calcium. If this were true, SOCE would play a vital role in growth cone turning, and indeed growth cone navigation throughout the development of the nervous system.

The central hypothesis of this thesis is that:

STIM1 is necessary for store-operated calcium entry in neuronal growth cones, and is required for correct axon guidance

1.7.1 Aims

To address the central hypothesis, a series of aims have been proposed to examine the role of STIM1 in SOCE, the regulation of L-type VGCCs, and cyclic nucleotide signalling within neuronal growth cones. These are to:

- 1. Confirm the presence and localisation of STIM1 and the Orai proteins within embryonic sensory neurons**

- 2. Determine whether STIM1 and Orai1 expression is spatially restricted within navigating growth cones**

- 3. Determine whether SOCE is functional within growth cones, and if it is mediated by STIM1 expression**

- 4. Determine whether STIM1 is required for correct growth cone guidance**

- 5. Determine if there is a functional interaction between STIM1 and L-type voltage-gated calcium channels in navigating growth cones**

- 6. Determine if STIM1 mediates cyclic nucleotide signalling during growth cone navigation**

The data presented throughout this thesis will aim to address each of the aims listed above, and to discuss the results within context of the previous literature, to determine if STIM1 is indeed necessary for SOCE within navigating growth cones.

Chapter 2:

**STIM1 and the Orai proteins are present in dorsal root
ganglia growth cones**

Chapter 2: STIM1 and the Orai proteins are present in dorsal root ganglia growth cones

2.1 Introduction

STIM1 is a crucial mediator of SOCE in many different cell types. Traditionally, much of the work on STIM1 and SOCE has been performed in non-neuronal cells, such as platelets and immune cells, providing most of the knowledge of STIM1 function. Recently, the study of the SOCE machinery, in particular STIM1 function, has begun to expand into the nervous system. It has been suggested that neurons harness SOCE for certain neuronal functions, including gene expression, synaptic transmission, and differentiation (Zufall et al., 2000, Emptage et al., 2001, Clapham, 2007, Venkiteswaran and Hasan, 2009, Gemes et al., 2011). Developing neurons require sustained intracellular calcium levels in order to regulate axonal outgrowth and growth cone turning, in response to calcium-dependent guidance cues (Gomez and Zheng, 2006). This leads to my hypothesis that growth cones require STIM1-mediated SOCE to sustain these rises in intracellular calcium, and replenish stores, in order to undertake these processes.

If SOCE is important in axon guidance, and particularly within growth cone turning, it follows that STIM1 and Orai proteins would need to be present within developing neuronal growth cones. In addition, expression of STIM1 and Orai proteins may be required within the periphery of growth cones where a response to guidance cues is initiated, and not just within the central region. Growth cones are known to turn towards the side of a growth

cone where calcium signals arise (Zheng, 2000). When caged calcium is released on one side of a growth cone, turning can be dictated by this release of calcium (Zheng, 2000). This suggests that calcium signals are spatially distributed throughout growth cones. As such, if SOCE is required at the site of turning within growth cones, STIM1 and Orai proteins would potentially need to be located on the leading edge of growth cones, within both lamellipodia and filopodia.

The structure of STIM1 is such that it contains domains that allow it to bind to, and cluster with itself, as well as other proteins (Williams et al., 2002, Baba et al., 2006, Huang et al., 2006). In non-neuronal cells, the distribution of STIM1 has been shown to change depending upon the internal calcium store status. When cells are at rest and internal calcium stores are full, STIM1 resides in a uniform, monomeric localisation throughout the ER membrane (Liou et al., 2005, Ji et al., 2008). In this form, STIM1 is bound to individual calcium ions and is inactive within the ER (Mercer et al., 2006, Dziadek and Johnstone, 2007). In this state, STIM1 expression appears as a diffuse pattern of protein expression (Liou et al., 2005, Soboloff et al., 2006b, Koh et al., 2009). As external signals activate store depletion, calcium is released from the ER, and STIM1 is depleted of calcium binding. This removal of calcium from STIM1 and the ER activates STIM1 to cluster together into oligomers, in areas of the ER membrane located close to the plasma membrane (Zhang et al., 2005, Baba et al., 2006, Luik et al., 2006, Wu et al., 2006, Liou et al., 2007). This pattern of STIM1 expression is described as punctate, where clusters of activated STIM1 are seen in areas of the cell close to the plasma membrane (Wu et al., 2006, Liou et al., 2007, Koh et al., 2009). Orai proteins are required for complete SOC

channel activation. The clustering and redistribution of STIM1 is the signal required for Orai to translocate along the plasma membrane into tetramers, forming the CRAC channel (Liou et al., 2007, Luik et al., 2008, Muik et al., 2008, Clapham, 2009) and activating SOCE, in a highly dynamic process. If STIM1 and Orai proteins are important for growth cone function and navigation, it would be expected that these proteins would display these dynamic distribution patterns as have been described within non-neuronal cells, and that they would be located on the growth cone leading edge.

In the work described in this chapter, thapsigargin was used to activate STIM1 puncta formation, and hence SOCE in growth cones. The discovery of thapsigargin as an agent to deplete ER calcium stores allowed for the concept of SOCE to be studied in detail, using electrophysiology and calcium imaging techniques (Putney Jr, 2007). Acute treatment of thapsigargin inhibits the SERCA pump on the ER membrane. Calcium constantly leaks out of the ER into the cytoplasm, and is constantly pumped back into the ER via the SERCA pump (Clapham, 2007). By blocking the SERCA pump with thapsigargin, the ER is effectively depleted of calcium (Takemura et al., 1989, Thastrup et al., 1990, Foskett and Wong, 1992, Gomez et al., 1995). Thapsigargin induced depletion of ER calcium activates STIM1 oligomerisation, and the activation of SOCE through the CRAC channel (Liou et al., 2005, Zhang et al., 2005, Huang et al., 2006, Mercer et al., 2006, Wu et al., 2006, Dietrich et al., 2007, Dziadek and Johnstone, 2007, Liou et al., 2007, Putney Jr, 2007).

The aim of this chapter was to examine the expression and localisation of STIM1 and two of its binding partners, Orai1 and Orai2 within cultured embryonic sensory neuronal growth cones. In order to determine whether STIM1, Orai1 and Orai2 were functional within the embryonic peripheral nervous system, depletion of ER calcium was employed by the use of thapsigargin. Given the dynamic nature of growth cones and SOCE, this study also sought to determine if SOCE machinery translocated throughout the growth cone to areas requiring increased intracellular calcium.

2.2 Materials and Methods

2.2.1 Ethical declaration

All animal procedures were approved by the Animal Ethics Committee of the University of Tasmania (Ethics number A0012322) and are consistent with the Australian Code of Practice for the Care and Use of animals for Scientific Purposes.

2.2.2 Materials

All materials used throughout this PhD were of analytical reagent grade.

STIM1 antibody and EGTA were purchased from Sigma-Aldrich (Missouri, USA). Orai1 antibody and peptide, Orai2 antibody and peptide and thapsigargin were purchased from Alomone Labs (Jerusalem, Israel). Alexa fluor 488 Phalloidin was purchased from Invitrogen, Life Technologies (California, USA). BDNF and semaphorin-3a were purchased from R+D Systems (Minneapolis, Minnesota, USA). Control, Orai1 and Orai2 siRNA were purchased from ThermoFisher Scientific (Massachusetts, USA).

2.2.3 SDS-PAGE and western blotting

Whole dorsal root ganglia (DRG) and cortex were dissected from E16-18 Sprague-Dawley rat embryos, lysed and triturated in radioimmunoprecipitation assay (RIPA) buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM phenylmethylsulphonyl fluoride (Sigma-Aldrich), 1mM ethylenediaminetetraacetic acid (EDTA), 5µg/ml leupeptin (Sigma-Aldrich), 5µg/ml aprotinin (Sigma-Aldrich), 1% NP-40 (Sigma-Aldrich), 1% sodium deoxycholate (Sigma-Aldrich), 0.1% sodium dodecyl sulfate (SDS)). Proteins were separated on 8% polyacrylamide-SDS gels, transferred onto 0.2µm PVDF membranes, and blocked overnight in 5% skim milk. Membranes were incubated with primary antibodies (STIM1, 1:1000; Orai1, 1:1000; and Orai2, 1:200), followed by HRP-conjugated secondary antibodies (1:5000 dilution). Antibody conjugates were detected using chemiluminescent substrate (Millipore) and imaged on a gel documentation system (Chemismart, Vilber-Lourmat).

2.2.4 Embryonic dorsal root ganglia (DRG) cell culture

Thoracic DRG were dissected from E16-18 embryos obtained from pregnant Sprague Dawley rats euthanized by CO₂ inhalation. DRGs were mechanically triturated, and cultured for 4-6 hr in sensory neuron medium (SNM) comprising Dulbecco's Modified Eagle's Medium/Hams F-12 medium 1:1 (Invitrogen), fetal calf serum (5% v/v), penicillin G (100 U/ml), streptomycin (100 µg/ml), nerve growth factor (50 ng/ml; Sigma-Aldrich) and N2 neural medium supplement (Invitrogen).

2.2.5 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) at 4°C, followed by permeabilisation and blocking with 0.4% Triton X-100 (Sigma-Aldrich) and 5% goat-serum (Sigma-Aldrich). Primary antibodies for STIM1 (1:1000; Sigma-Aldrich), Orai1 (1:400; Cell Signaling Technology) and Orai2 (1:300; Alamone Labs) were detected using Alexa Fluor 488/594 secondary antibodies (Invitrogen). Images were acquired with an Olympus BX50 microscope, cooled CCD camera (Photometrics) and software (NIS Elements, Nikon).

2.2.6 Orai1 and Orai2 antibody specificity

To assess antibody cross reactivity, Orai1 and Orai2 antibodies were preabsorbed with their respective peptides (Alomone) at a ratio of 1:1 (w/w) for 24 hours at 4°C. The preabsorbed antibody was applied instead of the primary antibody in both immunocytochemistry and western blotting protocols. The same has previously been done for the STIM1 antibody (Gasparini et al., 2009).

2.2.7 Analysis and Quantification of STIM1, Orai1 and Orai2 protein distribution

STIM1 and Orai1/2 protein distribution were determined by thresholding 8-bit images in ImageJ (NIH). Background was subtracted, and images were thresholded using the RenyiEntropy option, using pixel intensity. The lower level was set to 20 and the upper

level to 255. This threshold level was applied to all images across multiple experiments. The resultant images fell in to two main categories; the first, a speckled pattern where isolated pixels were visible with clear background occurring between them (punctate), and the second, a confluent pattern with no pixel isolation or background visible (diffuse). Simple counts were carried out on the resultant images, determining if images were punctate or diffuse. These were counted over 10 experiments, and percentages of diffuse or punctate were determined and analysed using unpaired t-tests (Graph Pad Prism).

2.2.8 Calcium depletion immunocytochemistry

DRG cultures were incubated for 4-6 hours in SNM. Calcium was depleted from the media by replacing the SNM with calcium and magnesium free Hank's Buffered Salt Solution (HBSS) containing nerve growth factor (50ng/ml), N2 neural supplement, penicillin G (100µg/ml), streptomycin (100µg/ml), ethylene glycol tetraacetic acid (EGTA; 300µM) and thapsigargin (50nM, Alomone). Cells were incubated in calcium free media for 5 min before fixation with 4% paraformaldehyde. Cells were subsequently processed for STIM1, Orai1 and Orai2 immunocytochemistry. All isolated growth cones on each coverslip were imaged. Identical threshold values were applied to images as described above in section 2.2.7, using ImageJ (NIH). Percentages of punctate versus diffuse growth cones were determined for STIM1, Orai1 and Orai2 in control conditions (SNM) and calcium depleted conditions, and analysed using unpaired t-tests (Graph Pad Prism).

For STIM1 and Orai1 puncta colocalisation experiments, colocalisation was demonstrated using pixel-by-pixel multiplication of STIM1 and Orai1 binary images. Numbers of puncta per growth cone were normalized for growth cone area and compared between control and EGTA/thapsigargin treated cultures. All image analyses were performed using ImageJ, and statistically analysed using unpaired t-tests (Graph Pad Prism).

2.2.9 Knockdown and quantification of endogenous Orai1 and Orai2 protein expression

Endogenous Orai1 or Orai2 expression was reduced with the addition of either Orai1 siRNA (5nM) or Orai2 siRNA (5nM) (Millennium Science, Dharmacon, Thermo Fisher Scientific, MA, USA). Orai1 or Orai2 siRNA were loaded during the whole DRG trituration step in the culture process (refer to section 2.2). DRG neurons were cultured as described in section 2.2, and left to grow for at least 6 hours, to allow the siRNA to take effect. Control, off-target siRNA (Thermo Fisher Scientific) were used at 5nM. Orai1 and Orai2 protein knockdown was confirmed with immunocytochemistry, and quantified with integrated pixel intensity in ImageJ. Quantification of Orai immunocytochemistry was used to assess protein expression after siRNA treatment in individual growth cones. In order to minimise bias, growth cones were selected by viewing the alternative antibody. Selection was based on isolation from other growth cones and axons. Image acquisition parameters were adjusted to avoid pixel saturation, and identical exposures were used for control and Orai1/2 siRNA treated cells. Integrated pixel intensities of Orai1/2 were measured for each

growth cone and normalised for growth cone area. Statistical analyses performed were unpaired t-tests (Graph Pad Prism).

2.2.10 Asymmetric protein distribution analysis

To determine the distribution of STIM1 and/or Orail during growth cone turning, cells were fixed with 4% paraformaldehyde after 15 min exposure to guidance cues, and processed for immunocytochemistry. Protein expression was assessed by comparing the near and far sides of the growth cone, with respect to the micropipette position. The growth cone was divided into 2 halves (see schematic, Fig. 2.10 C) based upon the final orientation of the axon. The integrated pixel intensity of each half was measured and normalised to area, to derive a pixel intensity near/far ratio using ImageJ software, as described by others (Leung et al 2006). Both axon extension and growth cone turning angles were analysed in ImageJ, to confirm turning responses to the guidance cues. Statistical analyses performed were ANOVA with post-hoc Tukey (Graph Pad Prism).

2.3 Results

2.3.1 STIM1, Orai1 and Orai2 proteins are present in DRG sensory neuronal growth cones

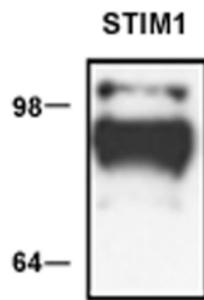
This study sought to determine whether endogenous expression of the SOCE proteins STIM1, Orai1 and Orai2 correlated with the status of internal calcium stores and growth cone motility within embryonic DRG sensory neurons. STIM1 and Orai1 have been demonstrated in DRG neurons previously, although their expression has not been described in detail (Gasperini et al., 2009, Gemes et al., 2011). Western blotting analysis was used to confirm STIM1, Orai1 and Orai2 expression in DRG neurons (Fig. 2.1 A-C). The western blots show staining at the expected bands for STIM1, Orai1 and Orai2. Orai3 was not examined due to unreliable results obtained with several Orai3 antibodies (data not shown). Rat cortex was included as a positive control for both Orai1 and Orai2 staining (Fig. 2.1 B and C). This study confirmed previous work (Gasperini et al., 2009, Gemes et al., 2011) demonstrating the presence and location of STIM1 and Orai1 in DRG, and also suggests that Orai2 is present within DRG of the embryonic peripheral nervous system.

While there is a large amount of western blot data within the literature demonstrating that STIM1 and Orai proteins are found within neurons, the subcellular location of these proteins has not been examined in detail within growth cones. Hence, it is not known whether these proteins reside in the growth cone in the same distribution patterns as seen in non-neuronal cells. Using immunocytochemistry, STIM1-immunoreactivity (IR) was

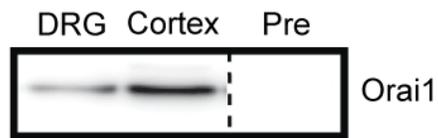
Figure 2.1. STIM1, Orai1 and Orai2 are present in whole DRG

A. Western blot analysis shows STIM1 present in DRG. A band is shown at approximately 90 kDa, the expected size of STIM1. **B.** Western blot analysis shows Orai1 present in DRG. Rat cortex is shown as a positive control (cortex). Orai1 preabsorbed antibody control is shown (pre). **C.** Western blot analysis shows Orai2 present in DRG. Rat cortex is shown as a positive control (cortex). Orai2 preabsorbed antibody control is shown (pre). *Figure 2.1 A was included in my honours thesis, Mitchell 2009 and was published in Gasperini et al., 2009. Figure 2.1 B and C were obtained during this PhD, and were published as part of figure 1 in Mitchell et al., 2012.*

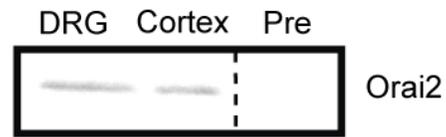
A



B



C



detected in DRG growth cones, as were Orai1 and Orai2 (Fig. 2.2). Further analysis revealed two distinct STIM1 expression patterns within growth cones. The first was a diffuse pattern, with little apparent discrete puncta. This pattern is similar to previous studies in non-neuronal cells showing STIM1 expression in resting cells with full internal calcium stores (Liou et al., 2005) (Fig. 2.2 A and C). The second pattern was highly punctate, with discrete aggregated puncta and some evident colocalisation. This pattern is similar to activated immune cells where internal calcium stores had been depleted (Liou et al., 2005) (Fig. 2.2 B and D). The pattern of Orai1-IR (Fig. 2.2 A and B) and Orai2-IR (Fig. 2.2 C and D) closely resembled that of STIM1-IR. When the pattern of STIM1-IR was punctate, so was the pattern of Orai1 and Orai2 expression (Figs 2.2 B and D). Similarly, when the expression pattern of STIM1-IR was diffuse, so was the pattern of Orai1 and Orai2 (Figs 2.2 A and C). Preadsorption with Orai antibodies abolished Orai1 and Orai2 immunoreactivity (Fig. 2.2 E and F). No immunoreactivity was observed in the 'no primary antibody' controls (Fig. 2.2 G).

Quantitative analysis of cultures was done using a thresholding algorithm in ImageJ. This revealed that $52.0 \pm 2.4\%$ of randomly growing, isolated growth cones stained for STIM1, $51.1 \pm 2.8\%$ of growth cones stained for Orai1 and $53.0 \pm 3.9\%$ of growth cones stained for Orai2 displayed the diffuse staining pattern (Fig. 2.3). Similarly, $48.0 \pm 2.4\%$ of growth cones stained for STIM1, $48.9 \pm 2.8\%$ of growth cones stained for Orai1 and $47.0 \pm 3.9\%$ of growth cones stained for Orai2 displayed the punctate staining pattern (Fig. 2.3). The pattern of STIM1 and Orai1/Orai2-IR also correlated with growth cone morphology. The majority (94%) of growth cones displaying diffuse STIM1 or Orai immunoreactivity were

Figure 2.2. STIM1, Orai1 and Orai2 are present in growth cones in diffuse or punctate expression patterns.

A-D. Representative growth cones probed for STIM1 (green) and Orai1 or Orai2 (red). Immunoreactivity expression patterns for STIM1, Orai1 and Orai2 were classed as either diffuse, with little apparent discrete puncta (**A and C**); or punctate, with discrete, aggregated puncta, and some co-localisation evident (**B and D**). Arrowheads show STIM1-only or Orai1/2-only puncta. Arrows show co-localised puncta. Threshold images illustrate the use of ImageJ to classify immunoreactivity patterns as either diffuse or punctate. **E.** Growth cone stained with preabsorbed Orai1 antibody control, with phalloidin staining (green) to outline growth cone. **F.** Growth cone stained with preabsorbed Orai2 antibody control, with phalloidin staining (green) to outline growth cone. **G.** No primary antibody control with both Alexa Fluor-594 and 488 secondary antibodies. Scale bar is 10 μ m and applies to all images. . *Growth cone B was included in my honours thesis, Mitchell 2009. The thresholding analysis was all done as part of this PhD work. Growth cones B, C and D were published as part of figure 1 in Mitchell et al. 2012.*

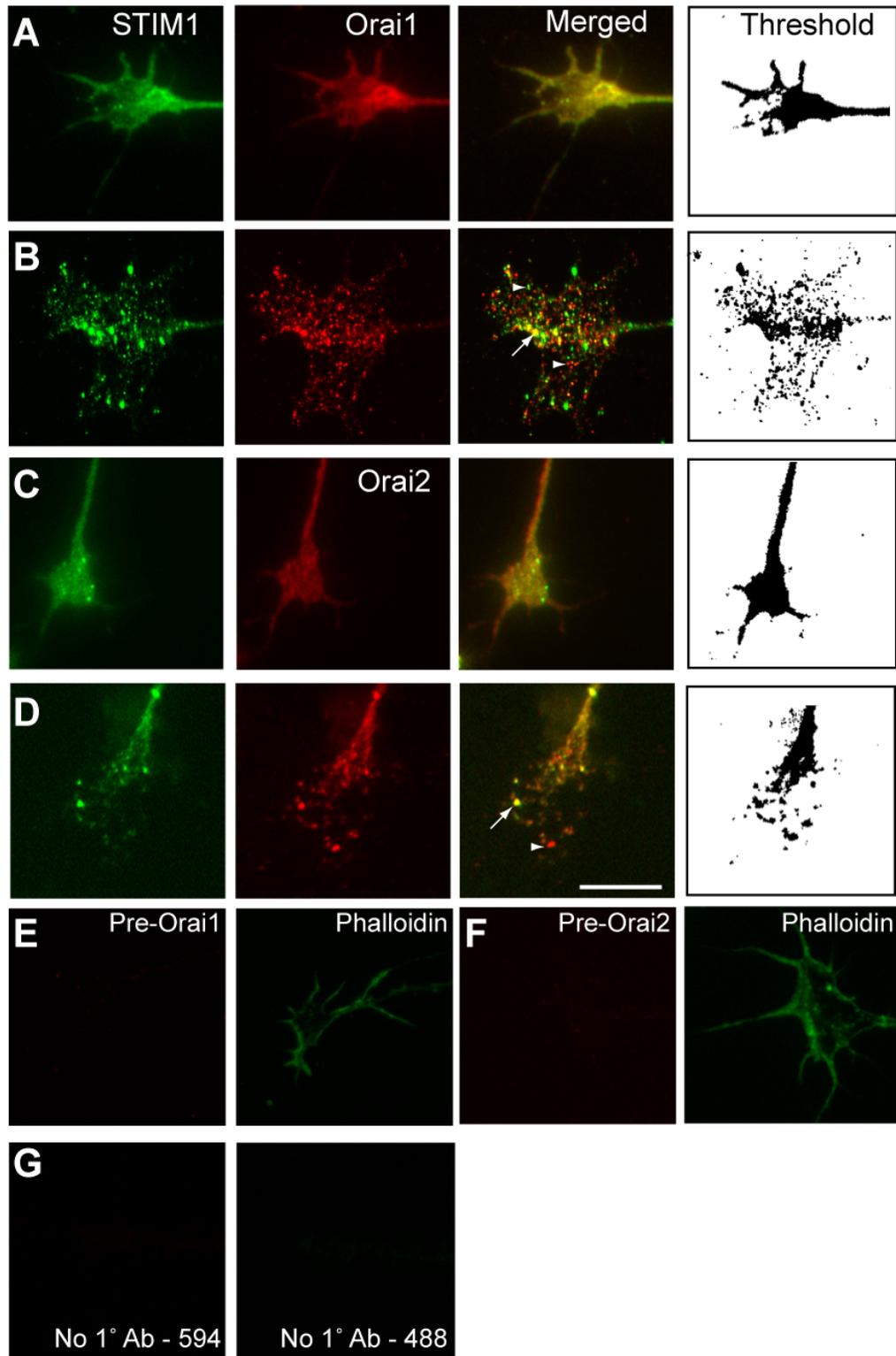
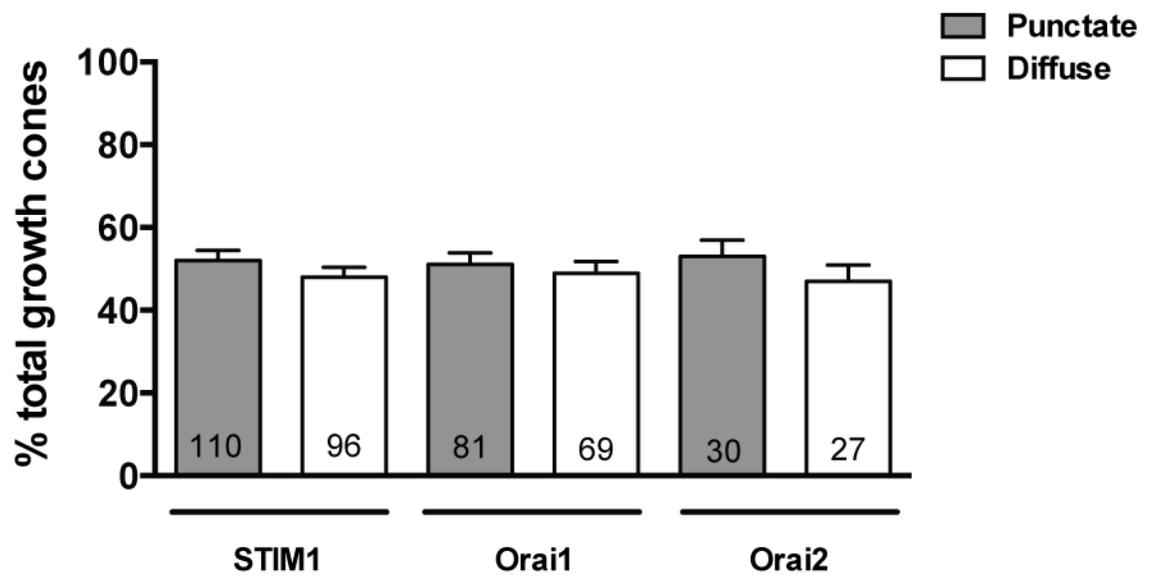


Figure 2.3. Quantification of STIM1, Orai1 and Orai2 immunoreactivity expression patterns, in randomly growing growth cones

Quantification of growth cones displaying either punctate or diffuse patterns of STIM1, Orai1 or Orai2 expression. The percentage of growth cones displaying punctate immunoreactivity patterns are shown in grey. The percentage of growth cones displaying diffuse immunoreactivity patterns are shown in white. Y-axis is the percentage of the total number of growth cones. The total number of growth cones counted per group are displayed in bars on graph (STIM1 n=206, Orai1 n=150, Orai2 n=57). Growth cones counted over 10 experiments. Error bars represent SEM and were determined per experiment.



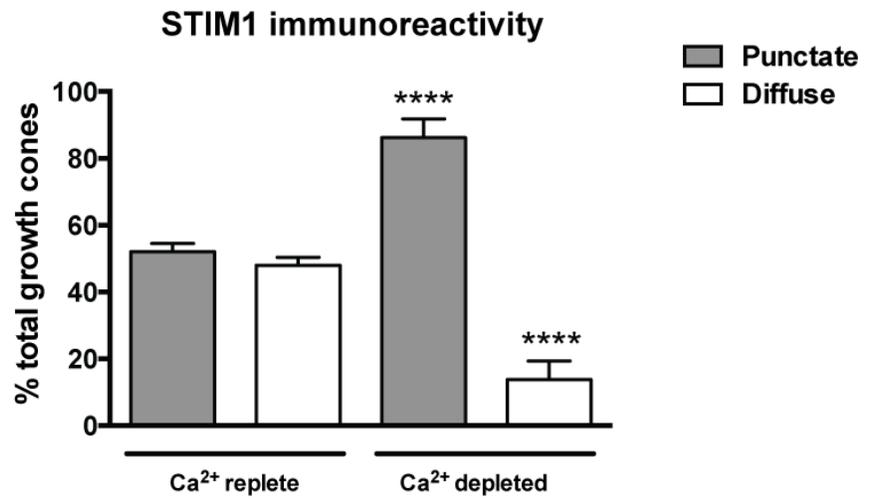
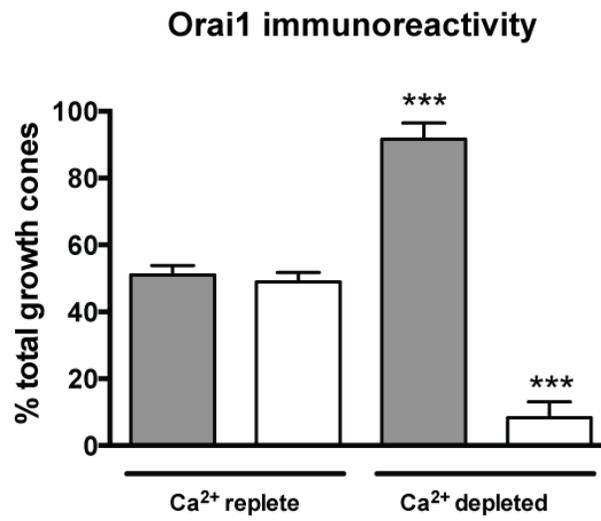
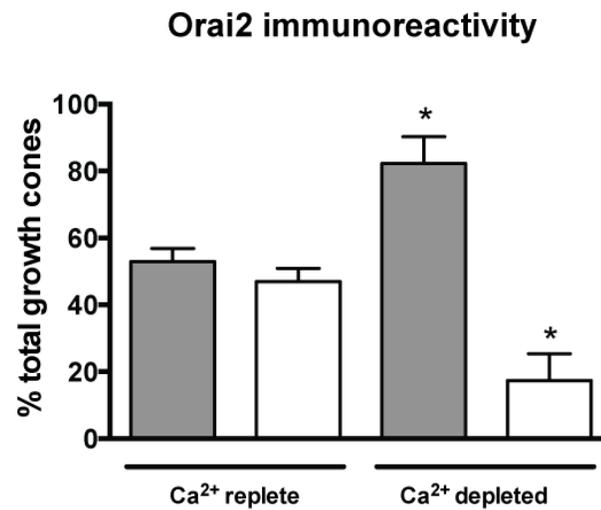
narrow, non-spreading, streamlined growth cones (Fig. 2.2 A, C). These growth cones tended to be smaller in area, ranging from 6 - 78 μm^2 . Conversely, growth cones displaying punctate STIM1 and Orai1/2 immunoreactivity exhibited spreading lamellipodia (Fig. 2.2 B and D) and tended to be larger in area, ranging between 33 - 247 μm^2 . The morphology of growth cones displaying a punctate pattern of STIM1/Orai immunoreactivity was reminiscent of growth cones pausing at decision regions (Tosney and Landmesser, 1985). Such growth cones have increased frequency of calcium transients (Gomez and Spitzer, 2000), which may require the activation of SOCE.

2.3.2 Depletion of calcium stores stimulates STIM1, Orai1/2 puncta formation, and STIM1-Orai1 co-localisation in growth cones.

In order to determine if the STIM1 immunoreactivity patterns observed above correspond to calcium store status, DRG cultures were treated with calcium-free media containing the calcium chelator EGTA, and the internal calcium store depletor thapsigargin. In randomly growing growth cones, store depletion significantly induced punctate expression patterns for STIM1 ($86.2 \pm 5.6\%$; $p < 0.0001$), Orai1 ($91.6 \pm 4.8\%$; $p < 0.001$) and Orai2 ($82.3 \pm 7.9\%$; $p < 0.05$) (Fig. 2.4), compared to calcium replete growth cones where punctate expression patterns for STIM1 ($52.0 \pm 2.4\%$), Orai ($51.1 \pm 2.8\%$) and Orai2 ($53.0 \pm 3.9\%$) were significantly lower (Fig. 2.4). In order to examine if there was an increase in co-localisation of STIM1 and Orai1 after thapsigargin treatment, the individual number of STIM1 and Orai1 puncta per growth cone were measured (Fig. 2.5 A and B). In a manner similar to an increase in total growth cones displaying punctate

Figure 2.4. STIM1-, Orai1- and Orai2-immunoreactive punctate growth cones increase after calcium store depletion.

Quantification of the expression patterns (punctate or diffuse) of STIM1, Orai1 or Orai2 in growth cones before and after treatment with thapsigargin and calcium free media. The percentage of growth cones displaying punctate immunoreactivity patterns are shown in grey, those with a diffuse immunoreactivity pattern are shown in white. **A.** Growth cones labelled for STIM1-IR in a calcium replete state (n=105) and in a calcium depleted state (n=54), over 5 experiments. **B.** Growth cones labelled for Orai1-IR in a calcium replete state (n=70) and in a calcium depleted state (n=26), over 4 experiments. **C.** Growth cones labelled for Orai2-IR in a calcium replete state (n=45) and in a calcium depleted state (n=28), over 3 experiments. Error bars represent SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Unpaired t-test.

A**B****C**

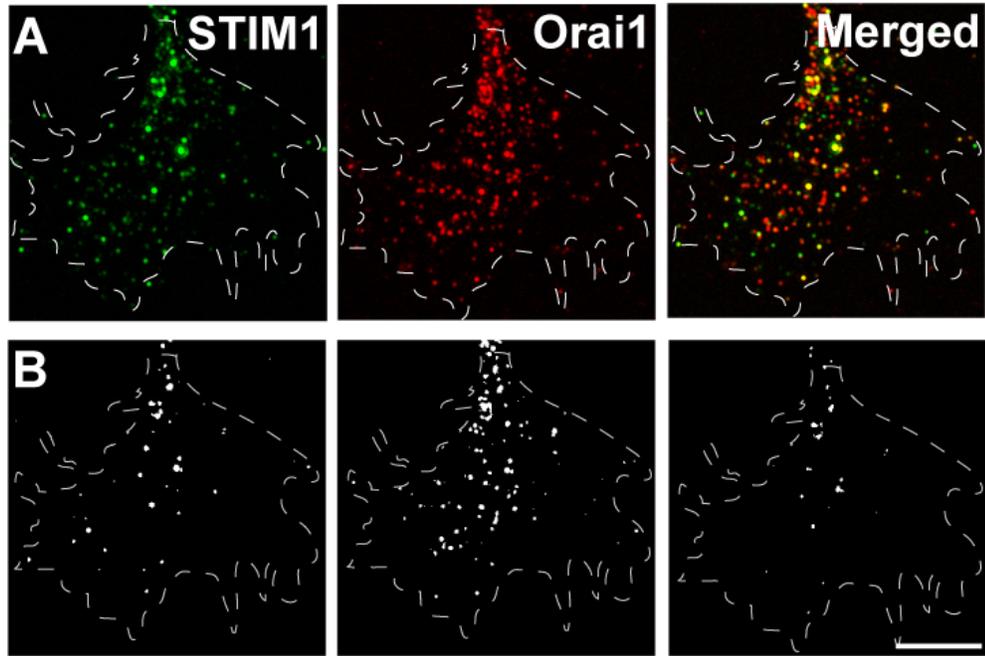
expression of protein after calcium depletion, the numbers of STIM1 and Orai1 puncta per growth cone area increased significantly after thapsigargin treatment when compared to vehicle control (Fig. 2.5 C; STIM1 $p < 0.05$, Orai1 $p < 0.01$). Additionally, there was also an increase in the number of co-localised puncta of STIM1 and Orai1 puncta after calcium depletion (Fig. 2.5 C; $p < 0.05$). The y-axis of Fig. 2.5 C represents the number of puncta per growth cone area (μm), so the numbers shown within the graph appear to be small, and are not representative of the whole growth cone. Consistent with these data, an increase in punctate STIM1 and Orai distribution has been previously demonstrated in non-neuronal cells after thapsigargin treatment (Liou et al., 2005, Soboloff et al., 2006b) suggesting that depletion of internal calcium stores activates STIM1- and Orai1/2-mediated SOCE within growth cones.

2.3.3 Orai1 is required for STIM1 and Orai2 oligomerisation upon calcium depletion.

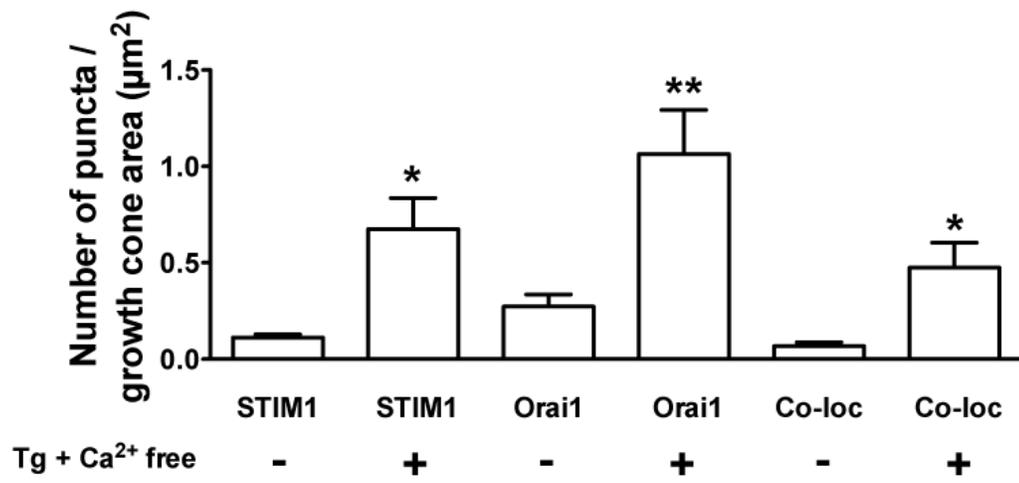
STIM1 is known to be the key mediator of SOCE. STIM1 activation is thought to be the primary event in the activation of SOCE, and precedes STIM1 oligomerisation and subsequent Orai activation (Wu et al., 2006). Previous work from our laboratory has demonstrated that upon the reduction of endogenous STIM1 levels, Orai1 and Orai2 puncta fail to form upon thapsigargin treatment, due to the lack of the primary STIM1 signal (Ainslie, Honours thesis, 2012). Reducing the endogenous levels of either Orai1 or Orai2 was hypothesised to have no effect on the primary event of SOCE, STIM1 activation and oligomerisation.

Figure 2.5. Aggregation and co-localisation of STIM1- and Orai1-immunoreactive puncta increases after calcium store depletion.

A. A representative growth cone immunolabelled for STIM1 (green) and Orai1 (red), after calcium store depletion with calcium free media and thapsigargin. **B.** Aggregated immunoreactive STIM1 and Orai1 puncta were quantified after thresholding for puncta size (ImageJ). **C.** The number of STIM1-, Orai1-immunoreactive and co-localised (co-loc) puncta per area of growth cone (μm^2) were quantified (from images as shown in B) in calcium replete (n=19) and calcium depleted (n=6) conditions (calcium free media and thapsigargin (Tg)) over 4 experiments. Scale bar equals 10 μm . Error bars represent SEM. Significance is compared between wildtype and treated. Dotted line shows outline of growth cone. * p < 0.05, ** p < 0.01. Unpaired t-test. *This figure was published as figure 2 in Mitchell et al., 2012.*



C



Specific Orai siRNA was utilised to reduce the expression of Orai proteins in growth cones, to assess the requirement of Orai proteins on STIM1 and Orai protein oligomerisation in response to calcium store depletion. Orai1 expression was targeted with 5nM Orai1 siRNA (Fig. 2.6 A), a concentration that does not show significant reduction in protein expression by immunocytochemistry. Work from our laboratory has shown that at 10nM a reduction in protein expression can be visualised, however even at the low concentration of 5nM, a functional phenotype was observed within growth cones (Ainslie, Honours thesis, 2012). Treatment with Orai1 siRNA also had no effect on STIM1 expression levels within growth cones (Fig. 2.6 B), but it did significantly increase the levels of Orai2 within growth cones by 2-fold (Fig. 2.6 C, $p < 0.01$), suggesting a built in redundancy of Orai2 within growth cones.

To determine if Orai1 was required for puncta formation of STIM1, and hence SOCE, calcium depletion was carried out on Orai siRNA treated growth cones. The depletion of calcium from the media caused a large increase in the number of growth cones displaying STIM1 punctate expression when treated with control siRNA ($86.2 \pm 5.6\%$, $p < 0.0001$; Fig. 2.7A) but only a small increase when treated with Orai1 siRNA ($66.7 \pm 4.2\%$, $p < 0.05$; Fig. 2.7A). The depletion of calcium from the media increased the number of control siRNA treated growth cones displaying Orai2 puncta ($82.3 \pm 13.8\%$, $p < 0.05$; Fig 2.7 B), however this did not occur upon reduction in Orai1 expression ($65.3 \pm 9.2\%$, $p > 0.05$; Fig. 2.7 B). These data suggest that Orai1 expression may be involved in the oligomerisation of Orai2 upon calcium depletion. These data suggest that Orai1 may be involved within the activation and oligomerisation of both STIM1 and Orai2 upon ER calcium depletion.

Figure 2.6. Orai1 siRNA reduces the endogenous level of Orai1, increases the endogenous level of Orai2, and has no effect on the expression of STIM1

A. Staining for Orai1-IR (red) reveals no apparent decrease in Orai1-IR after 6 hours Orai1 siRNA treatment. Quantification of Orai1-IR in control (n=71) and Orai1 (n=35) siRNA treated growth cones, over 4 experiments. **B.** Staining for STIM1-IR (green) reveals no decrease in STIM1-IR after 6 hours Orai1 siRNA treatment. Quantification of STIM1-IR in control (n=117) and Orai1 (n=80) siRNA treated growth cones, over 6 experiments. **C.** Staining for Orai2-IR (red) reveals a 2-fold increase in Orai2-IR after 6 hours Orai1 siRNA treatment. Quantification of Orai2-IR in control (n=46) and Orai1 (n=46) siRNA treated growth cones, over 4 experiments. Scale bar = 10 μ m and applies to all growth cones. The periphery of the growth cones are outlined with dashed lines. Error bars represent SEM. ** p<0.01. Unpaired t-test.

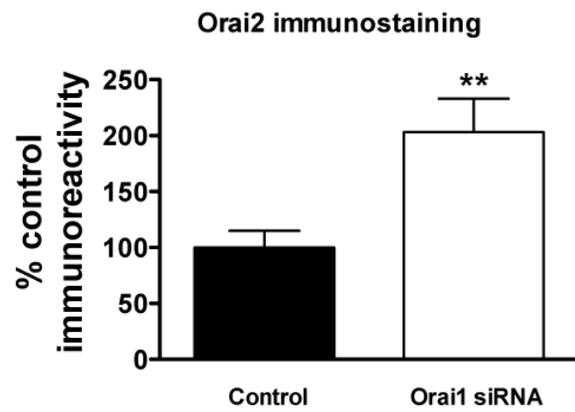
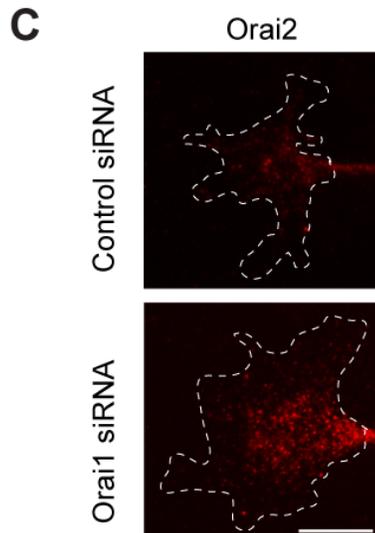
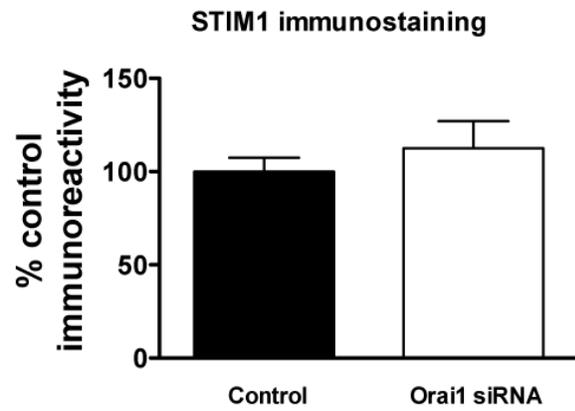
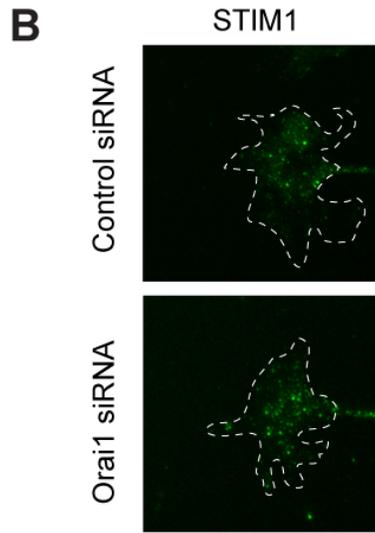
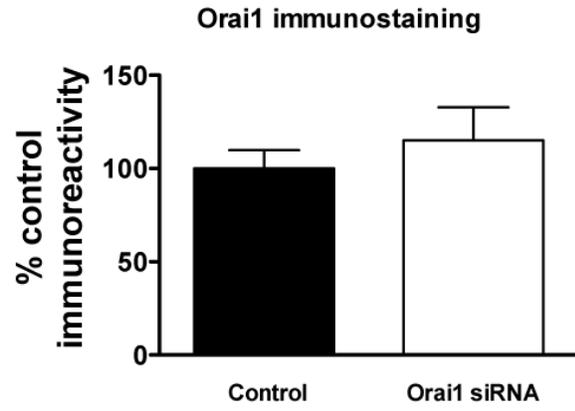
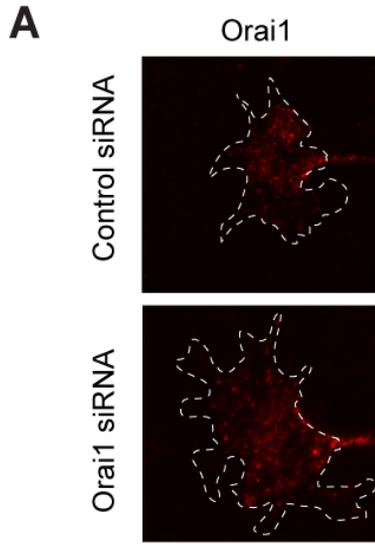
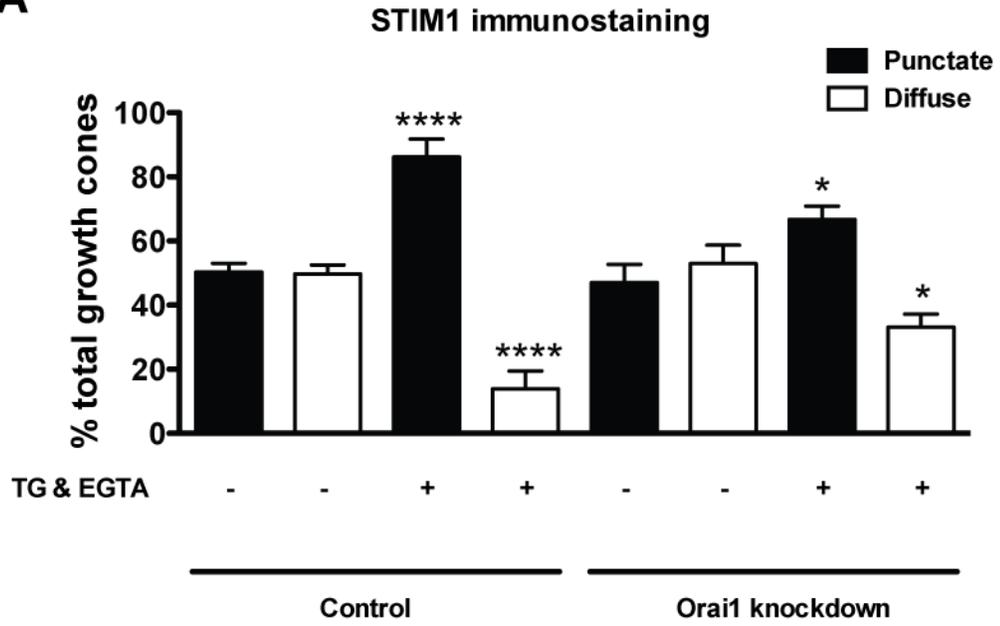
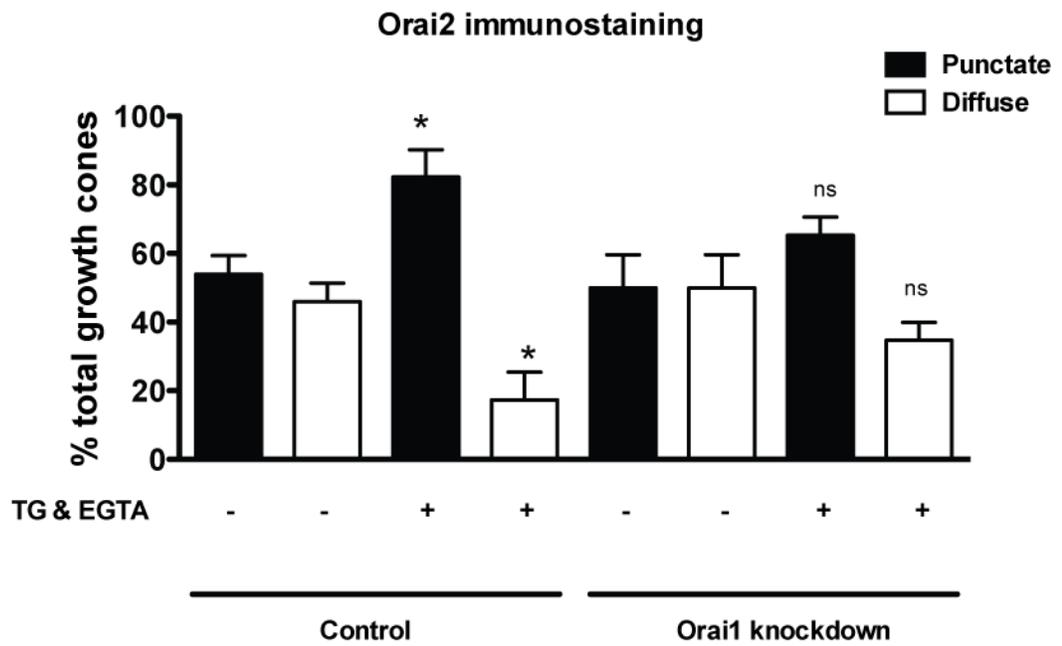


Figure 2.7. Orai1 knockdown decreases STIM1 and Orai2 puncta formation after calcium store depletion.

Quantification of growth cones displaying punctate versus diffuse STIM1 or Orai2 expression (control and Orai1 siRNA treated) analysed by thresholding (ImageJ). **A.** Percentages of growth cones displaying punctate (black bars) STIM1 expression versus diffuse (white bars) STIM1 expression in calcium replete and calcium deplete (Thapsigargin, EGTA and calcium free media) conditions, in both control and Orai1 siRNA treated growth cones. **B.** Percentages of growth cones displaying punctate (black bars) Orai2 expression versus diffuse (white bars) Orai2 expression in calcium replete and calcium deplete conditions, in both control and Orai1 siRNA treated growth cones. Protein expression is compared between growth cones treated with control siRNA in calcium replete media, and growth cones treated with control siRNA in calcium depleted media. Similarly, protein expression is compared between growth cones treated with Orai1 siRNA in calcium replete media, and growth cones treated with Orai1 siRNA in calcium depleted media. Error bars represent SEM. * $p < 0.05$, **** $p < 0.0001$. Unpaired t-tests.

A**B**

The data above suggests that Orai1 regulates Orai2 expression, and is required for STIM1 function in response to calcium depletion. I therefore repeated these experiments, but this time with Orai2 protein knockdown, in order to determine whether knockdown of Orai2 expression would perturb STIM1 and Orai1 protein aggregation, and hence the activation of SOCE. The siRNA for Orai2 appeared to be more effective by immunocytochemical analysis, than Orai1 siRNA. Orai2 siRNA produced a significant effect on the expression levels of Orai2 in growth cones. Immunoreactivity of Orai2 levels were reduced by 33% after Orai2 siRNA treatment (Fig. 2.8 A, $p < 0.05$). There was no change in the immunoreactivity levels of either STIM1 (Fig. 2.8 B) or Orai1 (Fig. 2.8 C) upon Orai2 siRNA treatment, suggesting that the Orai2 siRNA is specific for Orai2 protein only.

Upon calcium depletion from the media of Orai2 siRNA treated growth cones, the number of STIM1 puncta remained the same as in control siRNA treated growth cones when calcium was depleted ($87.0 \pm 3.4\%$ and $86.2 \pm 5.6\%$ respectively; Fig 2.9 A). Similarly, upon calcium depletion, the number of Orai1 puncta in Orai2 siRNA treated growth cones remained at similar levels as those in control siRNA growth cones ($87.2 \pm 5.6\%$ and $91.6 \pm 4.8\%$ respectively; Fig. 2.9 B). These data suggest that unlike Orai1, Orai2 is not normally functional in the activation and oligomerisation of STIM1 or Orai1 upon calcium store depletion in growth cones.

Figure 2.8. Orai2 siRNA reduces endogenous levels of Orai2, but has no effect on the expression of STIM1 or Orai1

A. Staining for Orai2-IR (red) reveals a decrease in Orai2-IR after 6 hours Orai2 siRNA treatment. Growth cones are outlined with dashed lines. Quantification of Orai2-IR in control (n=46) and Orai2 (n=65) siRNA treated growth cones, over 4 experiments. **B.** Staining for STIM1-IR (green) reveals no change in STIM1-IR after 6 hours Orai2 siRNA treatment. Quantification of STIM1-IR in control (n=117) and Orai2 (n=166) siRNA treated growth cones, over 6 experiments. **C.** Staining for Orai1-IR (red) reveals no change in Orai1-IR after 6 hours Orai2 siRNA treatment. Quantification of Orai1-IR in control (n=71) and Orai2 (n=72) siRNA treated growth cones, over 4 experiments. Scale bar = 10 μ m. Error bars represent SEM. * p<0.05. Unpaired t-test.

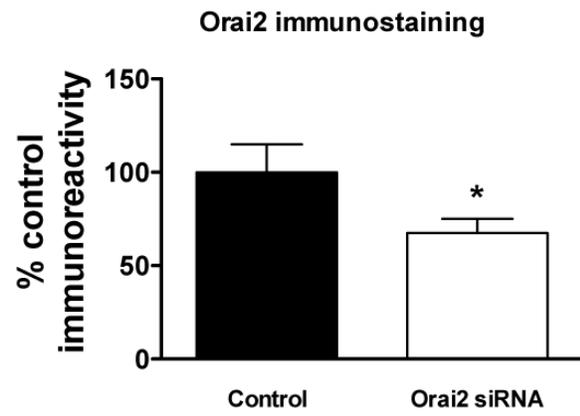
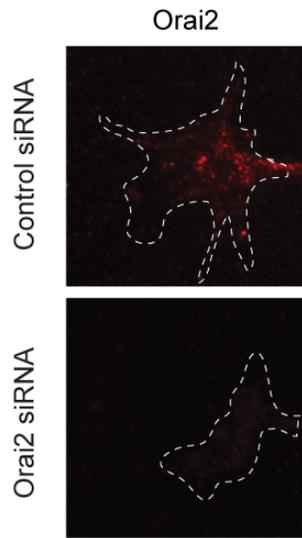
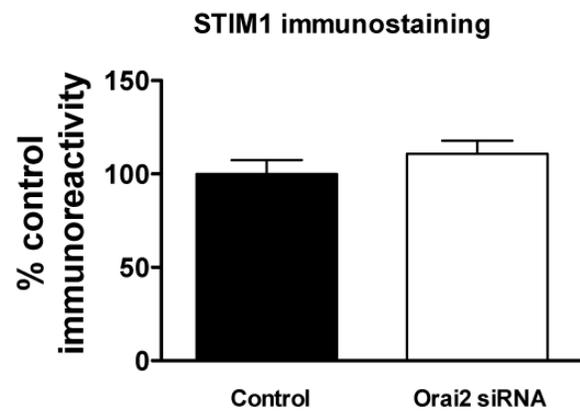
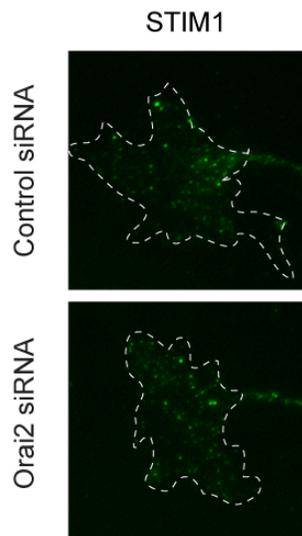
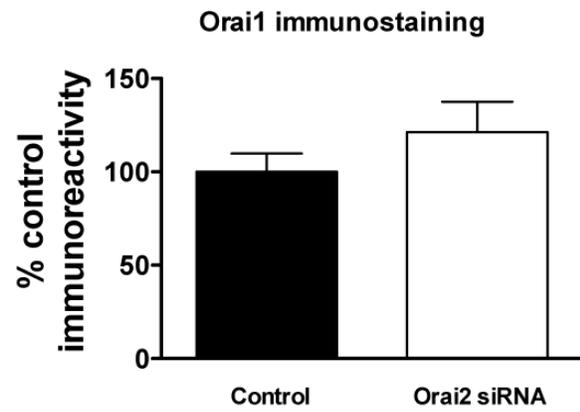
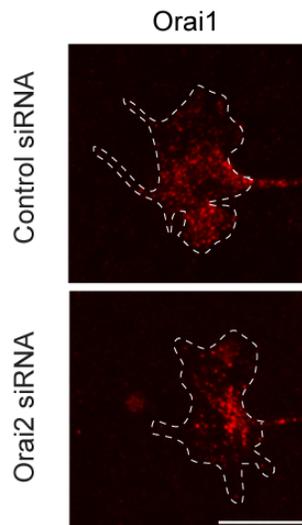
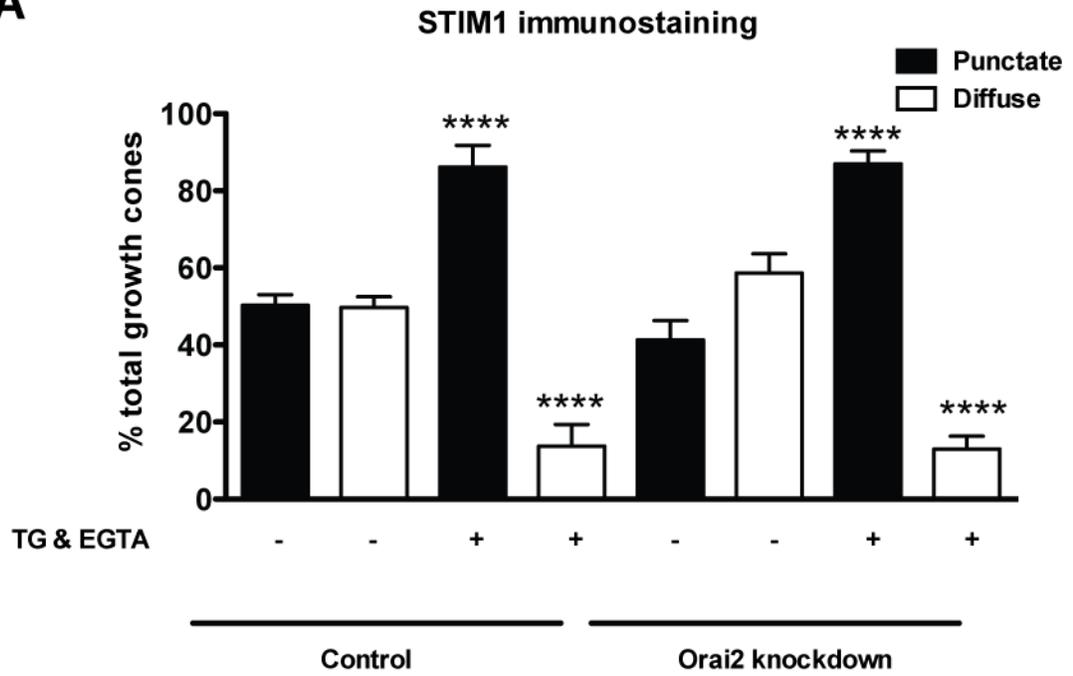
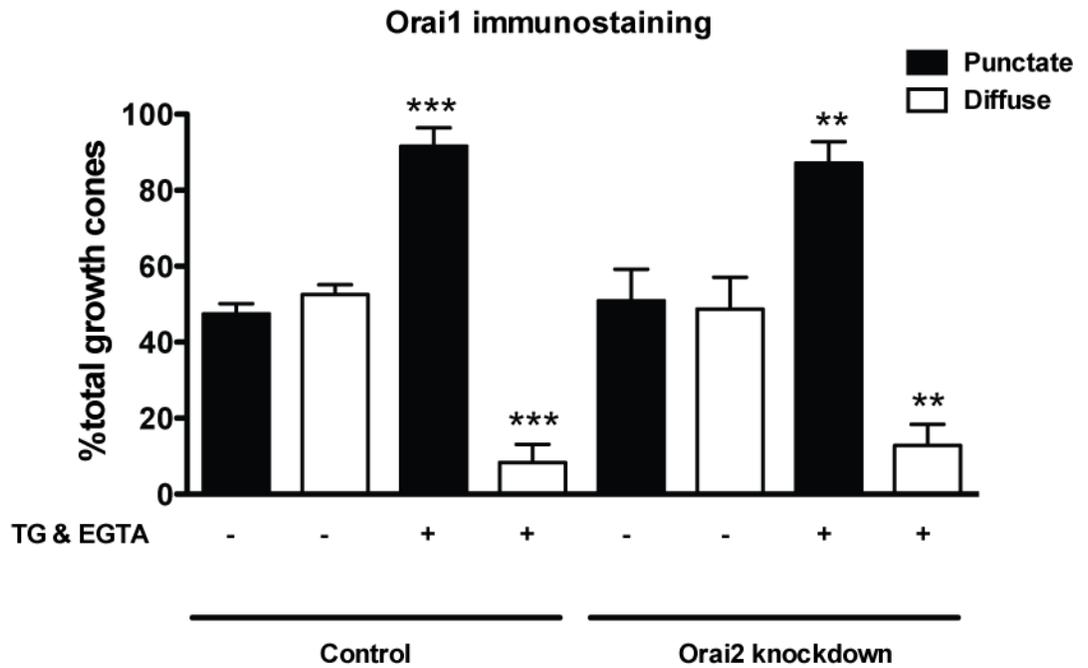
A**B****C**

Figure 2.9. Orai2 knockdown does not affect STIM1 or Orai1 puncta formation after calcium depletion.

Quantification of growth cones displaying punctate versus diffuse STIM1 or Orai1 expression (control and Orai2 siRNA treated) analysed by thresholding (ImageJ). **A.** Percentages of growth cones displaying punctate (black bars) STIM1 expression versus diffuse (white bars) STIM1 expression in calcium replete and calcium deplete (thapsigargin (TG), EGTA and calcium free media) conditions, in both control and Orai2 siRNA treated growth cones. **B.** Percentages of growth cones displaying punctate (black bars) Orai1 expression versus diffuse (white bars) Orai1 expression in calcium replete and calcium deplete conditions, in both control and Orai2 siRNA treated growth cones. Protein expression is compared between growth cones treated with control siRNA in calcium replete media, and growth cones treated with control siRNA in calcium depleted media. Similarly, protein expression is compared between growth cones treated with Orai2 siRNA in calcium replete media, and growth cones treated with Orai2 siRNA in calcium depleted media. Error bars represent SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Unpaired t-tests.

A**B**

2.3.4 Asymmetric localisation of STIM1 in turning growth cones

The data that has been presented so far has demonstrated that STIM1 and Orai proteins are present within neuronal growth cones, and that they cluster and form puncta after ER store depletion. Thapsigargin is an artificial activator of SOCE that growth cones are not exposed to *in vivo*. In order to determine if STIM1 and Orai1 protein localisation can be activated in response to physiological cues, STIM1 and Orai1 protein expression was examined in response to the physiological guidance cues. Guidance cues are present within the embryonic environment *in vivo* during development and are important in growth cone navigation. Spatial and temporal regulation of intracellular calcium is key to regulating growth cone motility (Zheng, 2000, Gomez et al., 2001, Gomez and Zheng, 2006, Jacques-Fricke et al., 2006, Tojima et al., 2011). Spatially restricted elevations of intracellular calcium alter growth cone turning towards the “high-calcium” side of the growth cone (Zheng, 2000). If STIM1 and Orai1 are important for growth cone navigation, it is predicted that the expression of STIM1 and Orai1 may be biased towards the side of the growth cone exposed to guidance cues. I hypothesised that STIM1 and Orai1 would be activated on the turning side of a growth cone in response to gradients of calcium-dependent, but not calcium-independent guidance cues. To test this hypothesis, growth cones were exposed to microgradients of phosphate buffered saline (PBS), BDNF or Sema-3a and then rapidly fixed as they were turning in response to the guidance cues (Fig. 2.10). BDNF is a calcium-dependent guidance cue, which relies on the activation of calcium ion channels, and release of intracellular calcium for its signalling effects (Song et al., 1997,

Song and Poo, 1999). Sema-3a is traditionally described as a calcium-independent guidance cue, as it does not rely heavily upon calcium for its intracellular signalling effects.

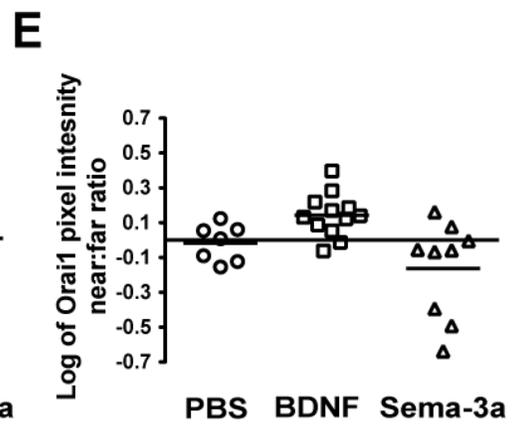
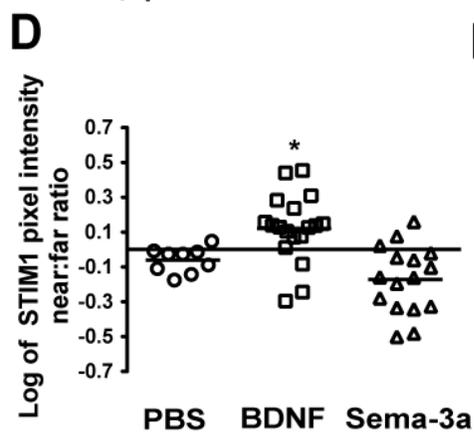
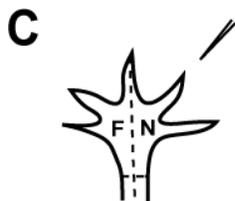
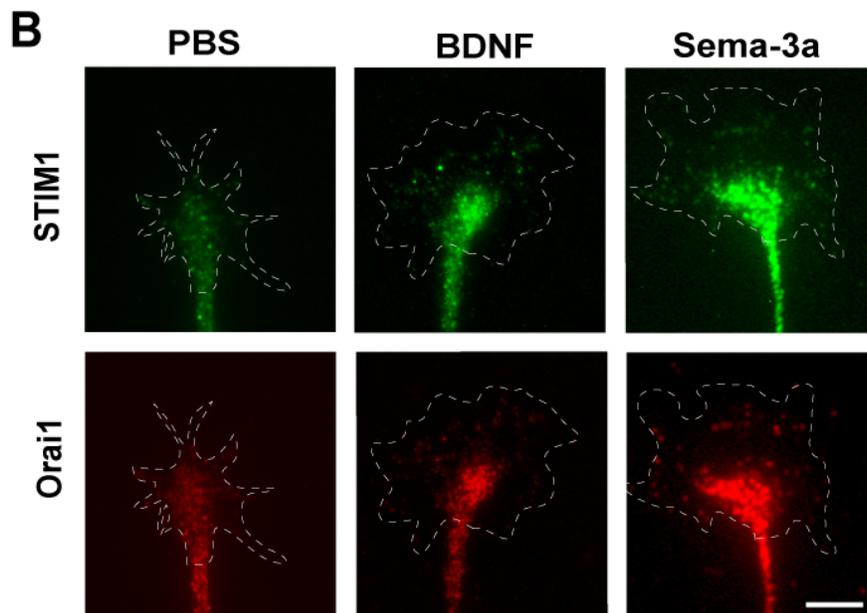
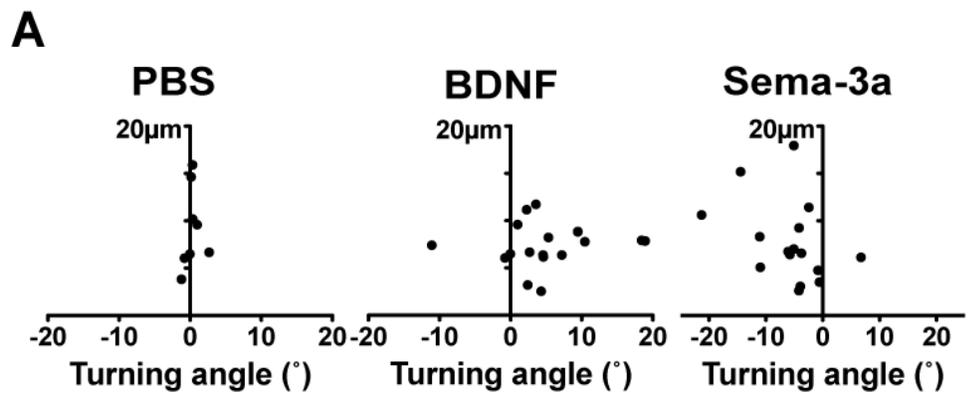
Growth cone extension and trajectory were compared with the spatial distribution of STIM1 and Orai1 immunoreactivity (Fig. 2.10 A and B). In response to PBS, the growth cones grew straight ahead, suggesting random growth ($0.5 \pm 0.4^\circ$; Fig. 2.10 A). When exposed to a gradient of BDNF, growth cones were attracted ($5.2 \pm 1.6^\circ$; Fig. 2.10 A), and in response to a gradient of Sema-3a, growth cones were repulsed ($-5.8 \pm 1.6^\circ$; Fig. 2.10 A). Immunoreactivity was measured on both the near and far sides of the growth cone, with respect to the direction of guidance cue gradients of PBS, BDNF or Sema-3a from the micropipette (Fig. 2.10 C). In response to BDNF, there was a significant increase in pixel intensity of STIM1 (0.12 ± 0.05 , $p < 0.01$; Fig. 2.10 D) but not Orai1 (0.14 ± 0.04 , $p > 0.05$; Fig. 2.10 E) on the attractive side (near side) of growth cones, compared to the PBS control (STIM1 -0.06 ± 0.02 , Fig. 2.10 D; Orai1 -0.02 ± 0.04 , Fig. 2.10 E). These data suggest that STIM1 expression is spatially reorganised in response to calcium-dependent guidance cue signalling, thereby potentially facilitating the spatial localisation of calcium within turning growth cones, and is consistent with previous reports (Zheng, 2000). Interestingly, there was a trend for STIM1 expression to be spatially reorganised in response to gradients of Sema-3a, although on the far side of the growth cone, as the growth cone turned away from the gradient of Sema-3a (-0.17 ± 0.05 , $p > 0.05$; Fig. 2.10 D). Orai1 localisation also appeared to trend towards the far side of the growth cone in response to Sema-3a (-0.16 ± 0.09 , $p > 0.05$; Fig. 2.10 E). These data suggest that components of STIM1-mediated SOCE

are actively recruited to the turning side of the growth cone in response to BDNF during growth cone navigation.

Figure 2.10. Asymmetric distribution of store-operated calcium entry machinery correlates with growth cone turning.

A. Scatter plots illustrate the data spread of final extension and trajectories of growth cones exposed to gradients of PBS, BDNF and Sema-3a. The micropipette would be positioned in the upper right corner. **B.** Representative immunocytochemistry images of individual growth cones turning in response to microgradients of PBS, BDNF and Sema-3a, respectively. Immediately post-turning, growth cones were fixed and immunostained for STIM1 (green) and Orai1 (red). Growth cone area is outlined with dashed lines. **C.** Schematic showing growth cones divided into near (N) and far (F) sides, with respect to the micropipette, for pixel intensity analysis. **D.** Logarithmic graph of STIM1 pixel intensity near:far ratio in response to PBS (n=9), BDNF (n=18) and Sema-3a (n=16). **E.** Logarithmic graph of Orai1 pixel intensity near:far ratio in response to PBS (n=7), BDNF (n=12) and Sema-3a (n=9). Scale bar equals 10 μ m. BDNF and Sema-3a treatments are compared to PBS control unless otherwise shown. * p<0.05, *** p<0.01. ANOVA with post-hoc Tukey.

This figure was published as figure 3 in Mitchell et al., 2012.



2.4 Discussion

This chapter has demonstrated that STIM1 and its binding partners Orai1 and Orai2 are expressed in growth cones and redistribute after store depletion. STIM1, Orai1 and Orai2 are expressed in two localisation patterns within sensory growth cones, depending upon ER calcium store status. When calcium stores are full, STIM1 and the Orai protein expression is diffuse, spread throughout the growth cone. Upon calcium store depletion, STIM1, Orai1 and Orai2 oligomerise into puncta and redistribute throughout both the ER and plasma membranes. Furthermore, there is an increase in STIM1 and Orai1 co-localisation, and expression is biased on the turning side of the growth cone, in response to a calcium-dependent guidance cue. Furthermore, this chapter demonstrated that while STIM1 appears to mediate the activation of Orai puncta upon calcium depletion in navigating growth cones, Orai1 also plays a regulatory role, as it is also required for the formation of STIM1 and Orai2 puncta after calcium depletion. Taken together, these data support the hypothesis that STIM1 regulates SOCE within growth cones

The expression pattern of STIM1, Orai1 and Orai2 within growth cones depends on calcium store status, and correlates with growth cone turning.

STIM1, along with Orai1 and Orai2 were found to be present within DRG of embryonic rats. These data confirm the presence of SOCE machinery within the peripheral nervous system, which is consistent with previous data (Dziadek and Johnstone, 2007, Gasperini et al., 2009, Klejman et al., 2009, Gemes et al., 2011). When examining STIM1 localisation in untreated, control growth cones there were two main patterns of localisation evident:

diffuse and punctate. In resting cells, the expression of STIM1 and Orai has previously been described as a diffuse, uniform pattern of localisation, until activated by a stimulus (Liou et al., 2005, Klejman et al., 2009, Koh et al., 2009). Calcium store depletion causes a rapid STIM1 redistribution into punctate cluster within areas of the ER membrane located close to the plasma membrane (Roos et al., 2005, Dziadek and Johnstone, 2007). However, growth cones differ from other cell types, as they are never fully resting. Growth cones are highly dynamic, motile structures that are constantly receiving and interpreting signals from the surrounding environment (Gomez et al., 1995, Gallo and Letourneau, 1999), hence the growth cone is hardly ever found in a true resting state. In culture, where physiological, *in vivo* directional guidance cues are absent, growth cones still interact with their environment as they extend, responding to contact with substrate and surrounding cells, activating signalling cascades and subsequent cytoskeletal rearrangements. During random growth in the culture dish, just over half the growth cones displayed a punctate pattern of STIM1 and Orai1 or Orai2 distribution, consistent with SOCE activation. Furthermore, after depletion of intraluminal ER calcium stores, the percentage of growth cones displaying diffuse STIM1 and Orai expression decreased, while the percentage of growth cones displaying punctate STIM1, Orai1 or Orai2 expression increased dramatically. The aggregation of STIM1 and Orai puncta upon calcium depletion were consistent with previous reports in non-neuronal cells (Liou et al., 2005, Chvanov et al., 2008, Koh et al., 2009). In addition, calcium depletion caused an increased level of STIM1-Orai1 puncta colocalisation within growth cones, consistent with the known activation of SOCE. The colocalisation of STIM1 and Orai1 is thought to represent the activation of the CRAC channel on the plasma membrane (Liou et al., 2005). Therefore, the presence of

punctate and diffuse expression patterns of STIM1 and Orai in growth cones would suggest that the punctate localisation of these calcium related proteins is indeed dependent on calcium store status, and that calcium stores are constantly being depleted and refilled in sensory growth cones, consistent with their dynamic, motile nature. If this were true the punctate localisation of STIM1 and Orai1 would be predicted to be biased on the turning side of a growth cone, in response to navigational guidance cues, consistent with previous work where spatially restricted calcium gradients have been shown to induce growth cone turning to one specific side of a growth cone (Zheng, 2000). The data in this chapter demonstrates that BDNF signalling mobilises STIM1 in a spatially and temporally regulated manner consistent with the spatial restriction of calcium signals to individual filopodia (Gomez and Spitzer, 2000) or the turning side of growth cones (Zheng, 2000, Henle et al., 2011). SOCE not only replenishes intracellular calcium stores, but also elevates cytosolic calcium. Given that STIM1 is a key mediator of SOCE, the asymmetric expression of STIM1 in turning growth cones would suggest that SOCE might be regulated in a spatially restricted manner, consistent with guidance cues eliciting localised increased levels of intracellular calcium. If SOCE is regulated in a spatially restricted manner, as these data suggest, then SOCE is predicted to contribute significantly to spatially restricted cytosolic calcium signals.

The spatial regulation of STIM1 in turning growth cones suggests that STIM1 is a dynamic component of growth cone motility machinery, and is actively recruited and reorganised asymmetrically in growth cones. While there was a tendency for Orai1 to also move to the turning side of the growth cone, this was not significant. These data suggest that multiple

Orai proteins, and/or TRPC proteins may be activated by STIM1 to affect SOCE in growth cones (Beech et al., 2003, Albert et al., 2007, Cahalan, 2009, Yuan et al., 2009, Cheng et al., 2011). There is evidence to suggest that in addition to binding and activating Orai proteins, STIM1 is also able to interact with TRPC channels, (Huang et al., 2006). TRPC channels, known to be active in axon guidance (Shim et al., 2005, Wang and Poo, 2005), were initially thought to be the CRAC channel before the discovery of Orai (Zhu et al., 1996, Mori et al., 2002, Philipp et al., 2003). Interestingly, there was also a trend towards asymmetric distribution of STIM1 on the far side of the growth cone, in response to Sema-3a microgradients. This was unexpected, since Sema-3a is a largely calcium-independent guidance cue that does not rely on large changes in calcium for signalling (Song and Poo, 1999). This may suggest that STIM1 plays a role in growth cone function that is independent of calcium signalling and SOCE. Alternatively, STIM1 puncta may be clustering in areas of growth cone expansion, due to the growth, or movement of the ER tubules in response to both BDNF and Sema-3a. There is currently little information on ER motility within growth cones, and particularly within response to guidance cues. As such, it is currently unknown whether the ER relocates first, and STIM1 translocation follows, or rather if STIM1 translocates to predetermined areas, and signals to, or ‘pulls’ the remainder of the ER to relocate to the site of STIM1 activation and translocation.

Orai1, but not Orai2, is required for calcium depletion-mediated oligomerisation of STIM1

STIM1 is believed to be the key mediator of SOCE, activated by the depletion of ER calcium stores (Luik et al., 2008). A reduction in STIM1 expression reduces CRAC channel activation (Spassova et al., 2006). In addition, upon introduction of a STIM1 E87A EF-hand mutant, which lowers the calcium binding affinity of STIM1, CRAC channel activation is also blocked (Spassova et al., 2006). In light of this, it could be predicted that upon the reduction of Orai proteins, STIM1 would still be activated and form puncta upon ER calcium depletion. Hence it was surprising to find that Orai1 expression was required for oligomerisation of STIM1 and Orai2 upon calcium store depletion. The percentage of growth cones displaying punctate STIM1 expression was significantly less when Orai1 expression was reduced, compared to controls. The formation of Orai2 puncta was abolished, with the percentage of growth cones displaying Orai2 puncta remaining at a similar level as when untreated. The same siRNA treatment has been used previously in our laboratory to perturb growth cone navigation (Ainslie, Honours thesis, 2012). These data suggest that Orai1 is necessary for the oligomerisation of STIM1 and Orai2 upon calcium depletion, and that these proteins do not function completely sequentially as previous thought, suggesting that there may be some crosstalk or feedback between STIM1 and Orai1, resulting in bi-directional signalling.

Interestingly, upon Orai1 reduction, Orai2 expression levels increased even though Orai2 function was diminished. When Orai1 expression was reduced, Orai2 puncta formation did

not increase upon calcium depletion. This suggests that Orai1 is the main functional Orai protein activated within growth cones, and that Orai2 is usually redundant but may be upregulated if required, for example, when Orai1 expression is reduced. Orai2 may have other functions since it failed to oligomerise. Alternatively, Orai1 and Orai2 may work in conjunction with each other, and if one is not present, then the function of the other is also perturbed. To examine this further, Orai2 siRNA was used to reduce the endogenous levels of Orai2. The reduction of Orai2 did not perturb the ability of STIM1 or Orai1 to form puncta upon calcium depletion, further supporting the hypothesis that Orai2 is not a functional member of the Orai family within growth cones. Consistent with these data, it was previously shown in our laboratory that Orai2 was not required for the function of growth cone navigation, in response to gradients of known guidance cues (Ainslie, Honours thesis, 2012). However, while these data suggest that in *in vitro* culture, Orai2 expression is upregulated, but not functional, within growth cones, it is possible that the level of protein reduction occurring with the Orai2 siRNA is not enough for a functional effect to be seen. However, most work from our laboratory has already demonstrated that a 30-50% reduction in protein expression is sufficient to perturb growth cone function (Gasperini et al., 2009, Mitchell et al., 2012).

Taken together, these data suggest that STIM1, Orai1 and Orai2 are present in neuronal growth cones. STIM1 and Orai proteins are expressed in both diffuse and punctate patterns of localisation, and these are dependent upon the status of the internal calcium store within the growth cone. While Orai1 appears to be necessary for STIM1 puncta formation, Orai2 appears to be upregulated in growth cones, and is not a functional member of the Orai

family within neuronal growth cones. In response to a physiological, calcium-dependent guidance cue, STIM1 protein expression appears to be biased towards the attractive, or turning side of the growth cone, suggesting that it is actively recruited in response to this cue. The data presented within the chapter has demonstrated that STIM and the Orai proteins display dynamic distribution patterns consistent with those described previously within non-neuronal cells. While the activation of STIM1 and Orai proteins occurs in response to both thapsigargin and physiological cues, it is unknown if this represents the activation of functional SOCE. The data presented here would suggest that SOCE is a functional process within growth cones, but whether SOCE is required for growth cone navigation is unknown.

Chapter 3:

**STIM1 mediates neuronal SOCE, growth cone turning,
and growth cone collapse**

Chapter 3: STIM1 mediates neuronal SOCE, growth cone turning and growth cone collapse

3.1 Introduction

Growth cones are key determinants in the connectivity of the complex neuronal array of the nervous system, as they direct extending axons towards their targets. Many guidance cues, such as netrin-1 and BDNF, alter growth cone motility by eliciting an asymmetric rise in calcium and subsequent second messenger signals across the growth cone (Tojima et al., 2011). Growth cone direction is biased towards the high calcium side of the growth cone (Zheng, 2000, Gomez et al., 2001, Gomez and Zheng, 2006). Understanding calcium signalling within the growth cone is a crucial element to understanding axon guidance. Hence, determining the source of calcium and the molecular basis of the spatial and temporal regulation of calcium signals within growth cones is key to understanding growth cone navigation and axon guidance.

Growth cone responses to netrin-1 and BDNF occur in a calcium-dependent manner (Ming et al., 1997, Hong et al., 2000, Ming et al., 2001), while growth cone responses to Sema-3a occur in a calcium-independent manner (Song et al., 1998, Song and Poo, 1999). Calcium-dependent guidance cues are able to alter growth cone turning direction via the activation of downstream calcium effectors, such as CaMKII and CaN (Wen et al., 2004). CaMKII and CaN operate in a molecular switch-like mechanism. CaMKII is activated by larger changes in cytosolic calcium concentrations, ultimately activating actin polymerisation. Smaller changes in calcium levels result in CaN activation, resulting in actin depolymerisation

(Wen et al., 2004). The activation of these specific effectors can determine the direction of growth cone motility, be it attraction (CaMKII) or repulsion (CaN).

Whether CaMKII or CaN gets activated depends on the relative change in cytoplasmic calcium concentrations. These changes in calcium concentration are directly linked to which calcium channels get activated. There are two major sources of calcium: extracellular and intracellular. In growth cones, extracellular calcium enters the cytoplasm through a range of calcium channels located on the plasma membrane, such as TRPC channels (Beech et al., 2003, Albert et al., 2007), VGCC (Hong et al., 2000, Berridge et al., 2003), and CRAC channels, which mediate SOCE. Intracellular calcium, which is stored within the ER, is released via ER membrane bound channels. This release of ER calcium is due to an initial influx from the extracellular source of calcium, via the activation of IP₃ (Li et al., 2005, Akiyama et al., 2009) and ryanodine receptors (Hong et al., 2000, Jin et al., 2005, Wang and Poo, 2005). When large changes in cytoplasmic calcium concentration occur, such as when calcium is released from the ER, CaMKII is preferentially activated (Wen et al., 2004). This release of calcium from the ER potentially activates the CRAC current and SOCE, resulting in a secondary influx of calcium into the cytoplasm, sustaining the activation of CaMKII. However, it remains unknown whether the CRAC channel functions within growth cones to modulate turning behaviour. In contrast, if only small amounts of calcium enter the cytoplasm, such as via TRPC channels, and CICR is not activated, CaN is activated instead of CaMKII (Wen et al., 2004).

The ER source of calcium is extremely important to growth cone motility, however little is known about the ER or the regulation of ER calcium within growth cones. This is despite the fact that it is well accepted that release of calcium from the ER can dictate growth cone turning behaviour. The requirement for CICR in growth cones has long been recognised (Gomez et al., 1995), although how ER stores are maintained in growth cones was unknown. STIM1 and Orai proteins have been shown to be present in the nervous system (Klejman et al., 2009, Skibinska-Kijek et al., 2009), and importantly within DRG growth cones (Fig. 2.2) (Gasperini et al., 2009). STIM1 and Orai are also important for normal neuronal development in *Drosophila melanogaster*, as the SOCE machinery is required for normal flight and the firing of flight motor neurons (Venkiteswaran and Hasan, 2009), as well as through the regulation of calcium spike firing in embryonic *Xenopus* spinal neurons (Chang and Spitzer, 2009). There is also some evidence for STIM1-mediated SOCE in neuronal cell bodies cells (Klejman et al., 2009). The requirement for SOCE in growth cone motility has been suggested (Hong et al., 2000, Li et al., 2005, Gasperini et al., 2009), however it has never been studied directly. Precise calcium regulation is crucial for normal growth cone function, and indeed motility. If STIM1 and SOCE do function within growth cones, this may have important implications for growth cone calcium regulation and motility.

The experiments described within this chapter aim to demonstrate the function of STIM1 within sensory growth cones with the use of a targeted knockdown approach. In order to address this aim, the levels of endogenous STIM1 expression will be lowered with the use of a targeted STIM1 morpholino. The functional knockdown of STIM1 will be assessed

with the use of ratiometric calcium imaging and thapsigargin treatment, to induce STIM1 activation and SOCE. To discover whether STIM1 is required for growth cone guidance, STIM1 morphants will be assessed in response to both calcium-dependent and calcium-independent guidance cues. The data in this chapter demonstrates that STIM1 is required for SOCE and growth cone attraction in response to calcium-dependent guidance cues, netrin-1 and BDNF. Furthermore, STIM1 is also shown to be necessary for turning in response to the calcium-independent guidance cue Sema-3a, and is necessary for Sema-3a-induced growth cone collapse.

3.2 Materials and Methods

3.2.1 Materials

STIM specific morpholino (5' GGGCAAGACGAGCGCACACATCCAT) and STIM1 control morpholino (5' GGcCAAcACcAGcCACAgATCCAT) were purchased from Gene tools (Philomath, Oregon, USA). STIM1 antibody, actin antibody and EGTA were purchased from Sigma-Aldrich (Missouri, USA). Thapsigargin was purchased from Alomone Labs (Jerusalem, Israel). BDNF, netrin-1 and semaphorin-3a were purchased from R+D Systems (Minneapolis, Minnesota, USA). Fura 2-AM, Alexa fluor 488 phalloidin were purchased from Invitrogen, Life Technologies (California, USA).

3.2.2 Embryonic DRG cell culture

DRG cell culture was performed as described in section 2.2.4

3.2.3 Quantification of STIM1 knockdown: Immunocytochemistry

Constitutive STIM1 expression was reduced by the addition of specific STIM1 morpholino (5' GGGCAAGACGAGCGCACACATCCAT), with STIM1 control morpholino (5 μ M) (5' GGcCAAcACcAGcCACAgATCCAT) used as a control (Gene tools). Morpholino antisense oligonucleotides provide a reproducible approach to reduce the level of

endogenous protein within DRG neurons (Gasperini et al., 2009). Morpholinos have been used extensively within non-mammalian models such as *Zebrafish* and *Xenopus* spinal neurons, and have recently begun to be used more often in mammalian cell culture models, particularly with in DRG cultures (Gasperini et al., 2009, Murray et al., 2009, Murray et al., 2012). Morpholinos were chosen for use in this study due to ease of delivery into DRG cultures, as well as the effective and easily reproducible knockdown that occurs within only a few hours (Gasperini et al., 2009).

Morpholinos were added during DRG trituration prior to culturing as per section 2.2.4. After 6 hours, cells were fixed with 4% paraformaldehyde and prepared for immunocytochemistry (refer to section 2.2.5). Cells were stained with a STIM1 antibody (1:1000), and phalloidin (1:40) to stain the actin cytoskeleton. Quantification of STIM1 immunocytochemistry was used to assess protein expression after morpholino knockdown in individual growth cones. Image acquisition parameters were adjusted to avoid pixel saturation, and identical exposures were used for control and STIM1 morphant cells. In order to minimise selection bias, growth cones were selected by viewing Alexa Fluor 488 phalloidin staining of actin (Invitrogen). Selection was based on spreading morphology and isolation from other growth cones and axons. Integrated pixel intensities of STIM1-IR were measured for each growth cone and normalised for growth cone area (ImageJ, NIH). Statistical analyses performed were unpaired t-tests (Graph Pad Prism).

3.2.4 Verification of STIM1 knockdown: western blotting

STIM1 knockdown was verified in whole cells (SH-SY5Y) using western blotting. Briefly, SH-SY5Y cells were scraped and loaded with morpholino, and incubated for 8 hours at 37°C, harvested and lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM phenylmethylsulphonyl fluoride (Sigma-Aldrich), 1mM EDTA, 5µg/ml leupeptin (Sigma-Aldrich), 5µg/ml aprotinin (Sigma-Aldrich), 1% NP-40 (Sigma-Aldrich), 1% sodium deoxycholate (Sigma-Aldrich), 0.1% SDS). Proteins were separated on 8% polyacrylamide-SDS gels, transferred onto 0.2µm PVDF membranes, and blocked overnight in 5% skim milk. Membranes were incubated with STIM1 (1:1000) and actin (1:25 000) antibodies, followed by HRP-conjugated secondary antibodies. Antibody conjugates were detected using chemiluminescent substrate (Millipore) and imaged on a gel documentation system (Chemismart, Vilber Lourmat).

3.2.5 Calcium imaging in DRG neurons

DRG neurons plated on glass coverslips were transferred to a room temperature solution of Fura-2 AM (5 µM, Invitrogen) in serum-free SNM. After 30 min, cells were washed once with SNM and incubated for a further 30 min at 37°C to allow complete de-esterification and mounted into an imaging chamber. Imaging buffer consisting of HBSS (Gibco) supplemented with calcium chloride (2mM), magnesium chloride (1.2mM), HEPES (10mM), dextrose (5mM), nerve growth factor (NGF, 50ng/ml) and N2 supplement (Invitrogen) was used for calcium imaging experiments. All experiments were carried out

at 37°C. Calcium-free experiments were performed in calcium-free imaging buffer supplemented with 300 μM EGTA (Sigma-Aldrich). Growth cones or cell soma were excited alternately with an attenuated (33% transmission) 340 and 380nm wavelength illumination source (Lambda DG-4, Sutter). Images were acquired at 510nm using an EMCCD digital camera (Evolve, Photometrics) and inverted microscope (Eclipse TiE; Nikon Instruments) using a 40xFluor-S oil-immersion objective (Nikon) equipped with DIC optics. Images from each growth cone were obtained at a frame rate of 1Hz and regions of interest (ROIs) were defined using NIS Elements 6D software (Nikon). After background subtraction, the fluorescence ratio (R) for each ROI was determined as the intensity of emission during 340 nm excitation divided by intensity of emission during 380 nm, on a pixel-by-pixel basis. Calcium concentrations were estimated using the formula $[\text{calcium}] = K_{\text{eff}} \times (R - R_{\text{min}}) / (R_{\text{max}} - R)$, where K_{eff} describes an effective binding constant K_d (224nM at 37°C) \times (F380 at saturable calcium / F380 at low calcium), R is the ratio at 340/380nm at the current time point, R_{min} and R_{max} are the calculated ratios at 340/380nm of cells at saturable and minimal cytosolic calcium concentrations as described previously (Grynkiewicz et al., 1985). Prism 5 (Graph-Pad Software) and Adobe Illustrator CS3 (Adobe Systems) were used for graphical and statistical analysis of data. Reagents used for calcium imaging experiments: Thapsigargin (2μM), BDNF (10μg/ml) and Sema-3a (20μg/ml).

3.2.6 Growth cone turning assay

Growth cone turning assays were performed as previously described (Gasperini et al., 2009). Briefly, isolated growth cones (selected as described in section 3.2.3) were exposed to gradients of BDNF (10 μ g/ml, R+D Systems), Netrin-1 (5 μ g/ml, R+D Systems), Sema-3a (20 μ g/ml, R+D Systems), or PBS. Gradients were created by pulsatile ejection through a fire-polished micropipette with a final tip diameter of 1.0-1.2 μ m. Micropipettes were positioned 80-100 μ m from growth cones, at a 45° angle to axon trajectories. Turning assays were performed on cells loaded with either control or STIM1 morpholino, as described in section 3.2.3. Growth cones were imaged using phase-contrast microscopy, and images acquired every 7 sec for 30 min using custom software (Matlab, Mathworks). Growth cones extending more than 10 μ m were included for analysis. Turning angles were determined by the change in axon trajectories of distal 10 μ m of axons over 30 minutes. Attraction was defined as a positive turning angle, repulsion was defined as a negative turning angle.

In calcium free experiments, calcium free media was used, consisting of calcium free Hank's Buffered Salt Solution (HBSS) containing nerve growth factor (50ng/ml), N2 neural supplement, penicillin G (100 μ g/ml), streptomycin (100 μ g/ml), fetal calf serum (5% v/v), and 300 μ M EGTA.

3.2.7 Semaphorin-3a collapse assay

DRG neurons were loaded with control or STIM1 specific morpholinos and plated onto glass coverslips. After 4 hours, Sema-3a (100ng/ml) was added for 30 min. Cells were fixed in 4% paraformaldehyde, and stained with Alexa Fluor 488 phalloidin. All growth cones were counted as either healthy with spreading lamellipodia, or collapsed, (no lamellipodia), and expressed as percentages of growth cones without spreading lamellipodia. During calcium free conditions, cultures were grown normally in SNM. Media was changed to a calcium free media (calcium free HBSS) containing nerve growth factor (50ng/ml), N2 neural supplement, penicillin G (100µg/ml), streptomycin (100µg/ml), fetal calf serum (5% v/v), and EGTA (300µM) for 30 mins. Sema-3a was then added to the calcium free media and left to incubate for an additional 30 mins before fixation.

3.3 Results

3.3.1. STIM1 expression is required for SOCE in growth cones

STIM1-specific oligonucleotide morpholinos were used to reduce STIM1 expression in DRG growth cones. STIM1 knockdown was confirmed by immunocytochemistry and western blotting 6 hours after plating (Fig. 3.1). STIM1 expression in growth cones treated with a mispaired morpholino (control morphants) was indistinguishable from wildtype growth cones (compare Fig. 3.1 A with Fig. 2.2 A-D). However, in growth cones treated with STIM1 specific morpholino (STIM1 morphants, Fig. 3.1 B), STIM1-immunoreactivity in growth cones was significantly reduced (51% of control \pm 13%, $p < 0.001$; Fig. 3.1 C). The efficiency of the STIM1 morpholino was confirmed using western analysis of STIM1-immunoreactivity in SH-SY5Y cells (Fig. 3.1 D).

While it had been suggested previously that growth cones utilise SOCE for their function, this had never been specifically shown. Thapsigargin is an inhibitor of the SERCA pump, which brings calcium back into the ER (Clapham, 2007), and subsequently activates SOCE (Liou et al., 2005). Thapsigargin was used to activate SOCE in DRG neurons. SOCE was measured by ratiometric calcium imaging to demonstrate the presence of SOCE within growth cones, and subsequently to confirm that STIM1 expression was required for SOCE within growth cones (Fig. 3.2 A-D). Acute treatment with thapsigargin (2 μ M) increased calcium levels in control morphant neurons by 562% \pm 123% (percentage of resting

Figure 3.1. Knockdown of endogenous STIM1 expression in neurons

Staining for STIM1 immunocytochemistry (red) and f-actin (phalloidin, green) reveals a decrease in STIM1-IR after 6 hours STIM1 morpholino treatment. **A, B.** Representative neurons were loaded with either control (**A**) or STIM1 (**B**) morpholinos. **C.** Quantification of STIM1-IR in control (n=35) and STIM1 morpholino (n=27) growth cones, over 4 experiments. **D.** Western blot analysis of SH-SY5Y cells loaded with control or STIM1 morpholino demonstrates STIM1 knockdown after 6 hours morpholino treatment (n=3). Scale bar in A and B equals 10 μ m. Error bars represent SEM. *** p<0.0001. Unpaired t-test. *Panels A and C were included in my Honours thesis, Mitchell 2009. This figure was published as part of figure 4 in Mitchell et al 2012.*

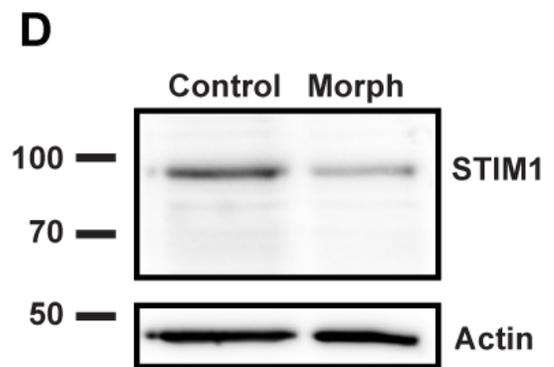
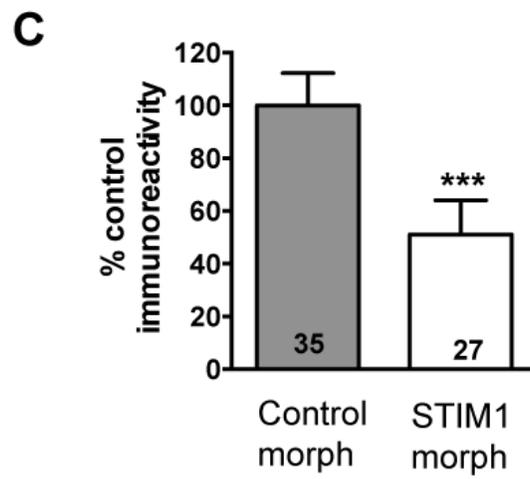
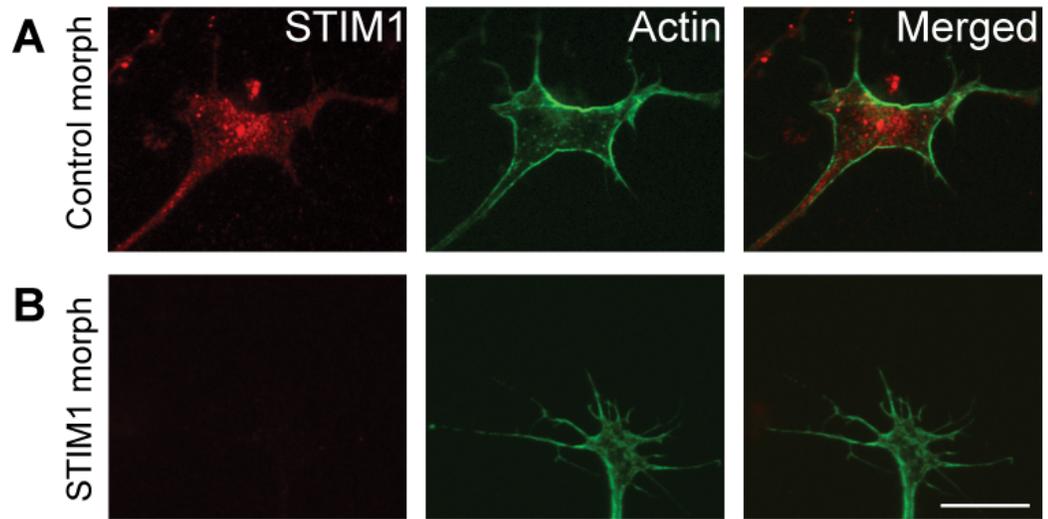
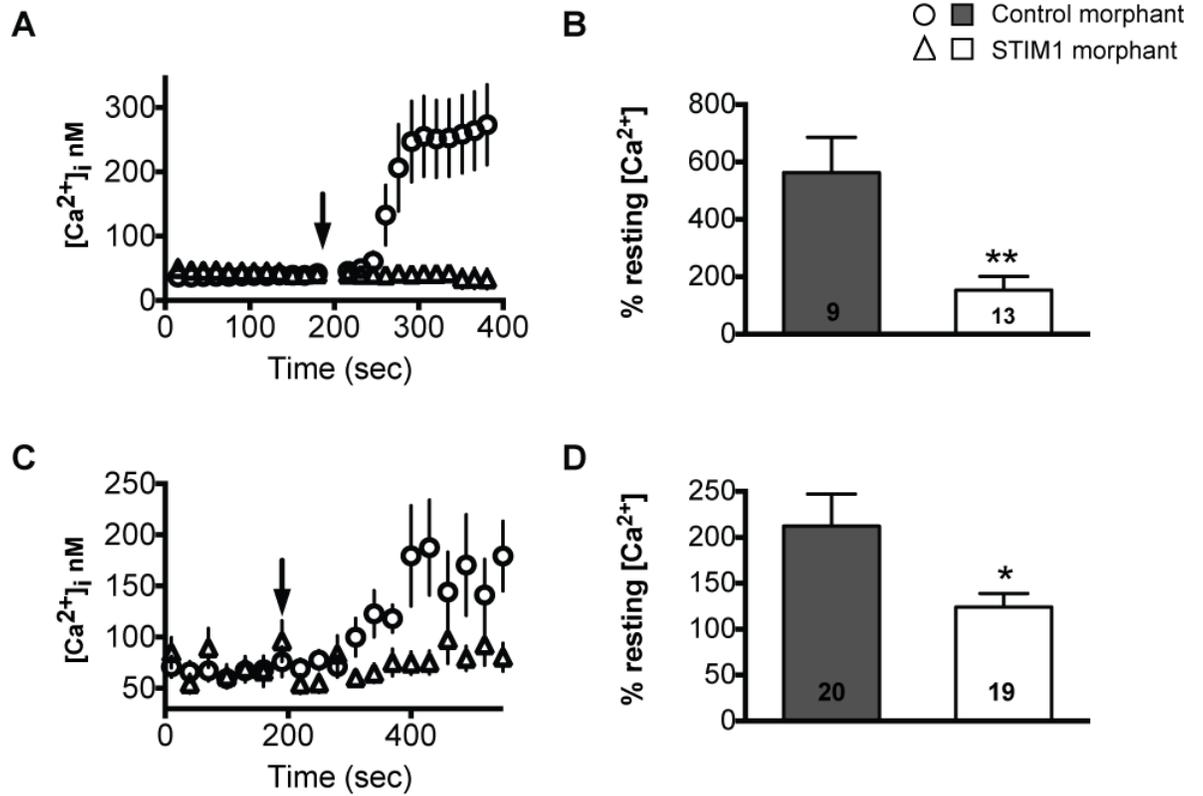


Figure 3.2. Knockdown of endogenous STIM1 expression abolishes SOCE in sensory neurons.

A, C. Traces of average intracellular calcium levels ($[Ca^{2+}]_i$ nM) of soma (**A**) and growth cones (**C**) loaded with control (circle) or STIM1-specific (triangle) morpholino. Traces show $[Ca^{2+}]_i$ before and after the addition of thapsigargin (black arrow). Control calcium levels in soma and growth cones increase while there is minimal $[Ca^{2+}]_i$ change in STIM1 morphant soma levels and little change in STIM1 morphant growth cone calcium levels. **B, D.** Percent of resting calcium levels is shown for both control (grey bars) and STIM1 (white bars) morphant soma (**B**) and growth cones (**D**) after thapsigargin activation. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney U-test). Numbers for each experiment are shown in the bars (B and D) and apply to A and C. *This figure was published as part of figure 4 in Mitchell et al 2012. Dr Robert Gasperini assisted in the collection of this data.*



calcium concentration) in the soma (Fig. 3.2 A and B, circles and grey bar) and $212\% \pm 35\%$ in growth cones (Fig 3.2 C and D, circles and grey bar). This thapsigargin induced calcium rise has been attributed to store leakage and activation of SOCE. Conversely, there was no statistically significant rise in intracellular calcium in STIM1 morphant neurons in response to thapsigargin treatment (soma, $124\% \pm 15\%$, $p < 0.01$, Fig. 3.2 A and B, triangles; growth cones, $153\% \pm 48\%$, $p < 0.05$, Fig. 3.2 C and D, triangles). These data confirm that SOCE can be activated as predicted in neuronal soma, but also in growth cones, which has never been shown before. This suggests that STIM1 is necessary for SOCE in both neuronal soma and growth cones.

3.3.2. STIM1-mediated SOCE is required for BDNF signalling, but not Sema-3a signalling

Thapsigargin is an artificial tool that is used to activate SOCE. As such, it is unknown whether physiological cues that growth cones come in contact with *in vivo* can also activate SOCE. Calcium imaging was used to determine whether STIM1-mediated SOCE is activated in growth cones as they respond to microgradients of guidance cues, such as BDNF or Sema-3a. BDNF is known to induce a rapid sustained rise in intracellular calcium within growth cones (Li et al., 2005, Gasperini et al., 2009). Accordingly, the application of a BDNF microgradient induced a rapid rise in intracellular calcium in control morphant growth cones (Fig. 3.3 A and B). This rise in intracellular calcium was significantly reduced in STIM1 morphant growth cones (Fig. 3.3 A). However, in some STIM1 morphant growth cones, a small rise in intracellular calcium was evident following

the initiation of the BDNF microgradient, although this rise was not sustained due to the lack of SOCE (Fig 3.3 B). Given that Sema-3a is a calcium-independent guidance cue, it was hypothesised that SOCE is not activated in growth cones in response to Sema-3a signalling, although this is not known. The data shown here illustrates a small to modest rise in intracellular calcium in response to Sema-3a in control morphant growth cones (Fig. 3.3 C and D), consistent with that described when cyclic nucleotide gated channels are activated by Sema-3a (Togashi et al., 2008b). This calcium rise appears to be attenuated in the STIM1 morphant growth cones, although it is not significantly different from the controls (Fig. 3.3 C and D), suggesting that STIM1-mediated SOCE is not activated in response to Sema-3a signalling.

3.3.3. STIM1 expression is required for growth cone turning

To test whether STIM1-mediated SOCE is necessary for growth cone navigation, growth cone turning responses to microgradients of BDNF, netrin-1 and Sema-3a were measured after STIM1 knockdown, using the classic *in vitro* growth cone turning assay developed by the Poo Laboratory (Lohof et al., 1992). The response of control morphant growth cones turning towards or away from PBS, BDNF, netrin-1 and Sema-3a (Fig. 3.4 A, B) did not differ from previously published data (Gasperini et al., 2009). However, the STIM1 morphant growth cones exhibited a dramatic reversal from attraction ($10.6 \pm 1.6^\circ$) to repulsion ($-6.8 \pm 2.3^\circ$; $p < 0.0001$) in response to BDNF (Fig. 3.4 A, B), and reversal from attraction ($8.8 \pm 2.1^\circ$) to repulsion ($-6.2 \pm 1.5^\circ$; $p < 0.0001$) in response to netrin-1 (Fig. 3.4 A, B). These data demonstrate that STIM1 is necessary for the classical calcium-dependent

guidance cue signalling, consistent with a switch from CaMKII to CaN signalling (Wen et al., 2004). Unexpectedly, the normal repulsive turning response of control growth cones to Sema-3a ($-7.8 \pm 2.2^\circ$) was abolished in STIM1 morphant growth cones, with repulsive turning converted to random turning ($1.0 \pm 2.5^\circ$; $p < 0.05$) (Fig. 3.4 A, B). The presence of the morpholino (control or STIM1) did not alter axon extension in response to any of the guidance cues, confirming that STIM1 knockdown affected only growth cone navigation and not normal axon growth (Fig. 3.4 C). While it was hypothesised that SOCE would be required for turning in response to BDNF and netrin-1 (Li et al., 2005, Gasperini et al., 2009), a role for STIM1 in growth cone turning in response to Sema-3a was not predicted. Given that reduced STIM1 expression had no significant effect on STIM1-mediated SOCE in response to Sema-3a, it was expected that reducing STIM1 would have no effect on Sema-3a growth cone turning.

To confirm that calcium influx was required for STIM1-mediated SOCE in growth cone turning, extracellular calcium was removed from the culture media. The removal of calcium from the media abolished growth cone turning in response to BDNF in both control ($1.0 \pm 1.9^\circ$, $p < 0.01$), as shown by others (Li et al., 2005) and STIM1 morphants ($0.5 \pm 3.4^\circ$, $p < 0.05$; Fig 3.5 A). Conversely, turning in response to Sema-3a in calcium-depleted media did not alter the turning responses in either control ($-4.9 \pm 2.0^\circ$, $p > 0.05$) or STIM1 morphants ($0.9 \pm 2.7^\circ$, $p > 0.05$; Fig. 3.5 A). Axon extension was not altered by the removal of calcium from the imaging media (Fig. 3.5 B). These data support the hypothesis that growth cone turning in response to BDNF, but not Sema-3a, requires the influx of extracellular calcium via STIM1-mediated SOCE. These data suggest that while calcium

Figure 3.3. STIM1 is required for BDNF activated SOCE in growth cones.

A. The average change in calcium ratio in growth cones exposed to a gradient of BDNF. Open circles represent control morphants (n=17) and open triangles represent STIM1 morphants (n=14). **B.** A representative trace of a growth cone response to a gradient of BDNF. The blue trace is from a control morphant, and the red trace is from a STIM1 morphant. **C.** The average change in calcium ratio, in growth cones exposed to a gradient of Sema-3a. Open circles represent control morphants (n=16) and open triangles represent STIM1 morphants (n=9). **D.** A representative calcium imaging trace of a growth cone response to a gradient of Sema-3a. The blue trace from a control morphant and the red trace is from a STIM1 morphant. The arrow in all panels represents gradient initiation. *This figure was published as part of figure 6 in Mitchell et al 2012. Dr Robert Gasperini contributed significantly to the calcium imaging data for this figure.*

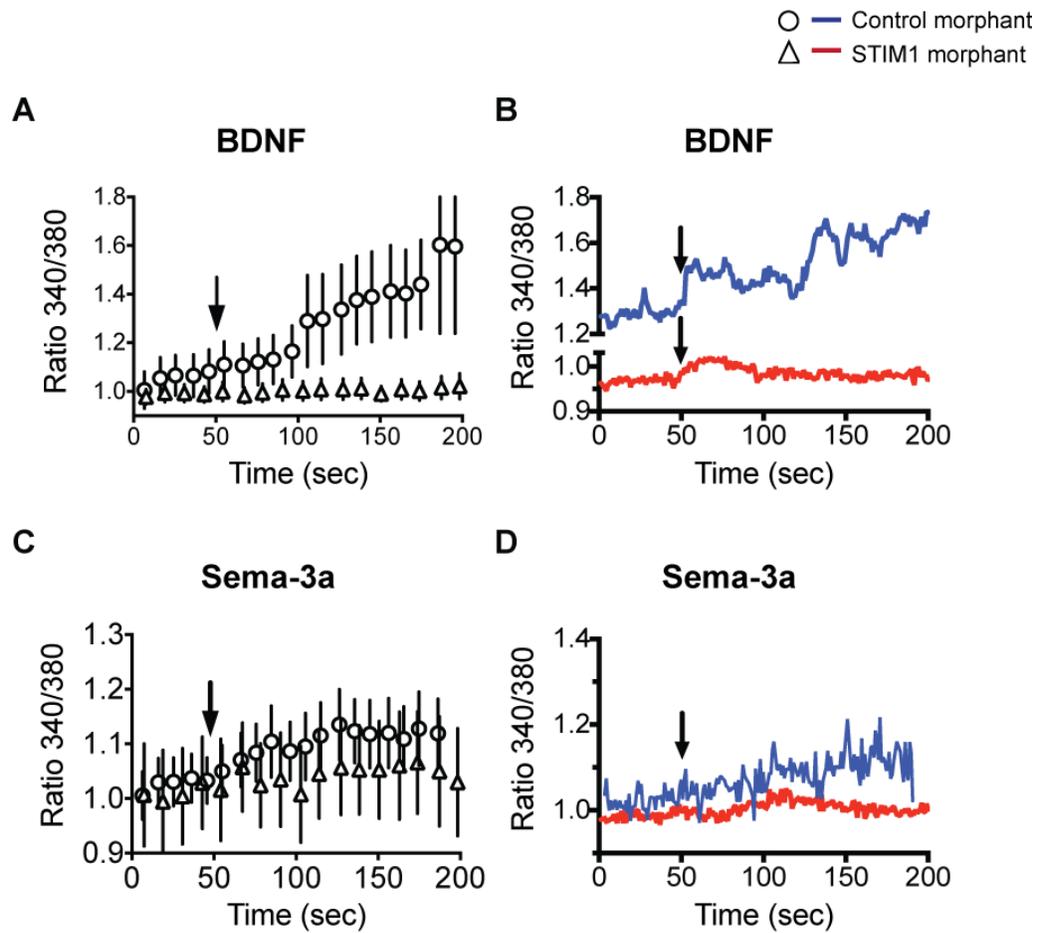


Figure 3.4. STIM1 expression is necessary for correct growth cone navigation.

A. Scatter plots illustrate the data spread for STIM1 and control morphant growth cone responses to PBS, BDNF, netrin-1 and Sema-3a microgradients, with total axon extension on the y-axis, and growth cone turning angle on the x-axis. **B.** Average growth cone turning angles of control (grey bars) and STIM1 (white bars) morphant growth cones after 30 minutes exposure to microgradients of PBS (control), BDNF, Netrin-1 or Sema-3a. Knockdown of STIM1 expression caused a reversal of turning in response to both BDNF and netrin-1, and abolished growth cone turning responses to Sema-3a. **C.** Control (grey bars) or STIM1 (white bars) morpholinos did not affect axon growth during the imaging period. Error bars represent SEM. **p < 0.01, ***p < 0.001. Mann-Whitney U-test. *This figure was published as part of figure 5 in Mitchell et al 2012.*

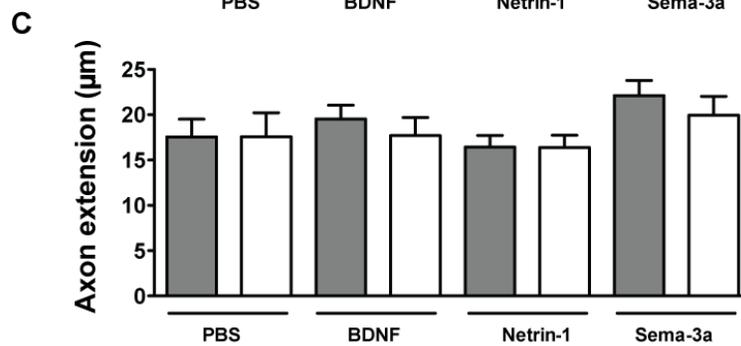
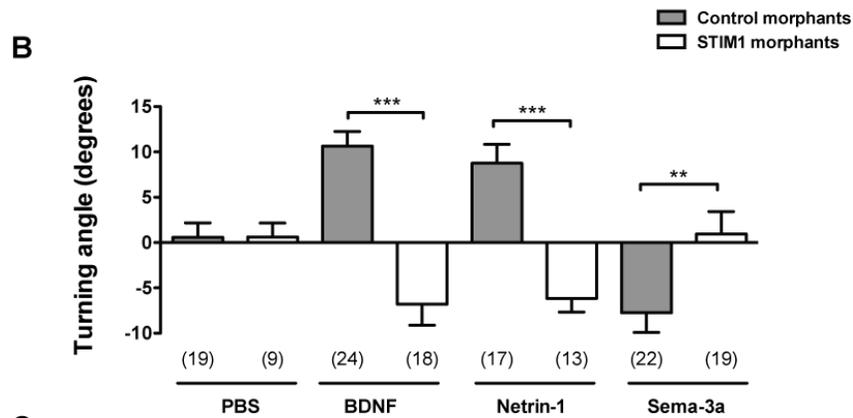
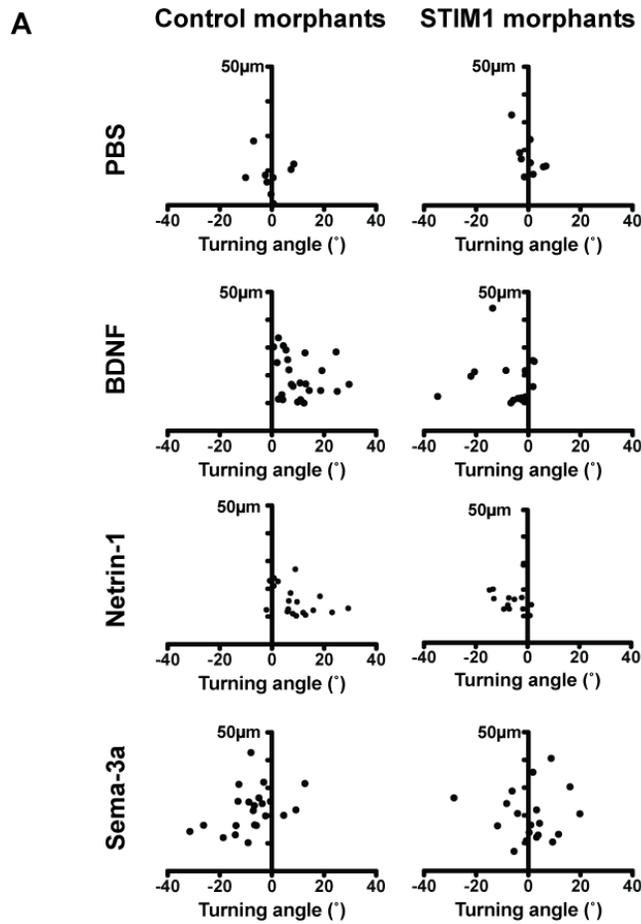
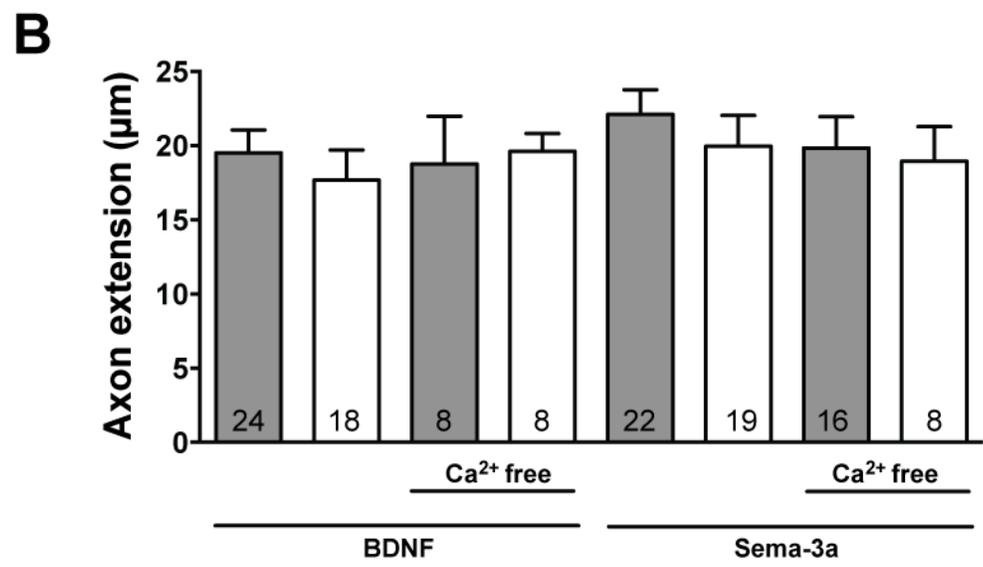
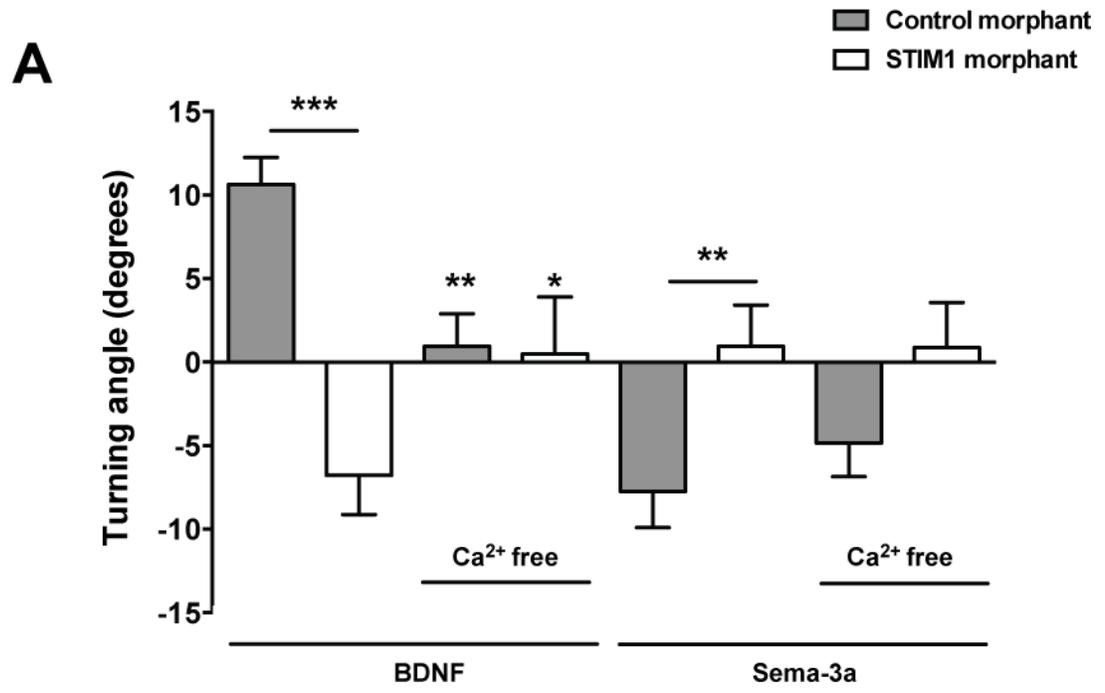


Figure 3.5. Extracellular calcium is necessary for STIM1-mediated growth cone turning in response to BDNF.

Graphs display average turning angles in response to guidance cues **A.** Average turning angles in response to BDNF and Sema-3a, in normal and calcium free conditions, for both control (grey bars) and STIM1 morphants (white bars). Calcium free conditions abolished turning to BDNF but did not affect turning in response to Sema-3a **B.** Calcium free conditions did not affect overall axon growth during the imaging period. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Mann-Whitney U-test. Error bars represent SEM. *This figure was published as part of figure 6 in Mitchell et al 2012.*



influx via STIM1-mediated SOCE is vital for growth cone responses to BDNF signaling, this calcium influx plays a relatively minor role in responses to Sema-3a signalling.

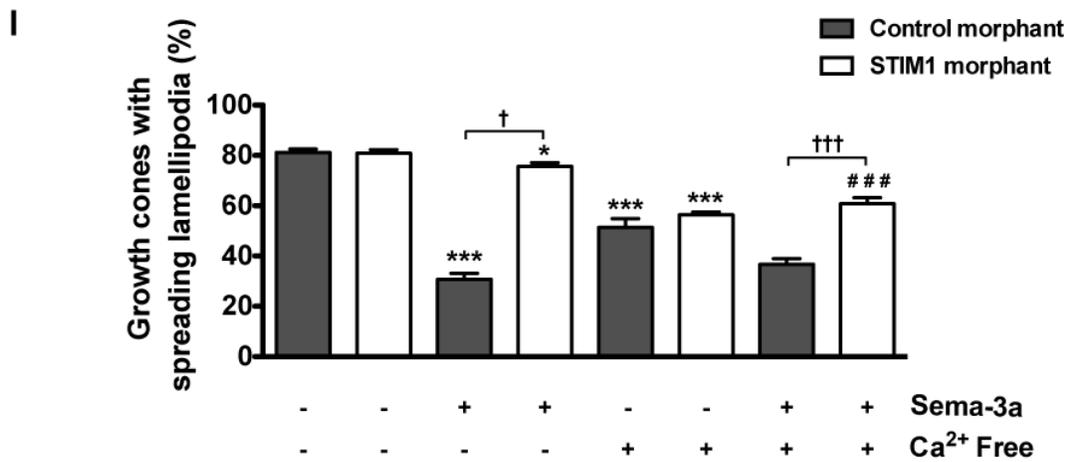
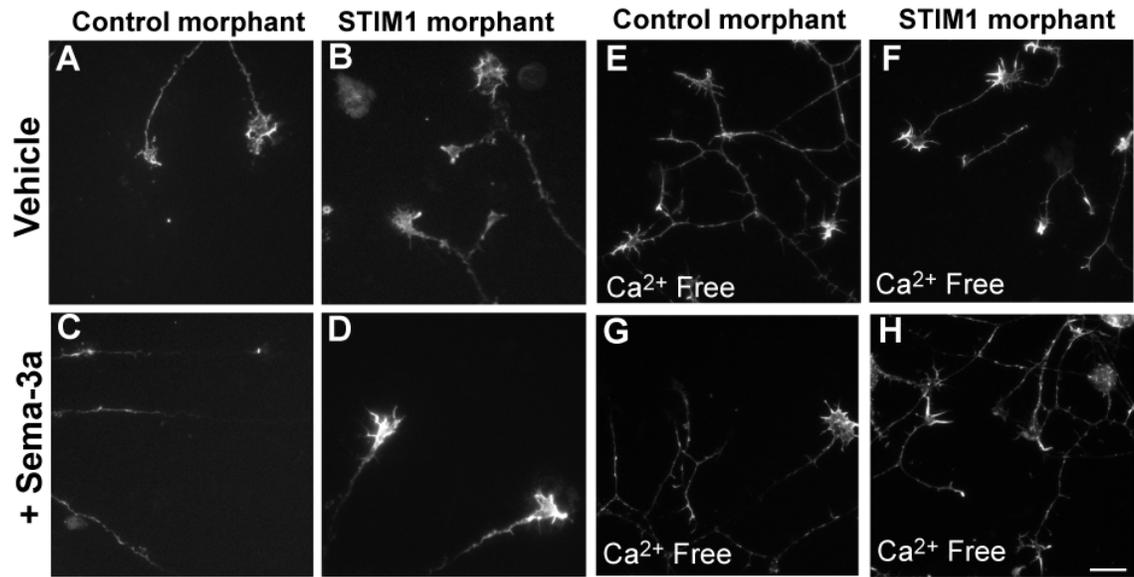
3.3.4. STIM1 is required for Sema-3a induced growth cone collapse.

Sema-3a is known to cause growth cone collapse as well as growth cone repulsion. Whether STIM1 is required for both of these functions of Sema-3a is not known. To test whether STIM1 was required for the collapsing effect of Sema-3a on growth cones, a Sema-3a collapse assay was performed on control and STIM1 morphant growth cones. Growth cones not exposed to Sema-3a exhibited a normal non-collapsed, spreading lamellipodial morphology in $81.4 \pm 1.6\%$ (control morphants; Fig. 3.6 A, I) and $81.1 \pm 1.7\%$ (STIM1 morphants; Fig. 3.6 B, I) of growth cones. The addition of Sema-3a reduced the percentage of growth cones displaying a spreading, lamellipodial morphology to $26.7 \pm 1.8\%$ in control morphant growth cones (Fig. 3.6 C and I; $p < 0.0001$). STIM1 morphants treated with Sema-3a did not collapse to the same extent as the control morphants, with $73.4 \pm 1.5\%$ of growth cones displaying a spreading morphology (Fig 3.6. D, I; $p < 0.05$). These data suggest that STIM1 is required both for the collapsing and repulsive turning effects of Sema-3a on growth cones. However, whether STIM1-induced calcium influx is required for growth cone collapse is unknown. Removal of calcium from the media reduced the number of growth cones displaying a spreading, lamellipodial morphology in both control and STIM1 morphant growth cones ($51.4 \pm 3.4\%$ and $56.4 \pm 1.2\%$ respectively; Fig 3.6 E, F and I). The addition of Sema-3a to the cultures further reduced the number of growth cones displaying a spreading, lamellipodial morphology in control morphant growth

cones ($36.7 \pm 2.3\%$, $p < 0.0001$; Fig 3.6 G and I). However the addition of Sema-3a to the culture had no effect on the STIM1 morphant growth cone morphology ($60.8 \pm 2.4\%$; Fig 3.6 H and I). These data demonstrate that a decrease in STIM1 expression prevents the signal transduction that would normally activate growth cone collapse in response to Sema-3a. Given that this effect persists in the absence of extracellular calcium this suggests that STIM1 operates downstream of Sema-3a signalling in a calcium-independent manner.

Figure 3.6. STIM1 is required for Sema-3a-induced growth cone collapse

A-H. Representative immunocytochemistry images of growth cones incubated in the absence or presence of Sema-3a and Ca^{2+} free media. Growth cone actin cytoskeleton was stained with phalloidin. **A, C.** Control morphant growth cones incubated in sensory neuron media (SNM) (A; n=915) or SNM containing Sema-3a (C; n=537). **B, D.** STIM1 morphant growth cones incubated in SNM (B; n=909) or SNM containing Sema-3a (D; n=1123). **E, G.** Control morphant growth cones incubated in calcium-free culture medium (E; n=929) or calcium-free medium containing Sema-3a (G; n=1693). **F, H.** STIM1 morphant growth cones incubated in calcium-free culture medium (F; n=1319) or calcium-free medium containing Sema-3a (H; n=1723). Scale bar equals 20 μm and applies to A-H. **I.** Mean percentages of control (grey bars) and STIM1 morphant (white bars) growth cones without collapse in the presence or absence of Sema-3a are graphed. Growth cones were grown in SNM or calcium-free media. Error bars represent SEM. * compared each to the control morphant in SNM and in the absence of Sema-3a. * $p < 0.05$, *** $p < 0.0001$. † $p < 0.05$, ††† $p < 0.0001$ (indicated by the bars). Data were collected over three different experiments. Treatments were compared using the Student's t-test. *This figure was published as figure 7 in Mitchell et al, 2012.*



3.4 Discussion

The data presented in this chapter demonstrates that STIM1 activates SOCE in sensory growth cones, and that STIM1 expression is required for growth cone navigation in sensory neurons in response to attractive and repulsive guidance cues. The activation of STIM1-mediated SOCE in growth cones is consistent with the known role of STIM1 in regulating SOCE in both neuronal and non-neuronal cells (Klejman et al., 2009, Park et al., 2010a, Gruszczynska-Biegala et al., 2011). While it has been suggested that SOCE may be important for growth cone motility (Hong et al., 2000, Li et al., 2005, Gasperini et al., 2009), these experiments are the first to demonstrate that STIM1-mediated SOCE is required for growth cone turning in response to calcium-dependent guidance cues. Unexpectedly, STIM1 expression is also required for chemorepulsion and growth cone collapse in response to the calcium-independent guidance cue Sema-3a, via a SOCE-independent mechanism. This result is significant and may represent a novel Sema-3a signaling pathway within growth cones.

STIM1 expression is required for SOCE in growth cones

The function of STIM1 is often examined with the use of experimental calcium depletion through the use of thapsigargin. Acute treatment of thapsigargin inhibits the SERCA pump on the ER membrane, mobilising calcium and preventing the uptake of calcium into the ER (Thastrup et al., 1990, Foskett and Wong, 1992, Gomez et al., 1995). This process activates the oligomerisation of STIM1 and the activation of SOCE through the CRAC channel

(Huang et al., 2006, Mercer et al., 2006, Dietrich et al., 2007). In order to examine if targeted STIM1 knockdown with morpholinos resulted in a functional reduction in STIM1 expression, thapsigargin treatment was used, in combination with ratiometric calcium imaging. Consistent with work from others, thapsigargin application rapidly increased intracellular calcium levels significantly above resting calcium levels, in both DRG neuronal somata and growth cones (Gomez et al., 1995). This was significantly reduced after STIM1 knockdown, confirming the functionality of STIM1 in DRG neurons, consistent with previous data from other cell types (Liou et al., 2005, Roos et al., 2005, Zhang et al., 2005, Lefkimiatis et al., 2009). These data demonstrate for the first time that SOCE operates in sensory neuronal growth cones, as it does in neuronal soma, and that this SOCE is mediated by the expression of STIM1.

Thapsigargin is an artificial activator of calcium that growth cones do not normally come in contact with *in vivo*. It was predicted that STIM1-mediated SOCE could be activated in growth cones with the use of physiological guidance cues that growth cones are exposed to within the embryonic environment throughout development. The data presented here demonstrate that in response to the calcium-dependent guidance cue BDNF, SOCE was activated and elevated intracellular calcium concentrations were sustained for the duration of the application of the guidance cue. In response to BDNF microgradients, two discrete calcium rises occurred within control growth cones. It is hypothesised that the first rise in intracellular calcium refers to CICR. The second, larger rise in calcium is SOCE, where STIM1 and Orai proteins interact to create the CRAC channel and subsequent calcium influx. This rise in calcium is sustained as long as the microgradient of BDNF is applied to

the growth cone. The activation of SOCE was significantly reduced after a reduction in STIM1 expression. Puffing of BDNF microgradients onto STIM1 morphant growth cones only caused a small initial rise in intracellular calcium concentrations. It is hypothesised that this calcium rise corresponds to the influx of calcium through TRPC channels, and the subsequent CICR from the ER (Li et al., 2005). This calcium rise does not continue to increase, and is unable to be sustained despite the presence of BDNF, due to the loss of STIM1-mediated SOCE. These data demonstrate the necessity of STIM1 for SOCE activation in response to a calcium-dependent guidance cue.

STIM1 is necessary for growth cone turning in response to BDNF and netrin-1

The reduction of endogenous levels of STIM1 abolished both thapsigargin-induced SOCE and calcium-dependent guidance cue-induced SOCE within growth cones. Based on these results, it was hypothesised that the loss of STIM1-mediated SOCE would alter growth cone turning behaviour in response to calcium-dependent guidance cues. This loss of SOCE would reduce cytosolic calcium and be predicted to activate the switch from CaMKII-mediated attraction to CaN-mediated repulsion.

Reduction of STIM1 expression in growth cones converted attraction towards BDNF and netrin-1 into repulsion. BDNF and netrin-1 are both calcium-dependent guidance cues that rely heavily on the influx of calcium through plasma membrane channels for their signalling mechanisms (Hong et al., 2000, Li et al., 2005, Wang and Poo, 2005). BDNF signals by binding to the TrkB receptor, activating TRPC channels for calcium influx,

which depolarises the plasma membrane, activating VGCCs (Li et al., 2005, Shim et al., 2005). Netrin-1 signals through the DCC receptor on the plasma membrane, activating similar signalling pathways, resulting in calcium influx into the cytoplasm (Hong et al., 2000, Wang and Poo, 2005). Growth cone turning behaviour depends on which downstream effectors are activated in response to calcium influx. CaMKII and CaN are two calcium effectors that are important for regulating cytoskeletal motility and hence growth cone behaviours. In the presence of a high CaMKII:CaN ratio, and relatively high calcium concentrations, growth cone attraction occurs. In the presence of a lower CaMKII:CaN ratio and lower calcium concentrations, growth cone repulsion occurs (Wen et al., 2004, Forbes et al., 2012). CaMKII has a lower affinity for calcium, so is activated when higher concentrations of calcium are present (Hudmon and Schulman, 2002, Wen et al., 2004). In response to both BDNF and netrin-1, CaMKII is preferentially activated, resulting in growth cone attraction (Song et al., 1997, Song et al., 1998, Wen et al., 2004, Gasperini et al., 2009). Activation of CaMKII results in growth cone attraction through actin polymerization, potentially through an increase in GAP43 phosphorylation (Williams et al., 1995, He et al., 1997, Dent and Meiri, 1998, Bolsover, 2005), and/or an activation of the specific RhoGTPases, Rac1 and cdc42, which participate in the assembly of actin filaments (Jin et al., 2005, Gomez and Zheng, 2006).

In contrast to CaMKII, CaN has a higher affinity for calcium, and requires a lower concentration of calcium for activation (Rusnak and Mertz, 2000, Wen et al., 2004). When STIM1 expression was reduced, SOCE was abolished causing growth cones to be repulsed in response to chemoattractive, calcium-dependent guidance cues BDNF and netrin-1. It is

likely that this is due to the activation of CaN, instead of CaMKII. Without the secondary influx of calcium mediated by SOCE, the initial rise in cytoplasmic calcium concentration due to CICR was not sustained, potentially activating CaN and hence resulting in growth cone repulsion. CaN activation causes growth cone repulsion potentially through the dephosphorylation of GAP43, capping actin filaments and leading to local retraction (Lautermilch and Spitzer, 2000, Bolsover, 2005), or the activation of RhoA, a RhoGTPase associated with repulsion (Gomez and Zheng, 2006). CaN also inhibits I1, which results in the downstream inhibition of CaMKII (Han et al., 2007). There is also crosstalk between the CaMKII-CaN system of activation, and cAMP signalling (Forbes et al., 2012). cAMP levels are elevated in response to increases in intracellular calcium concentrations. cAMP activates PKA, resulting in the activation of I1, which is normally inhibited by CaN. This means that cAMP helps to promote growth cone attraction, due to a reduction in the inhibition of CaMKII (Han et al., 2007). Taken together, the reduction of STIM1 abolishes SOCE in growth cones, and converts both BDNF and netrin-1 attraction to repulsion. This is likely occurring due to a switch from the activation of CaMKII, to CaN, changing growth cone turning behaviour.

STIM1 is required for Sema-3a repulsion and collapse, independent of calcium

It was surprising to find that while Sema-3a did not activate SOCE in growth cones, STIM1 was still required for growth cone turning in response to Sema-3a. Sema-3a is traditionally described as a calcium-independent guidance cue since Sema-3a-induced growth cone repulsion is not perturbed by low extracellular calcium (Song et al., 1998, Song and Poo,

1999). In addition, reduction of the calcium signalling mediator Homer1b/c has no effect on Sema-3a-induced growth cone repulsion (Gasperini et al., 2009). Homer proteins are scaffolding proteins with known roles in calcium homeostasis in synaptic plasticity (Tu et al., 1999) and axon guidance (Foa et al., 2001). Recently, Sema-3a-induced growth cone turning has been shown to rely on a small-modest calcium influx across the plasma membrane via cyclic nucleotide gated calcium channels (Togashi et al., 2008b). These channels conduct calcium at such a low (nanomolar) extracellular calcium concentration that the requirement of calcium for Sema-3a signalling would not have been detected in earlier studies (Song et al., 1998, Togashi et al., 2008b). The small rise in intracellular calcium levels in response to Sema-3a within control growth cones that was demonstrated in this work is consistent with those observed by Togashi et al. (2008), who demonstrated that Sema-3a signalling could increase cytosolic calcium levels by 5% via the cyclic nucleotide gated channels (Togashi et al., 2008b).

While reduction of STIM1 expression only partially attenuated the calcium response to Sema-3a, it abolished Sema-3a mediated repulsive turning. The removal of calcium from the culture media did not alter the already abolished turning response mediated by STIM1 reduction and Sema-3a signalling. This suggests that the Sema-3a effect on growth cones does not require substantial influx of calcium from extracellular sources. Removal of extracellular calcium also had no effect on Sema-3a turning responses in control growth cones. This is consistent with previous observations that Sema-3a can induce growth cone repulsion at very low levels of extracellular calcium (Song et al., 1998, Togashi et al., 2008b). Sema-3a is not just a growth cone chemorepellant guidance cue. It was originally

discovered as a growth cone-collapsing agent. If STIM1 was required for Sema-3a-mediated growth cone turning, it was likely to be required for Sema-3a-induced growth cone collapse as well. STIM1 appears to be necessary for the collapse of growth cones in response to Sema-3a. This requirement of STIM1 for Sema-3a-induced collapse also appears to occur independently of calcium, since removal of calcium from the media had no real effect on growth cone collapse. One interpretation of this Sema-3a data is that only very small changes in intracellular calcium levels are sufficient to perturb Sema-3a signalling and hence growth cone turning and collapse.

Another potential explanation for the requirement of STIM1 in Sema-3a-mediated growth cone turning and collapse is that STIM1 may function in a calcium-independent manner in response to Sema-3a. While the primary function of STIM1 is thought to be the activation of SOCE, this may not be the case in the presence of Sema-3a. The main function of STIM1 in response to Sema-3a may be to interact with phosphatase and tensin homolog (PTEN) or microtubules, (Iijima and Devreotes, 2002, Grigoriev et al., 2008, Henle et al., 2011, Shen et al., 2011), rather than activate SOCE. Sema-3a signalling has been shown to activate accumulation of PTEN to the leading edge of the growth cone membrane, which is necessary for Sema-3a induced growth cone collapse (Chadborn et al., 2006). PTEN accumulation at the leading edge would inhibit PI₃K activity and subsequent calcium-mediated attractive turning in growth cones (Henle et al., 2011). Furthermore, prior to membrane recruitment, PTEN is sequestered to microtubules (Chadborn et al., 2006) and STIM1 has been shown to interact directly with microtubules. STIM1 binds to the microtubule-plus-end tracking protein EB1, which facilitates the remodelling of the ER

along microtubules (Grigoriev et al., 2008). Taken together, these data suggest a novel mechanism whereby Sema-3a may activate ER and microtubule remodelling via PTEN and STIM1 to effect growth cone repulsion or collapse. Another alternative function of STIM1 has recently been proposed, whereby STIM1 regulates the levels of cAMP through the activation of adenylate cyclases and store-operated cAMP signalling (Lefkimmatis et al., 2009). This alternative function of STIM1 is examined in more detail in chapter 5 of this thesis.

Taken together, the data presented within this chapter demonstrate that STIM1 activates SOCE in growth cones, and that STIM1-mediated SOCE is necessary for growth cone navigation in response to calcium-dependent guidance cues. Furthermore, STIM1 is required for both growth cone turning and growth cone collapse in response to Sema-3a, and functions in a manner independent of extracellular calcium, suggesting that STIM1 is involved with multiple signalling pathways within growth cones.

Chapter 4:

**L-type voltage-gated calcium channels are not required
for STIM1-mediated growth cone navigation**

Chapter 4. L-type voltage-gated calcium channels are not required for STIM1-mediated growth cone navigation

4.1 Introduction

STIM1 is a promiscuous protein with many alternative functions and binding partners. Recently, $Ca_v1.2$, an important subunit that forms the L-type VGCC, was shown to be a negatively regulated target of STIM1 (Park et al., 2010a, Wang et al., 2010b). STIM1 was shown to reciprocally inhibit L-type VGCCs, while activating Orai1 (Park et al., 2010a). This STIM1 inhibition directly suppressed the depolarization-induced opening of $Ca_v1.2$ in neurons (Park et al., 2010a). In addition, knockdown of STIM1 reduced the store depletion-mediated inhibition of $Ca_v1.2$ (Wang et al., 2010b). Co-immunoprecipitation experiments demonstrated direct interaction between STIM1 and the C-terminus of $Ca_v1.2$ (Park et al., 2010a). The mechanisms leading up to the reciprocal inhibition are similar to the activation of Orai1, such that ER calcium depletion, resulting in STIM1 clustering within the ER membrane, causes the accumulation of $Ca_v1.2$ as well as Orai1. The accumulation of both of these proteins occurs in areas of the plasma membrane that are part of ER-PM junctions, where STIM1 clusters (Park et al., 2010a, Wang et al., 2010b).

$Ca_v1.2$ is the most abundant L-type VGCC within neurons. The primarily recognised role of L-type VGCCs in neurons is in synaptic transmission and plasticity (Tsien et al., 1988, Dunlap et al., 1995, Calin-Jageman and Lee, 2008, Catterall and Few, 2008, Catterall, 2011). L-type VGCC activation can cause changes in the regulation of gene expression, directly regulating neurotransmitter release (Tsien et al., 1988, Catterall, 2011), as well as

dendritic calcium spikes (Häusser et al., 2000, Sjöström and Nelson, 2002). L-type VGCCs are so called because they cause a long lasting calcium influx, as they have a slow voltage-dependent inactivation (Tsien et al., 1988). L-type VGCCs are known to be present in all regions of neurons, including neuronal soma, neurites and growth cones (Grinvald and Farber, 1981, Bolsover and Spector, 1986, Ross et al., 1986, Ross and Werman, 1987, Lipscombe et al., 1988, Obermair et al., 2004). L-type VGCCs have been shown to exist on growth cone membranes within the developing rat brain, with approximately 6 times more L-type VGCCs in synaptosomal membranes than growth cone membranes, suggesting that VGCCs increase upon synaptogenesis (Vigers and Pfenninger, 1991). Binding assays suggest that L-type VGCCs are enriched in growth cones that have been isolated from developing rat forebrains (Ohbayashi et al., 1998). L-type VGCCs often cluster together in hot spots within growth cones (Lipscombe et al., 1988, Silver et al., 1990). These hot spots are usually located at the base of filopodia that extend from the peripheral region of the growth cone, and are linked to the outgrowth of the growth cone margin (Silver et al., 1990). The greatest outgrowth of the growth cone margin occurs close to L-type VGCC hotspots, with a spatial correlation between hotspot, filopodia location, and growth cone outgrowth, suggesting that an increase in calcium through L-type VGCCs is required for the outgrowth of the growth cone margin (Silver et al., 1990).

L-type VGCC clusters are expressed on growth cones over the course of development in hippocampal neurons (Obermair et al., 2004). Before neurite outgrowth occurs, L-type VGCC clusters are evenly distributed over neuronal lamellipodia and filopodia. When the first neurites begin to emerge, and begin the process of outgrowth, these clusters are found

to be located on the surface of the neurites, as well as on growth cones, including filopodia tips. During the elongation of the single neurite that is to become the neuronal axon, the clusters of L-type VGCCs become particularly pronounced on the axonal growth cone, suggesting that L-type VGCC activity within growth cones is important for the early stages of axonal neurite outgrowth (Obermair et al., 2004). The activation of L-type VGCCs is thought to be required for the enhancement of neurite outgrowth, through activity within growth cones (Rogers and Hendry, 1990, Silver et al., 1990, Solem et al., 1995, Fukura et al., 1996, Ohbayashi et al., 1998). These effects have been discovered through the use of L-type VGCC agonists and antagonists in cultures from a variety of systems including mammalian and non-mammalian (Rogers and Hendry, 1990, Silver et al., 1990, Solem et al., 1995, Fukura et al., 1996, Ohbayashi et al., 1998). However, L-type VGCC activation is also known to contribute to the inhibition of neurite outgrowth in rat spinal cord slices (Bär et al., 1993), as well as growth cone arrest within PC12 neurites (Schindelholz and Reber, 2000). Taken together, these data suggest that L-type VGCC activation within growth cones may be important for the regulation of axon outgrowth.

Spontaneous calcium transients are important for growth cone motility. The calcium influx required for these spikes in calcium levels in growth cones occurs through non-VGCCs (Gomez et al., 1995, Gu and Spitzer, 1995). Addition of general calcium channel blockers La^{3+} and Ni^{2+} inhibited growth cone calcium transients. However, when a cocktail of VGCC blockers (conotoxin, verapamil and nifedipine) was used, the growth cone calcium transients continued (Gomez et al., 1995). Filopodia are the first area of the growth cone to detect changes within the external environment, and to respond appropriately, for the

growth cone to lead and direct axon pathfinding. Calcium transients can initiate in filopodia. In cultured *Xenopus* spinal neurons, the spontaneous calcium transients within growth cone filopodia were also found to be generated by calcium influx that occurs through non-VGCCs, suggesting that non-VGCC transients are necessary for growth cone navigation (Gomez et al., 2001). Taken together, these data suggest that growth cone calcium transients are not regulated by VGCCs. This leads to the question of whether L-type VGCCs are involved in growth cone navigation, or just neurite outgrowth and extension.

There is data from *Xenopus* spinal neurons to suggest that the L-type VGCCs are involved with growth cone navigation (Hong et al., 2000, Nishiyama et al., 2003). Netrin-1-induced attraction is dependent upon calcium influx through L-type VGCCs within *Xenopus* spinal neurons. When L-type VGCCs were blocked with nimodipine, netrin-1 induced attraction was converted to repulsion (Hong et al., 2000). Netrin-1 also induces an increase in the amplitude of L-type VGCC currents within turning *Xenopus* spinal neuron growth cones (Nishiyama et al., 2003). L-type VGCCs have also been shown to operate downstream of TRPC channels, in response to netrin-1 within turning *Xenopus* spinal neuron growth cones (Wang and Poo, 2005, Shim et al., 2009). While these data conclusively show that L-type VGCCs are required for netrin-1 induced growth cone turning, it is only within one specific cell type, *Xenopus* spinal neurons. It is still unknown whether L-type VGCCs are required for growth cone guidance within mammalian cells. For example, while BDNF-induced turning is dependent upon calcium influx, similar to netrin-1, it is unlikely that this influx occurs through L-type VGCCs. Inhibition of L-type VGCCs with nifedipine had no effect

on BDNF-induced attraction in rat cerebellar granule cells (Li et al., 2005). Similarly, spontaneous calcium transients in chick DRG growth cones are generated by a non-voltage gated calcium influx (Gomez 1995).

Data demonstrating a requirement for L-type VGCCs in axon guidance is inconclusive, and may be a cell type dependent effect. However, it is possible that L-type VGCCs do function in embryonic rat DRG turning, and that this role is regulated by STIM1. While data from DRG growth cones suggests that L-type VGCCs are not required for growth cone turning within these cells (Gasperini et al., 2009), this preliminary study examined the potential functional interaction between STIM1 and L-type VGCCs in growth cone turning. One potential explanation for the requirement of STIM1 in growth cone turning towards BDNF and netrin-1, is that in addition to mediating SOCE, STIM1 is interacting with L-type VGCCs. In order to address these aims, *in vitro* growth cone turning assay was used, in combination with targeted STIM1 knockdown, in the presence of an L-type VGCC agonist (BayK8644) and antagonist (nifedipine), to activate and inhibit L-type VGCCs respectively.

4.2 Materials and Methods

4.2.1 Materials

BayK8644 (referred to as BayK from here in) and nifedipine were purchased from Alomone Labs (Jerusalem, Israel). BDNF, netrin-1 and semaphorin-3a were purchased from R+D Systems (Minneapolis, Minnesota, USA).

4.2.2 DRG cell culture

Embryonic DRG cell culture was performed as described in section 2.2.4

4.2.3 Growth cone turning assay

Growth cone turning assays were performed as described in section 3.2.6 Pharmacological reagents used were BayK (5 μ M, Alomone) and Nifedipine (5 μ M, Alomone).

4.3 Results

To examine whether there is a functional interaction between STIM1 and L-type VGCCs in growth cone turning, pharmacological agents were used to activate and inhibit L-type VGCCs in the presence of normal or reduced STIM1 expression. BayK was used as a selective L-type VGCC agonist, and nifedipine was used as an L-type VGCC antagonist. After bath application of BayK, to activate L-type VGCCs, growth cone responses to microgradients of netrin-1 and BDNF, in the presence of STIM1 or control morpholinos, were measured. Activation of L-type VGCCs abolished attraction to netrin-1 in control growth cones, resulting in a random turning response ($-0.8 \pm 1.5^\circ$, $p < 0.0001$; Fig 4.1 A). Similarly, activation of L-type VGCCs abolished the repulsive turning response to netrin-1 in STIM1 morphant growth cones ($2.4 \pm 2.6^\circ$, $p < 0.05$; Fig. 4.1 A). Activation of L-type VGCCs with BayK increased axon extension in control growth cones in response to netrin-1 ($25.2 \pm 2.4 \mu\text{m}$, $p < 0.01$; Fig 4.1 B), but it did not affect extension in STIM1 morphant growth cones.

In response to microgradients of BDNF, activation of L-type VGCCs with BayK abolished growth cone attraction within control growth cones, resulting in a random turning response ($-0.6 \pm 2.9^\circ$, $p < 0.001$ Fig 4.1 C). The repulsive turning response to BDNF in STIM1 morphant growth cones was also abolished upon activation of L-type VGCCs ($-1.6 \pm 2.8^\circ$, $p < 0.05$; Fig. 4.1 C). Addition of BayK did not alter axon extension in response to BDNF in either control or STIM1 morphant growth cones (Fig. 4.1 D). These data suggest that activating L-type VGCCs increased the influx of calcium into the growth cone, changing

both the source of calcium, and the level of calcium within the cytoplasm during growth cone turning, hence perturbing downstream calcium signalling pathways.

The inhibition of L-type VGCCs after bath application of nifedipine had no effect on the turning angles of either control or STIM1 morphant growth cones, in response to gradients of either netrin-1 or BDNF (Fig. 4.2 A and C). While there was a trend of slightly decreased turning angles in response to netrin-1 (controls $4.7 \pm 1.4^\circ$, STIM1 morphants $-3.6 \pm 1.6^\circ$) and BDNF (controls $5.5 \pm 1.5^\circ$, STIM1 morphants $-2.7 \pm 2.0^\circ$) this was not significant in any case. Blocking L-type VGCCs increased axon extension in STIM1 morphants in response to netrin-1 ($21.9 \pm 1.8\mu\text{m}$, $p < 0.05$; Fig. 4.2 B), but not in control growth cones. Axon extension was not altered in control or STIM1 morphants in response to gradients of BDNF (Fig. 4.2 D). Taken together these data suggest that L-type VGCCs are not required for STIM1-mediated growth cone turning in sensory growth cones, although there does appear to be an interaction between STIM1 and L-type VGCCs, required for axon extension, in the presence of netrin-1. Importantly, these data suggest that the source of calcium is important for the function of navigating growth cones.

Figure 4.1. Activation of L-type voltage-gated calcium channels abolishes growth cone navigation

Graphs display average turning angles in response to guidance cues **A, C**. Average growth cone turning angles of control (grey bars) and STIM1 morphant (white bars) growth cones in response to netrin-1 (**A**) or BDNF (**C**) in the presence of BayK, to activate L-type VGCCs. **B, D**. Average axon extensions of control (grey bars) and STIM1 morphant (white bars) growth cones in response to netrin-1 (**B**) or BDNF (**D**), in the presence of BayK. The number of growth cones per treatment in the bars on graphs **B** and **D**, apply to graphs **A** and **C**, respectively. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to appropriate guidance cue-only control. Mann-Whitney U-test.

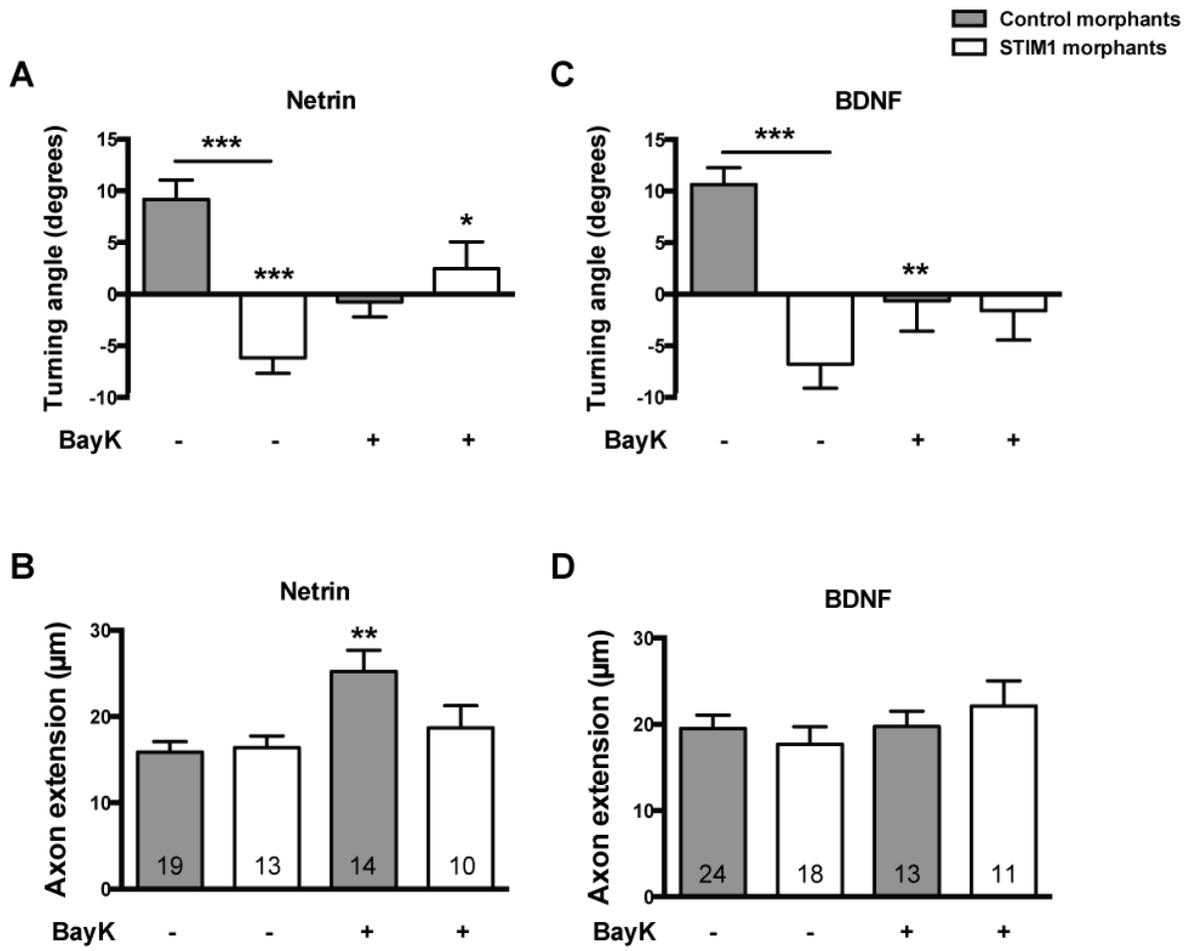
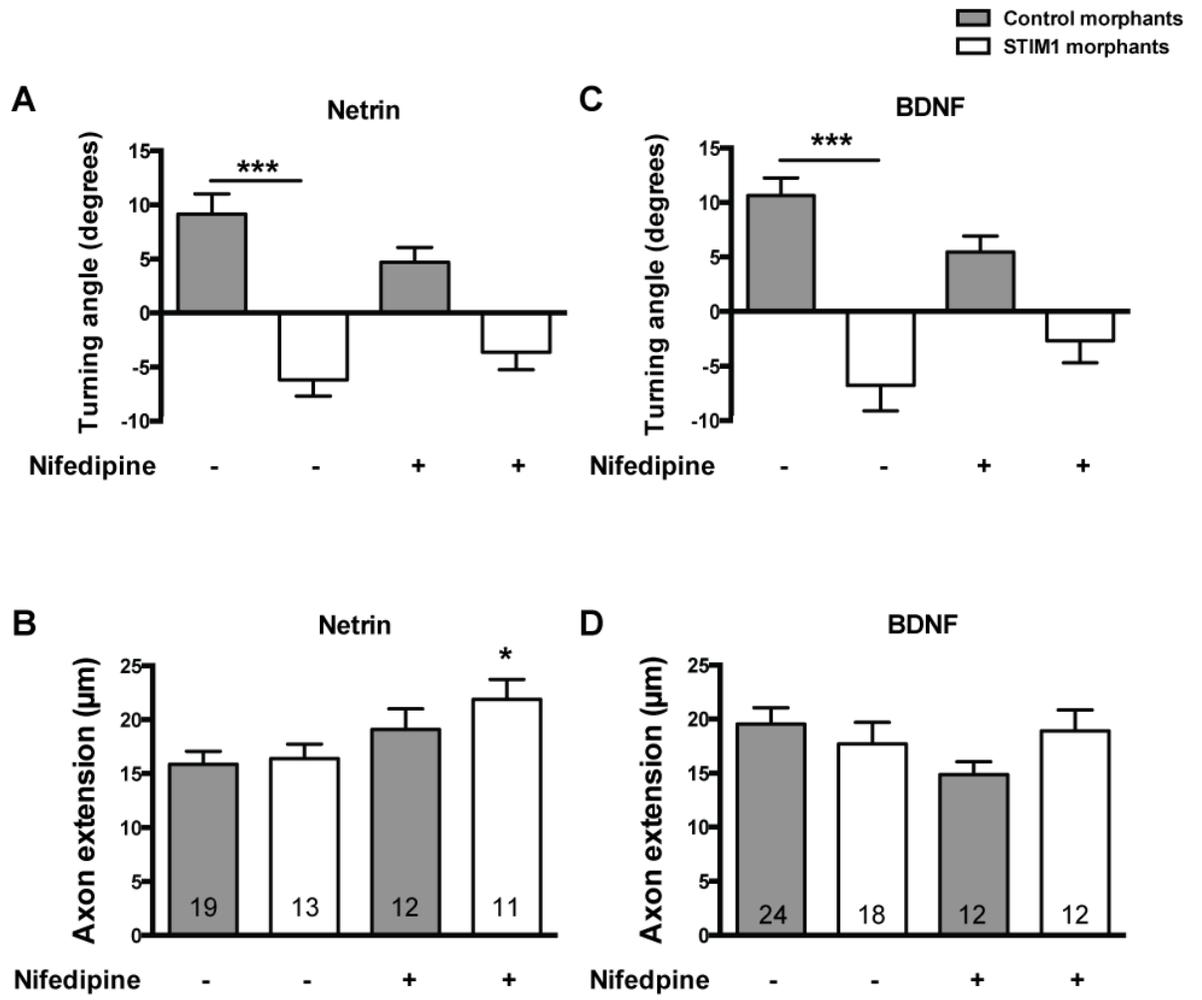


Figure 4.2. Inhibition of L-type voltage-gated calcium channels does not alter growth cone navigation

Graphs display average turning angles in response to guidance cues **A, C**. Average growth cone turning angles of control (grey bars) and STIM1 morphant (white bars) growth cones in response to netrin-1 (**A**) or BDNF (**C**), in the presence of nifedipine, to inhibit L-type VGCCs. **B, D**. Average axon extensions of control (grey bars) and STIM1 morphant (white bars) growth cones in response to netrin-1 (**B**) or BDNF (**D**), in the presence of nifedipine. The number of growth cones per treatment in the bars on graphs **B** and **D**, apply to graphs **A** and **C**, respectively. Error bars represent SEM. * $p < 0.05$, *** $p < 0.001$; compared to appropriate guidance cue-only control. Mann-Whitney U-test.



4.4 Discussion

The work in this chapter sought to determine whether a potential functional interaction exists between STIM1 and L-type VGCCs within turning growth cones, and whether this interaction could explain the requirement for STIM1 in growth cone turning towards BDNF and netrin-1. STIM1 has been reported to suppress voltage activation of neuronal L-type VGCCs in neurons, while simultaneously activating SOCE via Orai channels (Park et al., 2010a). While the requirement for L-type VGCC activity is vital in mature neurons, for example in dendritic depolarisation (Flavell and Greenberg, 2008), the necessity for signalling via L-type VGCCs appears to be non-essential in growth cone navigation. In this study, activation of L-type VGCCs abolished growth cone turning in response to both netrin-1 and BDNF, regardless of STIM1 expression, while blocking L-type VGCCs had no effect. This suggests that for growth cone navigation there is no functional interaction between STIM1 and L-type VGCCs. However, the activation of L-type VGCCs increased axon extension in control morphants in response to gradients of netrin-1, while inhibition of L-type VGCCs increased axon extension in STIM1 morphants in response to netrin-1, suggesting a role for STIM1 in netrin-1 mediated neurite extension.

STIM1-mediated growth cone turning does not require the activation of L-type voltage-gated calcium channels

Treatment with BayK activates L-type VGCCs, causing a large increase in channel activity. Due to this increase in channel activity, calcium flows across the membrane, elevating intracellular calcium concentrations within the growth cone (Hong et al., 2000). The

concentration of calcium within growth cones is crucial (Kater et al., 1988, Kater and Mills, 1991). Growth cones require an optimal range of calcium for normal function (Kater and Mills, 1991, Forbes et al., 2012). If too much calcium is present within the cytoplasm, growth cones collapse by withdrawing their lamellipodia and filopodia. If there is too little calcium within the cytoplasm, neurite outgrowth ceases (Kater and Mills, 1991). In control growth cones, where STIM1 and Orai proteins would be functional, mediating SOCE, activation of L-type VGCCs would cause additional calcium to enter the cytoplasm. Within these experiments, L-type VGCCs were activated globally with bath application of BayK. It would be assumed that all L-type VGCCs on growth cones are activated, saturating the cytoplasm with calcium influx. I hypothesised that under these conditions intracellular calcium was elevated above its optimal working range, and growth cone turning was abolished in response to both netrin-1 and BDNF. To fully address this hypothesis, future experiments would require calcium imaging of navigating growth cones, to determine the effect of L-type VGCC activation on growth cone calcium concentrations. In addition, L-type VGCCs could be activated in a localised, spatial manner, by puffing a microgradient of BayK onto growth cones. It would be predicted that this would activate L-type VGCCs in a smaller, localised area, which might provide more information as to the role of L-type VGCCs in growth cone navigation.

In response to calcium-dependent guidance cues, STIM1 function was required for SOCE and growth cone turning. I hypothesised that if L-type VGCCs function within growth cone navigation, then activating L-type VGCCs in STIM1 morphants would restore growth cone turning responses to both netrin-1 and BDNF. The influx of calcium via the L-type VGCCs

would replace the normal STIM1-mediated calcium influx. However, activating L-type VGCCs with BayK to increase calcium flow across the membrane did not restore attractive turning to either netrin-1 or BDNF in STIM1 morphants. These data suggest that the source of calcium is important in growth cone turning. Recently, studies have suggested that all calcium is not the same (Tojima et al., 2011). The source of calcium signals, through the gating of different sets of calcium channels, can determine growth cone turning direction. There are several studies which suggest that TRPC channels are activated first in response to BDNF signalling, dictating downstream calcium related signalling pathways to result in growth cone attraction (Li et al., 2005, Gasperini et al., 2009). Different ion channels allow different volumes of calcium to influx into the cell, due to their calcium specificity, as well as the duration of their opening. TRPC channels are transiently activated, are not selective for only calcium, which results in a small calcium influx (reviewed in Bon and Beech, 2013). L-type VGCCs are long-lasting in their activation, and allow large influxes of calcium into the cell (Tsien et al., 1988). Both the volume of calcium, and the location of entry of calcium are important for normal growth cone function. It is probable that global activation of L-type VGCCs would saturate the growth cone cytoplasm with large amounts of calcium (Kater and Mills, 1991). Future calcium imaging experiments would determine if this level of calcium would tip the balance towards growth cone collapse.

To further examine the potential functional interaction between STIM1 and L-type VGCCs within navigating growth cones, nifedipine was used as an L-type VGCC antagonist. Blocking L-type VGCCs had no effect on either control or STIM1 morphant growth cones, in response to gradients of either netrin-1 or BDNF. In previous L-type VGCC blocking

experiments in *Xenopus* spinal neuron growth cones, attraction to netrin-1 was switched to repulsion (Hong et al., 2000). This L-type VGCC activity in growth cone navigation may be cell type specific. In support of this concept, previous work has clearly demonstrated that upon pharmacological inhibition of L-type VGCCs, the turning responses of mammalian growth cones were not affected (Li et al., 2005, Gasperini et al., 2009). These data, in combination with the data presented in this study, support the hypothesis that L-type VGCCs do not play a role in growth cone turning within mammalian growth cones.

Interestingly, upon activation of L-type VGCCS with BayK, the axon extension of control growth cones in response to netrin-1 was increased significantly. These data suggest that L-type VGCCs may function in sensory neuron axon extension, instead of growth cone turning, consistent with previous data that has implicated L-type VGCCs within neurite and growth cone outgrowth (Goldberg, 1988, Silver et al., 1989, Pravettoni et al., 2000). Furthermore, inhibition of L-type VGCCs increased axon extension in STIM morphants in response to netrin-1, suggesting that there may be an interaction between STIM1 and L-type VGCCs within growth cones that regulates neurite extension. As there was no change in axon extension in response to BDNF signalling, this may suggest that the potential interaction between STIM1 and L-type VGCCs is only activated in response to specific guidance cues. However, to thoroughly test a potential interaction between STIM1 and L-type VGCCs in neurite outgrowth, axon extension would need to be examined over a much longer period of time than the 30 minute imaging period used for this growth cone turning assay.

Taken together, the data presented here supports the hypothesis that L-type VGCCs are not essential for growth cone navigation in mammalian cells. Indeed, it appears that if STIM1 does interact with L-type VGCCs within growth cones, it is to regulate axon extension. Given the propensity for spontaneous calcium transients in embryonic growth cones (Gomez and Spitzer, 2000, Gorbunova and Spitzer, 2002), it is likely that the interaction between STIM1, Orai and TRPC channels rather than L-type VGCCs is more important in growth cone navigation. However, the relative importance of the Orai-mediated CRAC current compared to TRPC activation in growth cone navigation remains to be determined.

Chapter 5:

STIM1 signals via cyclic nucleotides in growth cones

Chapter 5: STIM1 signals via cyclic nucleotides in growth cones

5.1 Introduction

While STIM1 is best known as both an ER bound calcium sensor and the key mediator of SOCE, there is evidence to suggest that STIM1 plays multiple roles within cells. STIM1 has been shown to interact with several other proteins and signalling pathways, which could be important in growth cone motility. One such protein is cAMP. STIM1 activates cAMP in a process termed store-operated cAMP signalling (Lefkimiatis et al., 2009). Store-operated cAMP signalling is a form of signalling that couples the ER calcium content directly with cAMP production, through the action of adenylate cyclases. STIM1 expression is required for the activation of this process, which occurs independently of fluctuations in cytosolic calcium concentrations, hence it is a store-operated mechanism (Lefkimiatis et al., 2009). The oligomerisation and relocation of STIM1 puncta to ER-plasma membrane junctions is essential for store-operated cAMP signalling. The authors used multiple mechanisms to block STIM1 function including reduction of endogenous STIM1 with ShRNA and siRNA, the expression of the STIM1 EF-hand mutant D76A, and the use of STIM1 translocation inhibitors ML-9 and 2-APB (Lefkimiatis et al., 2009). Each STIM1 inhibition mechanism significantly decreased cAMP production (Lefkimiatis et al., 2009). While store-operated cAMP signalling was first described within the NCM460 colonic epithelial cell line (Lefkimiatis et al., 2009), which reproduce the same biological properties of colonic crypt epithelial cells (Moyer et al., 1996), it has since been described to be functional within CaLu-3 cells (human airway epithelial cells) (Schwarzer et al., 2010) as well as within a model of mouse

cholangiocytes, implicating store-operated cAMP signalling in the pathogenesis of polycystic liver disease (Spirli et al., 2012). The process of store-operated cAMP signalling directly links ER calcium stores with the cAMP second messenger system (Maiellaro et al., 2012).

STIM1 regulation of store-operated cAMP signalling has the potential to profoundly affect growth cone motility. Both calcium signalling and cyclic nucleotide signalling operate in conjunction with each other within growth cones. Both regulation of calcium and cyclic nucleotide signalling are crucial for growth cone function and motility (Togashi et al., 2008b, Akiyama et al., 2009). Calcium signalling interacts closely with cyclic nucleotides, controlling the ACs, which are the enzymes that generate cAMP (Xia and Storm, 1997, Song and Poo, 1999). AC1 and AC8 are calcium-activated AC-isoforms, while AC5 and AC6 can be directly inhibited by cytosolic calcium. There is evidence to suggest that store-operated cAMP signalling is regulated preferentially by AC3 in colonic epithelial cells (Maiellaro et al., 2012).

Like calcium, there is an optimal range of cyclic nucleotides for normal function within growth cones. It has been shown that calcium-dependent guidance cues such as BDNF (Song et al., 1997) and netrin-1 (Ming et al., 1997) activate cAMP to cause growth cone attraction, while Sema-3a activates cGMP, which is associated with repulsion (Song et al., 1998, Togashi et al., 2008b). However, cyclic nucleotides do not work in isolation from each other. It is the ratio of cAMP to cGMP that is important for growth cone motility,

rather than the absolute levels of these molecules (Nishiyama et al., 2003). This cAMP:cGMP ratio is involved in determining how a growth cone will respond to certain guidance cues. If the cyclic nucleotide ratio is altered from its optimal range, altered guidance responses may occur.

The data presented in chapter 3 of this thesis demonstrated that STIM1 was necessary for growth cone turning in response to a range of calcium dependent and independent guidance cues. Sema-3a is a guidance cue that does not rely on intracellular stores of calcium for its signalling pathways, and does not activate SOCE within growth cones (see figure 3.4). This suggests that as well as functioning in SOCE, STIM1 also functions in calcium-independent signalling pathways within growth cones. The data presented within chapter 3 demonstrates that growth cone turning in response to Sema-3a, is unexpectedly abolished after the reduction of endogenous STIM1. Given the knowledge that Sema-3a effects growth cone turning through downstream cGMP signalling pathways, I hypothesised that upon reduction of STIM1 within growth cones, cAMP production is altered or decreased, perturbing the cAMP to cGMP ratio. This chapter explores this hypothesis, employing the growth cone turning assay, a growth cone collapse assay, and pharmacological manipulation of both cAMP and cGMP signalling.

The work in this chapter demonstrates that Sema-3a repulsion can be restored after STIM1 reduction by activating either cAMP or cGMP signalling within growth cones. The downstream cAMP pathways involved in this response are explored by activation and

inhibition of PKA and Epac. Similarly, growth cone collapse can be prevented in control growth cones by manipulating the cyclic nucleotide pathways. These data suggest that store-operated cAMP signalling functions within growth cones, particularly in the signalling pathways of calcium-independent guidance cues such as Sema-3a.

5.2 Materials and Methods

5.2.1 Materials

Sp-cAMPS, Rp-cAMPS, Sp-6-Phe-cAMPS, 8-pCPT-2'-O-Me-cAMPS, Sp-8-Br-PET-cGMPS, Sp-8-pCPT-cGMPS, Rp-8-Br-PET-cGMPS and Rp-8-pCPT-cGMPS were purchased from BioLog Life Science Institute (Bremen, Germany). Forskolin was purchased from Sigma-Aldrich (Sydney, NSW, Australia). Semaphorin-3a was purchased from R+D Systems (Minneapolis, MN, USA). Alexa fluor 488 phalloidin was purchased from Invitrogen, Life Technologies (Grand Island, NY, USA).

Table 5.1 Functions and concentrations of cyclic nucleotide pharmacological agents

Pharmacological agent	Function	Concentration used
Sp-cAMPS	Activates PKA	20 μ M
Forskolin	Activates adenylate cyclase	5 μ M
Rp-cAMPS	Inhibits PKA I and II	20 μ M
Sp-6-Phe-cAMPS (6-Phe-cAMPS)	Activates PKA (Epac not affected)	5 μ M
8-pCPT-2'-O-Me-cAMPS (8-Me-cAMPS)	Activates Epac (PKA not affected)	2 μ M

Sp-8-pCPT-cGMPS	Activates PKG 1 α and II Activates PKA II	20 μ M
Sp-8-Br-PET-cGMPS	Activates PKG 1 α and 1 β Inhibits cGMP-gated ion channels	20 μ M
Rp-8-pCPT-cGMPS	Inhibits PKG 1 α , 1 β , II Activates cGMP-gated ion channels	20 μ M
Rp-8-Br-PET-cGMPS	Inhibits PKG I α and 1 β Inhibits cGMP-gated ion channels	20 μ M

5.2.2 Embryonic DRG cell culture

Embryonic DRG cell culture was performed as described in section 2.2.4.

5.2.3 Growth cone turning assay

Growth cone turning was performed as described in section 2.2.4. Pharmacological agents were added to the media bath 30 minutes before imaging. Refer to table 5.1 for pharmacological agents used.

5.2.4 Sema-3a collapse assay

Sema-3a collapse assay was performed as described in section 3.2.7. Cyclic nucleotide analogues were added to the cultures for 30 min. Sema-3a was then added to the media containing the cyclic nucleotide analogues for an additional 30 minutes before cells were fixed for phalloidin staining. Cyclic nucleotide analogues were used at the same concentration as described in the growth cone turning assay (refer to table 5.1).

5.3 Results

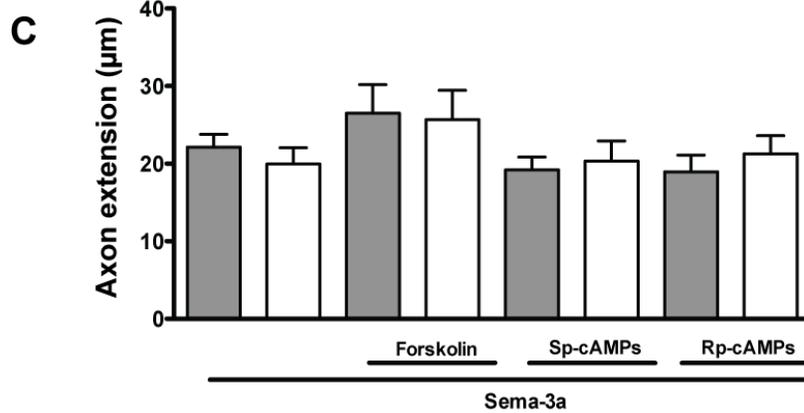
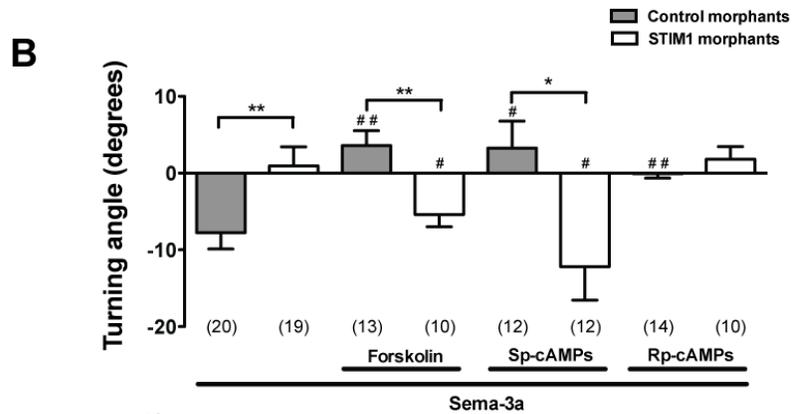
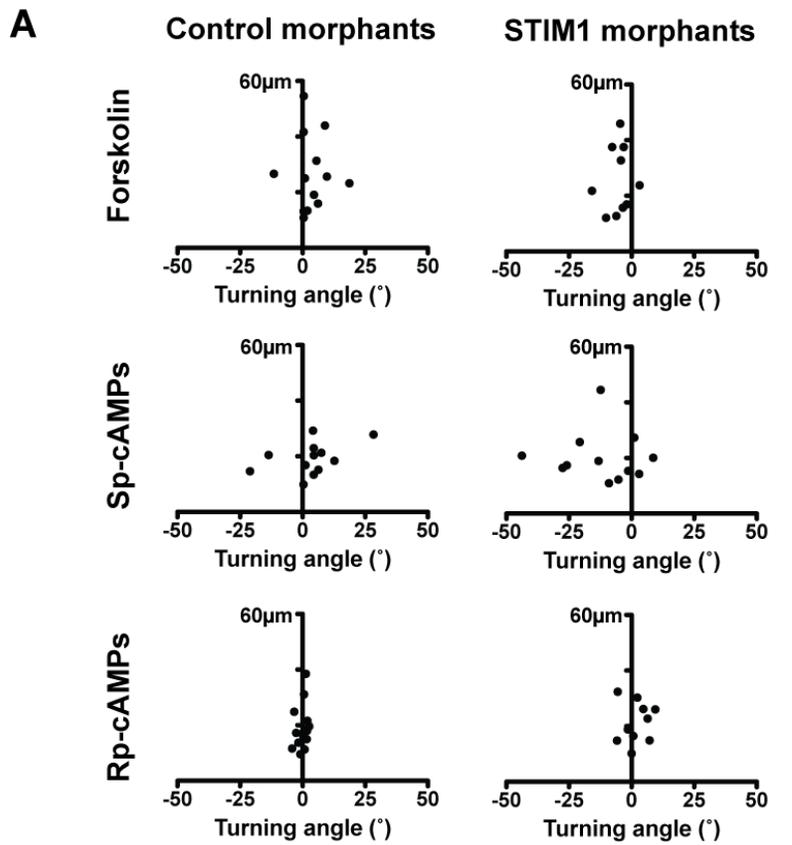
5.3.1. Activation of cAMP restores repulsive turning to Sema-3a after STIM1

knockdown

To test the hypothesis that a reduction in STIM1 would reduce the levels of cAMP within growth cones, the cAMP signalling pathway was artificially activated with pharmacological agents. Activation of cAMP signalling within STIM1 and control morphant growth cones was achieved with the addition of forskolin, an activator of adenylate cyclase, or Sp-cAMPS, a cell permeable cAMP analogue which activates PKA. The addition of forskolin or Sp-cAMPS rescued the repulsive response to Sema-3a in STIM1 morphants ($-5.4 \pm 1.6^\circ$ and $-12.2 \pm 4.4^\circ$, respectively; Fig. 5.1 A, B). Conversely, when adenylate cyclase or PKA were activated in control morphants, the turning responses to Sema-3a were pushed towards attraction ($3.3 \pm 3.5^\circ$ and $3.6 \pm 1.9^\circ$, respectively; Fig. 5.1 A, B). This is consistent with previous data demonstrating that manipulation of cyclic nucleotides can regulate growth cone turning (Ming et al., 1997, Song et al., 1997, Song et al., 1998, Murray et al., 2009). The inhibition of PKA with Rp-cAMPS abolished the turning affect of control morphants ($-0.1 \pm 0.6^\circ$, $p < 0.01$; Fig. 5.1 A, B), and had no significant effect on STIM1 morphants ($1.8 \pm 1.6^\circ$; Fig 5.1 A, B). The addition of activators and inhibitors of adenylate cyclase and cAMP had no effect on the axon extension of either control or STIM1 morphant growth cones in response to gradients of Sema-3a (Fig 5.1 C). These data demonstrate that STIM1 mediates cAMP signalling in response to Sema-3a signalling.

Figure 5.1. Activation of cAMP signalling restores repulsive turning to Sema-3a in STIM1 morphant growth cones.

A. Scatter plots illustrate the data spread for control and STIM1 morphant growth cone responses to Sema-3a, after pharmacological manipulation of cAMP. Total axon extension is represented on the y-axis, and growth cone turning angle is on the x-axis. **B.** Average growth cone turning angles of control (grey bars) and STIM1 morphant (white bars) growth cones, in response to Sema-3a, when treated with pharmacological agents forskolin, Sp-cAMPs, and Rp-cAMPs. **C.** Axon extension did not change upon treatment with pharmacological agents. Error bars represent SEM. Sample number included in **B** on turning angle graph relate to both **B** and **C**. Significance compared back to Sema-3a only controls; # $p < 0.05$, ## $p < 0.01$. Significance between morpholino treatment groups; * $p < 0.05$, ** $p < 0.01$. Treatments were compared with Mann-Whitney U-test.



5.3.2. Activation of cGMP restores Sema-3a-induced repulsion after STIM1

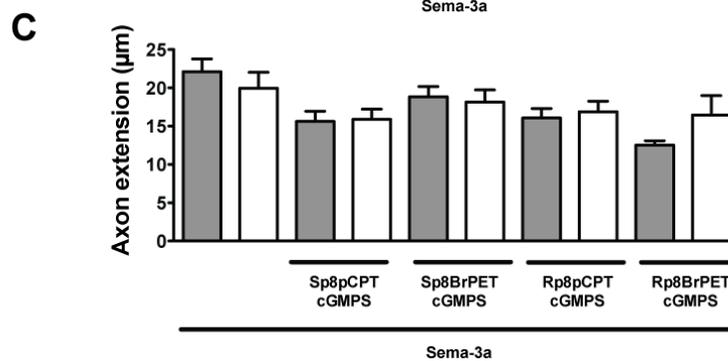
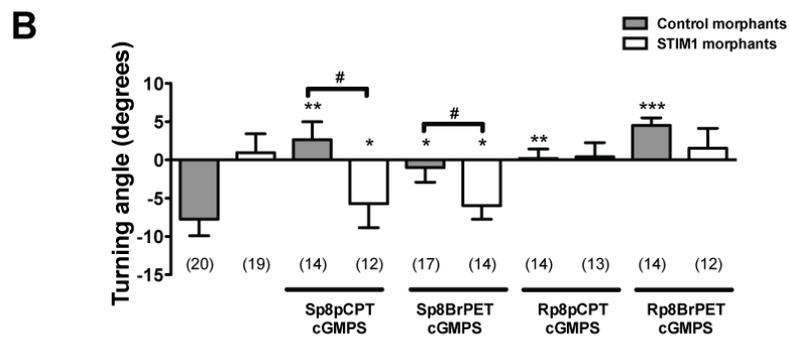
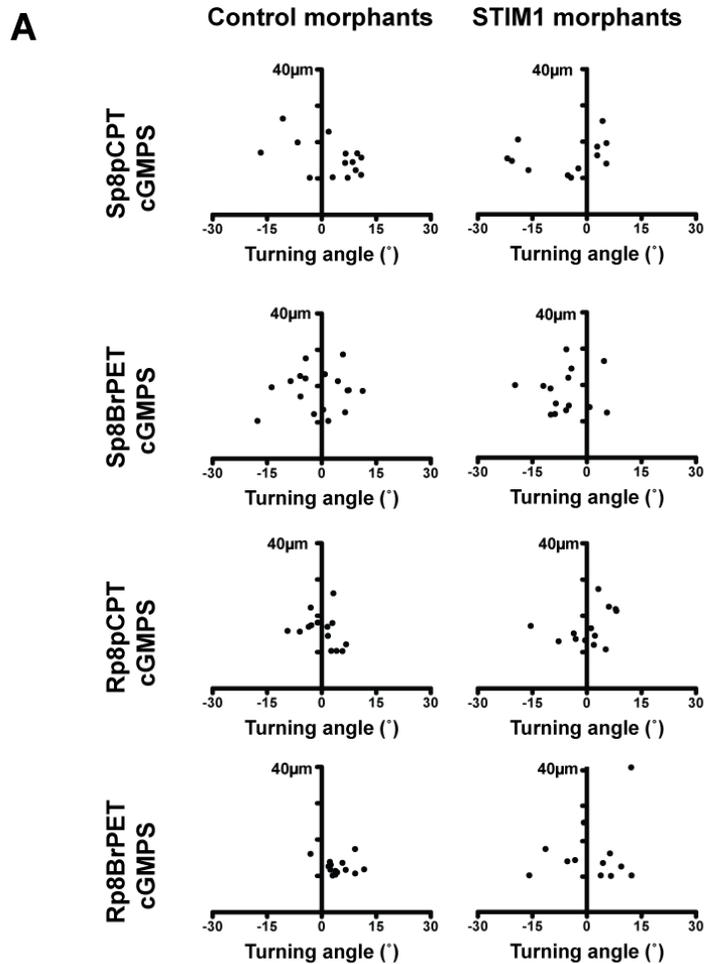
knockdown in growth cones

Sema-3a is well known to modulate growth cone motility via the downstream production of cGMP (Song et al., 1997). Cyclic nucleotides do not work independently of each other, with cAMP and cGMP working together within a ratio. If STIM1 knockdown perturbs cAMP signalling, then it could also perturb cGMP signalling. To test if the activation of cGMP could also rescue Sema-3a repulsion in STIM1 morphants, both cGMP activators and inhibitors were applied to growth cone cultures and examined in the turning assay. The reported actions of these agonists and antagonists are: Sp-8-pCPT-cGMPS activates PKG 1 α , PKG II and cyclic nucleotide gated channels, but it also activates PKA α . Sp-8-Br-PET-cGMPS activates PKG 1 α and PKG 1 β , but inhibits cyclic nucleotide gated channels, allowing for differentiation between kinase and channel activity. Two specific cGMP inhibitors were used. Rp-8-pCPT-cGMPS inhibits PKG 1 α , PKG 1 β and PKG II, while activating cyclic nucleotide gated channels. Rp-8-Br-PET-cGMPS inhibits PKG 1 α and PKG 1 β , and inhibits cyclic nucleotide gated channels.

Activation of PKG with Sp-8-pCPT-cGMPS or Sp-8-Br-PET-GMPS abolished the repulsive turning effect of Sema-3a on control growth cones ($2.7 \pm 2.3^\circ$, $p < 0.01$ and $-1.0 \pm 1.9^\circ$, $p < 0.05$ respectively; Fig. 5.2 A, B), resulting in a random turning effect. However, the repulsive turning effect of Sema-3a was restored in STIM1 morphants upon the activation of PKG with Sp-8-pCPT-cGMPS or Sp-8-Br-PET-GMPS ($-5.7 \pm 3.1^\circ$ $p < 0.05$ and -6.0

Figure 5.2 Activation of cGMP signalling restores repulsive turning to Sema-3a in STIM1 morphants

A. Scatter plots illustrate the spread of data in control and STIM1 morphant growth cones in response to Sema-3a, after pharmacological treatment with cGMP agonists and antagonists. Total axon extension is represented on the y-axis, and final growth cone turning angle is represented on the x-axis. **B.** Average turning angles of control (grey bars) and STIM morphant (white bars) growth cones in response to Sema-3a, after treatment with Sp-8-pCPT-cGMPS, Sp-8-Br-PET-cGMPS, Rp-8-pCPT-cGMPS, or Rp-8-Br-PET-cGMPS. **C.** Axon extension did not change in control or STIM1 morphants after treatment with cGMP agonists or antagonists. Error bars represent SEM. Sample numbers included on turning angle graph are related to both **B** and **C**. * Significance is compared to appropriate Sema-3a only morpholino control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. # Significance compared between morpholino treatment groups, # $p < 0.05$. Treatments were compared with Mann-Whitney U-test.



$\pm 1.8^\circ$, $p < 0.05$ respectively; Fig 5.2 A, B). There were no significant differences observed with the use of one cGMP activator over the other, with both restoring Sema-3a repulsion in STIM1 morphant growth cones. These data suggest that PKG activation, but not cyclic nucleotide gated channels, is sufficient to restore Sema-3a repulsion after STIM1 knockdown.

The repulsive turning response of Sema-3a was also abolished in control morphants after the inhibition of PKG, with Rp-8-pCPT-cGMPS ($0.2 \pm 1.2^\circ$, $p < 0.01$; Fig. 5.2 A, B), however, when PKG was inhibited in control growth cones with Rp-8-Br-PET-cGMPS the repulsive response to Sema-3a switched to attraction ($4.5 \pm 1.0^\circ$, $p < 0.0001$ Fig. 5.2 A, B). The inhibition of cGMP with either Rp-8-pCPT-cGMPS or Rp-8-Br-PET-cGMPS resulted in no change to the abolished turning effect of STIM1 morphants in response to Sema-3a. Axon extension did not change for either control or STIM1 morphants upon the addition of PKG activators or inhibitors (Fig. 5.2 C). These data reveal that the activation of cGMP signalling restores the repulsive turning response of Sema-3a after STIM1 reduction, suggesting that STIM1 regulates cGMP signalling pathways within growth cones.

5.3.3. Activation of cAMP is not sufficient to fully restore BDNF attraction after STIM1 knockdown

To determine if STIM1-cAMP signalling is a general phenomenon and common to all types of guidance cues, cAMP signalling was manipulated pharmacologically in growth cones, in

response to a gradient of BDNF. The activation of cAMP through the addition of forskolin and Sp-cAMPS attenuated the normal BDNF attractive turning response in control morphant growth cones, resulting in random turning ($3.3 \pm 1.8^\circ$, $p < 0.01$ and $2.1 \pm 1.9^\circ$, $p < 0.01$, respectively; Fig. 5.3 A, B), consistent with previously published data (Song 97). Similarly, the STIM1 morphant repulsive turning response to BDNF was abolished upon the addition of forskolin and Sp-cAMPS, resulting in random turning angles ($-0.4 \pm 2.1^\circ$ and $-0.8 \pm 1.2^\circ$, respectively; Fig. 5.3 A, B). When PKA was inhibited by addition of Rp-cAMPS, both control morphant and STIM1 morphant growth cones displayed abolished turning responses, resulting in random turning angles ($-1.4 \pm 1.2^\circ$ and $1.4 \pm 1.6^\circ$, respectively; Fig. 5.3 A, B). These data suggest that while cAMP signalling is important in the BDNF signalling response, that it is not the only downstream intracellular second messenger involved. This was predicted, given that STIM1-mediated SOCE was activated in growth cone turning in response to BDNF and netrin-1 gradients.

5.3.4. Activation of cGMP is not sufficient to restore BDNF attraction after STIM1 knockdown

To determine if cGMP signalling is involved in BDNF signalling, growth cone turning responses were measured after the activation and inhibition of PKG and cyclic nucleotide gated channels. Activation of PKG and cyclic nucleotide gated channels with Sp-8-pCPT-cGMPS had no effect on control morphant growth cones, with the normal attractive response to BDNF continuing ($8.4 \pm 2.4^\circ$; Fig. 5.4 A, B), potentially because this agonist also activates PKA, in addition to PKG. Conversely the activation of PKG and inhibition of

Figure 5.3. Activation of cAMP does not restore attractive BDNF turning in STIM1 morphant growth cones.

A. Scatter plots illustrate the spread of data for control and STIM1 morphant growth cone turning responses to BDNF after activation and inhibition of cAMP pathways. Total axon extension is represented on the y-axis, and final growth cone turning angle is represented on the x-axis. **B.** Average growth cone turning angles of control (grey bars) and STIM1 morphant (white bars) growth cones, in response to BDNF, when treated with pharmacological agents forskolin, Sp-cAMPS, or Rp-cAMPS (20 μ M). **C.** Axon extension did not change upon pharmacological manipulation of cAMP. Error bars represent SEM. Sample number included in bars of axon extension graph relate to both **B** and **C**. Significance compared back to BDNF only controls, ** $p < 0.01$, *** $p < 0.0001$. Significant between morpholino groups illustrated with bar, *** $p < 0.0001$. Treatments were compared with Mann-Whitney U-test.

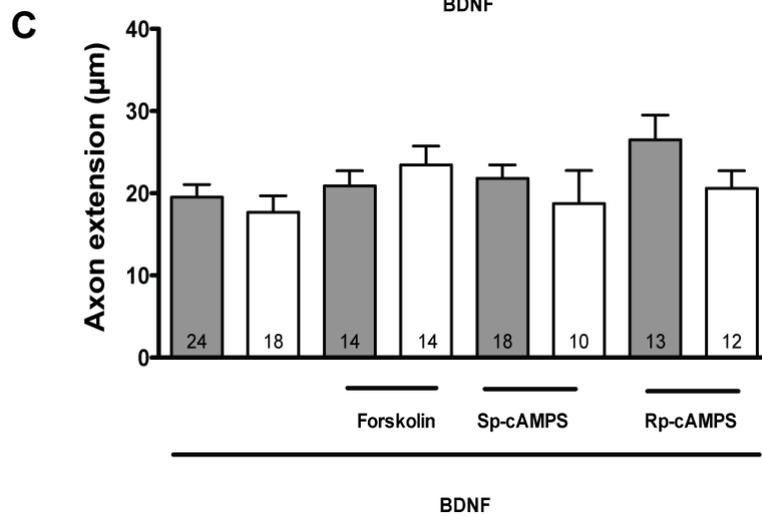
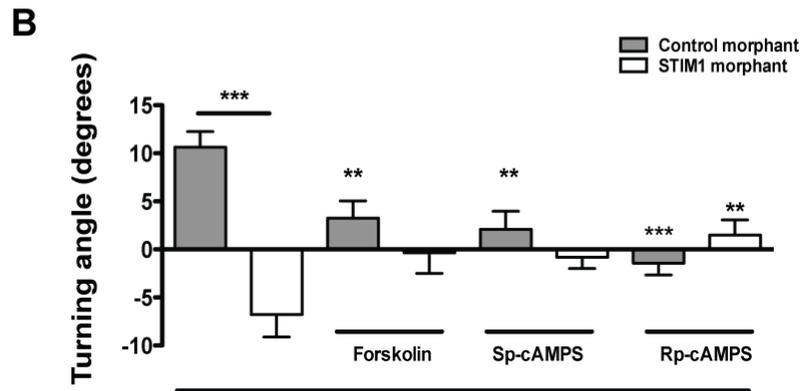
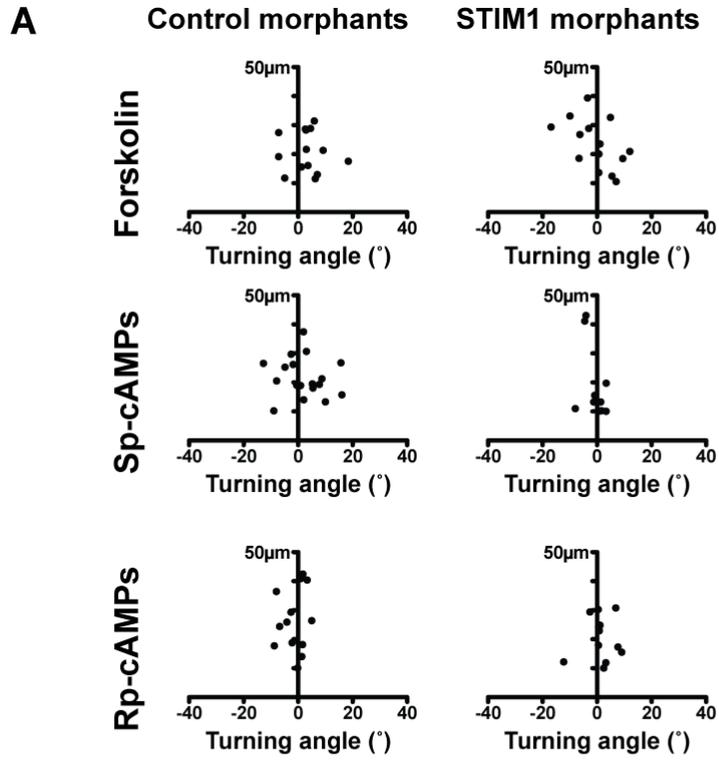


Figure 5.4 Activation of cGMP signalling alone is not sufficient to restore turning response to BDNF.

A. Scatter plots illustrate the spread of data in control and STIM1 morphant growth cones in response to BDNF, after pharmacological treatment with cGMP agonists and antagonists. Total axon extension is represented on the y-axis, and final growth cone turning angle is represented on the x-axis. **B.** Average turning angles of control (grey bars) and STIM morphant (white bars) growth cones in response to Sema-3a, after treatment with Sp-8-pCPT-cGMPS, Sp-8-Br-PET-cGMPS, Rp-8-pCPT-cGMPS, or Rp-8-Br-PET-cGMPS. **C.** Axon extension did not change in control or STIM1 morphants after treatment with cGMP agonists or antagonists. Error bars represent SEM. Sample numbers included on turning angle graph are related to both B and C. * Significance is compared to appropriate BDNF only morpholino control (control or STIM1), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Treatments were compared with Mann-Whitney U-test.

cyclic nucleotide gated channels with Sp-8-Br-PET-cGMPS attenuated the attractive turning response of control growth cones to BDNF ($0.7 \pm 2.7^\circ$, $p < 0.01$; Fig. 5.4 A, B), resulting in random turning. Activation of cGMP through the addition of either Sp-8-pCPT-cGMPS or Sp-8-Br-PET-cGMPS had no significant effect on the turning angle of STIM1 morphants in response to BDNF, however the mean suggests an abolished turning response ($1.9 \pm 4.0^\circ$ and $0.8 \pm 2.2^\circ$ respectively; Fig 5.4 A, B). Inhibition of PKG with Rp-8-pCPT-cGMPS or Rp-8-Br-PET-cGMPS abolished the turning response to BDNF in both control (-0.6 ± 1.0 , $p < 0.001$ and $1.6 \pm 1.6^\circ$, $p < 0.001$, respectively) and STIM1 morphants (-1.6 ± 2.0 , not sig. And -0.1 ± 1.6 , $p < 0.05$, respectively; Fig. 5.4 A, B). These data suggest that the repulsive response of STIM1 morphants to BDNF cannot be restored through the activation of cGMP alone. This is consistent with the BDNF-cAMP data above, that suggests while cyclic nucleotide signalling is important for BDNF signalling, STIM1-mediated SOCE is equally, if not more important for BDNF attraction.

5.3.5. Activation of PKA or Epac alone cannot rescue Sema-3a repulsion after STIM1 knockdown

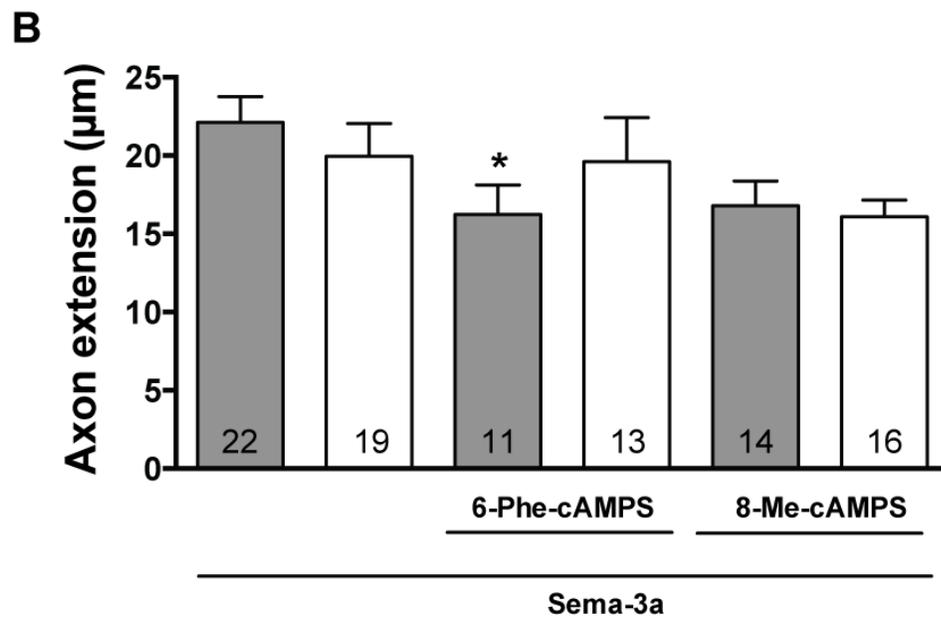
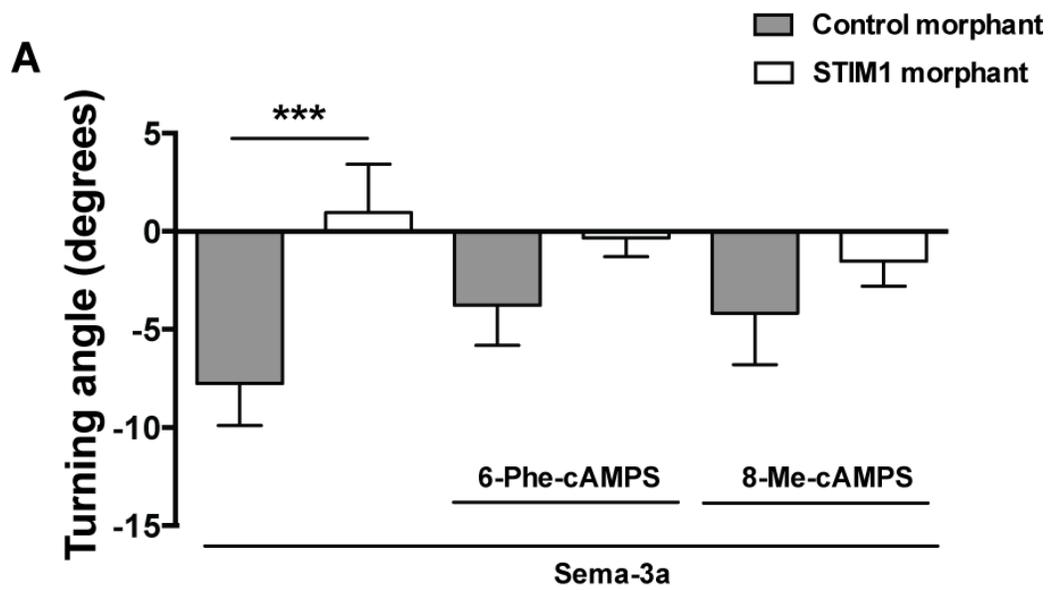
There are multiple downstream effector proteins required for cAMP signalling. Two of these are PKA and exchange protein directly activated by cAMP (Epac). It has previously been reported that there is a developmental switch between Epac and PKA in cAMP-dependent axon guidance. In embryonic growth cones, cAMP-dependent guidance is mediated via the Epac signalling pathway, while in mature growth cones it is mediated by the PKA signalling pathway (Murray et al., 2009). The data presented in this chapter

suggests that within growth cones STIM1-mediated cAMP signalling is required for growth cone turning away from Sema-3a. However, it is unknown which downstream effectors of cAMP are activated. To test the hypothesis of a cAMP developmental switch within growth cones, and to determine which pathway is activated by STIM1 in growth cones, PKA was specifically activated with Sp-6-Phe-cAMPS (6-Phe-cAMPS), and Epac was specifically activated with 8-pCPT-2'-O-Me-cAMPS (8-Me-cAMPS).

After addition of 6-phe-cAMPS (a PKA activator and Epac negative control), control morphant growth cones continued to display repulsive turning in response to Sema-3a ($-3.8 \pm 2.1^\circ$; Fig. 5.5 A), albeit with a smaller repulsive angle (not statistically significant). Similarly, when 8-Me-cAMPS (Epac activator, PKA negative control) was applied to control morphant growth cones, they also displayed a repulsive turning response to Sema-3a ($-4.2 \pm 2.6^\circ$; Fig. 5.5 A). After activation of PKA with 6-Phe-cAMPS, STIM1 morphant growth cones continued to display an abolished turning effect in response to Sema-3a, not significantly different from that when no pharmacological agents are added (-0.4 ± 1.0 ; Fig. 5.5 A). Similarly, after the addition of 8-Me-cAMPS to STIM1 morphant growth cones, abolished turning was also displayed in response to Sema-3a ($-1.5 \pm 1.3^\circ$; Fig. 5.5 A). Activation of PKA significantly shortened the axon extension in control growth cells ($p < 0.05$; Fig. 5.5 B), however no other changes in axon extension were observed. These data suggest that the individual activation of PKA or Epac alone is not enough to restore the normal repulsive turning response to Sema-3a, unlike when forskolin or Sp-cAMPS were applied to the cultures, suggesting that perhaps both downstream effectors of cAMP

Figure 5.5 Activation of PKA or Epac alone is unable to rescue Sema-3a repulsion in STIM1 morphants

A. Average growth cone turning angles of control (grey bars) and STIM1 morphant (white bars) growth cones, in response to Sema-3a, when treated with pharmacological agents 6-Phe-cAMPS (PKA agonist) and 8-Me-cAMPS (Epac agonist). The repulsive response of Sema-3a was unable to be rescued in STIM1 morphants upon activation of either PKA or Epac. **B.** Axon extension remained unchanged after pharmacological treatment, except for a decrease in extension seen in 6-Phe-cAMPS treated control growth cones. Error bars are SEM. Sample number included in bars of axon extension graph relate to both **A** and **B**. Significance compared to Sema-3a only controls, * $p < 0.05$. Significance between morpholino groups, *** $p < 0.0001$. Treatments were compared with Mann-Whitney U-test.



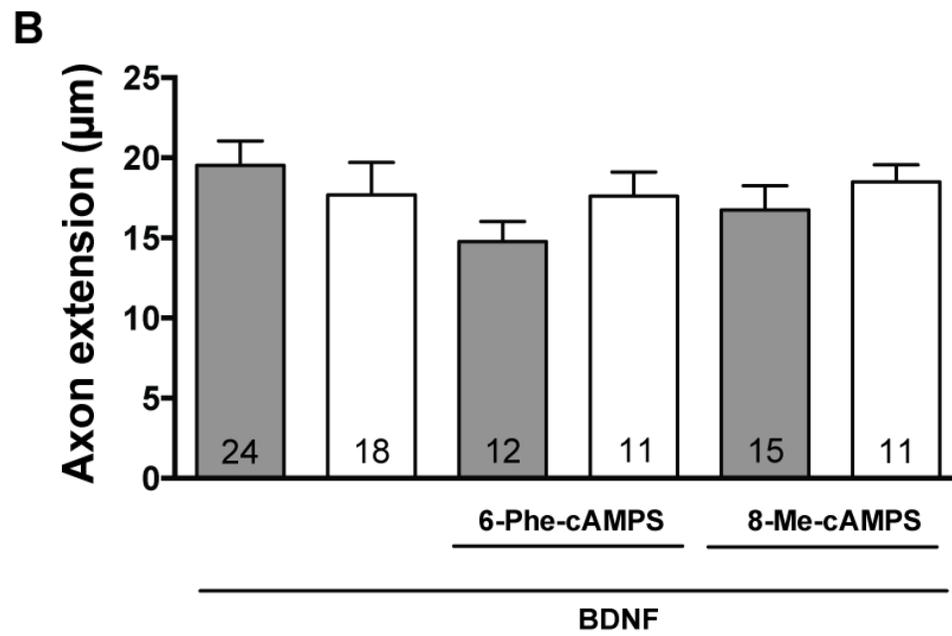
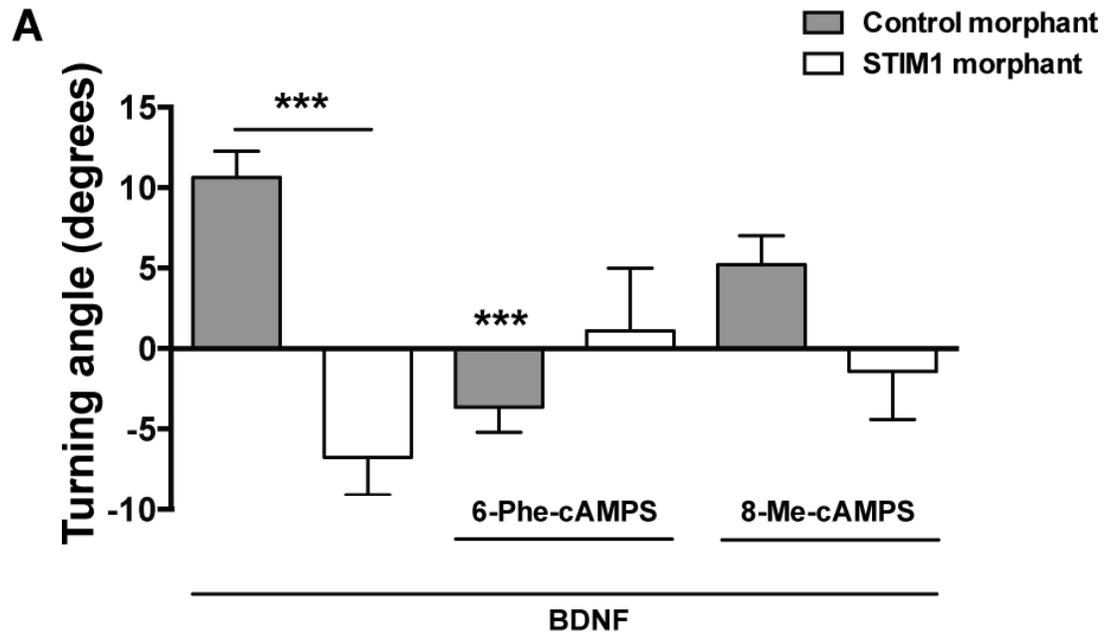
are required to be activated for the restoration of Sema-3a repulsion after a reduction in STIM1 protein expression. It has been shown previously that BDNF activates Epac in embryonic growth cones (Murray et al., 2009). I sought to determine whether activation of Epac or PKA could rescue STIM1 morphant turning in response to BDNF. After the activation of PKA with 6-Phe-cAMPS, both control and STIM1 morphant growth cones displayed an abolished turning response to a gradient of BDNF ($-3.7 \pm 1.5^\circ$ and $1.1 \pm 3.9^\circ$, respectively; Fig. 5.6 A). Similarly, after the activation of Epac with 8-Me-cAMPs, STIM1 morphant growth cones also displayed an abolished turning response to BDNF ($-1.4 \pm 3.0^\circ$, Fig. 5.6 A). After the activation of Epac, the turning response of control growth cones to BDNF was reduced, but not abolished ($5.2 \pm 1.8^\circ$, $p < 0.05$; Fig. 5.6 A). Activation of either PKA or Epac did not affect axon extension of either control or STIM1 morphants in response to BDNF (Fig. 5.6 B). These data suggest that in response to BDNF, Epac is normally activated. However, activation of Epac is not enough to restore BDNF attraction after STIM1 knockdown, supporting the cAMP turning data that STIM1-mediated SOCE is vital for BDNF signalling.

5.3.6. Correct cAMP levels are required for Sema-3a-induced growth cone collapse

The data in this chapter has provided evidence that cAMP activation restores repulsive turning in response to Sema-3a, after STIM1 knockdown. As shown previously (Fig. 3.6), STIM1 is not only required for growth cone turning, but also for Sema-3a growth cone collapse. In order to understand the mechanisms by which STIM1 is involved in Sema-3a

Figure 5.6 Activation of PKA or Epac alone does not restore STIM1 morphant turning towards BDNF

A. Average growth cone turning angles of control (grey bars) and STIM1 morphant (white bars) growth cones, in response to BDNF, when treated with pharmacological agents 6-Phe-cAMPS and 8-Me-cAMPS. **B.** Axon extension did not change after pharmacological treatment. Error bars are SEM. Sample number included in bars of axon extension graph relate to both **A** and **B**. Significance between morpholino groups (as shown by bar) and compared to BDNF only control, *** $p < 0.0001$. Treatments were compared with Mann-Whitney U-test.

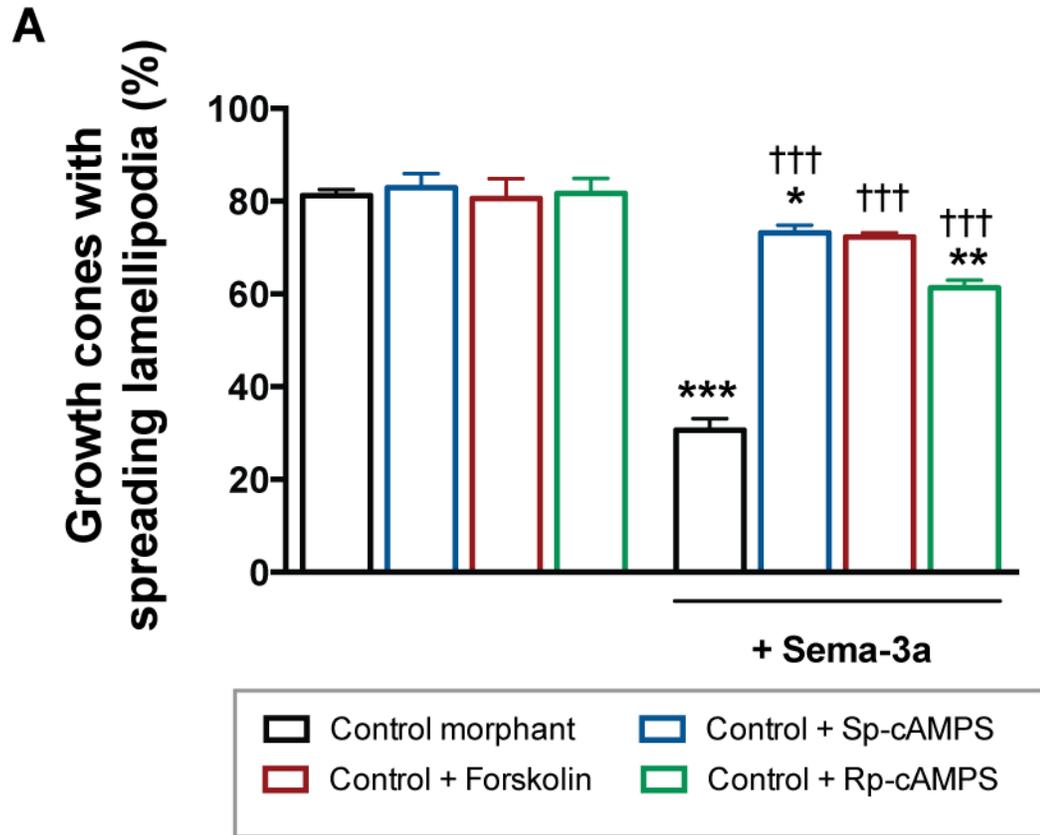


signalling, the Sema-3a collapse assay was utilised in combination with the addition of the cAMP analogues (see table 5.1). Control growth cones exhibited normal, healthy spreading lamellipodia morphology in 81.2 ± 1.4 % (control morphants; Fig. 5.7 A). Activation of adenylate cyclase or PKA did not alter the percentage of growth cones displaying healthy, spreading lamellipodial morphology in control growth cones (Fig. 5.7 A). Inhibition of cAMP with Rp-cAMPS also made no difference to the percentage of healthy, spreading growth cones in controls (Fig. 5.7 A). The addition of Sema-3a collapsed control growth cones, reducing the percentage of healthy spreading growth cones to 30.7 ± 1.6 % (Fig. 5.7 A, B $p < 0.0001$). Activation of both adenylate cyclase and PKA prevented Sema-3a-induced collapse in control growth cones (73.2 ± 1.6 %, $p < 0.0001$ and 72.3 ± 0.9 %, $p < 0.0001$ respectively; Fig. 5.7 A, B). Similarly, inhibition of PKA also prevented Sema-3a-induced collapse in control growth cones (61.3 ± 1.7 %, $p < 0.0001$; Fig. 5.7 A, B), but not to the same extent as when adenylate cyclase or PKA are activated. These data demonstrate that both activation and inhibition of cAMP signalling prevents growth cone collapse in control growth cones. These data suggest that Sema-3a-induced growth cone collapse is regulated by cyclic nucleotide ratios, and that the level of cAMP is important for Sema-3a function.

STIM1 morphant growth cones exhibited normal, healthy spreading lamellipodial morphology in 80.9 ± 1.4 % (Fig. 5.8 A). Activation of adenylate cyclase (forskolin) or PKA (Sp-cAMPS) did not alter the percentage of growth cones displaying spreading lamellipodia (Fig. 5.8 A). Inhibition of cAMP with Rp-cAMPS did not alter the percentage of spreading lamellipodial growth cones in STIM1 morphants (Fig. 5.8 A). The addition of Sema-3a did not collapse STIM1 morphant growth cones, with 75.7 ± 1.4 % of

Figure 5.7 Sema-3a-induced collapse can be prevented through the modulation of cAMP signalling in control growth cones

A. Percentage of control growth cones with spreading lamellipodial morphology in the presence or absence of Sema-3a, with cAMP activation or inhibition. Error bars are SEM. * compared each group to the representative control in the absence of Sema-3a. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. † compared to Sema-3a only (no drug), ††† $p < 0.001$. Data were collected over three different experiments, with sample sizes ranging from 674 growth cones to 1518 growth cones per treatment group. Treatments were compared using the Student's t-test. **B.** Table explaining the results from the graph in A, for treatment groups in the presence of Sema-3a. ✓ represents activation (for PKA) or the relative level of growth cone spreading morphology or collapse. ✗ represents inhibition of PKA.

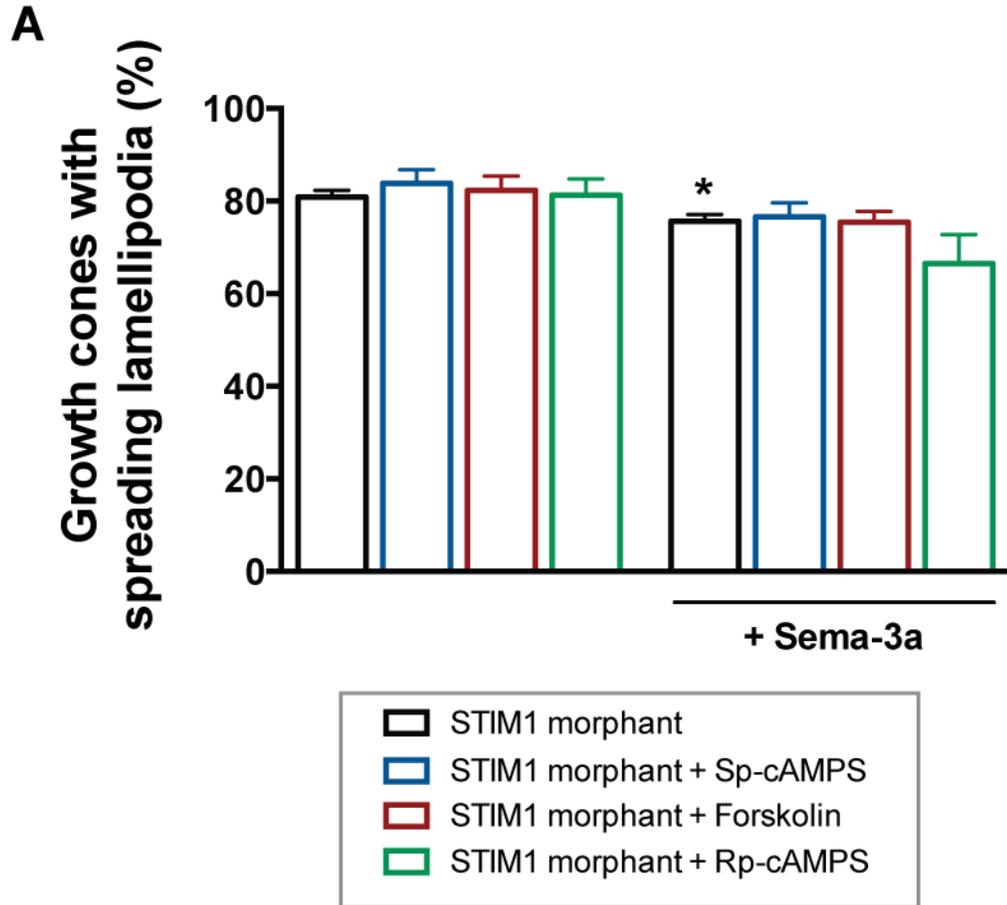


B

Sema-3a + Pharmacological Agent	PKA	Spreading	Collapsed
No drug	-	✓✓✓	
Sp-cAMPS	✓	✓✓	
Forskolin	✓	✓✓	
Rp-cAMPS	X	✓✓	✓

Figure 5.8 Sema-3a-induced collapse is not rescued in STIM1 morphants by manipulation of cAMP signalling

A. Percentage of STIM1 morphant growth cones with spreading lamellipodial morphology in the presence or absence of Sema-3a, with cAMP activation or inhibition. Error bars are SEM. * compares group to the representative control in the absence of Sema-3a. * $p < 0.05$. Data were collected over three different experiments, with sample sizes ranging from 560 growth cones to 1680 growth cones per treatment group. Treatments were compared using the Student's t-test. **B.** Table explaining the results from the graph in A, for treatment groups in the presence of Sema-3a. ✓ represents activation (for PKA) or the relative level of growth cone spreading morphology or collapse. ✗ represents inhibition of PKA.



B

Sema-3a + Pharmacological Agent	PKA	Spreading	Collapsed
No drug	-	✓✓✓	
Sp-cAMPS	✓	✓✓✓	
Forskolin	✓	✓✓✓	
Rp-cAMPS	✗	✓✓✓	

growth cones displaying a spreading morphology (Fig. 5.8 A, B, $p < 0.05$). The manipulation of cAMP signalling failed to rescue growth cone collapse in STIM1 morphants. There was no difference in the percentage of spreading growth cones in STIM1 morphants after activation of either adenylyl cyclase or PKA, or after the inhibition of PKA (Fig. 5.8 A, B). These data suggest that while STIM1 is necessary for Sema-3a-induced growth cone collapse, it may regulate this process through an alternative mechanism that does not involve cAMP.

5.3.7. Optimal levels of cGMP are required for Sema-3a-induced growth cone collapse

I next examined whether cGMP interacts with STIM1 to mediate Sema-3a-induced growth cone collapse. The Sema-3a collapse assay was utilised in combination with the addition of the cGMP analogues (see table 5.1). Control growth cones not exposed to Sema-3a exhibited normal healthy, spreading lamellipodial morphology (80.7 ± 1.1 %; Fig. 5.9 A). Activation of cGMP did not alter the percentage of growth cones displaying a spreading morphology in control growth cones (Fig. 5.9 A). Similarly, inhibition of cGMP did not alter the percentage of healthy, spreading growth cones in control morphants (Fig. 5.9 A). The addition of Sema-3a caused significant growth cone collapse, by reducing the percentage of healthy spreading growth cones to 31.2 ± 2.1 % in control morphants (Fig. 5.9 A, B, $p < 0.0001$). Activation of cGMP with Sp-8-Br-PET-cGMPS or Sp-8-pCPT-cGMPS prevented Sema-3a-induced collapse in control morphants (78.3 ± 3.2 %, $p < 0.0001$ and 65.3 ± 3.3 %, $p < 0.0001$; Fig 5.9 A, B). Inhibition of cGMP with Rp-8-Br-PET-cGMPS or Rp-8-pCPT-cGMPS also prevented Sema-3a-induced collapse in control morphants

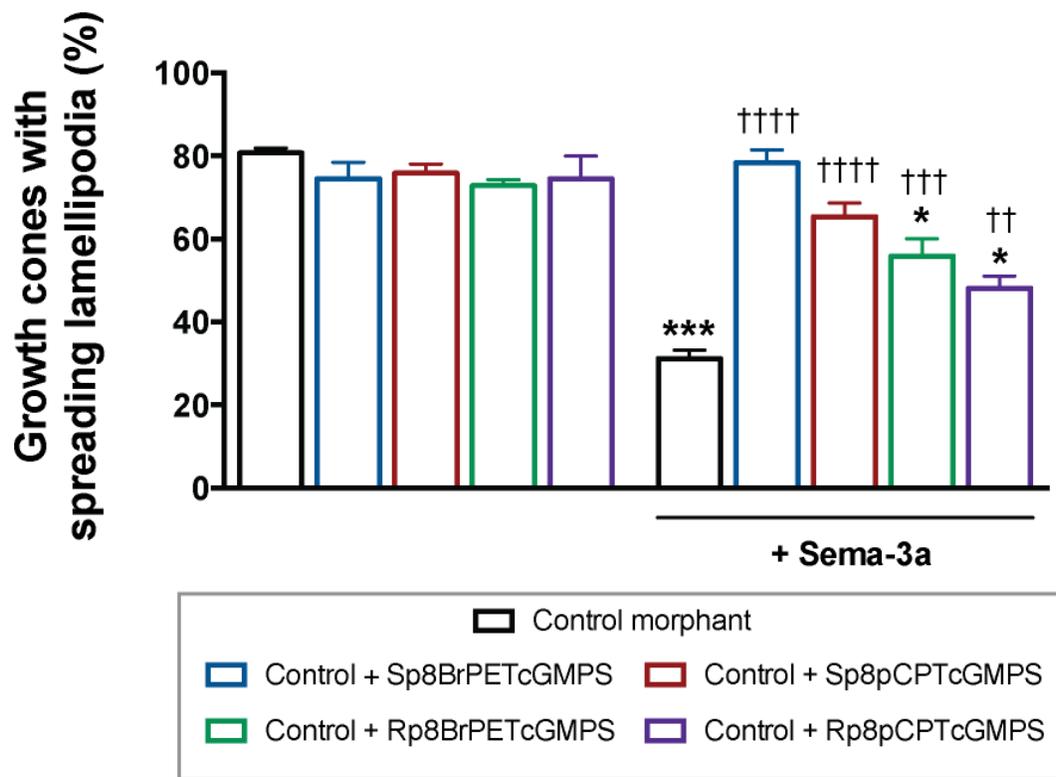
(55.9 ± 4.2 %, $p < 0.001$ and 48.1 ± 2.9 %, $p < 0.05$ respectively; Fig. 5.9 A, B), but not to the same extent as the cGMP activators. These data suggest that the levels of cGMP within growth cones may dictate the extent of Sema-3a-induced growth cone collapse.

STIM1 morphant growth cones in the absence of Sema-3a exhibited normal healthy, spreading lamellipodia (80.9 ± 1.4 %, Fig. 5.10 A). Activation of cGMP did not alter the percentage of spreading growth cones in STIM1 morphants (Fig. 5.10 A). Similarly, inhibition of cGMP did not alter the percentage of spreading lamellipodial growth cones in STIM1 morphants (Fig. 5.10 A). The addition of Sema-3a did not cause growth cone collapse in STIM1 morphants, with 75.7 ± 1.4 % continuing to exhibit a healthy, lamellipodial morphology. Activation of cGMP with Sp-8-Br-PET-cGMPS or Sp-8-pCPT-cGMPS in STIM1 morphant growth cones resulted in a small amount of Sema-3a-induced collapse (62.4 ± 5.5 %, $p < 0.01$ and 56.5 ± 3.3 %, $p < 0.0001$ respectively; Fig. 5.10 A, B), but not to the same extent as in control morphant growth cones with Sema-3a addition. Inhibition of cGMP with Rp-8-Br-PET-cGMPS did not cause growth cone collapse in STIM1 morphants, however the inhibition of cGMP with Rp-8-pCPT-cGMPS did cause collapse to a small extent in 66.7 ± 1.2 % of STIM1 morphant growth cones ($p < 0.01$; Fig. 5.10 A, B). These data suggest that STIM1 does not mediate Sema-3a-induced growth cone collapse through cGMP regulation.

Figure 5.9 cGMP levels dictate Sema-3a-induced growth cone collapse in control growth cones

A. Percentage of control growth cones with spreading lamellipodial morphology in the presence or absence of Sema-3a, with cGMP activation or inhibition. Error bars are SEM. * compares group to the representative control in the absence of Sema-3a. * $p < 0.05$, *** $p < 0.0001$. † compared to Sema-3a only (no drug), †† $p < 0.01$, ††† $p < 0.001$, †††† $p < 0.0001$. Data were collected over three different experiments, with sample sizes ranging from 219 growth cones to 822 growth cones per treatment group. Treatments were compared using the Student's t-test. **B.** Table explaining the results from the graph in A, for treatment groups in the presence of Sema-3a. ✓ represents activation (for PKG) or the relative level of growth cone spreading morphology or collapse. ✗ represents inhibition of PKG.

A



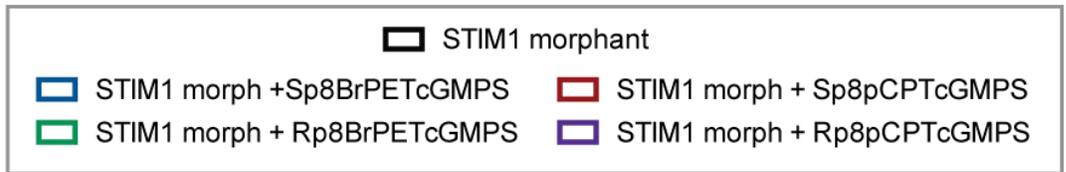
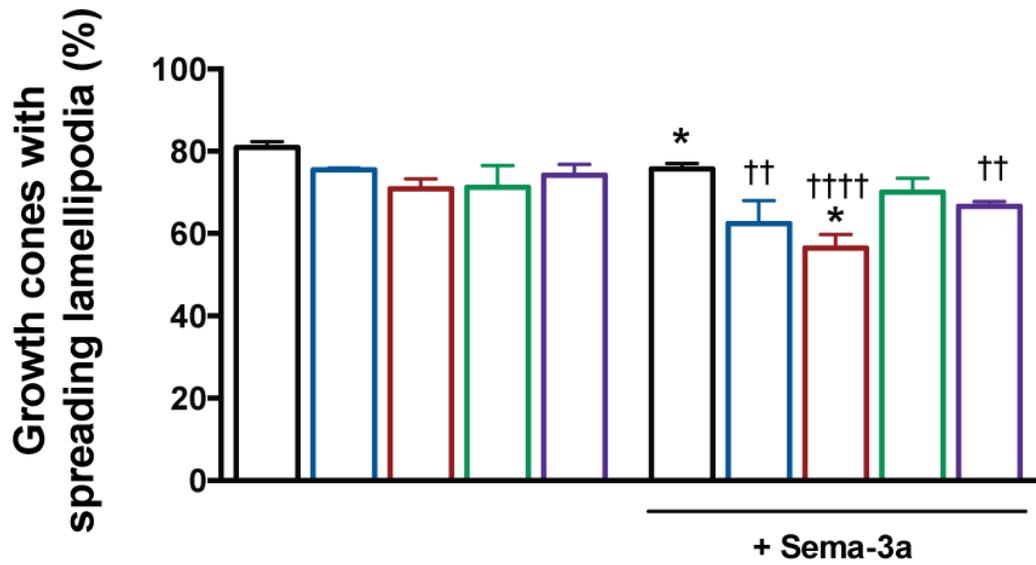
B

Sema-3a + Pharmacological Agent	PKG	CNGC	Spreading	Collapsed
No drug	-	-		✓✓✓
Sp-8-Br-PET-cGMPS	✓	✗	✓✓✓	
Sp-8-pCPT-cGMPS	✓	✓	✓✓✓	
Rp-8-Br-PET-cGMPS	✗	✗	✓✓	✓
Rp-8-pCPT-cGMPS	✗	✓	✓	✓

Figure 5.10 Activation of cGMP is unable to restore Sema-3a-induced growth cone collapse in STIM1 morphants

A. Percentage of STIM1 morphant growth cones with spreading lamellipodial morphology in the presence or absence of Sema-3a, with cGMP activation or inhibition. Error bars are SEM. * compares group to the representative control in the absence of Sema-3a. * $p < 0.05$. † Compared to Sema-3a only (no drug), †† $p < 0.01$, †††† $p < 0.0001$. Data were collected over three different experiments, with sample sizes ranging from 246 growth cones to 934 growth cones per treatment group. Treatments were compared using the Student's t-test. **B.** Table explaining the results from the graph in A, for treatment groups in the presence of Sema-3a. ✓ represents activation (for PKG) or the relative level of growth cone spreading morphology or collapse. ✗ represents inhibition of PKG.

A



B

Sema-3a + Pharmacological Agent	PKG	CNGC	Spreading	Collapsed
No drug	-	-	✓✓✓	
Sp-8-Br-PET-cGMPS	✓	✗	✓✓	✓
Sp-8-pCPT-cGMPS	✓	✓	✓✓	✓✓
Rp-8-Br-PET-cGMPS	✗	✗	✓✓	
Rp-8-pCPT-cGMPS	✗	✓	✓✓	

5.4 Discussion

The data in this chapter demonstrated that the novel STIM1-mediated cAMP regulation pathway, store-operated cAMP signalling, is functional within growth cones; particularly in growth cones navigating away from the guidance cue Sema-3a. In addition, these data also demonstrated that STIM1 does not only regulate cAMP, but rather regulates both cAMP and cGMP signalling in growth cones, consistent with known cAMP-cGMP interactions that are required for normal growth cone motility. However, these data also demonstrate that STIM1-mediated cyclic nucleotide signalling is complex, and that the STIM1 signalling mechanisms activated in growth cone turning are not the same as in growth cone collapse. These data demonstrate that STIM1 activates multiple signalling pathways in growth cones, including STIM1-mediated SOCE and STIM1-mediated cyclic nucleotide signalling.

Activation of cAMP restores Sema-3a repulsion in STIM1 morphant growth cones

Sema-3a is traditionally described as a calcium-independent guidance cue, as it does not rely on large increases in intracellular calcium levels for its signalling cascades to occur. Given that Sema-3a signalling did not activate SOCE (Fig. 3.3), it was unexpected that reduced STIM1 expression would alter turning in response to Sema-3a (Fig. 3.4). However, since STIM1 has been shown to regulate cAMP levels in store-operated cAMP signalling in colonic epithelial cells (Lefkimiatis et al., 2009, Maiellaro et al., 2012), I hypothesised that STIM1 may alter growth cone turning in response to Sema-3a, via a regulation of the cAMP:cGMP ratio within growth cones. A decrease in cAMP via STIM1 reduction would

activate a reciprocal increase in cGMP, perturbing the cAMP:cGMP ratio (Shelly et al., 2010). Consistent with this hypothesis, artificial elevation of cAMP restored the repulsive turning effect of Sema-3a in STIM1 morphants, suggesting that the activation of cAMP is able to return the cAMP:cGMP ratio back to a normal range. Inhibition of cAMP with Rp-cAMPS abolished turning in control growth cones, while STIM1 morphant turning remained abolished. These data support the hypothesis that decreased STIM1 expression reduced the cAMP:cGMP ratio, perturbing the normal turning response to Sema-3a. These data support the novel idea that STIM1 not only regulates SOCE within growth cones, but that it can also regulate cyclic nucleotide levels in response to Sema-3a signalling.

In control growth cones, when cAMP was artificially activated with either forskolin or Sp-cAMPS, repulsive turning in response to Sema-3a was abolished. This is consistent with previous data, perturbing the ratio of cAMP:cGMP (Song et al., 1998, Hong et al., 2000, Nishiyama et al., 2003). It is thought that a low cAMP:cGMP ratio is required for normal growth cone repulsion, however there is also an optimal range in which cyclic nucleotides must function for normal growth cone motility (Nishiyama et al., 2003). Activation of cAMP results in a relative decrease in cGMP levels, shifting the growth cone turning response towards an attractive response (Song and Poo, 1999, Tojima et al., 2011). In addition, Sema-3a signals by binding to the neuropilin1/plexinA1 receptor complex, generating the downstream production of cGMP, which normally acts to cause growth cone repulsion (Song et al., 1998, Song and Poo, 1999, Togashi et al., 2008b). The combination of cGMP activation due to Sema-3a signalling, and the artificial activation of cAMP, would

be predicted to perturb the cAMP:cGMP ratio, and abolish growth cone turning (Song et al., 1998, Nishiyama et al., 2003).

Deciphering the downstream effectors of cAMP: Activation of PKA or Epac alone cannot rescue Sema-3a repulsion in STIM1 morphants

Traditionally cAMP was thought to signal only through protein kinase A (PKA). However, a novel family of proteins, the exchange proteins directly activated by cAMP (Epac), were discovered to operate independently of PKA, downstream of cAMP (De Rooij et al., 1998, Kawasaki et al., 1998). Recently, it was shown that Epac, as well as PKA, is important in the regulation of cAMP-dependent growth cone turning (Murray and Shewan, 2008). Important for growth cone navigation, a developmental switch was shown to exist between embryonic and adult DRG growth cones, where Epac mediates the turning responses of cAMP-dependent guidance cues in embryonic growth cones, while PKA mediates these responses in adult growth cones (Murray et al., 2009). The data in this chapter demonstrated that while the activation of cAMP through forskolin and Sp-cAMPS was able to restore growth cone repulsion to a gradient of Sema-3a after STIM1 knockdown, activating PKA or Epac alone could not rescue Sema-3a growth cone turning, in either control or STIM1 morphants. This suggests that in response to Sema-3a, which depends on cGMP production, these growth cones require both downstream effectors of cAMP for full cyclic nucleotide signalling to operate.

Activation of cGMP restores Sema-3a repulsion in STIM1 morphant growth cones

If STIM1 expression can modulate the cAMP:cGMP ratio, then I hypothesised that activating cGMP could also potentially rescue turning in response to Sema-3a, after STIM1 reduction. Interestingly, in control growth cones activation of cGMP with Sp-8-Br-PET-cGMPS or Sp-8-pCPT-cGMPS abolished the turning response to Sema-3a. Sema-3a signalling cascades activate cGMP (Song et al., 1997, Ming et al., 1999, Togashi et al., 2008b), resulting in a reciprocal decrease in cAMP and growth cone repulsion. An additional artificial increase in cGMP levels is thought to push the cAMP:cGMP ratio out of a functional range, resulting in random turning. In STIM1 morphants, cGMP activation restored the repulsive turning response to Sema-3a by shifting the cyclic nucleotide ratio to favour cGMP. Consistent with my hypothesis, inhibition of cGMP with Rp-8-Br-PET-cGMPS abolished control growth cone turning away from Sema-3a. The inhibition of cGMP would shift the cAMP:cGMP ratio towards cAMP, and hence growth cone attraction. The turning response to Sema-3a in STIM1 morphants remains abolished when cGMP is inhibited, due to a perturbed cAMP:cGMP ratio. These data suggest that STIM1 regulates cGMP levels within growth cones, either directly, or indirectly as a result of STIM1-mediated cAMP signalling.

Activation of STIM1-mediated cAMP signalling fails to rescue STIM1 morphant turning towards BDNF

While STIM1-mediated cyclic nucleotide signalling could rescue turning towards Sema-3a, the same result is not observed for BDNF turning. The data presented in this chapter

demonstrates that BDNF signalling depends on both STIM1-mediated SOCE, as well as STIM1-mediated cAMP signalling. Within both control and STIM1 morphants, activation of cAMP signalling pathways abolished BDNF-induced growth cone turning. The abolished turning response in control growth cones is consistent with the concept that an optimal cAMP:cGMP ratio is required for the regulation of growth cone responses (Nishiyama et al., 2003). Interestingly, activation of cAMP signalling pathways abolished the repulsive response to BDNF observed in STIM1 morphants, but was not sufficient to restore STIM1-mediated attractive turning in response to BDNF. These data suggest that STIM1-mediated SOCE is also crucial for BDNF-induced growth cone navigation. It would be of interest to determine if SOCE alone is sufficient to rescue BDNF turning in STIM1 morphants, or if STIM1-mediated cyclic nucleotide signalling is required in addition to SOCE activation. In order to address this, the CRAC channel, and hence SOCE, would need to be activated directly, independently of STIM1. Currently, the only available activators of SOCE work through the depletion of ER calcium stores (Bogeski et al., 2010, Putney, 2010), or by the overexpression of the CAD peptide. Depleting the ER stores would confound these experiments since it could potentially activate other SOC channels, such as the TRPC channels. The CAD peptide interacts with Orai proteins in order to cluster them to activate the CRAC current (Song and Poo, 1999, Muik et al., 2008, Kawasaki et al., 2009, Park et al., 2009, Yuan et al., 2009, Tojima et al., 2011). At the present time, there are no pharmacological activators of the Orai proteins, or the CRAC channel, that work independently of STIM1 activation, rendering this experiment unachievable. However, if such an activator were available, I would predict that the activation of SOCE alone could restore attractive BDNF turning in STIM1 morphants.

Furthermore, in order to establish if STIM1-mediated cyclic nucleotide signalling is normally functional in response to BDNF signalling, experiments using FRET constructs for downstream cAMP effectors (Depry et al., 2011, Huang et al., 2012) could be used in growth cone turning experiments, in the presence and absence of STIM1 morpholinos. This would allow direct assessment of whether cAMP activation is normally triggered by STIM1 activation in BDNF signalling.

Deciphering the downstream effectors of cAMP: Activation of PKA or Epac alone is unable to restore BDNF-induced attraction in STIM1 morphants

To examine the downstream effectors of STIM1-mediated cAMP signalling, PKA and Epac were manipulated in combination with BDNF signalling. After activation of PKA or Epac alone, growth cone turning responses to BDNF were perturbed in control growth cones. Activation of either PKA or Epac on their own were unable to rescue the attractive turning response to BDNF in STIM1 morphants. Instead, turning was abolished and random growth occurred. This was consistent with the failure of forskolin or Sp-cAMPS to restore BDNF turning, suggesting that STIM1 activation of SOCE is also required for full rescue of BDNF attraction. In control growth cones, Epac activation attenuated the attractive turning response to BDNF, consistent with the known role of Epac in growth cone attraction (Murray et al., 2009). Epac is also known to stimulate CICR (Kang et al., 2003) and modulate CaMKII activity (Pereira et al., 2007), suggesting that Epac activation is able to regulate calcium signalling pathways, which mediates the attractive response to BDNF (Murray et al., 2009). Conversely, when PKA is activated in control growth cones,

the turning response to BDNF switched from attraction to repulsion, consistent with a developmental Epac-PKA switch, where PKA is not normally activated in embryonic growth cones (Murray et al., 2009). This suggests that by artificially activating PKA, signalling pathways that are not normally active are stimulated, resulting in growth cone repulsion instead of attraction. These data suggest that in response to BDNF, Epac is normally the cAMP effector that is activated. However, the activation of Epac alone is unable to restore BDNF-induced attraction in STIM1 morphants, suggesting that STIM1 activation of SOCE is also essential for BDNF chemoattraction.

Activation of STIM1-mediated cGMP signalling fails to rescue STIM1 morphant turning towards BDNF

cGMP signalling pathways were also examined in response to BDNF signalling, yielding similar results to the manipulation of cAMP. Activation of cGMP with Sp-8-Br-PET-cGMPS abolished the turning response in both control and STIM1 morphants. Interestingly, activation of cGMP with Sp-8-pCPT-cGMPS did not alter attraction in controls, potentially due to the fact that this compound also activates PKA as well as PKG. All turning responses were also abolished with the inhibition of cGMP. Taken together with the BDNF-cAMP data, these data suggest that while cyclic nucleotide levels are important for BDNF signalling, STIM1 regulation of cyclic nucleotides may not function in response to BDNF, while the regulation of calcium through SOCE is directly activated BDNF signalling.

Sema-3a-induced growth cone collapse is mediated by optimal levels of cAMP and cGMP

STIM1 expression was shown to be required for growth cone turning in response to Sema-3a, and similarly STIM1 was also necessary for Sema-3a-induced growth cone collapse. Given this, experiments were performed to determine if the inhibition of Sema-3a-induced growth cone collapse after STIM1 knockdown, could be rescued by cyclic nucleotide activation.

Upon treatment with Sema-3a in control cells, growth cones withdrew their lamellipodia and filopodia and appeared collapsed, as described previously (Kapfhammer and Raper, 1987). Elevation of cAMP levels with either forskolin or Sp-cAMPS prevented Sema-3a-induced growth cone collapse. This is consistent with previous data where Sema-3a growth cone collapse was suppressed by the elevation of cytosolic cAMP levels in chick DRG growth cones (Chalasanani et al., 2003). These data differ from other work where treatment with Sp-cAMPS did not alter the rate of Sema-3a-induced collapse of rat DRG growth cones (Song et al., 1998). However, differing concentrations of Sp-cAMPS were used in these experiments (0.5 and 5mM; Song et al., 1998) compared to 20 μ M used in the experiments described in this chapter. This suggests that the inhibition of Sema-3a-induced growth cone collapse, mediated by an increase in cAMP levels may be a dose-dependent response. It has previously been reported that elevated levels of cAMP can rescue the repulsive activity of axons in response to the guidance cue MAG (Qiu et al., 2002), as well as facilitate in axonal regeneration of the spinal cord after injury in adult rats (Neumann et

al., 2002). In combination with suppression of Sema-3a collapse due to an elevation in cAMP (Chalasani et al., 2003), it has been proposed that elevating the levels of cAMP within mammalian neurons may not mediate enhanced growth of growth cones and neurons, but may actually reduce the responsiveness of neurons to repulsive guidance cues (Snider et al., 2002, Chalasani et al., 2003).

Reduced STIM1 expression prevented Sema-3a-induced growth cone collapse, with STIM1 morphant growth cones displaying spreading lamellipodia and filopodia. Given that elevation of cAMP within growth cones was able to restore repulsive turning in response to Sema-3a microgradients after STIM1 knockdown, I hypothesised that cAMP elevation would restore growth cone collapse in STIM1 morphants. However, this did not occur. Growth cones continued to retain their spread lamellipodia and filopodia morphology. These data suggest that STIM1 actually plays a different role in Sema-3a-induced growth cone collapse, than what it does in Sema-3a-mediated growth cone turning.

Elevation of cGMP with PKG activators prevented Sema-3a-induced growth cone collapse in control growth cones. This is consistent with previous data where elevation of cGMP prevented Sema-3a collapse in mouse DRG growth cones (Schmidt et al., 2002). Previously, Sema-3a collapse has also been inhibited in rat DRG growth cones with activation of cGMP (Song et al., 1998), however not to the same extent as demonstrated here. The differences can be explained with the use of newer, more cell permeable and specific PKG agonists (refer to table 5.1). In addition, a lower concentration of cGMP

agonist was used (20 μ M) compared with previous experiments (0.5 and 5mM; Song et al., 1998), which may suggest that Sema-3a collapse prevention with cGMP activation is dose-dependent, as was the case with cAMP activation, supporting the hypothesis that an optimal cAMP:cGMP ratio is required. There is evidence to suggest that cGMP-dependent protein kinase cGK1 (also known as PKG1) is not involved in the Sema-3a signalling cascade that causes growth cone collapse, however its activation, which may have occurred here, can counteract Sema-3a collapse (Schmidt et al., 2002). Since Sema-3a signals via a downstream production of cGMP, this suggests that Sema-3a-induced cGMP elevation can cause collapse, however excess activation of PKG may inhibit growth cone collapse, consistent with the hypothesis that optimal ranges of cyclic nucleotides are required for normal growth cone function. Furthermore, inhibition of cGMP with multiple antagonists reduced the rate of growth cone collapse. This is likely due to a reciprocal decrease in cGMP and increase in cAMP levels, preventing growth cone collapse. Less PKG activation could potentially result in more growth cone collapse (Schmidt et al., 2002), suggesting that the levels of cGMP within growth cones can dictate the responsiveness of growth cones to Sema-3a, supporting the concept of a dose-dependent response.

Elevation of cAMP and cGMP within STIM1 morphant growth cones were able to restore repulsive turning to Sema-3a gradients. Hence it was predicted that elevating cGMP within STIM1 morphants would rescue Sema-3a-induced collapse. When cGMP was activated, only small levels of collapse were observed. This was potentially due to a change to the ratio of cAMP:cGMP, which was already lowered by STIM1 knockdown, resulting in a reciprocal rise in cGMP levels (Shelly et al., 2010). If cGMP levels rise enough, PKG can

be activated. PKG can induce membrane depolarisation, opening VGCCs on the plasma membrane, causing high-amplitude excessive calcium influx (Nishiyama et al., 2008, Tojima et al., 2011). Inhibition of cGMP in STIM1 morphants did not rescue Sema-3a-induced collapse, suggesting that STIM1 function in Sema-3a-induced growth cone collapse is not via the regulation of cyclic nucleotides.

In conclusion, the data presented in this chapter demonstrated that STIM1 regulates both cAMP and cGMP signalling, either directly or indirectly, within navigating growth cones. Sema-3a signalling is heavily dependent on an optimal STIM1-mediated cAMP:cGMP ratio for its proper function within growth cones. While STIM1 may mediate cyclic nucleotides in response to BDNF signalling, BDNF is also critically dependent on STIM1-mediated SOCE for its functions. In addition, these data demonstrated that while Epac operates downstream of BDNF, the activation of either PKA or Epac alone was unable to restore normal growth cone responses to Sema-3a, suggesting that both effectors of cAMP are required for Sema-3a turning in growth cones. Furthermore, these data demonstrated that the levels of cAMP and cGMP are critical for Sema-3a-induced growth cone collapse in control growth cones. Taken together, these data demonstrate that the novel STIM1-mediated cyclic nucleotide signalling pathway does function in growth cone turning, particularly in response to Sema-3a. However, these data also demonstrate that while STIM1 is required for Sema-3a collapse, it is not via cyclic nucleotide signalling.

Chapter 6:

Conclusions and future directions

Chapter 6. Conclusions and future directions

STIM1 is most well known for the role it plays as the key mediator of CRAC channel activation and SOCE. However, STIM1 is a promiscuous protein that can interact with other proteins and have effects on a range of targets, some related to, and some beyond the scope of SOCE. Some of these include binding to TRPC channels to regulate other SOC channels (Huang et al., 2006), direct inhibition of $Ca_v1.2$ L-type VGCCs (Park et al., 2010a, Wang et al., 2010b), interactions with the microtubule-plus end tracking protein EB1 to regulate ER movement (Grigoriev et al., 2008), direct interactions with Golli, a negative regulator of SOC channels (Walsh et al., 2010), and interactions with cAMP signalling pathways (Lefkimmiatis et al., 2009). This thesis has examined three of these functions within navigating growth cones: the traditional function of STIM1 in SOCE, the functional interaction of STIM1 with L-type VGCCs, and the function of STIM1 in regulating cAMP signalling. The work presented within this thesis provides strong evidence that STIM1 function is crucial for the normal function and correct guidance of sensory neuronal growth cones.

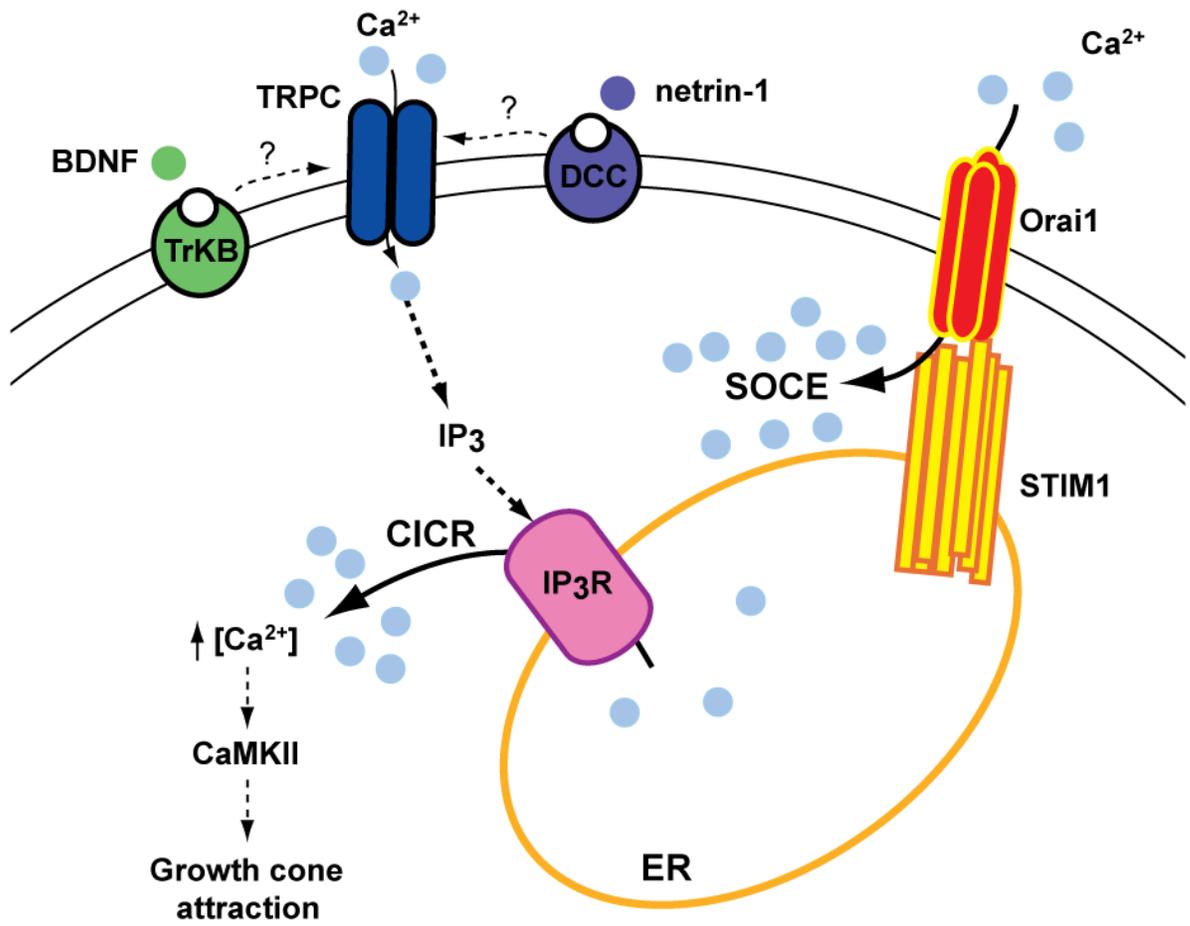
This thesis has demonstrated that STIM1 is present within the central and peripheral domains of growth cones, as well as extending filopodia, supporting the evidence that the growth cone ER is a motile structure that can move and extend into all regions of a growth cone, including the filopodia (Landis, 1983, Dailey and Bridgman, 1989, Deitch and Banker, 1993). Furthermore, in response to the calcium-dependent guidance cue BDNF,

STIM1 expression was biased towards the attractive, turning side of the growth cone, placing it in the ideal position to regulate spatial and temporal restricted calcium signalling in turning growth cones. Further exploration demonstrated that STIM1-mediated SOCE was a functional process in navigating growth cones, in response to both an artificial cue (thapsigargin) and a physiological guidance cue (BDNF). Furthermore, STIM1-mediated SOCE was required for correct growth cone turning responses to gradients of BDNF and netrin-1, with STIM1 knockdown switching growth cone attraction into repulsion. This is consistent with the CaMKII/CaN switch model, where relatively high increases in intracellular calcium concentration activate CaMKII and growth cone attraction, and relatively low increases in calcium concentration activate CaN and growth cone repulsion (Wen et al., 2004). I hypothesised that when STIM1 is present within growth cones, calcium-dependent guidance cues (BDNF and netrin-1) activate calcium release from the ER, which is sustained through the activation of STIM1 and SOCE, resulting in the activation of CaMKII and growth cone attraction (Fig. 6.1). Conversely, when STIM1 expression is reduced, SOCE does not occur, and CICR from the ER is not sustained. CaN is activated instead of CaMKII, resulting in growth cone repulsion.

The level of intracellular calcium is crucial for growth cone behaviour. If there is too much calcium, growth cones collapse, but if there is too little calcium, growth cone motility is halted (Kater and Mills, 1991). STIM1 has previously been demonstrated to interact with, and inhibit L-type VGCCs in order to regulate SOCE (Park et al., 2010a, Wang et al., 2010b). In my work presented here, activation of L-type VGCCs abolished growth cone

Figure 6.1. STIM1-mediated store-operated calcium entry sustains CICR to mediate growth cone attraction.

A model depicting the basic downstream signalling effects of calcium-dependent guidance cues BDNF and netrin-1. BDNF and netrin-1 bind their receptors, TrKB and DCC, activating calcium channels on the growth cone plasma membrane. Small volumes of extracellular calcium influx into the cytoplasm, and activate the IP₃ signalling pathway, to produce IP₃. IP₃ binds to the IP₃ receptor (IP₃R) on the ER membrane to release stored intracellular calcium into the cytoplasm, through calcium-induced calcium release (CICR). Release of ER calcium activates STIM1 monomers to cluster close to the plasma membrane, activating Orai1 tetramers and SOCE. The large volume of calcium influx from SOCE sustains CICR, resulting in the activation of CaMKII and growth cone attraction.

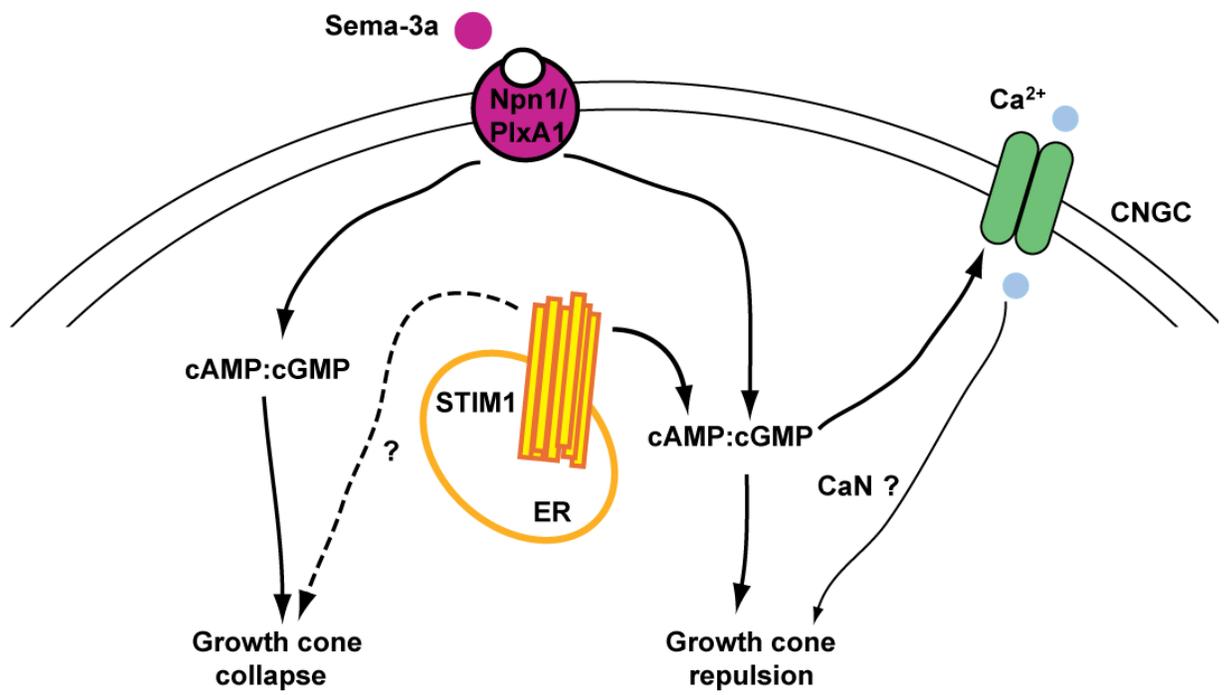


turning in response to calcium-dependent guidance cues, suggesting that L-type VGCCs are not required for normal growth cone attraction, and that the source and volume of calcium influx into the growth cone is essential for correct navigation. Interestingly, a functional interaction between STIM1 and L-type VGCCs may be required for axon extension in response to netrin-1, suggesting a novel role for STIM1 in the mediation of axon extension in response to netrin-1, in combination with L-type VGCCs.

Surprisingly, STIM1 was demonstrated to be necessary for both growth cone repulsion and growth cone collapse in response to the calcium-independent guidance cue *Sema-3a*, suggesting a novel calcium-independent function of STIM1 in the regulation of growth cone function. In order to address this further, the novel STIM1-mediated store-operated cAMP signalling pathway (Lefkimmatis et al., 2009) was examined within growth cones. In the absence of STIM1 expression, growth cone repulsion to *Sema-3a* could be restored with the activation of either cAMP or cGMP signalling pathways, suggesting that STIM1 regulates the cAMP:cGMP ratio within growth cones, either directly or indirectly through activation of STIM1-mediated store-operated cAMP signalling (Fig. 6.2). These findings implicate a novel STIM1-mediated cyclic nucleotide signalling pathway in normal growth cone turning. STIM1 was also demonstrated to be required for *Sema-3a*-induced growth cone collapse. Given the involvement of STIM1 in cyclic nucleotide signalling in *Sema-3a*-mediated growth cone repulsion, the activation of both cAMP and cGMP were examined to determine if they are required for STIM1-mediated growth cone collapse. Collapse could not be restored with cyclic nucleotide activation, suggesting that STIM1 functions through a different, unknown mechanism in order to mediate *Sema-3a* collapse (Fig. 6.2).

Figure 6.2 STIM1 signalling regulates cAMP:cGMP ratio for Sema-3a-induced growth cone repulsion, but regulates growth cone collapse via a different, unknown mechanism.

A model depicting proposed mechanisms of calcium-independent Sema-3a signalling in growth cones, to cause repulsion and collapse. Sema-3a binds the Npn1/PlxA1 receptor complex on the growth cone membrane. This binding results in downstream production of cGMP through multiple mechanisms. cGMP activates cyclic nucleotide gated channels (CNGC) which allow for a small influx of extracellular calcium, which results in growth cone repulsion, potentially through calcineurin (CaN). Activation of STIM1 regulates cAMP production, and the cAMP:cGMP ratio, which when at an optimal range results in growth cone repulsion. The cAMP:cGMP ratio is also important in the regulation of growth cone collapse, but this is not regulated by STIM1. However, STIM1 is required for growth cone collapse, but the mechanisms by which this occur are currently unknown.



These data have provided strong evidence that the regulation of STIM1 is crucial to the normal function of sensory growth cones. Given this, changes to the expression levels of STIM1 and STIM1-mediated regulation of SOCE are likely to have important ramifications for axon guidance, and indeed neuronal development. Errors in axon guidance are well known to be significant contributors to neurodevelopmental disorders such as autism and mental retardation (Li et al., 2009, Sbacchi et al., 2010, Chow et al., 2012). Furthermore, STIM1 has recently been shown to be upregulated in both animal and human cases of epilepsy, a disorder of excessive neuronal excitation (Steinbeck et al., 2011). There is also evidence that SOCE is upregulated after painful nerve injury, and that injured neurons have an elevated dependency on SOCE (Gemes et al., 2011). STIM1 and SOCE are also known to be required for flight in *Drosophila melanogaster* (Venkiteswaran and Hasan, 2009). Knockdown of STIM1 results in phenotypes that are flightless, and unable to generate normal rhythmic firing required for flight, suggesting that STIM1 and SOCE are important for the formation of neural circuits, and are critical for normal neuronal function.

Given the importance of STIM1 and SOCE within normal neuronal function, it is important that STIM1 function is understood in detail within the developing nervous system. This thesis has provided insight and knowledge into the function of STIM1 in growth cone guidance, in an *in vitro* model. In order to further assess the role of STIM1 in neuronal development, further experiments would be required, using *in vivo* models of development. While STIM1 knockout mice perish *in utero* (reviewed in Cahalan, 2009), there are conditional STIM1 mutants available (Oh-hora et al., 2008). These mice can be crossed with specific transgenic mice to conditionally ablate both copies of the *Stim1* gene in a

specific population of cells of interest. This would allow for the study of STIM1 function within specific neuronal populations. Zebrafish also provide an excellent *in vivo* model for axon pathfinding and neuronal development. STIM1 expression can be reduced embryonically with morpholino injections, and neuronal subpopulations can be examined over the course of development. This would determine if there are any defects within the axon pathfinding process and the development of the zebrafish nervous system in the absence of STIM1.

The data described in this thesis support the hypothesis that STIM1 is necessary for SOCE in sensory growth cones, and is required for correct axon guidance. These data have also demonstrated a novel pathway by which STIM1 can also mediate cyclic nucleotide signalling within navigating growth cones, and subsequently regulate calcium-independent signalling. The results of this thesis reveal that STIM1 is a key regulator of intracellular second messenger pathways that are vital for the normal function of growth cones, providing an insight into the complex mechanisms that regulate growth cone guidance and indeed axon pathfinding.

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