Structural and functional role of CADM1 on mast cell-neuron cross-talk

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Author's declaration

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Rania Magadmi

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Abstract

Neuroimmune interactions are important in the pathophysiology of many chronic inflammatory diseases, particularly those associated with alterations in sensory processing and pain (Grace et al., 2014). Much of our understanding of these interactions comes from studies on mast cell- sensory neuron interactions in diseases such as irritable bowel syndrome (IBS) (Barbara et al., 2004) . Mast cells and neurons are connected to each other both functionally and structurally. Cell adhesion molecule 1 (CADM1) has been identified as an adhesion molecule between mast cells and sensory neurons (Ito et al., 2008). However, it remains to be unclear if CADM1 is essential in mast cell-neuron functional cross-talk.

The aim of this study is *to investigate the role of CADM1- mediated adhesion between mast cell and neuron on IgE- mediated mast cell signalling and pro-inflammatory mediator secretion.* Using the *in vitro* co-culture system of mouse bone marrow mast cells (BMMC) with sensory neurons isolated from dorsal root ganglions (DRG), expression and cellular distribution of CADM1 was determined. The adhesion between BMMC and sensory neurons was quantified using a fluorescent assay. Application of a CADM1 blocking peptide or knockdown CADM1 in BMMCs were found to significantly decrease both the BMMC attachment to sensory neurites, as well as, the enhancement of IgE-mediated BMMC degranulation in the presence of neurons. Furthermore, CADM1 Knockdown attenuates the enhancement of IL-6 release from BMMC in co-culture. Importantly, this study also showed that the CADM1-dependent enhancement of IgE-mediated mast cell response is specific for neurons. It is suggested that neurons may mediate mast cell enhancement through action potential (AP)-independent neuropeptide release.

In conclusion, my data have been revealed for first time the direct role of CADM1 in functional mast cell-sensory neuron cross-talk. *Elucidating the precise mechanisms of CADM1 in IgE signaling is imperative for understanding many disease processes*. The results of this study would contribute to the available knowledge about role of CADM1 in neuroimmune interactions and hold promise as a therapeutic target in various diseases such as IBS.

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Abbreviations

Ab	Antibody
Ag	Antigen
Ag-Indep	Ag-independent
AM	Acetoxy-methyl group
ANOVA	Analysis of Variance α alpha
AP	Action potential
Ara-C	Cytosine β- D -arabinofuranoside
ASM	Airway smooth muscle
ATP	Adenosine 5'-triphosphate
b-Hex	b-Hexaminidase
BFA	Paraformaldehyde
BMMC	Bone marrow derived mast cell
BSA	Bovine serum albumin
c48/80	Compound 48/80
Ca^{2+}	Calcium
CADM1	Cell adhesion molecule 1
CAM	Cell adhesion molecule
Cap	Capsaicin
CASK	Calcium/Calmodulin-Dependent Serine Protein
CBMC	Umbilical cord blood mast cell
CD	Custer of differentiation
CGRP	Calcitonin gene-related peptide
CNS	Central nerveous system
CSF	Colony- stimulating factor.
CTMC	connective tissue type
CTX	Toxin cocktail
DAG	Diaglycerol
DAPI	4',6-diamidino-2-phenylindole
DC	Denteric cells
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNP	Dinitrophenol Ag
DRG	Dorsal root ganglion
DRR	Dorsal root reflexes
ECM	Extracellular membrane
EGF	Epidermal growth factor.
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum

ETDA	Ethylene-diamineteraacetic acid
FACS	Fluorescence Activated Cell Sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FceRI	Fc epilson high affinity receptor for IgE
FGFR	Fibroblast growth factor receptor
Gab2	GRB2-associated binding protein 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GIT	Gastrointestinal tract
GPCRs	G protein–coupled receptors
GRB2	Growth factor receptor-bound protein
HBSS	Hank's balanced salt solution
HEK	Human Embryonic Kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HLMC	Human lung mast cell
HMC-1	Human mast cell line
HUVEC	Human umbilical vein endothelial cells
IASP	International association for the study of pain
IBS	Irritable bowel syndrome
ICAM	Intercellular adhesion molecule
ICC	Immunocytochemistry
IgE	Immunoglobulin E
IgSF	Immunoglobulin superfamily
IL-	Interleukin
IMDM	Iscove's Modified Dulbecco's Media
IP3	Inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
KCl	Potassium chororide
kDa	Kilodaltons
LAD	Laboratory of allergic diseases cell line
LAT	Linker to activated T-cell
LT	Leukotrienes
m.w	molecular weight
MAPK	Mitogen-Activated Protein Kinases
Mcl-1	myeloid cell leukemia 1
MCp	Mast cells progenitors
MCPV-1	Mast cell progenitor Vienna-1 cell line
MC_T	Tryptase- positive mast cell
MC_{TC}	Tryptase and chymase-positive mast cell
MFI	Mean fluorescence intensity
MITF	Microphthalmia transcription factor

MMC	Mucosal-like mast cells
mPCMC	Mouse peritoneal cell-derived mast cells
mRNA	Messenger ribonucleic acid
Na	sodium
NaV	Voltage Gated Sodium Channel
NBA	Neurobasal A medium
NCAM	Neural cell adhesion molecule
Necl-2	Nectin-like molecule-2
NFAT	Nuclear Factor of Activated T Cell
NFκB	Nuclear translocation of the transcription factor
NGF	Nerve growth factor
NIH/3T3	Fibroblast cell line
NK	Neurokinin
ns	non- significant
p	Probability values
P2X	purinergic receptor
PAF	Platelet- activating factor
PAR	Protease-activated receptor
PBS	Phosphate buffer solution
PDAC	Pancreatic ductal adenocarcinoma
PFA	Paraformaldehyde
PG	Prostagladin
PI3K	Phosphotidylinositol 3-OH Kinase
PIC	Protease inhibitor cocktail III
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PVDF	Polyvinylidinefluoride transfer membrane
RBL	Rat basophilic leukemia cell line
rmIL-3	Recombinant mouse IL-3
rmSCF	Recombinant mouse SCF
RNA	Ribonucleic acid
Scarm	Scrambled
SCF	Stem cell factor
SCG	Superior cervical ganglion
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error of the mean
SgIGSF	Spermatogenic immunoglobulin superfamily
ShRNA	Short hairpin RNA
SNAP	Synaptosomal associated protein

Src kinase	Sarcoma kinase
STAT	Signal Transducer and Activator of Transcription
STIM1	Stromal interaction molecule 1
subP	Substance P
Syk	Spleen tyrosine kinase
SynCAM	Synaptic cell adhesion molecule1
TG	Trigeminal ganglia
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNFR1	Tumor necrosis factor receptor 1
TrkA	Tropomyosin kinase receptor A
TRPC-1	Transient receptor potential canonical channel 1
TRPV1	Transient receptor potential vanilloid
TSLC1	Tumor suppressor in lung cancer-1
TTX	Tetrodotoxin
VEGF	Vascular endothelium growth factor
WB	Western blot
WT	Wild-type

Chapter 1: Introduction

1.1 Mast cell biology and relevance

Mast cells are long-lived, highly granulated immune cells. They are best acknowledged for their major role in defence against pathogens and responses to allergens. Mast cells are considered as both sensory cells and effector cells because they are activated by various stimuli, and release a plethora of potent mediators.

1.1.1 Mast cell development and the importance of microenvironment

In contrast to other hematopoietically-derived cells, mast cells depart the bone marrow as immature progenitor cells that complete their maturation within tissues. Mast cells originate from multipotential $CD34^{+}$ stem cells in bone marrow (Kirshenbaum et al., 1991) and circulate as immature mast cells progenitors (MCp) expressing $CD34^{+}$, Kit^{+} and $Fc\varepsilon RI^{-}$ (Agis et al., 1993) (Chen et al., 2005). MCp migrate into various tissues under a regulating process of locally-produced cytokines. Within tissues, they remain as a homeostatic pool or differentiate into mature mast cells under the influence of specific micro-environmental signals and growth factors (Galli, 1990). Although mast cells can be found in various organs, their distribution and density are highest at the surfaces exposed to the environment like mucosal surfaces in the airways and gastrointestinal tracts (Abraham and St John, 2010). Moreover, they ultimately dwell in close to nerves, blood vessels, and smooth muscle cells (Galli and Tsai, 2008). Mature mast cells are long-lived cells (Padawer, 1974). Indeed, they have the ability to re-granulate in response to additional stimuli after recovery from initial degranulation (Xiang et al., 2001).

Mast cell development, survival, maturation, and functional responses are influenced by locally tissue-derived growth factors. The most critical factor to control mast cell

differentiation from MCp is interleukine-3 (IL-3) . IL-3 induces differentiation of MCp into mucosal-like mast cells (MMC) (Razin et al., 1984). However, it may also act synergistically with other factors like IL-4 to induce differentiation into connective tissue -like mast cells (CTMC) (Schmitt et al., 1987). Other interleukins such as IL-9 or IL-10 enhance mast cell proliferation only when used synergistically with IL-3 (Hültner et al., 1990) (Rennick et al., 1995). Furthermore, nerve growth factor (NGF) has a synergistic effect with IL-3 on the development of CTMC-like cells (Matsuda et al., 1991). Therefore, the effect of IL-3, alone or in combination with other micro-environmental factors plays a crucial role in determining the final phenotype of mast cells in a particular tissue (Hu et al., 2007).

The other important factor for mast cell migration and growth is stem cell factor (SCF) (Galli et al., 1993). *In vivo,* SCF is released from stromal cells (Galli et al., 1993) like fibroblasts (Hogaboam et al., 1998), and endothelial cells (Mierke et al., 2000). It is either expressed on the cell surface or released as a soluble protein. In both ways, it binds receptor tyrosine kinase (c-Kit) on mast cells. c-Kit receptor belongs to immunoglobulin (Ig)-superfamily of proteins. It has five (Ig)-like motifs in its extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain (Galli et al., 1994). SCF promotes mast cells migration by two mechanisms. Serum SCF recruits MCp from bone marrow to blood (Okumura et al., 1996) while tissue-derived SCF acts as chemotactic for MCp (Nilsson et al., 1994). Although mast cells *in vitro* can be cultured without SCF, they can't be *in vivo.* $Kit^{Sl}/Kit^{Sl\text{-}d}$ mice and Kit^{W}/Kit^{Wv} mice that lack SCF and functional c-kit, respectively, lack mast cells in their tissues (Kitamura et al., 1978) (Kitamura and Go, 1979). More interestingly, SCF doesn't only influence MCp but also influences mast cell function after complete maturation. SCF can augment IgE-dependent mast cell degranulation and cytokine release (Hundley et al., 2004). In addition, SCF increase mast cells adhesion to fibronectin, and integrin (Kinashi and Springer, 1994).

Indeed extracellular matrix (ECM) proteins also have a vital role in regulating mast cell biology like differentiation and maturation. *In vivo*, mast cell progenitors migrate from circulation into tissues to complete their development. This process requires them to physically interact with ECM proteins and other cells. Mature mast cells are always found within tissues attached to other structures or ECM. Thus, mast cell adhesion molecules have a vital role in their development and functions. In order to facilitate the migration of mast cell progenitors into tissues where they mature and residence, mast cells express a diversity of adhesion receptors. These include several members of integrins (Sperr et al., 1992), cell adhesion molecule-1 (CADM1) (Ito et al., 2003), and N-cadherin (Suzuki et al., 2004). These molecules are discussed in greater detail below (Section 1.4).

1.1.2 Mast cell activation

A fundamental feature of mast cells is their ability to secrete and produce mediators in response to certain stimulants. Mature mast cells can be stimulated by two primary mechanisms. IgE-dependent or IgE-independent mechanisms. In the former, mast cell activation is initiated by antigen (Ag). While in the later, mast cell activation is initiated by exposure to complement factors, bacterial components, and neuropeptides (Figure 1.2)

1.1.2.1 IgE-dependent mast cell activation

Mast cell expresses high-affinity IgE receptors (FcεRI) on their cell surfaces. FcεRI is a tetrameric protein complex comprising of the IgE binding α- chain, the membrane-tetraspanning β chain and two disulphide-linked γ chains (Blank et al., 1989). IgE-dependent activation is initiated upon binding of Ag to specific IgE that binds to FcεRI α chain on mast cells via its Fc portion (Hakimi et al., 1990) (Metcalfe, 2008). The crosslinking of surface-bound IgE by specific antigens leads to aggregation of FcεRI. This results in a sequence of phosphorylation events that recruit and activate specific signaling molecules (Figure 1.1) (Metcalfe et al., 1997). As reviewed by (Gilfillan and

Rivera, 2009), aggregation of FcεRI receptor stimulates Src (sarcoma) tyrosine kinase (Lyn) that bound to β subunit. Lyn trans-phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on γ chains of the FcεRI which act as major docking sites for other Src tyrosine kinase (Syk). Activated Syk phosphorylates the adaptor molecule LAT1 (linker for activation of T cell- 1) and recruits adaptor molecules such as Gab2 and Grb2 to assemble a signaling complex (Rivera and Olivera, 2007). This complex phosphorylates phospholipase Cγ (PLCγ) that hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP2) to procedure soluble inositol-1,4,5-triphosphate (IP3). By binding to its receptor IP3R on endoplasmic reticulum membrane, IP3 induces the release of the "first wave" intracellular calcium stores. Once endoplasmic calcium stores are emptied, the calcium sensor on endoplasmic reticulum called stromal interaction molecule 1 (STIM1) interacts with Orai1 channels (the store- operated calcium channel) on plasma membrane to facilitate "the second wave" of calcium influx from the extracellular space. Increased intracellular calcium activates protein kinase C (PKC) and, eventually, initiate degranulation (Wu, 2011). Also, the increase in intracellular calcium dephosphorylates the nuclear factor for T cell activation (NFAT) to cross nucleus membrane and regulates several cytokine genes transcription including TNFα (Klein et al., 2006). In addition, Syk- LAT1 complex activates MAPK (mitogen-activated protein kinase) and phospholipase A2 (PLA2). PLA2 releases arachidonic acid from cell membrane to generate arachidonic acid mediators .

A parallel pathway, aggregation of FcεR1 receptor phosphorylates other Src tyrosine kinase (Fyn). Fyn activates and recruits adaptor proteins such as linker for activation of T cell-2 (LAT2), and Gab2 that form signaling complex (Rivera and Olivera, 2007). This results in activates the signaling enzyme phosphoinositide 3-kinase (PI3K) that phosphorylates PIP2 to IP3 and mediate degranulation. PI3K also induces nuclear translocation of the transcription factor (NFκB) by degrading its inhibitor IκB that bind to it. In addition, Fyn- LAT2 activation results in formation of phosphotyrosine -induced STAT5 dimers that transfer into the nucleus and stimulate cytokine production (Pullen et al., 2012). MAPK/STAT (Akira, 1997) and NFκB (Kalesnikoff et al., 2002) are

important in IL-6 production. Fyn also induces calcium influx through transient receptor potential canonical channels (TRPC-1) channel (Gilfillan et al., 2009).

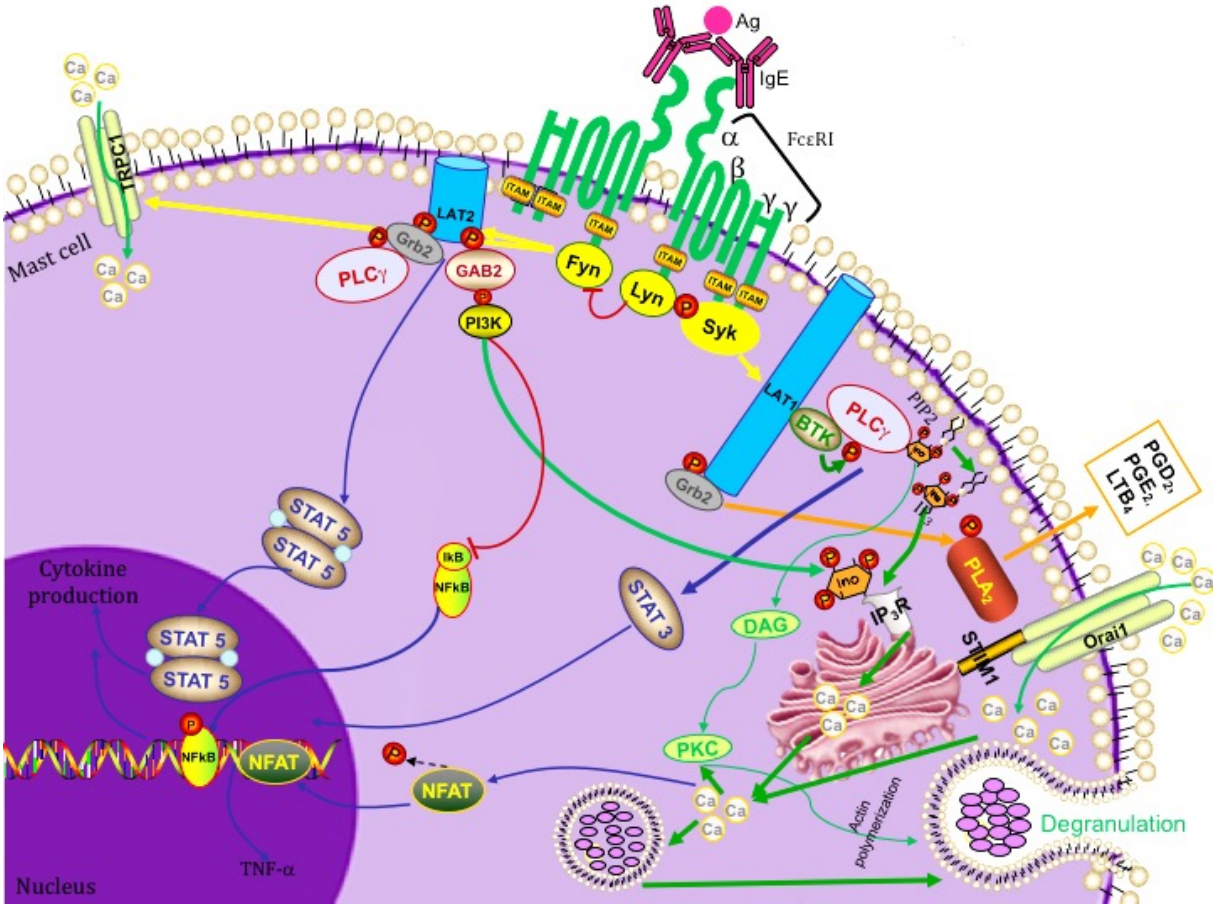


Figure. 1.1 FcεRI signalling in mast cells

Upon FcεRI cross linking by IgE, the Src-family kinases Lyn, Syk and Fyn induce downstream signaling cascade that activate PLCγ and ultimately gives rise to large increases in cytosolic Ca^{2+} and degranulation. The production of eicosanoids occurs following activation of the mitogen-activated protein kinase (MAPK) pathway. Nuclear factor activation lead to cytokines generation.

1.1.2.2 IgE-independent mast cell activation

The other primary mast cells activating pathway is IgE- independent and achieved by activating G-protein coupled receptors (GPCRs). Mast cells express various GPCRs . Among many, polybasic secretagogue of mast cells compounds such as the pan-mast cell activator compound 48/80 (C48/80) (Shefler et al., 1999). C48/80 interacts with membrane proteins to directly activate GPCRs through PI3-kinase (Byrne et al., 2007).

In addition, neuropeptides such as substance P (SubP) can induce histamine release from mast cells (Ebertz et al., 1987), as well as, calcitonin gene-related peptide (CGRP) (Bauer and Razin, 2000). These mediators will be covered in detail in an upcoming section (Section 1.2.2) of this thesis.

The extracellular adenosine 5'-triphosphate (ATP) can also activate mast cells by binding to purinoceptors (P2). Two subtypes of P2 are expressed by mast cells; the ligand-gated ion receptors (P2X), and (P2Y) which is GPCR. Mast cells phenotype and anatomical source determine the subtype expression of P2. Similarly, the mast cell responses to ATP are distinct between different species (Bulanova and Bulfone-Paus, 2010). For example, It has been reported that ATP induces histamine and prostaglandin (PG) release from rat peritoneal mast cells through P2X and P2Y receptors. This effect is mediated by PKC downstream signaling (Jaffar and Pearce, 1990). Contrary, HLMCs don't degranulate in response to ATP, but enhances IgE-mediated histamine release (Schulman et al., 1999).

Apart from activating mast cells by means of GPCRs, mast cells can also be directly activated by diverse array of reagents. Calcium ionophores (ionomycin) activate mast cells by increasing intracellular calcium release and extracellular influx (Morgan and Jacob, 1994). Pathogens can also directly activate mast cells by binding Toll-like receptors (TLRs). Among 11 different TLRs, mast cells known to express TLR2, TLR3, TLR4, TLR6 and TLR8 (Sandig and Bulfone-Paus, 2012). Each receptor has a district effect on mast cell function and mediator release (Varadaradjalou et al., 2003). C3a and C5a anaphylatoxins which are complement components also induce calcium mobilization and mediators production in mast cells (Venkatesha et al., 2005). Mast cells express C3aR and C5aR that couple to the Gαi family of heterotrimeric GPCRs (Nilsson et al., 1996).

In conclusion, the sensitivity of mast cells to wide range of distinct stimuli reflects the variety of mast cells roles *in vivo.*

1.1.3 Mast cell mediators

Upon activation, mast cells release and synthesize a diverse array of mediators that have diverse biological functions as shown in (Figure 1.2). They are categorized into three major categories (Metcalfe et al., 1997): **(1)** preformed active mediators that are stored in the cytoplasmic granules, **(2)** *de novo* synthesized lipid mediators, and **(3)** *de novo* synthesized cytokines, chemokines and growth factors. ***Table 1.1*** lists mast cell mediators and their biological functions. However, it is not necessary mediators from all three categories are released together or happen always as some mast cell activation can cause release of certain cytokines without preceding with degranulation. In addition to the fact that mast cell mediators induce a broad range of biological effects, the mediators overlap in their biological effects and sometimes opposite their effects. Therefore, mast cell effects are stimulant-specific, mediator-specific, and tissue-specific.

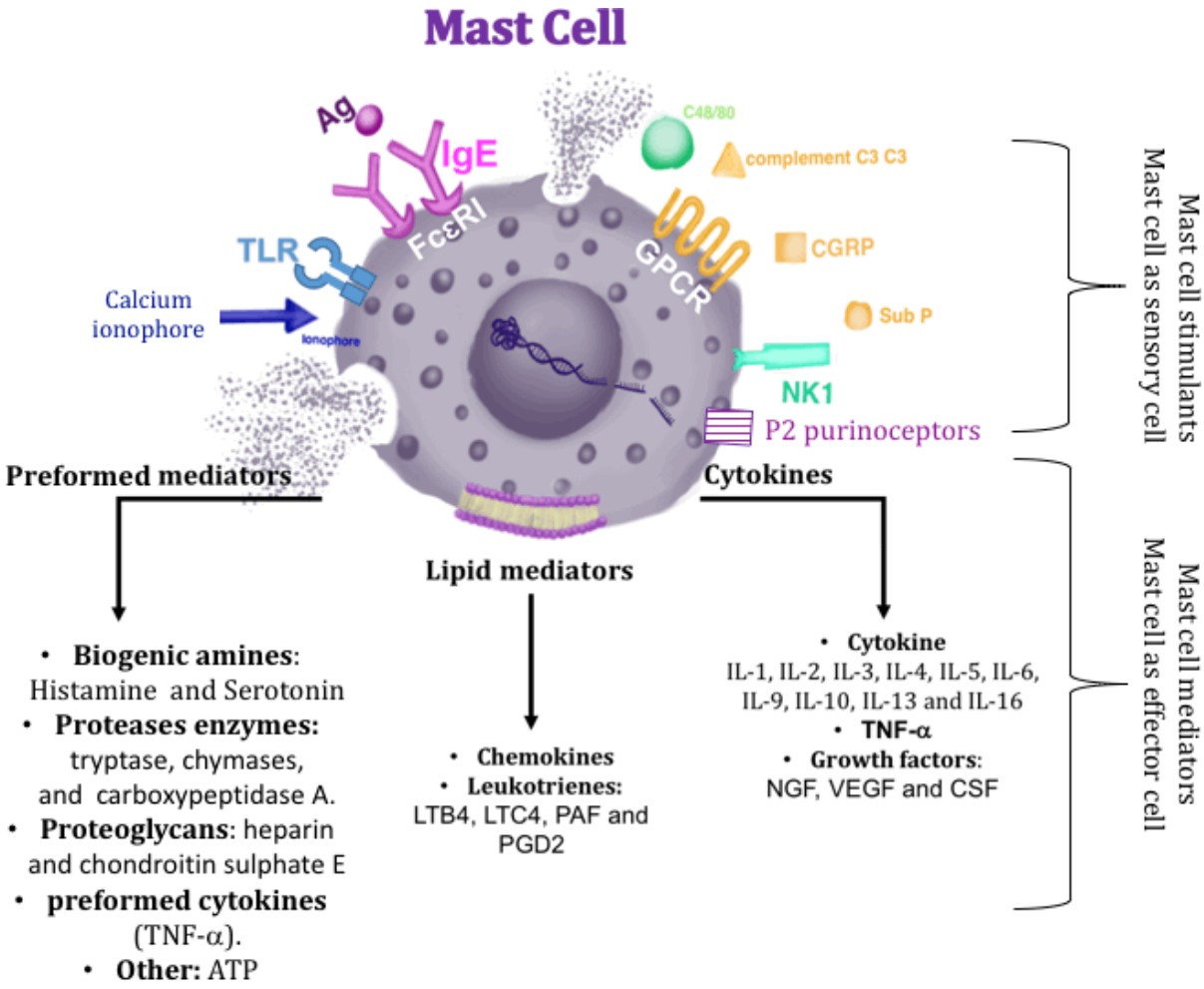


Figure.1.2 Mast cell activation and mediator release.

Mast cells express a diversity of cell surface receptors. Mast cells can be activated either by IgE-dependent or IgE-independent mechanisms which results in synthesize and release of a diverse array of mediators. **Ag,** Antigen; **FcεRI**, high affinity IgE receptor; **TLR**, Toll-like receptor; **c48/80**, compound 48/80; **CGRP**, calcitonin gene-related peptide; **SubP**, substance P; **NK-1**, neurokinin 1; **GPCR**, G-protein coupled receptor; **TNF-α,** tumor necrosis factor α; **ATP,** Adenosine 5'-triphosphate; **LT**, leukotrienes; **PAF**, platelet- activating factor; **PGD2**, prostaglandin D2; **IL**, interleukine; **NGF**, nerve growth factor; **VEGF**, vascular endothelium growth factor; and **CSF**, colony- stimulating factor.

1.1.3.1 Preformed granule- stored mediators

The largest category of mast cells mediators are the preformed granule-stored mediators. These mediators include biogenic amines, protease enzymes, proteoglycans, and preformed cytokines like tumor necrosis factor-α (TNFα). Within minutes after activation, they are released from cytoplasmic granules through a process of degranulation. Increased Ca^{2+} influx after activation mediates granule movement and fusion to the cell membrane. Selective release of only certain mediators from the granules by exocytosis is called piecemeal degranulation (Dvorak and Morgan, 1997) while the classic granule extrusion is called anaphylactic degranulation (Dvorak et al., 1991).

The most well-known biogenic amine in mast cells is histamine. It is formed from decarboxylation of histidine in the Golgi apparatus of mast cells (Metcalfe et al., 1997). Although it is released from different cells such as basophils (Ishizaka et al., 1972), its major source is mast cell (RILEY and WEST, 1952). Histamine exerts its biological function by binding to its G-protein coupled receptors called histamine receptors (H1-H4) (MacGlashan, 2003). Histamine receptors are widely expressed by different cell types. The fast release of histamine and wide-spread expression of its receptors explain the paracrine effect of histamine. Therefore, it promotes many physiological functions including increased vascular permeability and systematic vasodilation (Bachert, 2002). Serotonin is also present in mast cell granules (Kushnir-Sukhov et al., 2007). The major biological functions of serotonin are vasoconstriction and pain (Theoharides et al., 2007).

The second group of preformed granule-associated mediators are the protease enzymes such as tryptase, chymases, and carboxypeptidase A. The effects of tryptase on other cell types have been extensively investigated, with a specific focus on epithelium, smooth muscle cells and fibroblasts (Gruber et al., 1997). It mediates its effects by activating protease-activated receptor 2 (PAR-2) (Akers et al., 2000). Tryptase is found in MCC and CTMC while, chymase is specific for CTMC (Metcalfe et al., 1997). Unlike

phenotype-specific tryptase and chymases, hexosaminidase is known to be found in lysosomes of all mast cell subpopulations. Therefore, it is frequently used in *in vitro* studies to quantify degranulation (Lundequist and Pejler, 2011). Mast cells also release granule ATP upon Ag stimulation (Marquardt et al., 1984) (Suzuki et al., 2007). Because extracellular ATP is quickly degraded by ecto-nucleotidases enzymes (Colgan et al., 2006), ATP mediates autocrine or paracrine signaling in mast cells and nearby cells, respectively, by binding to P2 (Abbracchio and Burnstock, 1998) (Novak, 2003). As mentioned above (section 1.1.2.2), It produces a diverse array of biological effects depending on which P2 receptor subtype binds to (Bulanova and Bulfone-Paus, 2010).

Another group of preformed granule-associated mediators is the proteoglycans. They have a unique molecular structure consisting of a central protein core of repeating serine and glycine residues connected to glycosaminoglycans which are unbranched carbohydrate side chains (Metcalfe et al., 1997). This structure gives proteoglycans the ability to regulate and stabilize other preformed secretory granule mediators. The most commonly found mediators in mast cells are heparin and chondroitin sulphate E. (Metcalfe et al., 1997). Heparin is known for its ability to inhibit blood coagulation.

1.1.3.2 de novo synthesized lipid mediators

Beside their ability to exocytose preformed granule-derived mediators, activated mast cells also respond to stimuli by synthesizing and releasing lipid-derived substances like PGs and leukotrienes (LT). Membrane phospholipids are broken down to arachidonic acid by phospholipase A_2 enzyme. Then, arachidonic acid is metabolized by either cyclooxygenase to generate PGs and thromboxanes, or by lipoxygenase to generate leukotrienes (Metcalfe et al., 1997). Because gene transcription is not required to synthesize lipid mediators, they are released from mast cells within 5-30 minutes of activation.

1.1.3.3 de novo synthesized cytokines mediators

Mast cells may also respond to activation by induction of gene transcription and production of a wide-range of cytokines, chemokines and growth factors. Which of these factors are synthesized is dependent upon the mast cells' microenvironment and phenotype (Bradding et al., 1995), and the kinetics of second messenger signaling cascades evoked as a result of receptor activation (Gilfillan and Tkaczyk, 2006). In contrast to lipid mediators, cytokine release from mast cells occurs hours after activation. In general, cytokines and chemokines regulate recruitment and function of local immune cells (Metcalfe et al., 1997). Major cytokines expressed and released by activated mast cells include IL-3, IL-6, IL-10 and TNFα (Kobayashi et al., 2000).

Class	*Mediators*	*Main biological function*
Preformed mediators	**Biogenic amines:**	
	Histamine	Vasodilation, increase vascular permeability and pain
	Serotonin	Vasoconstriction and pain
	Enzymes:	Remodel tissue matrix
	Tryptase	Degradation of fibrinogen, pro-inflammatory, pain
	Chymase	Degradation of basal membrane substance, conversion of Angiotensin
	Carboxypeptidase A	Peptide processing
	Proteoglycan:	
	Heparin	Angiogenesis, NGF stabilization
	Chrondroitin sulfate	Cartilage synthesis, anti-inflammatory action
	Others: ATP	Extracellular signaling by binding to purinergic receptor
Lipid mediators	**Chemokines:**	
	Leukotriene B4	Leukocyte chemotaxis
	Leukotriene C4	Vasoconstriction, pain
	Platelet-activating factor	Platelet activation, vasodilation
	Prostaglandins D2	vasodilation, pain
Cytokines	**Interleukins** (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and IL-16)	Pro-inflammatory, leukocyte migration, pain
	Tumor necrosis factor a	Pro-inflammatory, vascular adhesion molecule expression
	Growth Factors	
	Nerve growth factor (NGF)	Peripheral sensitization
	Vascular endothelial growth factor (VEGF)	Neovascularization, vasodilation
	Colony stimulating factor (CSF)	Promote eosinophil production

Table 1.1. Mast cell as an effector cell

"adapted from **(Theoharides, 2002)"**

1.1.4 Heterogeneity of mast cell phenotypes

As mentioned above (section 1.1.1), MCp arise from the bone marrow and are recruited to different tissues to mature under the control of micro-environmental factors. *In vivo*, mature mast cells exist as a heterogeneous population depending on their anatomic location, function and biochemical properties (Moon et al., 2010). The heterogeneity of mast cells was first reported when mouse mast cells from mucosal tissues were found to histochemicaly differ from mast cells in connective tissues. Since then, mouse mast cells have been classified into two major subpopulations based on their anatomical locations (**Table 1.2**): MMC are found beneath the mucosal surface of the respiratory and gastrointestinal tract while, CTMC mostly localize in the submucosa of the gastrointestinal tract (GIT), peritoneum, and in the skin.

Mast cell subpopulations differ not only in their immunohistochemical properties, but also in the mediators they release in response to stimuli and in their responses to inhibitory drugs. Immunohistochemical studies have revealed that MMC are immunoreactive to chymase and CTMC are immunoreactive to both tryptase and chymase (Xing et al., 2011). Alcian blue/safranin histochemical staining can also be used to distinguish between MMC and CTMC (Kitamura, 1989). MMC stain blue with Alcian blue, while CTMC stain red with safranin. CTMC produce more histamine in response to activation than MMC (Purcell et al., 1989). On the other hand, MMC synthesize higher amounts of all arachidonic acid metabolites except PGD2, compared to CTMC (Levi-Schaffer et al., 1987). Regards their responsiveness to different stimuli, CTMC respond robustly to c48/80 by releasing more histamine than MMC (Galli, 1990), but CTMC respond better to the stabilizing effect of Na- cromolyn (Pearce et al., 1989).

In contrast to mice mast cell phenotypes, human mast cells from different anatomical sites strongly express tryptase, but not all of them express chymase. Therefore, human mast cells are classified into two subpopulations based on their granular proteases (Irani et al., 1986) as tryptase (MC_T), or tryptase and chymase (MC_{TC}) positive. This classification is believed to be equivalent to mice mucosal and connective tissue

phenotypes. However, these are no distinct anatomical localizations for these subpopulations *in vivo.* Human mast cells from the same site may differ in their phenotype. For example, human mast cells in GIT mucosa are mainly MC_T, but some of the mast cells are MC_{TC} phenotype (Irani et al., 1989). Interestingly, mast cell phenotypes show a high level of plasticity *in vivo* and *in vitro*. Changing the surrounding tissue environment may change mast cell phenotype. Thus, the model of mast cells used in experiments must be considered when studying mast cell functions (Kitamura, 1989).

	MMC	*CTMC*	*References*
Tissue localization	Mucosa of respiratory and GI tracts	Submucosa of respiratory and GI tracts, Skin, and peritoneum	(Galli, 1990)
T-cell dependent in development	No	Yes	(Razin et al., 1984)
Staining			
Formaldehyde fixation	Sensitive	Resistant	(Craig et al., 1988)
Alcian/blue/Sarfanin O	Blue	Red	(Kitamura, 1989)
Berberine sulphate	-	+	(Kitamura, 1989)
Mediators			
Histamine	<1 pg/ cell	1-30 pg/cell	(Purcell et al., 1989)
Proteoglycan	Condroitin sulphate	Heparin, chondroitin sulphate E	(Razin et al., 1982b)
Protease Tryptase	-	MCP-6, and -7	(Xing et al., 2011)
Chymase	MCP-1 and -2	MCP-4, and -5	
carboxypeptidase	-	+	
LTC4	>25 ng/10^5 cells	n.d	(Razin et al., 1982a)
LTB4	3-12 ng/10^5 cells	n.d	(Levi-Schaffer et al., 1987)
PGD2	1-5 ng/10^5cells	n.d	(Levi-Schaffer et al., 1987)
Response to secrtagouges			
IgE/Ag	+	+	(Kitamura, 1989) (Galli, 1990)
Substance P	+	+	(Kitamura, 1989) (Galli, 1990)
Compound 48/80	-	+	(Kitamura, 1989) (Galli, 1990)
Inhibited by Na Chromoglycerate	No	Yes	(Pearce et al., 1989)

Table 1.2. Mast cell heterogeneity in mouse.

1.1.5 Mast cell functions in health and diseases

Although mast cells are best recognized for their major contribution in allergies, a growing body of literature has highlighted the contribution of mast cells in homeostasis and promoting the pathology of different diseases (Moon et al., 2010) (Weller et al., 2011). Understanding mast cells' strategic distribution, response to various stimuli, variety of mediators, and plasticity leads to better appreciation of mast cells in biology and pathology.

1.1.5.1 Mast cell functions in health

1.1.5.1.1 Mast cells in homeostasis

The fact that no living human being can exist lacking mast cells (Weller et al., 2011) reflects the important role of mast cells in maintaining health. Since mast cells are potent inflammatory cells, and inflammation is a homeostatic response in principle then it is acceptable to consider mast cells as players in homeostasis. By using mast cell-deficient ($MC^{-/-}$) mice, the physiological functions of mast cells have been defined (Wershil, 2000). Mast cells contribute to hair growth, bone remodeling and wound healing as $MC^{-/-}$ mice have impaired hair follicle cycling (Maurer et al., 1997), osteopenia (Silberstein et al., 1991) and significant delay wound healing (Noli and Miolo, 2001). The effect of mast cells on bone remodeling is mediated by release of osteopontin (Nagasaka et al., 2008), IL-1 and histamine which act to promote osteoclast development (Chiappetta and Gruber, 2006). Similarly, mast cells promote epithelial cell and fibroblast proliferation by producing of many growth factors such as NGF, platelet-derived growth factor, and fibroblast growth factors (Noli and Miolo, 2001). In the gut, mast cells and their mediators are important for gut homeostasis. Mast- cell-chymase-deficient mice showed alteration in chloride and water transport, mucous section from epithelial cells, vascular permeability and intestinal motility (Groschwitz et al., 2009).

1.1.5.1.2 Mast cells in host defence

The strategic position of mast cells near to surfaces exposed to external environment makes them the first "defense-barrier" to recognize the invading microbes (Echtenacher et al., 1996). Pathogens activate TLR receptors expressed on mast cells leading to release of a diversity of mediators including chymase (Knight et al., 2000) and tryptase (Huang et al., 2001), TNFα, LTB4 and other mediators that recruit neutrophils (von Köckritz-Blickwede et al., 2008). By using mast cell-deficient ($MC^{-/-}$) mice, mast cells were shown to play a critical contribution to host defense against different pathogens including parasites (Knight et al., 2000) (Maurer et al., 2006, Sasaki et al., 2005) and bacteria (Xu et al., 2006). Indeed, it has been shown in a mouse cecal ligation model that mast cell- derived TNFα is necessary to survive acute septic peritonitis (Echtenacher et al., 1996).

1.1.5.2 Mast cells in disease

In principal, mast cells mediate the same functions in physiological and pathological conditions. However, mast cell functions can become harmful when they respond to innocuous stimuli or when their response is prolonged, as is the case in allergy and chronic inflammation, respectively. The increase in mast cell numbers within tissues is reported in many pathophysiological conditions such as asthma (Brightling et al., 2002), multiple sclerosis, (Ibrahim et al., 1996) rheumatoid arthritis, (Huang et al., 2002), irritable bowel disease (O'Sullivan et al., 2000) (Park et al., 2003) (Barbara et al., 2004), congestive heart failure (Hara et al., 2002), and cancer (Terada and Matsunaga, 2000) (Molin et al., 2002). For the purpose of this thesis, it is useful to focus on the contribution of mast cell in allergy and pain-related diseases.

1.1.5.2.1 Asthma and allergy

The role of mast cells in allergic diseases is well-defined (Bradding et al., 2006). The number of mast cells has been reported to be significantly increased in allergic lesions. In lungs of asthmatic patients, number of mast cells that settle in direct contact with key anatomical structures for asthma such as airway smooth muscle, mucus glands and epithelium is remarkably increased (Carroll et al., 2002). Mast cells' location close to external environment helps in them becoming sensitized with IgE against specific antigens. Mast cell-derived mediators contribute in pathogenesis of the chronic inflammation of asthma either directly or by promoting tissue remodeling. The acute allergic symptoms that range from a runny nose to lethal anaphylactic shock are due to the rapid release of preformed mediators such as histamine, tryptase, PGD2, and LTC4 from activated mast cells. As I mentioned earlier (section 1.1.3), these mediators act on neighbor cells and induce vasodilation, bronchoconstriction and mucus secretion. Mast cell derived mediators also account for late-phase reactions by producing de novo synthesized cytokines like IL-4, IL-5, and IL-13 that control leucocyte recruitment (Yu et al., 2006).

1.1.5.2.2 Functional gastrointestinal disorder and IBS

The contribution of mast cells in the pathophysiology of irritable bowel syndrome (IBS) has recently been reviewed (Wouters et al., 2016). IBS is the most prevalent diagnosis among the functional gastrointestinal disorders. It is presented clinically with bloating, changes in bowel habits and recurrent abdominal cramp or pain which underlies visceral hypersensitivity (Longstreth et al., 2006). Many researchers have reported the same observation of increased mast cells numbers in the GIT mucosa of IBS patients (Weston et al., 1993) (O'Sullivan et al., 2000) (Guilarte et al., 2007) (Wang et al., 2007) (Goral et al., 2010), with an apparent preferred association with nerve fibers (Park et al., 2003). Moreover, it has been observed by electron microcopy that the number of degranulated mast cells in close contact with nerves is also increased in biopsies of IBS patients (Barbara et al., 2004). This number correlates to abdominal pain severity in IBS (Park et al., 2006) (Di Nardo et al., 2014). This strongly implicates a crucial role of mast cells in the pathophysiology of visceral hypersensitivity in IBS (Ohman and Simrén,

2010). Mast cells in mucosal biopsies of IBS patients showed not only increases in their number but also increases in their mediator content (Guilarte et al., 2007). Supernatants of mucosal biopsies and stool samples of IBS patients have increased levels of mast cell mediators like tryptase, histamine, serotonin, PGE_2 and IL-6 (Barbara et al., 2007) (Liebregts et al., 2007) (Buhner et al., 2009, Annaházi et al., 2009). These mediators may sensitize neurons. Indeed, supernatants isolated from mucosal biopsies of IBS patients evoked spike discharge in human submucosal neurons (Buhner et al., 2009) and rat DRG (Barbara et al., 2007). Pre-incubation with histamine and serotonin receptor antagonists or protease inhibitor block IBS supernatants- mediated response. IL-6 also plays a role in visceral hypersensitivity (O'Malley et al., 2011). Supernatant from colon of maternal separation rat as an animal model for IBS evoked calcium response in submucosal neurons. Pre- incubation with anti- IL-6 antibody blocked this response (O'Malley et al., 2011).

Interestingly, IBS patients with atopic background have more increase in intestinal permeability compared to non-atopic IBS patients (Lillestøl et al., 2010). Furthermore, atopic IBS patients show significantly increased numbers of IgE-coupled mast cells (Lillestøl et al., 2010) as well as the higher serum IgE level correlates with GIT complain severity (Vara et al., 2016). *All together, these data suggest that mast cell-IgE- mediated signaling could be a major player in the pathophysiology of IBS. Understanding the key role of mast cells in IBS is likely to reveal potential therapeutic targets*.

1.1.5.2.3 Migraine

Migraine is a chronic disorder that is characterized by recurrent episodes of moderate to severe headache associated with visual and sensory symptoms. Although the precise pathophysiology of migraine remains incompletely understood, it is highly accepted that neuro-sensitization and cerebral vaso-dilation have the primary roles (Spierings, 2003). Remarkably, mast cell mediators have been suggested to contribute in the both effects (Theoharides et al., 2005). It has been shown that systematic administration of c48/80 into rats resulted not only in dural mast cells degranulation but also sensitization of

meningeal nociceptors (Levy et al., 2007). Further studies identified mast cell mediators involved in sensitization of meningeal nociceptors namely: ATP (Giniatullin et al., 2008) PG, tryptase, TNFα and IL-6 (Aich et al., 2015).

1.1.5.2.4 Autoimmune diseases

It has been shown that mast cells contribute in the pathogenesis of many autoimmune diseases. The observation of increased mast cell number with increase their mediator secretion at sites of inflammation is common in many autoimmune diseases such as rheumatoid arthritis. It is an autoimmune, chronic joint disease that characterized by leukocyte –mediated cartilage and bone destruction. Mast cells accumulate in synovial fluids of affected joints (Tetlow and Woolley, 1995). Mast cell- deficient mice are resistant to the development of rheumatoid arthritis (Lee et al., 2002). Mast cell-derived IL-1 is a key mediator in initiation of autoantibody-mediated arthritis (Nigrovic et al., 2007).

In conclusion, the ability of mast cells to migrate to different tissues, and the potent mediators can be released from them reflect the diversity of mast cells functions. Mast cells responses are essential in homeostasis, but also contribute in the pathogenesis of a diverse array of diseases. Controlling mast cell roles in these diseases is an attractive therapeutic target. Interestingly, the role of mast cells in different diseases is, in part, indirect through their continuous communication with various cells and the surrounding environment. *Understanding these intercellular communications is essential to advance the science of mast cell-directed therapies.*

1.1.6 Models for studying mast cells *in vitro*

Unlike other immune cells that are found in peripheral blood, mature mast cells can be only isolated from tissues (Metcalfe et al., 1997). Thus, it is challenging to isolate pure primary mast cells from either humans or rodents. Despite this limitation, available human and mouse models for primary mast cells have been developed for *in vitro*

studies (Table 1.3). Because mast cells display a strong species and organ-specific heterogeneity even after maturation, one should keep in mind that the biochemical properties and functional responses of mast cells are critically dependent on the micro-environmental culturing conditions. Thus when studying mast cell functions *in vitro*, cell source and culturing conditions must be carefully considered (Bischoff, 2007) (Moon et al., 2010).

1.1.6.1 Primary mast cells

Primary Mouse mast cells

Primary mouse mast cells can be generated either as bone marrow-derived mast cells (BMMCs) or mouse peritoneal cell-derived mast cells (mPCMC). BMMCs are the most widely used model. BMMCs are considered to be phenotypically equivalent to MMC mast cells (Razin et al., 1984). BMMCs are cultured from bone marrow-derived progenitors till maturation, with support of IL-3. After 4 weeks, generated cultures have >98% pure $FcεRI^{+}$ Kit^{+} mast cells that can survive in culture for 6 weeks (Razin et al., 1982b). BMMCs are the most extensively used primary mast cells because their isolation protocol is relatively easy and it generates large quantities of mast cells. More importantly, they have the advantage that they can be successfully used in reconstitution experiments in mast cell- deficient mice and they are relatively easy to transfect (Otsu et al., 1987). This allows researchers to expand work from basic *in vitro* cell biology and investigate specific mast cell function in complex *in vivo* settings. A limitation though, is that BMMCs represent only one phenotype that is MMC. Because different mast cell phenotypes response differently, another model is required when CTMC responses in particular are under investigation. mPCMC are isolated as mature CTMC from mouse peritoneum and cultured in the presence of SCF only for two weeks. This method can generate cultures have >98% pure $FcεRI^{+}Kit^{low}$ mast cells that can survive for more than two months (Czarnetzki and Behrendt, 1981). However, only 5% of recovered peritoneal cells are mast cells, and thus quantities recovered may be insufficient for some types of experiments. A disadvantage of mPCMC is that they have no equivalent in humans, who don't have peritoneal mast cells in physiological

condition (Bischoff, 2007).Thus, translation of findings from mPCMC to human biology is questionable.

Primary Human mast cells

Mature human mast cells are difficult to be isolated in large number because they are not found in blood. However, they can be cultured either as mast cell progenitors from blood or as mature mast cells from solid tissues. Mast cell progenitors can be obtained from peripheral blood or umbilical cord blood (CBMC) (Saito et al., 1995) and must be cultured in the presence of SCF for 6-12 weeks while they mature. Thy are depend on SCF for their survival which can make working with them cost intensive, however, they can be produced in large quantities. After maturation, peripheral blood mast cells are tryptase^{+} chymase^{+} (Rottem et al., 1994) while CBMCs are tryptase^{+}chymase.$^{-}$ CBMCs express less FcεRI that upregulated after incubate them with IgE and IL-4 (Yamaguchi et al., 1999).

Primary human mast cells can also be obtained from solid tissues such as lung (Schulman et al., 1982), intestine (Lorentz et al., 2015) and skin (Gibbs et al., 2001). Isolated mast cells keep the phenotype of their tissue of origin. Although tissue-derived mast cells are mature mast cells and can be used immediately, they are limited in number and need multiple steps for purification. The lack of availability of large quantities of fresh human tissues, usually obtained from surgical specimens, is the most common reason for abandoning their use.

1.1.6.2 Mast- cell lines:

Because the low yield and the time constraints and cost factors for primary cultures, transformed mast cell lines have been considerably used as an alternative. They can be of either human or murine origin. Currently, there are 4 human cell lines: the human leukemic mast cell line-1 (HMC-1), laboratory of allergic diseases cell line (LAD1-2), cell line (LUVA) and mast cell progenitor Vienna (MCPV-1). HMC-1 has been established

from peripheral blood of a patient suffering from mast cell leukemia (Butterfield et al., 1988) . Mast cells from HMC-1 are fast growing, and SCF-independent because Kit is permanently phosphorylated. However, they are immature mast cells and don't express FcεRI on their surface even after incubation with IgE, which makes them not suitable for studying allergic responses (Moon et al., 2010) . In contrast, LAD2 cells are $FC\varepsilon RI^{+}$ and more mature. They respond to Ag and release β-hexosaminidase, histamine, tryptase and chymase. Although LAD2 is established from the bone marrow of a patient suffering from mast cell sarcoma with a KIT mutation, mast cells derived from LAD2 have normal KIT (Kirshenbaum et al., 2003) . Thus, they depend on SCF for survival as do primary human mast cells. The major disadvantage of this cell line is its long doubling time (15-20 days). Another cell line (LUVA) was established from $CD34^{+}$ purified peripheral blood cells of a donor suffering from aspirin-exacerbated respiratory disease but without clinical symptoms of mastocytosis or leukemia (Laidlaw et al., 2011). Despite their normal KIT expression, they are SCF-independent. Initially, they express FCεRI and respond to Ag by release of β-hexosaminidase, protease and cytokines. However, they lose FCεRI expression after long-term culture. Recently, MCPV-1 cell line was designated by lentiviral immortalization of cord blood-derived mast cell progenitors (Hoermann et al., 2014). The aim of this cell line was to generate a cell line reflecting systemic mastocytosis *in vitro*. Thus, MCPV-1 express CD52, the molecular target in systemic mastocytosis.

Regarding murine mast cell lines, the most extensively used one, is the rat basophilic leukemia-2H3 cells (RBL-2H3). This cell line was established from chemically-induced leukemia in rat, then modified to secrete histamine. Because of RBL-2H3 –derived mast cells express FcεRI, they are extensively used to answer questions related to FcεRI signaling pathways and related degranulation. However, they share typical characteristics of basophils which could influence the interpretation of the results (Passante et al., 2009). MC/9 is another murine mast cell line. Unlike RBL-2H3 mast cells, MC/9 mast cells are IL-3 dependent. Both of these cell lines can respond to different stimulants such as Ag/IgE, calcium ionophore and other secretagogues (Musch

and Siegel, 1986). They can be easily grown in large numbers. However, as transformed cells their normal cell functions may be altered. Another less known murine mast cell line is IC-2. This cell line is established from mouse BMMCs. It depends on IL-3 only on their survival and doesn't express Kit (Koyasu et al., 1985).

In a conclusion, there is no perfect model to represent mast cell functions in human. The most suitable mast cell-model to be used in a study must be decided according to the research question and available facilities.

Model	*Murine*	*Human*	*Limitations*
Primary cultures of mast cells from progenitor cells	• BMMC	• Cord-blood-derived mast cell • Peripheral-blood-derived mast cell	• Long-lasting process; development of mast cells requires progenitor cell cultures for 6-12 weeks , and addition of a cocktail of cytokines • It is currently questionable whether full mast-cell maturation can be achieved by this means
Primary cultures of tissue mast cells	• Peritoneal mast cells • Isolated mucosal or skin mast cells are more difficult to obtain (small amounts of tissue and low mast-cell densities)	• HLMC • Human skin mast cells	• Murine peritoneal mast cells might differ from tissue mast cells in the peritoneal cavity in humans under normal conditions • Murine mucosal mast cells occur only at small numbers in normal mucosa (mouse<rat<human) • Human tissue mast cells are limited cell yield ($\sim 10^4$ -10^5 mast cells per gram of tissue), cumbersome isolation procedures and purification techniques
Transformed mast-cell lines	RBL and IC-2	HMC-1,LAD-1, LAD-2, LUVA, and MCPV-1.	• Transformation substantially alters normal cell function (e.g KIT is permanently phosphorylated in HMC-1 cells, which therefore become independent of SCF ; by contrast, LAD cells require SCF for survival and therefore might be more appropriate as a human mast-cell model)

Table 1.3. Models for studying mast cell *in-vitro*

"adapted from (Bischoff, 2007)" **BMMC,** bone marrow mast cell; **HLMC**, human lung mast cell; **RBL**, Rat basophilic leukemia; **HMC-1**, human leukemic mast cell line-1; **LAD1**, Laboratory of allergic diseases cell line; and **MCPV-**1, mast cell progenitor Vienna.

1.2 Sensory neurons

1.2.1 Pain and nociception

Pain is defined by the international association for the study of pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage", and nociception as "the neural process of encoding noxious stimuli" (www.iasp-pain.org). Three different types of noxious stimuli, mechanical, thermal and chemical, could initiate nociception at the terminals of nociceptive sensory neurons. Their cell bodies lie within dorsal root ganglia (DRG) and trigeminal ganglia (TG) (Woolf and Ma, 2007). DRG contain 3 subtypes of sensory neurons: Aβ-fibers, Aδ- fibers and C- fibers. These fibers are classified based on their conduction velocity which positively correlates with the diameter and degree of myelination. Aβ-fibers with a large diameter and thick myelination have the fastest conduction velocity. They respond only to innocuous stimuli such as light touch. Therefore, they are classified as non-nociceptive neurons. While c-fibers, and Aδ- fibers are nociceptive neurons. Aδ- fibers with medium-diameter and thinly-myelinated have faster conduction velocity than c-fibers with small and unmyelinated axons (Hunt and Mantyh, 2001) (Basbaum et al., 2009). Nociceptors can further be divided based on their neuropeptide expression. Peptidergic nociceptors express and release subP or CGRP. In contrast, non-peptidergic nociceptors don't express neuropeptides but bind to their specific marker isolectin IB4 (Carlton, 2014).

Painful stimuli usually activate first Aδ- fibers that are responsible for the immediate sharp pain (Bishop, 1958). When stimulus strength increases, c-fibers are activated and mediate slower diffuse pain. As shown in Figure 1.3, two physiological consequences follow nociceptor activation, first, activated nociceptors generate action potentials (AP) that synapse in the dorsal horn of the spinal cord. APs are transmitted to second order nociceptive neurons via the excitatory neurotransmitter glutamate (Petrenko et al., 2003). Then, nociceptive signals ascend contra-laterally in the spino-thalamic and spino-reticulothalamic tracts to the somatosensory cortex where nociceptive signals are processed in cognitive, emotional and motivational ways (Garland, 2012). In addition,

nociceptors' cell bodies within DRG synthesize and release several neuropeptides and inflammatory mediators upon activation. These include SubP, CGRP, serotonin and ATP. Released mediators at the nerve terminals result in neurochemical changes in the local environment that activate nociceptors or lowers their activation threshold, and eventually leads to peripheral sensitization.

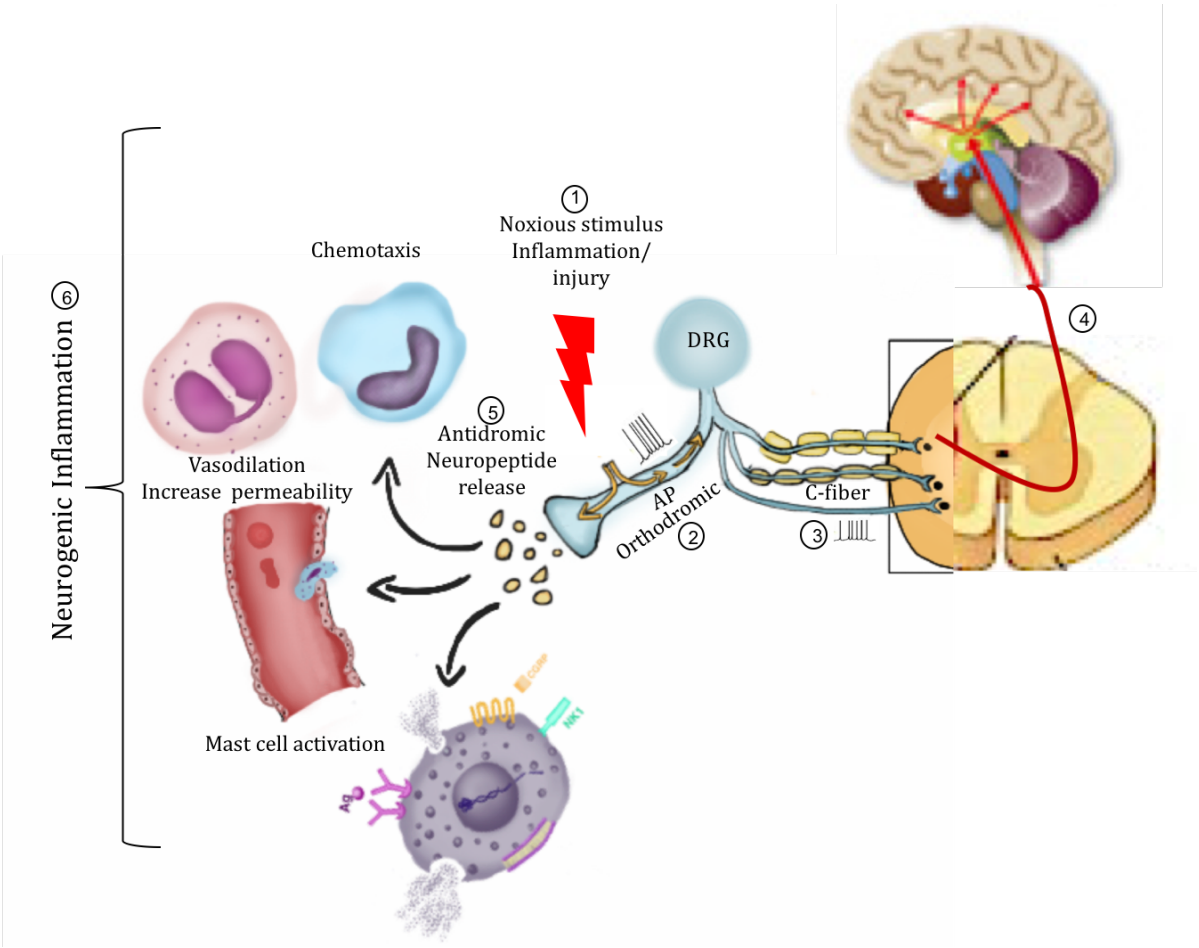


Figure.1.3 The nociceptive pain pathway and neurogenic inflammation

(1) Activation of nociceptors by noxious stimuli generates orthodomic and antidromic signals. **(2)** Orthodromic signals travel to the dorsal root ganglion (DRG). **(3)** Then, action potential (AP) synapses in dorsal horn of the spinal cord**. (4)** From the dorsal horn, the signals are carried along the ascending pain pathway or the spinothalamic tract to the thalamus and the cortex. **(5)** Antidromic signals induce neuropeptide release. Neuropeptides cause recruitment of inflammatory cells, vasodilatation and mast cell activation (neurogenic inflammation**)(6).**

1.2.2 Neuropeptides:

Within DRG, cell bodies of activated peptidergic nociceptors synthesize several neuropeptides including subP, and CGRP (Ebersberger et al., 1999) and other peptides such as vasoactive intestinal peptide. Besides their role in nociception, sub P, and CGRP act as pro-inflammatory mediators .

The most extensively studied neuropeptide is subP. It is found mainly in nociceptor neurons that have their cell bodies in the DRG and TG (Severini et al., 2002). However, it is also found in some immune cells, including macrophages (Pascual and Bost, 1990), and eosinophils (Aliakbari et al., 1987) which reflects its inflammatory functions (Black, 1994). It is involved in development of inflammation either directly or by activating other inflammatory cells like mast cells. It binds to neurokinin (NK)-1 receptor on endothelial cells and induces vasodilation (Bhatia, 2010). It promotes leucocyte recruitment to sites of inflammation and facilitates leukocytes adhesion to endothelium (Maggi, 1997). It activates mast cells and induces degranulation (Suzuki et al., 1999).

Another neuropeptide released from activated nociceptors is CGRP (Maggi, 1995). CGRP mediates its effects by binding to its receptor that has two components. One component is GPCR called calcitonin receptor-like that is a seven transmembrane protein. The other component is a member of receptor activity-modifying protein-1 (McLatchie et al., 1998). Like subP, CGRP is involved in inflammation (Brain and Williams, 1985). It is a potent vasodilator neuropeptide by activating its receptor on endothelial cells (Brain et al., 1985). Although CGRP doesn't affect vascular permeability, it enhances the effect induced by SubP when both neuropeptides are released concomitantly (Maggi, 1995). However, CGRP has more long-acting effects than SubP (Ebersberger et al., 1999). CGRP is important in regulating hypertension because it has a positive inotropic and chronotropic effect on the heart, besides its vasodilation effect (Deng and Li, 2005). CGRP also induces secretion of pro-inflammatory cytokines like IL-1, IL-6, and TNFα from mononuclear cells (Cuesta et al., 2002) and mast cells (Ottosson and Edvinsson, 1997).

1.2.3 Neurogenic inflammation

Sensory neurons are highly complicated structures (Carlton, 2014). They are best known as an afferent for noxious stimuli. However, because they are able to synthesize and release neuropeptides that induce biological effects, they could be considered as efferent as well (Maggi and Meli, 1988). This efferent function is known as dorsal root reflexes (DRR) which is thought to contribute to disease pathology (Carlton, 2014). As shown in Figure 1.3, the anti-dromic activity of nociceptors results in release of their neuropeptides: subP and CGRP (Maggi, 1995). Neuropeptides mediate vasodilation that results in redness, hotness and increased vascular permeability that causes swelling. Collectively, these are signs of inflammation. Since this inflammation is evoked by activation of nociceptors, it is defined as neurogenic inflammation (Schmelz and Petersen, 2001). The subpopulation of nociceptors that specifically mediate neurogenic inflammation are capsaicin-sensitive nociceptors (Holzer, 1988) which are a subtype of c-peptidergic fibers (Weng and Dougherty, 2005). Indeed, destruction of these nociceptors suppresses neurogenic inflammation. Although, the nociceptors are the major players in neurogenic inflammation, other immune cells that are activated by neuropeptides such as mast cells are important to feedback and prolong the inflammation (Schmelz and Petersen, 2001).

It has been suggested that neurogenic inflammation has a potential role in the pathophysiology of neuroimmune diseases such as migraine (Malhotra, 2016) asthma (Pisi et al., 2009), allergic rhinitis (Klimek and Pfaar, 2011), and inflammatory bowel disease (Renzi et al., 2000)

1.2.4 Sensory neuron *in vitro* model (DRG culture)

DRG cultures have been widely used to study cellular and molecular mechanisms involved in pain signaling, and pathogenesis of the peripheral neuropathy disorders. Moreover, they provide a valuable tool to identify and validate new potential analgesic drugs.

Different types of sensory neuron cultures have been developed and proved as a powerful tool (Melli and Höke, 2009). However, the research design is the most important factor to determine the best type to answer the research questions. Sensory neuronal cell cultures could be either primary or cell lines.

1.2.4.1 Primary DRG

The most important advantage of primary DRG cultures over neuronal cell lines are that they are more likely to reflect the i*n vivo* biology. Primary sensory neurons are easily isolated from DRG of various animals (Melli and Höke, 2009). Rodent DRG cultures have been extensively used to study sensory nerve development, function, neuronal survival, electrical signaling (Harper and Lawson, 1985a), and neurite outgrowth (Gavazzi et al., 1999). They are also used as an *in vitro* model of peripheral neuropathies disorders, such as diabetic neuropathy (Vincent et al., 2007) and inflammatory pain (Kallenborn-Gerhardt and Schmidtko, 2011).

Different protocols can be used to culture sensory neurons from mouse DRG. They can be isolated from embryonic (Horie and Kim, 1984) or adult (Scott, 1977) mice and they can be either dissociated by enzymatic trypsinization or seeded as explants. Two advantages are unique for embryonic DRG culture (Melli and Höke, 2009). The high yield of cells make embryonic DRG an ideal model for neuroprotective drug screening (Melli et al., 2006a) (Melli et al., 2008). The second advantage is that the growth of neurites is highly dependent on NGF, and they undergo degeneration by withdrawing NGF (Unsain et al., 2014). This feature makes embryonic DRG an ideal model for studying axonal degeneration in diseases such as diabetes (Taiana et al., 2014) and HIV (Melli et al., 2006b). Furthermore, embryonic DRG sensory neurons are suitable to be used with Campenot chambers to study axon development. Campenot chambers are compartmentalized so that axons can extend separately and grow away from cell bodies and be examined independently (Campenot, 1977). However, NGF-dependent neurite

survival limits the use of embryonic DRG cultures in experiments when the effect of other chemical factors is investigated.

In contrast, adult DRG can survive independently of NGF and serum, as well. Therefore, they can be used in experiments with defined media (Melli et al., 2006a). In addition, adult DRG cultures provide a model of completely developed sensory neurons that more closely reflect their biology *in vivo*. Adult DRG cultures are commonly used in studying the effect of inflammatory mediators on nociceptive signaling (Lin et al., 2006).

The main difficulty with primary DRG cultures, in general, is the heterogeneity of the population due to the presence of different neuronal subtypes as well as non-neuronal cells (Petruska et al., 2000). A recent single-cell, RNA sequencing experiment showed that one DRG could represent potentially 11 functionally distinct neuronal subtypes (Usoskin et al., 2015).

1.2.4.2 DRG cell lines

The limited cell numbers of primary neuronal cultures make them unsuitable for a high-throughput screening of analgesic drugs. Therefore, sensory neuronal lines are used. 50B11 is a cell line derived from embryonic rat DRG (Chen et al., 2007). Neurons from this cell line can be differentiated and produce high cell numbers. They have typical characteristic receptors for nociceptive sensory neurons. They express DRG neuronal markers such as transient receptor potential vanilloid (TRPV1) and receptors for NGF. However, data generated from this cell line must be validated in primary DRG cultures. Another cell line derived from Mouse Embryonic DRG is MED17.11(Doran et al., 2015). They differentiate quickly, and neurite outgrowth can be detected within hours. Although the cells express sodium-voltage channel 1.7 (NaV1.7) and respond to nociceptor stimulants such as capsaicin and veratridine, they fail to express NaV1.8. Their unique advantage is that they don't form aggregates like other cell lines making them an excellent model for high-throughput drug screening.

1.3. Mast cell- neuron interactions

Mast cells and neurons are found to communicate with each other both functionally and structurally (Forsythe and Bienenstock, 2012). They are found in close association with each other, where activation of one cell type influences the other activities (Figure 1.4). These mast cell-neuron cross-talk has been demonstrated in diverse tissues like GIT, respiratory system, skin, and brain in health and disease.

1.3.1 Mast cell- nerve functional interactions

The functional connection between mast cells and neurons has been studied extensively and reveals that there is bi-directional communication between these cells (van Diest et al., 2012). Activated mast cells release mediators that feed back onto the activating neurons. Activated neurons release neuropeptides that could further activate mast cells. This functional crosstalk is important in the regulation of normal homeostasis and pathophysiological conditions.

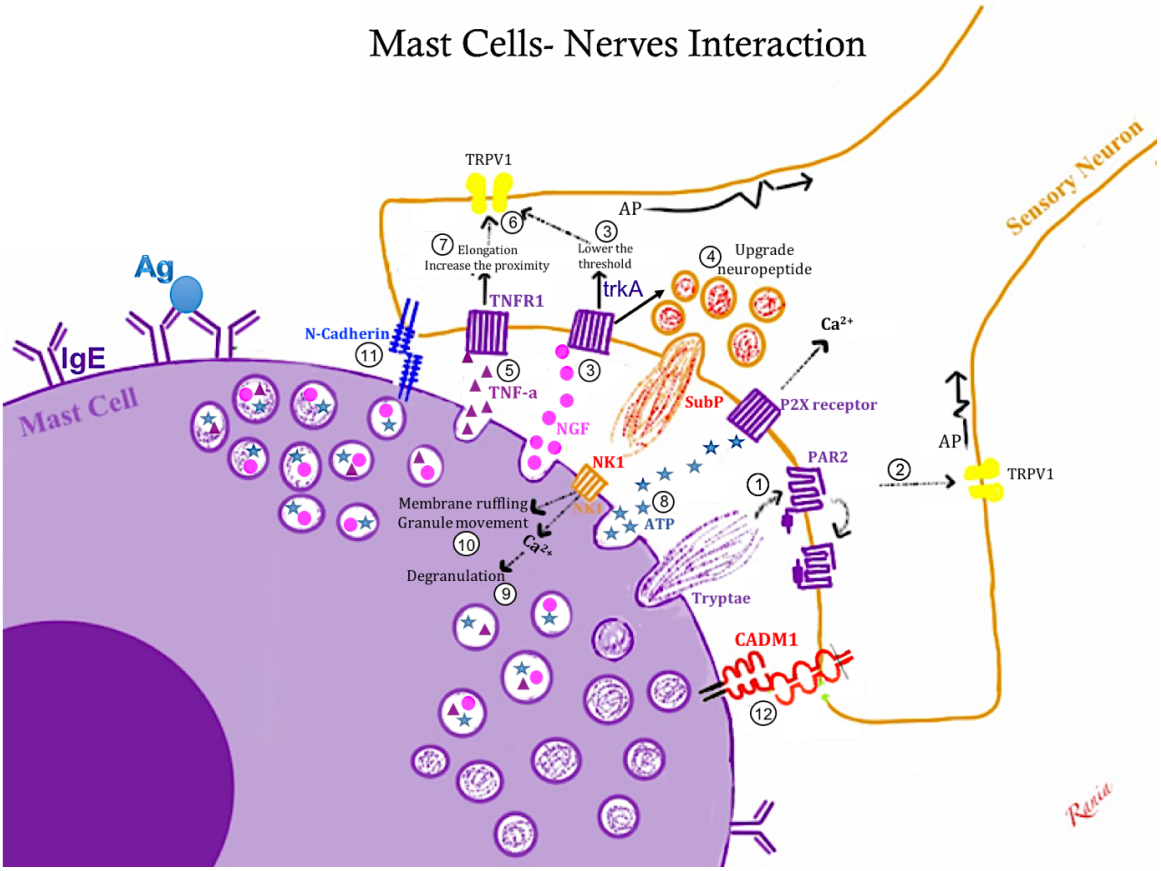


Figure.1.4. Mast cell- nerve interaction.

Mast cell-nerve proximity is mediated by adhesion molecules: cell adhesion molecule-1 (CADM1) and N-cadherins. This proximity facilities the functional bidirectional communication. Mast cell releases or synthesizes mediators, which act on the relevant receptors expressed by sensory neurons. Sensory neuron responses by release neuropeptides that activate mast cells. Encircles numbers represent references. **(1)** (Linden et al., 2001); **(2)** (Amadesi et al., 2004); **(3)** (Leon et al., 1994); **(4)** (Lindsay and Harmar, 1989); **(5)** (Kakurai et al., 2006); **(6)** (Hensellek et al., 2007) (van Diest et al., 2012) **(7)** (Kakurai et al., 2006); **(8)** (Suzuki et al., 2007); **(9)** (Maggi, 1997) (Suzuki et al., 1999) **(10)** (Mori et al., 2002); **(11)** (Suzuki et al., 2004); **(12)** (Furuno et al., 2005) (Hagiyama et al., 2011) (Furuno et al., 2012). **Ag**, antigen; **IgE**, immunogloubulin E; **PAR2**, Protease activated receptor 2; **NGF**, nerve growth factor; **trkA** , high-affinity tropomyosin kinase receptor A; **TNF-α,** tumour necrosis factor -α ; **TNFR1**, tumour necrosis factor receptor 1; **ATP**, adenosine tri-phosphate; **P2X**, purinergic 2X receptor; **SubP**, substance P; **NK-1**, neurokinin 1; **TRPV1**, transient receptor potential vanilloid ;**AP**, action potential.

1.3.1.1 Mast cell signaling to neurons

Mast cells synthesize, store and release a diversity of mediators that induce direct functional responses in neurons or sensitize nociceptors indirectly through activation of co-expressed receptors (van Diest et al., 2012). These mediators include all classes of mast cell mediators. Preformed mediators like tryptase, histamine, serotonin and ATP. Synthesized lipid mediators such as PGs and cytokines such as NGF, and TNFα.

As shown in Figure 1.4, mast cells secrete tryptase upon stimulation. Tryptase cleaves and activates PAR2 expressed on sensory neurons (Linden et al., 2001) and sensitizes TRPV1, the most studied receptor involved in sensory pain perception (Amadesi et al., 2004). As a result, neuropeptides subP and CGRP are released from neurons causing hyperalgesia (Amadesi et al., 2004) and neurogenic inflammation (Cenac et al., 2003). Likewise, other preformed mast cell mediators have been reported to modulate TRPV channels as well. Histamine and serotonin sensitize TRPV1 and TRPV4 by binding to their receptors on nociceptors (Van Steenwinckel et al., 2009). Pre-incubation of DRG with histamine and serotonin enhance calcium response induced by TRPV agonist *in vitro*. On the same way, they enhance the visceral hypersensitivity induced by colorectal distension in rat (Cenac et al., 2010). Excitingly, systematic administration of histamine H1 receptor antagonist effectively reduces visceral hypersensitivity in maternal separation model for IBS in comparable to other analgesic drugs (Stanisor et al., 2013).

Mast cells synthesize and release cytokines and growth factors that influence neuronal activation. Mast cell-derived IL-1β and IL-6 cytokines evoke neuronal excitatory responses of small intestinal submucosal neuron *of guinea pig* (Xia et al., 1999). Moreover, mast cells synthesize, store, and release NGF (Leon et al., 1994). In addition to the vital role of NGF on survival and differentiation neurons (Matsushima and Bogenmann, 1990), it has been reported that local injection of NGF lowers the threshold of sensory neurons and induces long-lasting hypersensitivity in human (Rukwied et al., 2010) and rat (Mills et al., 2013). This effect may be due to its ability to up-regulate SubP and CGRP expression in DRG (Lindsay and Harmar, 1989). Interestingly, NGF has

autocrine effect on mast cells. It activates high-affinity tropomyosin kinase receptor A (TrkA) expressed by mast cells (Tam et al., 1997) and induces CTMC degranulation (Stempelj et al., 2003) and IL-6 and PGE2 production *in vitro,* in rat (Marshall et al., 1999). In addition, NGF promotes mast cell migration *in vitro* (Sawada et al., 2000). TNFα is also secreted from activated mast cells and acts on sensory neurons either directly or indirectly. Mast cell- derived TNFα can bind directly to TNFR1 on sensory neurons that leads to nerve elongation. In an animal model of contact hypersensitivity, oxazolone failed to induce cutaneous nerve elongation in TNF-deficient mice (Kakurai et al., 2006). It doesn't only promote nerve elongation: one interesting finding in Kakurai's work is that mast cell- derived TNFα enhances the close proximity between mast cells and nerve fibers (Kakurai et al., 2006). TNFα also increases nerve susceptibility to excitatory stimuli such as capsaicin (van Houwelingen et al., 2002). Conversely, mast cell- derived TNFα can act on neurons indirectly via activation of TNFR1 on fibroblasts, which leads to fibroblast production of NGF that in turn acts on sensory neurons. Both TNFα, and NGF are involved in sensitization and up-regulation of TRPV1 nociceptors in sensory neurons (Winston et al., 2001) (Hensellek et al., 2007). Moreover, ATP secreted from Ag- activated mast cells induces calcium signal in sensory neuron in co-culture and applying ATP antagonist (apyrase)reduces this effect (Suzuki et al., 2007). Sensory neurons express P2X and P2Y purinergic receptors (Ruan and Burnstock, 2003), and purinergic signaling plays a role in pain transmission pathways (Burnstock, 2009). P2X signaling contributes in pain pathway by provoking glutamate release from nociceptors which leads to fast synaptic transmission (Gu and MacDermott, 1997). While, P2Y contributes in pain transmission by its interaction with TRPV1 (Moriyama et al., 2003).

1.3.1.2 Neuron signalling to mast cells

As explained in (section 1.2.3), sensory neurons can influence mast cell activation through DDR- related mechanism. Upon electrical stimulation of rat vagus nerve, the amount of intestinal mast cell-derived histamine is increased (Gottwald et al., 1995). Indeed, capsaicin – activated c-fiber neurons also showed the same effect. This suggests that noxious stimuli activate c-fiber neurons that response as efferent by releasing

neuropeptides that activate mast cells (Suzuki et al., 1999) (Mori et al., 2002). Mori examined the effect of subP at the site of mast cell contact with neurons. He found that stimulation of the neurons leads to the release of subP which then causes mast cell membrane ruffling and granule movement (Mori et al., 2002). In this regard, subP is the most extensively investigated neuropeptide. SubP induces mast cell- derived mediators like histamine (Ottosson and Edvinsson, 1997) and cytokines such as:IL-1 (Laurenzi et al., 1990), IL-6 (Gagari et al., 1997), IL-10, TNF-α (Lee et al., 1994) and NGF (Maggi et al., 1997) (Cuesta et al., 2002). Interestingly, NK1 receptor-deficient mice failed to develop stress-induce asthma (Pavlovic et al., 2008). In a model of stress-induced atopic dermatitis, there was increased number of degranulated mast cells in contact to subP-positive nerves in the skin of mice (Peters et al., 2005). Collectively, this is proposing the important role of subP in mast cell-mediated inflammation.

Several lines of evidence indicate that subP is a functional messenger between neurons and mast cells and has a major function in the pathogenesis of pain, neurogenic inflammation and stress-induced diseases. This has been proven using mast cell- neuron co-culture system. Activated neurons release subP that causes either piecemeal- or classic degranulation of mast cells (Maggi, 1997). Scorpion venom- induced neuronal activation increases calcium mobilization in mast cells in co-culture. This effect is abolished by subP antibody or NK-1 receptor antagonist (Suzuki et al., 1999). SubP activates mast cells via both NK1 receptor-dependent and a receptor-independent pathway. NK1 expression in mast cells seems to depend on microenvironment factors. Human intestinal mast cells don't express NK receptors in physiological conditions, but they are upregulated after IgE-mediated mast cell activation (Bischoff et al., 2004). Furthermore, IL-3 dependent BMMC don't express NK1 that is upregulated when BMMCs are co-cultured with pro-inflammatory cytokines IL-4 and SCF (van der Kleij et al., 2003) (Okabe et al., 2006), and as a result these mast cells acquire the sensitivity to subP (Karimi et al., 2000). This suggests that mast cells don't express NK receptor in normal physiological conditions, which is why higher concentrations of substance P is needed to activate mast cells through receptor-independent pathway. By contrast, lower concentrations of subP can activate mast cells directly through NK1 receptors that are expressed during inflammation (van der Kleij et al., 2003).

It has been suggested that a picomolar concentration of subP that does not induce mast cell degranulation in physiology could increase the sensitivity of mast cells to subsequent stimuli (Suzuki et al., 1999). In contrary to this suggestion, small dose of subP has been reported to downregulate FcεRI expression in human primary mast cells and LAD2 cell line (McCary et al., 2010). However, pre-senstized mast cells with IgE have been shown to prevent subP- induced FcεRI downregulation. This is of great interest clinically because it suggests that SubP-mediated FcεRI downregulation is a physiological process to protect mast cells from further activation. However, this downregulation is not happened in allergic patients with IgE- sensitized mast cells. That could explain the increased incidence of neurogenic inflammation disorders in atopic patients (Jones et al., 2014). More importantly, this proposes that *nerve cell-derived neuropeptides not just activate mast cells, but they regulate their functions in health and diseases.*

In contrast to subP, little data are available regarding the effect of CGRP on mast cells, and there is discrepancy whether CGRP could induce mast cell degranulation. Although CGRP evokes an increase in Ca^{2+} signaling in BMMCs (De Jonge et al., 2004), it produces only small amount of mMCP-1 without affecting degranulation (Rychter et al., 2011). CGRP also can't induce degranulation in LAD2 (Kulka et al., 2008). On the other hand, CGRP was shown to increase histamine release from dura mater mast cells in rat, although it doesn't induce peritoneal mast cells degranulation in rats (Ottosson and Edvinsson, 1997). The histamine released from CGRP-activated dura mater mast cells is blocked by CGRP receptor antagonist suggesting receptor mediated mechanism. However, more works needs to be done to explore the role of CGRP in mast cell- neuron cross-talk.

1.3.2 Mast cell- nerve structural interactions

Many studies are providing conclusive evidence for the juxtaposition of mast cells and nerves *in vivo*. Indeed, mast cells have shown preference to attach to subP and CGRP-positive sensory neurons in human and rat intestine (Stead et al., 1987), respiratory tract (Alving et al., 1991) dura matter (Rozniecki et al., 1999), and other tissues (Spanos et al., 1997). By electron microscopy, it has been shown that the gap between mast cells and nerves is not larger than 20nm (Stead et al., 1987). Moreover, it is reported that the number of contacts between mast cells and neurons is increased during infection (Stead et al., 1987), allergic conditions (El-Nour et al., 2005), and inflammatory conditions such as irritable bowel syndrome (Barbara et al., 2004).

In vitro co-culture, superior cervical ganglion (SCG) with RBL cell line showed that mast cells attach to neurites although they don't form typical characteristics of synapses (Blennerhassett et al., 1991) (Furuno et al., 2005). Although many studies showed the mast cell- neuron bi-directional communications, few studies examined the effect of intimate attachment of mast cells to neurites on their communications. Apart from one study showed that the movement of mast cells granules and membrane ruffling were restricted at the point of contact between mast cell and neuron as seen by electron microscopy (Mori et al., 2002), one study clearly compared the response between attached and non-attached mast cells to neurites (Suzuki et al., 2005). After IgE-activation of mast cells, both Ca^{+2} responses and FcεRI expression were increased significantly in mast cells attached to neurites compared to non-attached mast cells in the same co-culture. *These data indicate that physical contact between mast cells and neurons may augment mast cell responses to antigen. However, this attractive suggestion needs to be scientifically investigated to explore the novel subsequent event for mast cell-neuron cross-talk.*

1.4 Cell adhesion molecules (CAMs)

Localization and function of different cells are controlled by cell-cell or ECM-cell adhesions that mediated by CAMs. Cell adhesion is a fundamental process for normal tissue architecture and intercellular communication. In some cases, the cytoplasmic domain of CAMs interact with protein kinases to induce intracellular signaling which involves cell functions such as cell growth, degranulation, and chemotaxis (Hynes, 1992). CAMs can mediate either homotypic adhesion, in which the same CAMs bind to each other in both cells, and heterotypic adhesion, in which CAM from one class bind CAM from different class on the surface of another cell (Figure 1.5). Here, I will review some examples for CAMs involved in protein-protein interactions

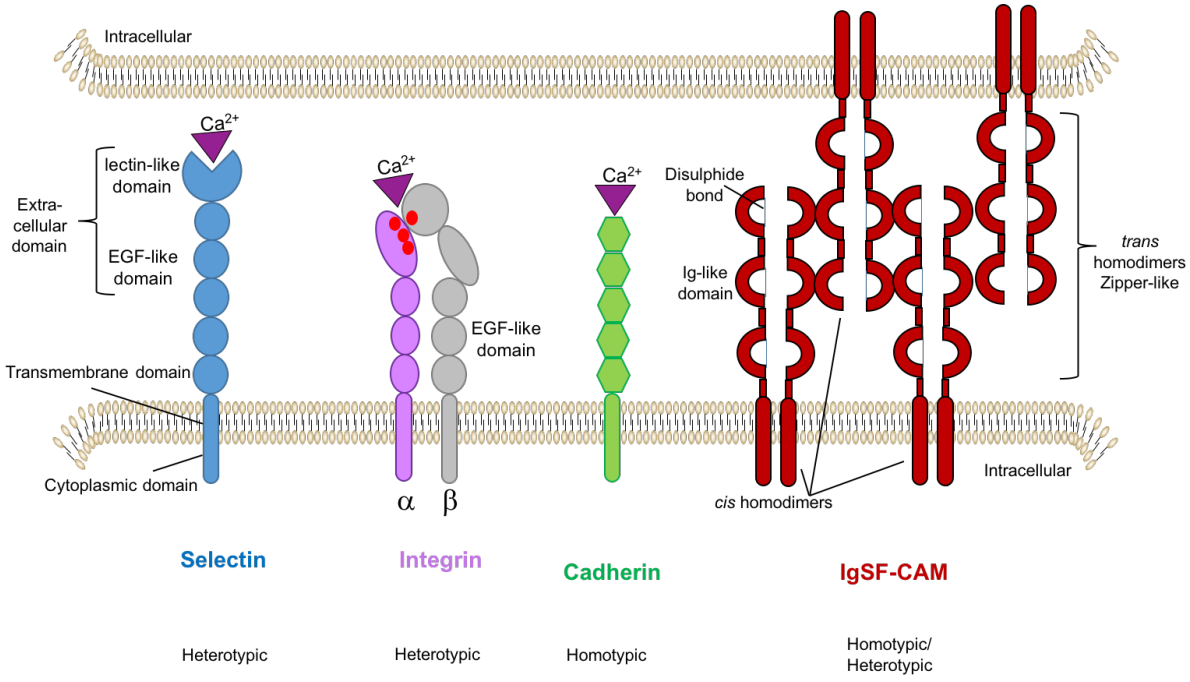


Figure.1.5 Main families of cell adhesion molecules

General structural characteristics for: Selectins, Integrins, Cadherins and Immunoglobulin superfamily adhesion molecules (IgSF-CAM) are shown. Selectin, integrins and cadherins are calcium (Ca^{2+})- dependent CAMs, while IgSF-CAMs are calcium-independent. Unlike cadherins, selectins and integrins can mediate heterotypic cell adhesion. IgSF-CAMs mediate both homo-and heterotypic cell adhesion. **EGF**= epidermal growth factor.

1.4.1 CAM families

1.4.1.1 The selectins

Structurally, selectins have an extracellular domain that consists of an N-terminal, lectin-like subunit (C-type), and an epidermal growth factor (EGF)-like subunit, followed by a transmembrane domain, and a cytoplasmic tail (Figure. 1.5) (St Hill, 2011). The selectins have three members E-, L-, and P-selectins which named after the cell-type expressed on endothelial cells, leukocytes and platelets; respectively. They are important in leukocytes homing in sites of tissue inflammation. Thus, they are implicated in the pathogenesis of cancer and inflammatory diseases like asthma and arthritis (Kelly et al., 2007).

1.4.1.2 The Integrins

The integrins have a unique structure among CAMs. They present as heterodimers of non- covalently linked α and β transmembrane subunits with globular extracellular domain (Figure. 1.5) (Hynes, 2002). They mediate cell-ECM interactions, as well as, heterotypic cell-cell interactions. Intriguingly, integrins also induce intracellular signaling upon binding to ECM. This signaling regulate cell growth, and proliferation(Hynes, 2002) and impaired signaling can contribute in the pathogenesis of thrombotic disorders and cancer (Guo and Giancotti, 2004) (Avraamides et al., 2008).

1.4.1.3 The Cadherin

Cadherins are transmembrane calcium-dependent homotypic CAM. Their extracellular domain has five repeated subdomains, EC1 to EC5 (Figure. 1.5) (Takeichi, 1995). The classic cadherins are named after the first cell-type identified to express prominently in, although it is not exclusive. For example, E-cadherin is mostly present in epithelial cells, N-cadherin in the nervous system, P-cadherin in placenta cells and VE-cadherin is specific for the vascular endothelial cells (Gumbiner, 2005). Cadherins form cis homodimers of alike molecules that trans interact to counter-receptors on other cells in zipper- like manner. Because of their homotypic binding specificities, they are important

in selective adhesion between embryonic cells(Tepass et al., 2002). Furthermore, cadherins can induce intracellular signaling (Gumbiner, 2005). Loss of cadherins can lead to increase metastatic potential in malignancies. Thus, it is used as a prognostic biomarker in solid cancers (Richardson et al., 2012).

1.4.1.4 Immunoglobulin superfamily of cell adhesion molecules (IgSF-CAMs)

The IgSF-CAM superfamily is the largest group with a distinct range of cell adhesion molecule subfamilies. Members of this family are characterized by the presence of one or more Ig-like extracellular domains with single transmembrane glycoproteins (Figure. 1.5). For example, Intercellular adhesion molecule 1 (ICAM1) has five Ig- like domains, ICAM2 has only two Ig- like domains, while CADMs and nectins have three Ig- like domains. IgSF-CAMs interact in trans both homotypically and heterotypically with other IgSF-CAMs members, as well as, other CAMs classes including integrins, cadherins and tyrosine kinase receptors. They mediate Ca^{2+}-independent cellular adhesion (Cavallaro and Christofori, 2004).

An example for IgSF-CAM subfamily is the nectins. Nectins colocalize with N-cadherin in synapses of the developing nervous system (Mizoguchi et al., 2002) (Okabe et al., 2004). Although the number of synapses in the hippocampus is decreased in nectin-deficient mice, no change in in the properties of synaptic transmission is detected in those mice. Therefore, it seems that formation of synapses is not entirely dependent on nectins (Honda et al., 2006).

Among IgSF-CAM molecules, I found CADM1 is interesting. CADM1 is multifunctional immunoglobulin adhesion receptor. It participates in mast cell adhesion to fibroblasts (Ito et al., 2003) (Moiseeva et al., 2013b), airway smooth muscle cells (ASM) (Yang et al., 2006) (Hollins et al., 2008) (Moiseeva et al., 2013b) and nerves (Furuno et al., 2005) (Hagiyama et al., 2011).

1.4.2 Cell adhesion molecule 1 (CADM1)

The CADM1 is a member of IgSF-CAMs subfamily which has four members: CADM1, CADM2, CADM3, and CADM4 (Biederer, 2006). All of them have three Ig-like domains of the V-, C1-, and I-set subclasses, a transmembrane domain and a cytoplasmic tail that is highly conserved among them. However, their protein structures are different in the presence or absence of O-and N- glycosylation sites in the extracellular domain. In addition, the CADM genes are remarkably different in size (Biederer, 2006).

1.4.2.1 CADM1 expression, function and alternative nomenclature

Extraordinarily, CADM1 is known in literature with different nomenclatures due to the parallel identification of the same molecule by various research groups with different research goals. Table 1.4 lists of alternative nomenclatures for the CADM1. Eventually, its nomenclature was standardized to CADM1 by the HUGO gene nomenclature committee (Koma et al., 2008), the name that has been used in this thesis. Therefore, CADM1 has been cross-referenced in literature.

The diversity of nomenclatures for CADM1 reflect the numerous cell types that express it and its broad range of functions (As shown in table 1.4). Interestingly, CADM1 expression in BMMC has been confirmed to be regulated by the microphthalmia transcriptional factor (MITF) (Ito et al., 2003), a member of the basic-helix-loop-helix-leucine zipper family that involve in signaling pathways required for mast cell survival (Hodgkinson et al., 1993).

CADM1 has been reported to express in several cell types including nerves (Biederer et al., 2002), mast cells. (Ito et al., 2003), spermatogonia (Wakayama et al., 2001), pancreatic secretory (Koma et al., 2008) and pulmonary alveolar cells (Pletcher et al., 2001).

Name	Protein function	Reference
Synaptic cell adhesion molecule1 (SynCAM)	synaptic adhesion molecule that expressed throughout the brain It drives assembly of synaptic cleft Involved in CNS formation and development	(Biederer et al., 2002)
Tumor suppressor in lung cancer-1 (TSLC1)	Tumor suppressor gene in lung cancer	(Masuda et al., 2002) (Pletcher et al., 2001)
Spermatogenic immunoglobulin superfamily (SgIGSF)	spermatogenesis, Male sexual development Bile duct/ductule formation in the pancreas Mast cell adhesion protein	(Wakayama et al., 2001) (Ito et al., 2003)
IgSF4	Synapsis formation	(Watabe et al., 2003)
Nectin-like molecule-2(Necl-2)	Localization of transmembrane proteins in epithelial cells	(Shingai et al., 2003)
CADM1	Tumor suppression Apoptosis Nerve-islet cell interactions	(Koma et al., 2008)

Table 1.4: Alternative nomenclature for the CADM1 molecule

1.4.2.2 CADM1 structure and isoforms

As other CADMs, CADM1 has three extracellular Ig-like domains, a single transmembrane region, and a short cytoplasmic domain (Ito, 2010). CADM1 is a 442 amino acid protein. The extracellular domain comprises of 373 amino acids; the transmembrane domain comprises of 23 amino acids, and the cytoplasmic domain has 46 amino acids. CADM1 undergoes O-linked glycosylation on the extracellular domain. Its intracellular domain has protein 4.1 and PDZ-binding motifs that act as a molecular scaffold for cytoskeleton and CASK, syntenin, and focal adhesion kinase (FAK), respectively (Watabe et al., 2003) (Figure 1.6).

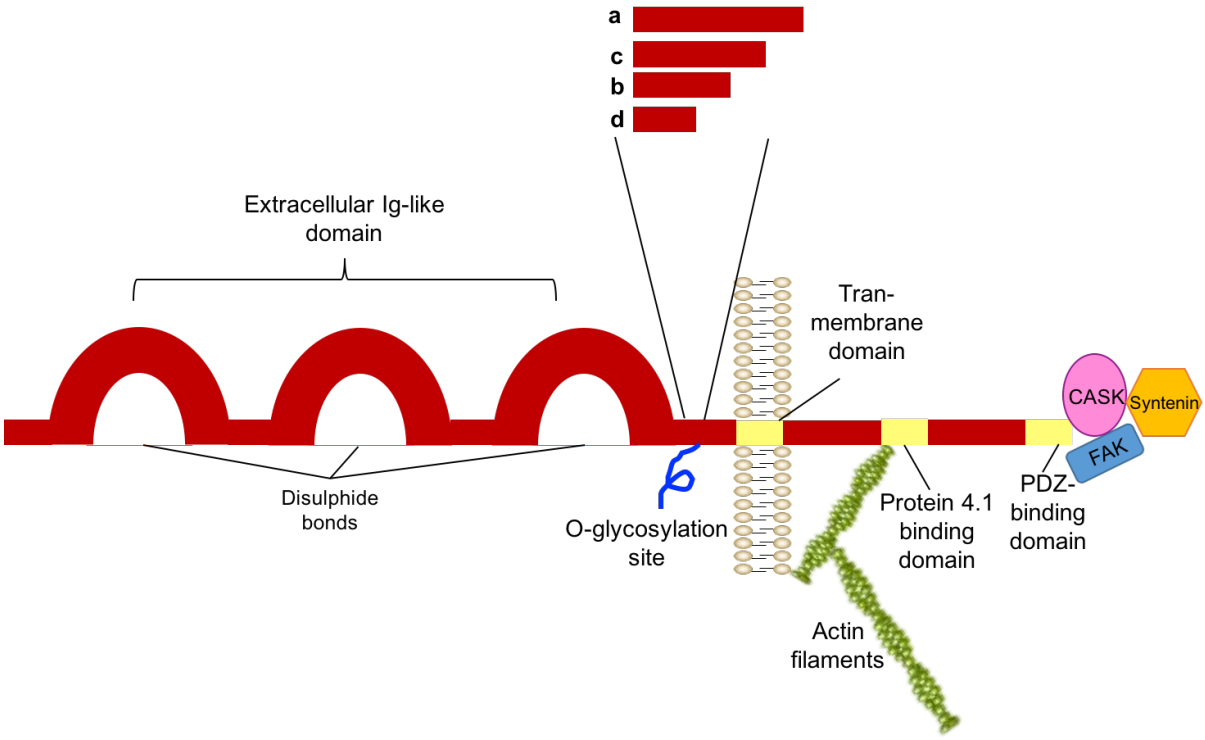


Figure.1.6 S**tructure of cell adhesion molecule1 (CADM1)**

CADM1 has three extracellular Ig-like domains, a single transmembrane region, and a short cytoplasmic domain with protein 4.1 and PDZ motif. As a result of mRNA alternative splicing, four CADM1 isoforms (a-d) are generated.

Depending on the type of the cell expressing CADM1 and binding partner on attached cells, *cis* homodimers of CADM1 can *trans* bind either homotypically or heterotypically in zipper- like manner (Ito, 2010) (Figure 1.5). The binding that occurs among neuronal cells is an example of homotypical binding (Biederer et al., 2002), while the heterotypic adhesion is between mast cells and fibroblasts (Ito et al., 2003) and between spermatogenic and Sertoli cells (Wakayama et al., 2001). The adhesion partners could be CADM1 itself, CADM2, CADM3, nectin3 and CRTAM (Ito, 2010)

CADM1 gene is mapped on chromosome 9 in mouse and 11 in human. It consists of 12 exons in mouse (Biederer, 2006). As a result of alternative mRNA splicing between exons 7 and 11, CADM1 has four isoforms (CADM1a-CADM1d). Those isoforms differ in the juxtamembranous extracellular region, that is, downstream of the exon encoding the third Ig-like loop and upstream of the exon encoding the transmembrane region (Biederer, 2006) (Figure 1.6).

1.4.2.3 CADM1 in diseases

CADM1 is essential for health and development. However, modulation of its expression has been implicated in several diseases. CADM1- knockout mice developed lymphoma and leukaemia, in addition to, some solid tumours such as lung, stomach and hepatocellular carcinomas (van der Weyden et al., 2012). Clinically, It has been recommended to use CADM1 as diagnostic and prognostic markers for osteosarcoma (Inoue et al., 2013) and colon cancer (Zhang et al., 2012), respectively. Furthermore, its expression is reduced in pathogenesis in autism (Zhiling et al., 2008) and venous thrombosis (Hasstedt et al., 2009), while is increased in the neurodevelopmental disorder Rett syndrome (Nectoux et al., 2010).

1.4.2.4 Adhesion molecules in mast cell-neuron contact

Remarkably, little is known regarding the molecular mechanisms involved in the close anatomical relationship between mast cells and neurons. As a result of the success of *in-*

vitro co-culture system of mast cells and neurons, efforts were spent to investigate the molecules involved in this functional unit (Forsythe and Bienenstock, 2012). Tow adhesion molecules have been proposed to be the responsible of mast cell- neuron interaction.

CADM1 was recently shown to be expressed by both mast cells (Ito et al., 2003) and neurons (Biederer et al., 2002). CADM1 was found to mediate the attachment of mast cells to fibroblasts (Ito et al., 2003, Moiseeva et al., 2013b) and mast cells to airway smooth muscle cells (ASMCs) (Yang et al., 2006) (Moiseeva et al., 2013b). CADM1 is also expressed in the adhesion site of mast cells to neurons (Furuno et al., 2005) (Hagiyama et al., 2011) and blocking CADM1 by peptide disrupts adhesion between BMMC and SCG neurons in a dose-dependent manner (Furuno et al., 2005).

Interestingly, it is proposed that CADM1 doesn't promote only the adhesion but also enhances the functional crosstalk between mast cells and sensory neurons (Ito et al., 2008). This suggestion was based on the observation that the mast cells lines (IC-2) that express all other adhesion molecules except CADM1 response less efficiently to neuronal activation compared to BMMCs, which express endogenous CADM1 (Furuno et al., 2005). Indeed, ectopic expression of IC-2 cells with CADM1 normalized their response. Consistent with this observation, the number of degranulated mast cells in mice mesentery after nerve stimulation is decreased in CADM1-deficient mice compared to wild type (Ito et al., 2007). All these evidence suggest the crucial role of CADM1 in upgrading the level of functional communication between mast cells and sensory neurons. *However, further investigations are needed to validate this hypothesis.*

Furthermore, when CADM1 molecules were blocked with pretreatment with anti-CADM1, there was a small but appreciable number of mast cells attached to neurons. This observation suggests that, in addition to the major role of CADM1 in mediating the intercellular attachment between mast cells and neurites, other adhesion molecules are also involved in this communication. Since N-cadherins are expressed by mast cells and

neurites, it is proposed that N-cadherins are accountable for the residual adhesion of mast cells to neurites (Furuno et al., 2005). N-cadherins are homophilic, calcium-dependent adhesion proteins which are known to have a role in neural synapse formation (Goda, 2002) (This molecule will be covered in detail below). In BMMC monoculture, N-cadherins are expressed as an intracellular protein. However, they traffic to the plasma membrane after co-culture BMMC with SCG (Suzuki et al., 2004). This suggests that N-cadherins are involved in contact between mast cells and neurons. However, mast cells from IC-2 cell lines and BMMC from microphthalmia transcription factor (MITF) mutant mice are lacking endogenous CADM1 but express a normal level of all other adhesion molecules including N-cadherins (Ito et al., 2003). Because these mast cells without CADM1 attach less to neurites, compared to wild-type BMMC and because there is no evidence of the involvement of N-cadherin in mast cell-neuron functional interaction, CADM1 apparently play a key role in mast cell- neuron interaction and the role of N-cadherin is less critical. *This speculation needs to be carefully investigated.*

1.5 Thesis aims and outlines:

The role of expression, function and interactions of cell adhesion molecules in normal development and pathogenesis is a rapidly advancing research area (Mandai et al., 2015). However, it remains unclear if adhesion is essential in mast cell-neuron cross-talk. Therefore, we tested the hypothesis that *the functional cross talk between mast cells and sensory neurons is modulated by CADM1 by up-regulating their mediators and/or receptors involved in this intercellular communication.*

Thus, the objectives of this thesis were:

1. To characterise and optimize the BMMC and DRG cultures as models for primary mast cells and sensory neurons, respectively, are described in in chapter 3 and 4.
2. To investigate the impact of neurons on IgE-mediated mast cell signalling using *in vitro* co-culture system (chapter 4).
3. To identify and characterise CADM1 expression in primary murine mast cells and sensory neurons and verify its involvement in mast cell-sensory neuron adhesion (chapter 5).
4. To examine the effect of CADM1 adhesion on IgE- mediated mast cell signalling and pro-inflammatory mediator secretion in the presence of neurons (chapter 5).
5. To explore the potential mechanisms of adhesion-mediated neuronal effect on mast cell functions (chapter 6).

Final conclusions and how my results could be transferred into human health and diseases are summarized in chapter 7.

Chapter 2. Material and Methods

2.1 Primary cell cultures and co-culture

2.1.1 Bone Marrow Derived Mast Cells (BMMC) Isolation and Culturing

Mouse bone marrow-derived mast cells (BMMCs) are a valuable model to investigate the function of mucosal- type mast cells (Razin et al., 1982b). BMMCs were isolated from 8-12 week-old C57BL6 wild type mice (Suzuki, 2004)

2.1.1.1 Preparation and isolation of mouse BMMCs

After scarifying the mouse, its fur was wet with 70% ethanol and its intact femur and tibia were removed. Then, all the tissue was scraped away. The remaining steps were carried out under sterile condition in tissue culture hood. The epiphysis of femur and tibia were cut off to expose the bone marrow. Then, bone marrow cells were extracted by repeatedly flushing the femur and tibia using a 27-G needle and 10 ml syringe filled with calcium and magnesium free phosphate buffer solution (PBS, PAN Biotech GmbH, Cat no. P04-53500). The cells thus obtained were washed by centrifuging at 340xg for 10 min at 4°C. Then, red blood cells were lysed using lysis buffer (Ery-lyse-buffer, 0.83% ammonium chloride 4.15g/500ml 0.168% Na carbonate 0.84g/500ml 1mM ethylene-diaminetera acetic acid (EDTA) (1ml of 0.5 M stock)/500 ml pH 7.3 filtered sterile) for 10 min at room temperature. To stop the reaction, 10 ml of complete Iscove's Modified Dulbecco's Media (IMDM) medium was added. Table 2.1 shows contents of complete IMDM medium. Then, cell suspension was centrifuged at 340xg for 10 min at 4°C. After discarding the supernatant, cell pellet was re-suspended in 5 ml of complete medium. Then, the cells were collected by passage through 20µM mesh filter (Falcon) into a 50 ml tube. Then, 5 ng/ml mouse recombinant IL-3 with or without 10 ng/ml recombinant mouse stem cell factor (rmSCF) were added. These cells were collected in a 75cm^2 tissue culture flask and cultured at the density of 0.5x10^6 -1x10^6 cells/ml in 7.5% CO2 at 37 °C

for 4 weeks until they differentiated into BMMCs. At this stage, BMMC purity was confirmed with flow cytometry (FACS) (see below).

Complete IMDM Reagent	Amount	Concentration	Supplier
IMDM with HEPES, without L-glutamine	50 ml		Lonza12-726F
Heat inactivated fetal bovine serum (FBS)	5 ml	10%	Gibco 10500-064 Lot 07F2645K
L-Glutamine with Penicillin/Streptomycin 100X	500 µl	100 u/ml	PAA Laboratories P11-01
MEM Vitamin 100X	500 µl	1%	Gibco 11120-037
2-mercaptoethanol	50 µl	50 µM	Gibco 31350-010
Sodium pyruvate 100 mM	500 µl	1%	Gibco 11360-039
Non-essential amino acid X100	500 µl	1%	Gibco 11140-035

Growth Factors Reagent	Amount	Concentration	Supplier
Mouse SCF 10 µg/ml	25 µl	10 ng/ml	R&D systems 455-MC-010
Mouse IL-3 10 µg/ml	12.5 µl	5 ng/ml	R&D systems 403-ML-010

Table 2.1. Contents of BMMC medium

2.1.1.2 maintenance of BMMC culture.

Every week, non- adherent cells were transferred to 50 ml falcon tube and spun X340xg for 10 min at 4°C. Then, supernatant was discarded and cell pellet was re-suspended with fresh complete IMDM medium supplemented with 5 ng/ml rmIL-3 with or without

10 ng/ml rmSCF. Cells were retained for further culturing in new culture flask while adherent cells were discarded.

2.1.1.3 Cell Counting

Because specific cell numbers were needed for each experiment, cells were counted using a haemocytometer. 100 µl of a cell suspension was diluted with 400 of medium (1:4). Then, 45 µl of Trypan Blue solution (Gibco, Invitrogen) was added to determine viable and non-viable cells. 10 µl of this solution was pipetted under each end of the haemocytometer's coverslip to fill the chamber by capillary action. Using a light microscope at x10 magnification, the number of cells present was counted in each of the four divided corners of haemocytometer. The following equation was used to determine the total viable cell number:

Cell number/ ml = (four chamber viable cell number/4) x (dilution factor) x 10^4

To work out the required volume for a particular number of cells:

Volume of cell suspension for required cell number (ml) = (required cell number/actual total cell number) x volume of actual total cell suspension

Figure 2.1 shows BMMC growth curve for 24-hour after maturation.

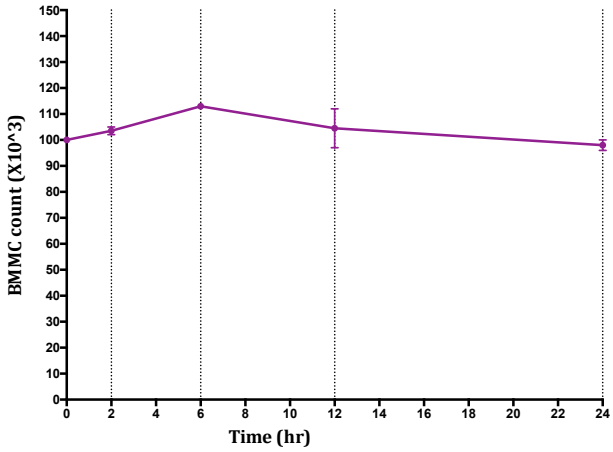


Figure. 2.1. BMMC growth rate after maturation

After 4 weeks of culturing in complete medium, BMMCs were counted at different time points (2-6-12-24 hour) to estimate BMMC growth rate after maturation.

2.1.2 Dorsal Root Ganglion (DRG) culturing

Adult mouse DRG neurons cultures were used as a model to study sensory neurons functions. (Melli and Höke, 2009) (Sleigh et al., 2016)

2.1.2.1 Preparation of Matrigel-coated well

Matrigel (growth factor reduced, phenol red free, BD, 356231) is a solubilized basemembrane preparation, extracted from the Engelbreth-Holm-Swarm mouse sarcoma. Its major components include laminin and collagen IV. Depending on the experiment; wells with glass coverslip or without were coated with matrigel (thin gel method) before seeding DRG. 50µl aliquot of matrigel slowly thawed on ice (at least 2 hours). Glass coverslips were placed in a 24-well plate and cooled in fridge with box of tips. By using cold tips, 50 µL of defrost matrigel was diluted in 450 µl of cold medium (1:9) and mix. 50 µl of diluted matrigel was added to the middle of each well. Then, it removed quickly by cold pipette. Plate was incubated at 37°C for 20-25 minutes and cells were immediately seeded onto the middle of coverslips. Wells were flooded after 1 hour and cultured overnight prior to subsequent experiments.

2.1.2.2 DRG isolation and culturing

Adult (from 8- 12 weeks) C57BL male mouse was used for DRG isolation. After killing the animal with cervical dislocation, the spinal column was isolated from the back of the mouse and cut longitudinally by scalp to identical two halves. Then the spinal cord and meninges were removed. Under stereomicrosope, around 30 DRGs were collected in small petri dish (35mm, Nunc, thermo scientific) filled with PBS on ice. Then quickly DRGs were trimmed off all attached fibers (Sleigh et al., 2016). After that, DRGs were transferred to 1ml of PBS with 0.06µg/ml collagenase XI (Sigma) and 0.1 µg/ml Dispase for enzymatically digestion. DRGs were incubated for 1 hour in 37°C, 7.5% CO_2. After a time, DRGs were gently triturated by pipetting with narrow tip pipette until cloudy and all DRGs were dissociated. For selective isolation of neurons, gradient centrifuge technique with 15% bovine serum albumin (BSA) in medium was used. Cells were

carefully added to the top layer of 15% BSA solution. Cells were then spun at 450xg (1900rpm) for eight minutes without deceleration. After collecting the neurons from 15% BSA solution; neurons were washed with complete Neurobasal A medium (NBA, Gibco) containing 2% B-27 supplement (Gibco), 2mM Glutamax (Gibco), 1% penicillin /streptomycin (Gibco). Finally, neurons were resuspended in small amount of complete NBA medium (50 µl/ well) and seeded on 16 mm matrigel (BD) - coated glass coverslips (see above). Wells were incubated in 37°C, 7.5% CO_2. After 1hour, cells were flooded with complete NBA medium supplemented with 10ng/ml NGF (sigma) and 1 µM Cytosineβ-D-arabinofuranoside (Ara-C, Sigma) and incubated for one day before using in co-culture. NGF and Ara-C were used only for first day of DRG culture. Ara-C is anti-mitotic agent that used to control the number of proliferating non-neuronal cells and improve the purity of DRG culture without effect neuronal viability (Schwieger et al., 2016).

Complete NBA Reagent	**Amount**	**Concentration**	**Supplier**
NBA	10 ml		Gibco 10888-022
B-27	200 µl	2%	Gibco 17504044
Glutamax 200mM	100 µl	2 mM	Gibco 35050-061
Penicillin/Streptomycin	100 µl	1 %	PAA P11-013
Ara-C (1mM)	10 µl	1 µM	Sigma C1768

Growth Factors Reagent	**Amount**	**Concentration**	**Supplier**
Mouse NGF (10 µg/ml)	10 µl	10 ng/ml	Sigma N2513

Table 2.2. Contents of NBA media

2.1.3 BMMC-DRG Co-culture

BMMC were isolated as mentioned above (section 2.1.1). After 4 weeks, the purity of BMMCs were assessed by flow cytometry (see below) and were used only if the purity >95%. On the other hand, DRGs were isolated as mentioned in (section 2.1.2) and seeded on matrigel-coated wells. After 1 day of DRG cultured in complete NBA medium, DRG media were removed and (1-3X10^5/50µl/well depend on the experiment) BMMCs suspensed in co-culture medium was added to DRG. Co-cultures were incubated in 37^{o}C with presence of IL-3 (5ng/ml) for different time points. For separation experiment (Figure 4.5), transwells (Costar; Corning) with a 0.4-µm insert were used. DRG were cultured in the lower chamber, while BMMCs were added in the insert. Supernatants were collected and analyzed for mediators' release.

50% of Complete IMDM Reagent	Amount	Concentration
IMMD	10 ml	50%
FCS	1 ml	10%
L-Glutamine with Penicillin/Streptomycin 100X	100 µl	100 u/ml
MEM Vitamin 100X	100 µl	1%
2-mercaptoethanol	10 µl	50 µM
Sodium pyruvate 100 mM	100 µl	1%
Non-essential amino acid X100	100 µl	1%

50% Complete NBA Reagent	Amount	Concentration
NBA	10 ml	50%
B-27	200 µl	2%
Glutamax 200mM	100 µl	2 mM
Penicillin/Streptomycin	100 µl	1 %

Growth Factors Reagent	Amount	Concentration
Mouse IL-3 10 µg/ml	10 µl	5 ng/ml

Table 2.3. Contents of BMMC-DRG co-culture medium

2.2 HEK 293 cell line

2.2.1 Maintenance of HEK 293 cells

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma D5796) supplemented with 10% heat- inactivated FBS, 1mM sodium pyruvate and 100 µg/ml streptomycin, and 100 units/ml penicillin at 37°C, 5% CO2 with humidity control.

2.2.2 Sub-culturing of HEK 293 cells

When HEK 293 cell monolayer reached around 75% confluence, flasks were sub-cultured. Confluent cells were washed with 5 ml pre-warmed DMEM (for T-25 flasks). Then, cells were treated with 1 ml trypsin-EDTA (Sigma, T4049) for one min at 37°C. 4 ml pre-warmed complete medium was added to the flask to stop trypsinization. The detached cells were collected into 15-ml Falcon tube. Cells were centrifuged 1000 rpm for 5 minutes at room temperature. Pellet was resuspended in 10 ml DMEM, and then seeded into T-25 flasks at the desired density. HEK 293 cell cultures were sub-cultured eight to ten times before being discarded and a new cryopreserved cell aliquot thawed.

2.2.3 BMMC- HEK co-culture

HEK cells were plated in 96-well plate in their medium. When HEK cells reach 75%-80% confluence, media were removed and mature BMMCs were added and co-cultured in the same way as for BMMC-DRG co-culture (see section 2.1.3).

2.3 Mediators secretion assays

After co-culturing BMMC and DRG for various time points, cells were stimulated either in IgE- dependent or IgE independent pathways. After stimulation, specific assay was used for each mediator.

1. Preformed mediator release and degranulation: β-hexosamindase.
2. Newly transcript cytokines: TNFα and IL-6 using enzyme-linked immunosorbent assay (ELISA) commercial kits.

2.3.1 BMMC stimulation

Two major pathways can activate exocytosis in mast cells. Antigen (Ag)- activated and non Ag-activated pathways.

2.3.1.1 Ag-activated BMMC stimulation

Ag stimulates mast cells by aggregation of the high affinity receptor for IgE (FcεRI). Thus, BMMCs were incubated at $1X10^6$ cells/ ml in their complete culture medium with 0.5 µg/ml of murine anti-dinitrophenol (DNP) IgE (Sigma, D8406, clone SPE-7) at 37°C and 7.5% CO_2 overnight. Next day, cells were transferred to 15-ml falcon tube, centrifuged 340xg for 5 min and washed twice with complete IMDM or co-culture medium, depend on the experiment, to wash excess IgE. After last wash, cells were resuspended with 90 µl/ well and added to 96 well-plate and allowed to equilibrate at 37°C before stimulation. In case of studying BMMC activation in co-culture, BMMC added to 1-day-old DRG culture and co-cultured for specific time 2, 6, or 24 hours. To stimulate BMMC with DNP Ag (Sigma, A6661), 10µl of 10X DNP Ag diluted in same culture medium was added. Final concentration of DNP was from (0-10-30-100-300 ng/ml). To measure spontaneous release (which will be referred by constitutive/ Ag-independent degranulation throughout this thesis), 10µl of only medium was added to control wells. Cells were challenged for various period of time in 37°C depend on mediators wanted to measure.

2.3.1.2 Non-Ag- activated BMMC stimulation

As mentioned in introduction (section 1.1.2.2), some compounds trigger mast cell exocytosis by directly act on G proteins such as amine compound 48/80 (c48/80).

Compound 48/80

BMMCs were challenged with different concentration of c48/80 ranging from (10-100 µg/ml) for 30 minutes at 37°C before collecting supernatants. Supernatants were used to measure β-Hexonaminidase (β-Hex) (See 2.3.3.1). The dose response curve (Figure 2.2) showed that EC_{50} was 30µg/ml. Thus, we used this submaxium concentration in subsequent experiments.

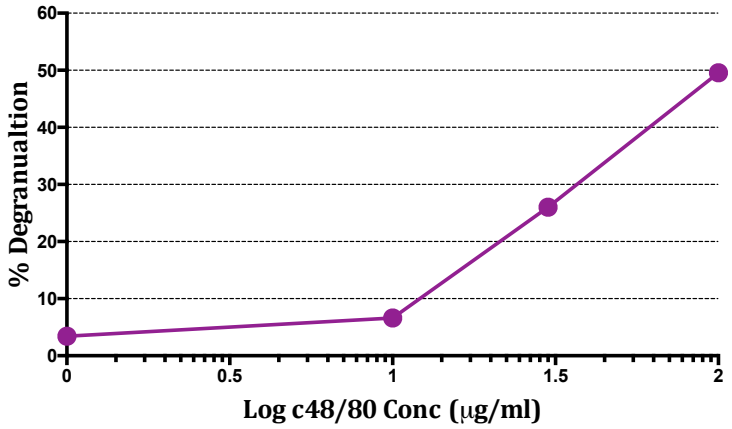


Figure. 2.2. Log dose-response curve of Compound 48/80.

Percentage of BMMC degranulation upon stimulation with different concentration of c48/80 (10, 30 and 100 µg/ml).

2.3.2 DRG stimulation

For mediator secretion assay, nociceptive neurons in coculture were stimulated with 1 µM of capsaicin (Sigma, 12084).

2.3.3 Measuring of mast cell degranulation by β-hexosaminidase release assay

β-Hexonaminidase (β-Hex) is lysosomal associated enzyme that is secreted into cell supernatants upon mast cell degranulation. β-Hex enzyme activity can be monitored by using 4-Nitrophenyl N-acetyl-β-D-glucosaminide as substrate. β-Hex convert 4-Nitrophenyl N-acetyl-β-D-glucosaminide to 4-nitrophenol that can measured at absorbance of 405nm using spectroscopy (Wendeler and Sandhoff, 2009).

For optimization β-Hex assay in respect of BMMCs cell numbers, the absorbance reading from lysates of different cell number of BMMCs were compared (Figure 2.3). On my hand, the assay was unable to detect B-Hex amount from cell culture with less than $1X10^5$ BMMCs. Thu, $2.5X10^5$ BMMCs/ well were used for all subsequent β-Hex experiments.

$2.5X10^5$ BMMCs/well were stimulated with indicated agonists (or medium for spontaneous release) for 30 minute in 37°C. Then, plate was centrifuged at 340xg for 5 min at 4ºC and 50µL of supernatant were transferred to a 96- well plate and incubated with 50µl of 2mM of substrate solution 4-Nitrophenyl N-acetyl-β-D-glucosaminide (Sigma, N-9376) diluted in 0.2 M citrate buffer (PH 4.5) (Table 2.4) for 2 hours at 37 °C. The total β-Hex value was determined by lysis the cell pellet (from each well) with 0.5% triton- X 100 in medium for 10 minutes at room temperature. Then, the plate spun again and 50µL of lysate was transferred to another substrate plate and incubate for 2 hours as the same manner as supernatant plate. The reaction was stopped by adding 90µl of 1M Trizma-HCl (sigma, T3253) buffer (pH 9.0). The absorbance of the mixture was measured at 405 nm using a microplate reader (Optima). Background absorbance readings (b) were determined from wells containing all buffers except supernatant. Experiments were carried out in duplicate in a single experiment and each experiment was repeated at least with 3 cultures.

The β-Hex activity was calculated using the following formula: d*egranulation (%) = ((supernatant -b)/(Total-b)) x100.*

Fold change of enhancement in degranulation was calculated by dividing the percentage of degranulation in tested condition to the percentage of degranulation in control condition.

Results are displayed as mean ±SEM. Significance was assessed using paired two-tailed *t*-test.

Reagent	Amount	Concentration	Supplier
Citric acid	2.34g	0.2M	Sigma, C1909
Na Citrate	2.588g	0.2M	BDH 301284C
Autoclaved ddH_2O	100 ml		
pH	4.5		

Store at room temperature

Table 2.4. Recipe of citrate buffer

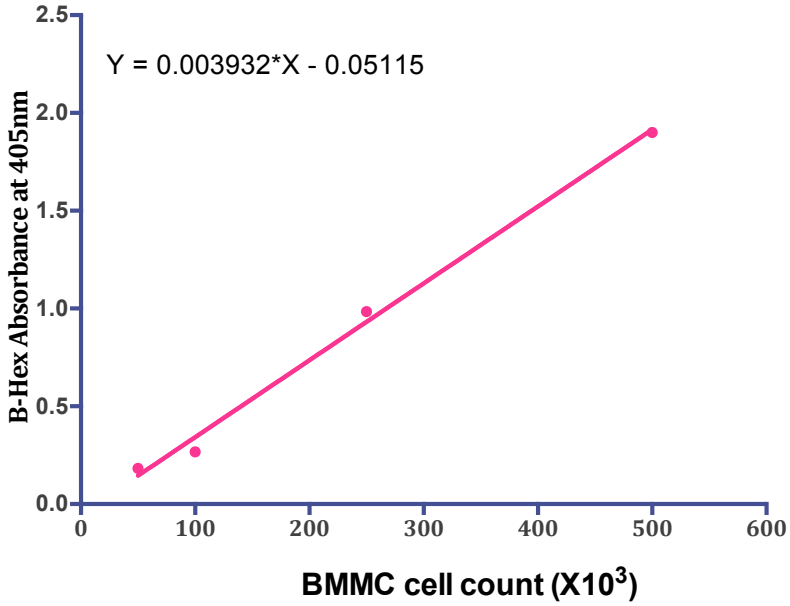


Figure. 2.3. Correlation between B-hex absorbance and number of BMMCs.

For optimization of B-Hex assay, different cell number of BMMCs were lysed with 0.5% tritonX100 and supernatants were collected.

2.3.4 Measuring cytokines release by ELISA (IL-6, and TNFα)

IL-6, and TNFα are cytokines important for both homeostatic and inflammatory functions of mast cells. To prepare lysate for the assay, $1X10^5$ BMMCs/well were stimulated with DNP for 6 hours in 37°C. Then, cell suspension was transferred to 1.5 ml

Eppendorf tubes and spun at 5000xg for 2 min at 4ºC. Supernatants were carefully collected and transferred to new tubes and stored at -20 ºC. Then ELISAs were performed according to the manufacturer's protocol (see Table 2.5 for kit numbers). Briefly, 50µl of assay diluent was added to required number of pre-coated wells. Then, 50µl of the samples and standards was added and incubated for 2 hours at room temperature. After 5 times washing, 100 µl of specific conjugate was added to each well and incubated for 2 hours at room temperature. After another 5 times washing, 100 µl of substrate solution was added to each well and incubated for 30 minutes (in dark) at room temperature. Finally, reaction was stopped by adding 100µl of stop solution to each well and absorbances determined using OPTIMA plate reader set to 450nm and corrected wavelength 540nm.

Sample concentrations were determined from the standard curve. Samples and standards were performed in duplicate and each experiment was repeated 3 times. The serial dilutions from the standard were prepared from 1000pg/ml to 7.8pg/ml, then the standard curve was constructed. The mean absorbance for each standard against concentration were plotted on the y-axis and x-axis, respectively. Then, sample concentration could be detriments from the curve. Results are displayed as mean ±SEM. Significance was assessed using two-tailed paired t-test.

Cytokine	**Supplier**	**Cat.no.**
IL-6	R&D	M6000B
TNFα	R&D	MTA00B

Table 2.5. Cytokines ELISA kits

2.4 Flurometric Calcein- adhesion assay

Calcein-AM (Vybrant Cell Adhesion Assay Kit (V13181), Invitrogen, Life Technologies) is nonfluorescent lipophilic dye. Once entered into cells, it is cleaved by endogenous esterases to calcein, a highly green fluorescent and well-retained dye. It is considered as a viability maker because it is quenched upon loss of cell viability (Lichtenfels et al., 1994).

2.4.1 BMMCs labelling with Calcein-AM

$1X10^6$ BMMCs/ ml of serum-free medium were labelled with 5µM Calcein-AM for 30 minutes at 37 °C and carefully washed three times with pre-warmed medium. After washing, cell pellet was resuspend at density of $1X10^5$ BMMCs/ 100µl/ well and used for subsequent experiments.

2.4.2 BMMC –DRG adhesion assay

BMMC –DRG adhesion assays were performed as shown in (Figure 2.4). DRG culture was seeded at density of $1.5X10^3$ cells/ well in matrigel-coated 96-well plates and incubated for 1 day. Next day, $1X10^6$ BMMCs/ ml of serum-free medium / well were labelled with 5µM Calcein-AM (Invitrogen, Life Technologies) for 30 minutes at 37 °C and carefully washed three times with pre-wormed medium. Subsequently, calcein- labelled BMMCs were resuspended with co-culture medium at density of $1X10^5$ BMMCs/ 100µl/ well and co-culture with DRG for 2 hours. Unattached BMMCs were washed out by spinning the plate upside down at 20xg for 2 minutes. Wells were filled up with co-culture medium again. The fluorescent signal was measured by plate reader before and after washing step for total and attached readings, respectively. Calcein excitation wavelength used is 485 nm and emission wavelength is 520 nm. experiments were run in duplication. Background fluorescence from DRG only wells was subtracted. Adhesion was presented as percentages of adherent BMMCs to total. For comparison control, calcein-labeled

BMMCs were seeded on 1-day old matrigel coated wells in same co-culture medium and for the same time like the ones with DRG co-culture.

When CADM1 blocking peptide was used, calcein labeled BMMCs or DRG were pre-incubated for 30 minutes prior to co-culture with 1-30 µg/ml of recommended protein against extracellular domain of CADM1.This functional blocker is fused with human IgG FC (9D2, Medical & Biological Laboratories). Peptide incubation period is followed by co-culture step without washing the peptide. All experiments were repeated at least three times.

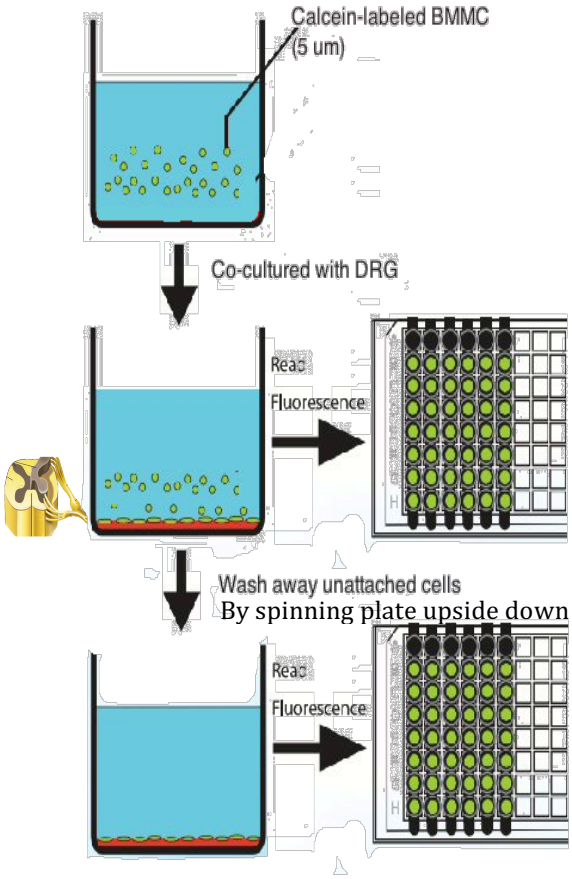


Figure. 2.4. calcein adhesion assay.

Calcein-labeled BMMCs were co-culture with 1-day old DRG for 2 hours. un-attached BMMCs were removed by spinning plate upside down (20xg) for 2 minutes. Calcein fluorescence was detected with excitation wavelength of 485 nm and emission wavelength 520 nm by plate reader.

2.4.3 BMMC –HEK adhesion assay

BMMC –HEK adhesion assay was performed in the same way as for BMMC-DRG adhesion assay except for the step of removal of non-adherent BMMCs. Because HEK cells are more delicate than neurons, using spinning method to remove non-attached BMMCs results in removing HEK cells as well. Thus, we removed non-attached BMMCs from BMMC-HEK cells co-culture by gently washing with 200 µl of PBS and filliping the plate for 3 times.

2.5 Techniques to study protein expression

2.5.1 Flowcytometry

BMMC surface protein expression was examined using flow cytometry. $1X10^6$ BMMCs/ sample were used. BMMCs were washed two times with cold PBS, at 340g for 5 min. Cells were resuspended in cold FACS buffer in concentration of $1X10^6$ cells / 100 µl/ sample. All following procedures were carried out on ice and all antibodies were diluted in FACS buffer (2 mMol EDTA in PBS with 2% FBS). BMMCs were blocked with 1:100 of Fc Block (CD16/32) (eBioscience) for 15 minutes on ice to prevent non-specific binding of antibodies. After washing with FACS buffer twice, BMMCs were incubated with the optimal concentrations of conjugated antibodies for 20 minutes on ice (Table 2.6 for list of antibodies). Next, BMMCs were washed with 0.5 ml of FACS buffer twice and spun at 340xg for 2 minutes at 4°C in between. After spinning, cell pellet was resuspended with 300 µl of FACS buffer/ tube. Fluorescence was detected with FACSCalibur (BD Biosciences) at emission wavelengths of 488 nm for Alexa Fluor® 488 labeled samples, 660 nm for APC labeled samples or 585 nm for PE labeled samples. For analysis, the viability was first gated based on side scatter and electronic volume from unstained sample. Only viable cells from gated population were included in fluorescence measurements.

For CADM1 intracellular expression, BMMCs were fixed and permeablized before staining with antibodies. To do so, cells were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20 minutes at 4°C. Then, permeablized with 0.1% tritonX100 for 10 minutes.

All antibodies used for flow cytometry experiments were conjugated except CADM1. Thus, indirect immunofluorescence staining was performed. Alexa flour @488 anti-rabbit (Invetrogen) is used as secondary antibody. For all experiments, two markers were measured simultaneously in the same samples. One of them was c-kit as a specific marker for BMMCs. All experiments were repeated 3 times.

FACS antibodies	Dilution	Supplier
APC-anti- mouse c-Kit (CD117)	1:100	eBioscience (17-1171)
PE- anti-mouse FCeRI	1:100	eBioscience (12-5898)
Rabbit anti-CADM1 Polyclonal IgG (H-300)	1:100	Santa Cruz sc-33198 lot F0407
Alexa Fluor@ 488 donkey anti-rabbit	1:1000	Invitrogen A-21206

Table 2.6. FACS antibodies

2.5.2 Immunocytochemistry

2.5.2.1 BMMC seeding

BMMC were seeded on matrigel-coated glass coverslip 16mm (see 2.1.2.1 for matrigel coating). But, because BMMCs are difficult to adherent to glass coveslip, the dilution of matrigel used for BMMC seeding was (1:3). After coating the well with matrigel, BMMC cultured for 4-6 weeks were counted (2X 10^3 cells/ well), washed and resuspended in 50µl/ well of complete IMDM. 50µl of BMMCs were loaded in the middle of each well.

Plate was incubated for 30 min then wells were flooded with complete IMDM and incubated for further 1-2 hours to ensure BMMC were well attached.

2.5.2.2 BMMC, DRG and co-culture immunocytochemical staining

Seeded BMMC and DRG on wells were washed with PBS twice. Then, fixed with 4% PFA for 10 minutes. To enhance the permeability, cells were incubated with 0.1% tritonX100 in PBS for 10 minutes. Then, incubate for 1 hour with blocking buffer (2% normal donkey serum (sigma), 0.2% Fish serum gelatine (FSG) Sigma, G7765) and 0.01% TritonX100) in PBS). After that, cells were stained with primary antibodies with specific concentration (see table 2.7 for complete list of primary antibodies) and incubated for overnight at 4°C. On next day, dishes were washed 3 times with blocking buffer. Each time for 5 minutes. Then, cells were stained with secondary antibodies with specific concentration (see table 2.7 for complete list of secondary antibodies) for 2 hours in dark at room temperature. After time, dishes were washed 3 times (each time 5 minutes) with PBS and one time with dH_2O. Glass coverslips were mounted onto microscope slides using mounding medium (Vectashield Hard set H1500, Vector) with 4',6-diamidino-2-phenylindole (DAPI) to stain the nucleus. For negative control, some wells were stained with only secondary antibodies.

For surface staining: After washing cells with cold PBS, primary antibody was added slowly to the living cells in cold PBS, and incubated for 30 minutes at 4 ^{o}C to prevent endocytosis of any added antibody. Then, dishes were washed 3 times with cold blocking buffer (without tritonX100). Each time for 5 min. Then, cells were stained with secondary antibodies for 2 hours in dark at room temperature on ice. After washing the wells 3 times with cold PBS, cells were fixed with 4% PFA at room temperature for 15 minutes. After that, coverslips were mounted as above.

Primary Antibodies	Dilution	Supplier
Rabbit anti-periphrin	1:1000	Sigma P5117
Guinea pig anti-SubP	1:100	Abcam ab10353
Mouse anti-CGRP	1:100	Abcam Ab81887
Mouse anti-B III Tubulin monoclonal IgG	1:1000	R&D MAB1195 clone TuJ-1 lot HGQ0113121
Rabbit Anti-CADM1 Polyclonal IgG (H-300)	1:300	Santa Cruz sc-33198 lot F0407
Mouse anti-Tryptase	1:300	Abcam ab2378
Goat anti FcεRIγ (A-18) polyclonal IgG	1:300	Santa Cruz (sc-33496 lot: I2811)
Alexa Fluor@ 488 anti-mouse c-Kit	1:100	Biolegend (6861)

Secondary Antibodies	Dilution	Supplier
Alexa Fluor® 488 donkey anti-rabbit	1:1000	Invitrogen A-21206
Alexa Fluor® 594 donkey anti-mouse	1:1000	Invitrogen A-21203
FITC-donkey anti-guinea pig	1:100	Millipore
Alexa Flour® 647 chicken anti-goat IgG	1:500	Invitrogen A21449 lot 1700331

Table 2.7 Antibodies List

2.5.2.3 Fluorescence and Confocal microscopy

Images were viewed using a 40X and 60X oil objective (N.A. 1.42) on Nikon A1 TIRF confocal microscope. Samples were illuminated at the required wavelength using 405nm, 488nm, 561nm lasers. Stacks of 0.5 μm thickness (z – axis) were collected for each cell studied.

2.5.2.4 Image Analysis

3 coverslips were prepared for each condition. At least 4 fields with X20 or X40 magnification from each coverslip were captured. Experiments were repeated at least three times using cultures prepared from different animals. Each experiment was designated as an "N" of one and "n" represents number of total cells. Images were analyzed using Fiji-Image J (Schneider et al., 2012).

Neurons subtype quantifications: Specific subtype immunoreactive cells were counted manually using cell counter plugin. Only neurons with a visible nucleus that were not touching the edge of the image were counted.

Neuronal morphology: To calculate soma diameter, area and neurite length, DRG cultures were stained with anti-β III Tubulin antibody to identified neurons and neurites. Soma diameter was calculated by measuring the largest axes containing the nucleus. Soma area was calculated by measuring the area of region of interest that was selected using the freehand selections tool around the edge of each soma. Neurite length was defined as the length of neurites that are initiated from one neuron. To quantified neurite length, first neurites were traced by simple neurite tracer plugin (Image J) for all neurons in the field to generate total neurite length (in μm). Then, total neurites length was divided over the number of neurons in the field (Longair et al., 2011). Neurites complexity was assessed using Sholl analysis plugin (Fiji-ImageJ) that calculated number of neurite crossing points of a concentric circle set with radii increasing by 20μm (Stanko et al., 2015).

Fluorescence intensities plots were generated in Fiji using RGB profiler plugin. (http://rsb.info.nih.gov/ij/plugins/rgb-profiler.html). After merging of two different channel images, line was drawn across cell of interest. Then, RGB profiler plugin was lunched.

For co-localization quantification, Coloc 2 plugin (Fiji) was used. After select region of interest (ROI) from 2 color channel images, Coloc 2 plugin was lunched. Colocalization significance was judged based on calculated Pearson's R coefficients. R=1 indicates a perfect colocalization and R=0 a complete exclusion.

To calculate surface FcεRI mean fluoresce intensities (MFI), particle analysis function from Fiji software was used. First, background was subtracted and threshold was adjusted before MFI plugin was used.

2.5.3 Western blot

One way of protein detection in cells is by western blotting. It consists of gel electrophoresis, a way to separate proteins based on their molecular sizes. Then, proteins are transferred onto a membrane and incubated with antibodies to allow the protein detection. Finally, protein detection is visualized using chemiluminescent or fluorescent techniques. All immunoblots presented throughout this thesis have been repeated at least 3 times. Representative examples are shown in figures.

2.5.3.1 Preparing cell lysis

For BMMCs and HEK cells, $1X10^6$ of 4- week old BMMCs and 80% confluent HEK cells were washed twice with 2 ml of cold PBS then resuspended with 200 µl of cold lysis buffer (50mM Tris-HCl, 150mM NaCl, 0.3% TritonX100, PH 8) with 1% of protease inhibitor cocktail III (PIC) (Fisher Scientific). Whereas, DRGs were isolated and all cells were cultured in one well for 24 hours to allow for neurite outgrowth. Next day, cultures were washed twice with cold PBS. Then 100 µl of cold in lysis buffer and PIC added and adherent cells were scalped by cold plastic cells scraper. Then cell suspension was collected into 0.5ml-eppendorf tube. Lysates from BMMC, HEK cells and DRG were

incubated with lysis buffer and PIC for one hour at 4°C with rotation. The insoluble debris was removed by spinning at 14,000 g for 20 minutes at 4°C. Supernatants were aliquot in new tubes (10 µl each) and stored at -80°C or proceed to Bradford assay.

2.5.3.2 Determination of protein concentration

The protein concentration of each lysate was measured before use it to obtain the same amount of each sample. To do so, Bradford protein assay was used. The color of Bradford dye is changed differently in response to varying protein concentrations. Protein concentrations for samples were calculated from a standard curve based on bovine serum albumin (BSA) standard (Sigma). Eight serial dilutions of BSA (1mg/ml) were prepared. 5 µl of diluted lysate or BSA was added to the middle of 96 well plate. Then 250 µl Bradford reagents (Sigma) was added. the absorbance measured at 595 nm with plate reader. BSA standard absorbance values were exported into Prism Graphpad and plotted against its different concentration. Samples concentrations were calculated from the curve then multiply by its dilution factor to give the protein concentration in mg/ml.

2.5.3.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The resolving and stacking acrylamide gels (Proto gel, National diagnostic) were made as described in (Table 2.8 and 2.9), respectively. For separation of CADM1 protein, 10% resolving gel was used. After cleaning the glass plates with ethanol and assembly, the resolving gel was loaded between the glass plates. Water was added to top of the gel to flatten the gel. Once the resolving gel set, water was removed and the stacking gel was added with the comb in place. Once the stacking gel set, the glass plates were transferred to running unit (Biorad) and ensure its assembly with the smaller plate inside. Then, the comb was removed carefully and the running buffer (Table 2.10) was added slowly. 5-7 µl of Pre-Stained ladder marker (Fisher Scientific) was loaded into the first well. Equal amount of protein (5-10 µg/lane) was added to 4X Lameli loading buffer (Sigma) and boiled for 5 minutes at 95°C to denature the proteins. Then, samples were carefully loaded into the wells of the stacking gel using loading tips. The gel was run at a constant

voltage of 75V till the ladder was showed up then the voltage increased to 100V for 2 hours at room temperature.

Resolving Gel	**10%**
Water	4.1 ml
Tris pH 8.8	2.5 ml
30% Acrylamide	3.3 ml
10% sodium dodecyl sulphate (SDS)	100 µl
10% Ammonium Persulfate (APS)	50 µl
Tetramethylethylenediamine (TEMED)	10 µl

Table 2.8 Resolving gel

Stacking Gel	**4%**
Water	6.1 ml
Tris pH 6.8	2.5 ml
Acrylamide	1.3 ml
10% sodium dodecyl sulphate (SDS)	100 µl
10% Ammonium Persulfate (APS)	50 µl
Tetramethylethylenediamine (TEMED)	10 µl

Table 2.9 Stacking gel

10X Running Buffer	Final Concentration	Calculated weight
Tris	250mM	30.3g
Glycine	2M	144g
sodium dodecyl sulphate (SDS)	0.1%	10g
Water		1L
	pH 8.3 store @4°C	
To make 1L of 1X: 100ml of 10X was topped up to 1 L with water		

Table 2.10. Running buffer

2.5.3.4 Electroblotting

Before the running is finished, 1L of 1X transfer buffer was prepared (Table 2.11). Nitrocellulose transfer membrane (Biorad) was soaked in transfer buffer along with sponges and filter papers. Once the transfer had finished, the gel was removed carefully from the glass and rinsed in transfer buffer. Transfer holder was placed black side on the bench and transfer sandwich was constructed in following order downside up: sponge-filter paper-gel-membrane-filter paper-sponge attention was taken to minimize air bubbles in each layer. the holder was then secured and inserted (black side of the holder to the black side of the tank) into the transfer tank with ice block. The tank then topped up slowly with transfer buffer. Transfer was performed at 85V for 90 minutes at 4°C (protein of interest 110kDa).

10X Transfer Buffer	Final Concentration	Calculated weight
Tris	250mM	30.3g
Glycine	2M	144 g
Water		1L
To make 1L of 1X: 100ml of 10X and 100ml of methanol were topped up to 1 L with water		

Table 2.11 transfer buffer

Meanwhile, 5% milk blocking buffer in TBST (Table 2.12) was prepared. Complete dissolving of milk was ensured. After finishing the transfer, the membrane was incubated for 1 hour with 5% milk blocking buffer on an orbital shaker. Then, the membrane was transferred to plastic wallet. The appropriate concentration of primary antibodies were prepared in milk blocking buffer (see table 2.13 for list of antibodies). 2ml of diluted primary antibodies was pipetted into the wallet and the wallet was sealed. The membrane was incubated with primary antibodies overnight at 4^{o}C on an orbital shaker. The following day, the membrane was washed with TBST for 3 times. Each time was for 10 minutes on orbital shaker. Then, the membrane transferred to plastic wallet again and incubated with diluted secondary antibodies in 5% milk for 2 hours (see table 2.13 for list of antibodies). In case of using Li-cor system, the following steps were carried out in dark. The membrane washed again with TBST in the same way like for primary Abs. For Licor system, the membrane was allowed to dry then the fluorescent signals were detected immediately. For chemiluminescent, the membrane was wrapped in cling film in cassette. ECL Western Blotting Detection Reagents (ThermoScientific) was used for 1-3 minutes to develop the membrane. Then, it exposed to X-ray films (Fuji) and developed using x-ray developer.

10X TBS Buffer	Final Concentration	Calculated weight
Tris	200mM	24.3g
NaCl	1.37M	80g
Water		1L
	pH 7.4	
To make 1L of 1X: 100ml of 10X was topped up to 1 L with water. then, 0.1% tween 20 was added		

Table 2.12 *TBST buffer*

Antibody	Concentration	Supplier
Mouse anti-mouse GAPDH	1:5000	Fisher Thermo
Mouse anti-mouse α-Tubulin	1:5000	Sigma
Rabbit anti-mouse CADM1	1:300	Santa Cruz
HRP- conjugated Goat- anti Rabbit IgG	1:2000	Sigma
HRP- conjugated Goat anti-mouse IgG	1:2000	Sigma
Goat IRDye 800 anti-Rabbit	1:5000	Licor
Goat IRDye 700 anti-Mouse	1:5000	Licor

Table2.13. *List of Antibodies used in western blot*

2.6 Calcium imaging

Fluorescent calcium imaging was used to quantify changes in intracellular calcium after activation. In this study, Fura-2 AM was used as a ratiometric calcium indicators. The excited advantage of ratiometric indicators is that they reduce the problems of photobleaching, uneven dye loading (Paredes et al., 2008). The peak absorbance of not bounded Fura-2 is 380nm. When it bounds to Ca^{+2}, its absorbance shifts to 340nm. This shift can be observed by measuring emission at 510nm. Therefore, using the ratio of 340/380 provides an accurate measurement of intracellular calcium regardless to the concentration of Fura-2 inside the cell. Moreover, to prevent the leakage commonly associated with fluorescent indicators, Fura-2 AM (acetoxy-methyl) was used. Form with AM group is membrane permeable. This allows for the fluorescent dye to enter and trapped into the cells. The intracellular non-specific esterases cleave off the acetoxy-methyl groups (AM) and convert the dye to the free lipophobic pentacarboxylic Fura-2 form that is membrane impermeable. All together allows accurate measurement of intracellular calcium.

2.6.1 Loading of cells with Fura-2 AM

Cultures were seeded on 16-mm glass coverslips. Coverslips were incubated with 2.5 μM Fura-2AM (frozen aliquotes of 1mM made up in Dimethyl sulphoxide (DMSO)) (Invitrogen) in medium for 45 minutes in 7.5% CO2 at 37^{o}C. Then the cells were washed with medium for 15 minutes in 7.5% CO2 at 37^{o}C, followed by 15-minute wash with external solution at room temperature. Coverslips were protected from light at all stages.

2.6.2 Experimental set up and image acquisition

Coverslips were placed into a recording chamber (bath dimensions (LxWxH) 24x13x4.1mm, volume by depth 133μl/mm) (RC-25F, Warner instruments). Coverslips were mounted onto an inverted fluorescent microscope (Axiovert S100 TV, Zeiss) and

viewed using a 40X oil immersion objective (NA 1.3, Zeiss). The external solution was perfused to recording chamber from a reservoir bottle at constant rate. Syringes on a perfusion rack were used to apply drugs when required. The outflow from the recording chamber was connected to a length of silicone tubing, with a suction pump (Watson Marlow SciQ 323) to draw the fluid into a waste bottle. To ensure carrying out the experiments at 37^{o}C, tubes containing solutions were passed through a heating block before application to cells, and a thermometer was placed in the perfusion chamber to check bath temperature.

Labeled cells were alternately illuminated at 340 and 380nm using a monochromator (Polychrome IV, TILL Photonice LPS-150). The emitted light was passed through a 510 nm filter and detected by CCD camera (Roper scientific, Photometrics). Images were analysed by regions of interest being placed around individual cells and acquired using Metamorph® software (Meta Imaging). The data were further analysed and graphed using Prism (GraphPAD software). The background fluorescence was subtracted from the fluorescence value for each wavelength. Then, the ratio of (340/380) was calculated using Microsoft excel. Δchange was also calculated using this formula (The maximum of ratio 340/380 in the presence of stimulant) – (the mean of 5 frames of ratio 340/380 prior to stimulant application).

In DRG silence experiments (Section 6.2.4), different system was used. Experiments were carried on using an inverted microscope (Olympus, IMT-2) and a 40X oil immersion objective (Olympus, 160/-, DPlanApo40UVPL). All recordings were performed at room temperature and a thermometer was placed next to the recording rig to monitor the room temperature at all times. Cells were alternately illuminated at 2 second intervals at 350 and 380nm using a Cairn Dual OptoLED light source. Images were acquired using *Simple PCI 6* software. The ratio of (350/380) was also calculated by the software.

2.6.3 Imaging solution

10mM Hepes (pH 7.4), 142mM NaCl, 5mM $NaHCO_3$, 16mM Glucose, 2mM KCl, 1mM $MgCl_2$ and 2mM $CaCl_2$, 0.1% BSA.

2.6.4 Stimulants used for calcium imaging

Pre-sensitized BMMCs were stimulated with 100 ng/ml DNP (Sigma, A6661). While, neurons were stimulated with either capsaicin (Sigma, 12084) or high-potassium solution (85mM NaCl, 5mM $NaHCO_3$, 62mM KCl, 1mM $MgCl_2$, 10mM glucose, 10mM HEPES, 2.5mM $CaCl_2$, pH 7.4).

In DRG silence experiments (Section 6.2.4), toxin cocktail (CTX) compose of 1μm from each tetrodotoxin (TTX) (Almone labs, T-500), ω–conotoxin MVIIC (Almone labs, C-150) and ω–conotoxin GVIA (Almone labs, C-300) was used. To examine the effectiveness of CTX to block AP in neurons, DRG cultures were stimulated by KCl. It has been reported that there is no significant difference in calcium signals from sequential KCl stimulations of neurons (up to 3 stimulations) (Unpublished data, Zainab Mohammed and Mohammed Nassar, 2015).

2.7 Amaxa nucleofection of BMMC

Transfection is a process of introducing exogenous nucleic acids into cells. It is a powerful tool to study gene function and intracellular signaling pathways by modifying protein production. Nucleofection is a new efficient non- viral transfection method for primary and hard–to–transfect cells. Comparing with other non-viral transfection methods, nucleofection has shown better transfection efficiency than other chemical techniques as lipofection (Jacobsen et al., 2006) and higher cell viability than other physical techniques as electroporation.

The exciting advantage of nucleofection technique is its ability to deliver the negatively charged exogenous material straight to the nucleus by using both the chemical and physical transfection principals. The chemical principal is by bounding the exogenous molecule to a nuclear-localization signal protein to form a complex in a cell–type specific solution. On the other hand, the physical principle is by using specific electrical shocks to form small pores on the cell membrane. These pores allow the molecule–protein complex to transfer into the cells. Another advantage of nucleofection is that it is independent on cell division because the exogenous material is introduced straight into the nucleus. All together make nucleofection is good choice for non-divided and hard–to–transfect cells (Hagemann et al., 2006). BMMCs are difficult to transfect most likely due to their low proliferative rate. Therefore, nucleofection by Nucleofector II (Amaxa, Lonza) was used in this study. ShRNA's used in nucleofection are listed in (Table 2.14). 3 unique constructs of CADM1 ShRNA plasmids and non-targeting scramble control (Figure 2.5) from (Genecopoeia) were used. The important of scramble control ShRNA is to test the possibility of non-specific effect of transfection.

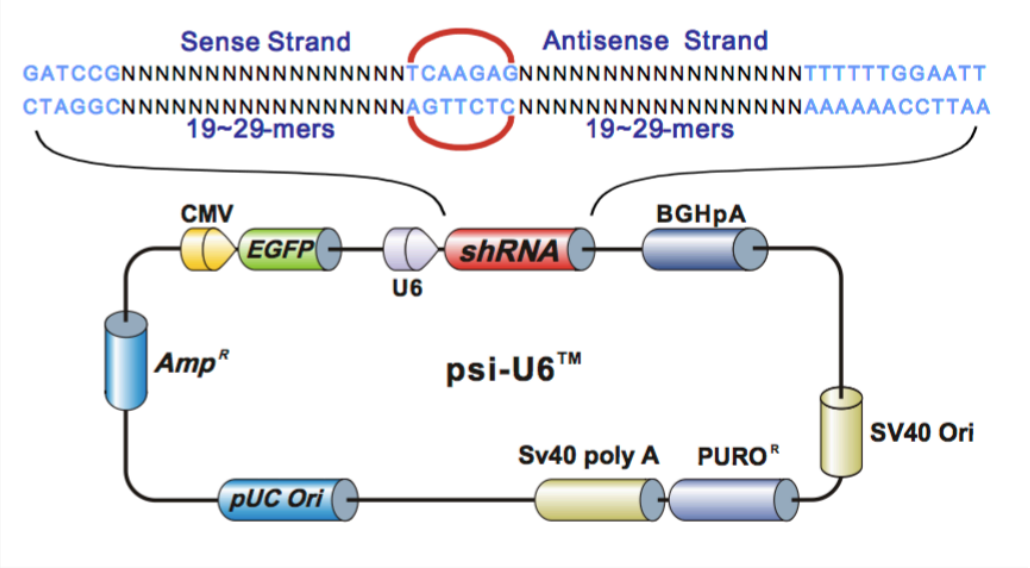


Figure. 2.5 Plasmid used for shRNA knockdown experiments

http://www.genecopoeia.com/wp-content/uploads/oldpdfs/product/shrna/psi-U6.pdf

	CAT NUMBER	PROMOTER	SELECTING MARKER	REPORTER	VECTOR	TARGET SEQUENCE
CADM1-ShRNA1	MSH031688-31	U6	Puromycin	eGFP	psi-U6	ggacagaatctgtttactaaa
CADM1-ShRNA2	MSH031688-32					cctccacgtaacttgatgatc
CADM1-ShRNA3	MSH031688-33					ggagattgaagtcaactgtac
Scramble-ShRNA	CSHCTR001					

Table 2.14. List of CADM11 ShRNA plasmid constructs

2.7.1 Transfection optimization and Efficiency analysis

The nucleofection was conducted in accordance to the manufacturer's instructions. Basic Fibroblasts nucleofector kit (VPI-1002, 90279050) and recommended programs (X-001 or T-016) were suggested by a representative from Lonza to use for BMMC. To choose the correct program, we transfected BMMCs first with 2µg pMax-green fluorescent protein (pmaxGFP) control plasmid provided with Nucleofector Kit. After 48 hours, the transfection efficacy was judged by counting GFP positive BMMC using fluorescent microscope at 488 nm wavelength (Figure 2.6). Three positions of each well of each transfection were captured and pmaxGFP positive BMMC were counted manually using Image J. Transfection efficacy for both programs was almost the same 58.2% and 53.75% for X-001 and T-016, respectively. Moreover, the viability of cells from both programs that evaluated by 0.4% trypan blue was 70%. However, cells from T-016 program look smaller. Based on this results, X-001program was chosen for subsequent transfections.

For more accurate evaluation for transfection efficiency, FACS was also conducted using a 488-nm laser (Beckman). Viability was gated based on side scatter. only viable cells were included in fluorescence measurements. Transfection efficiency was measured by the percent of total population that is expressing pmaxGFP (Figure 2.6). Results of FACS also supported the effectiveness of Amaxa nucleofection with more than 50% transfection.

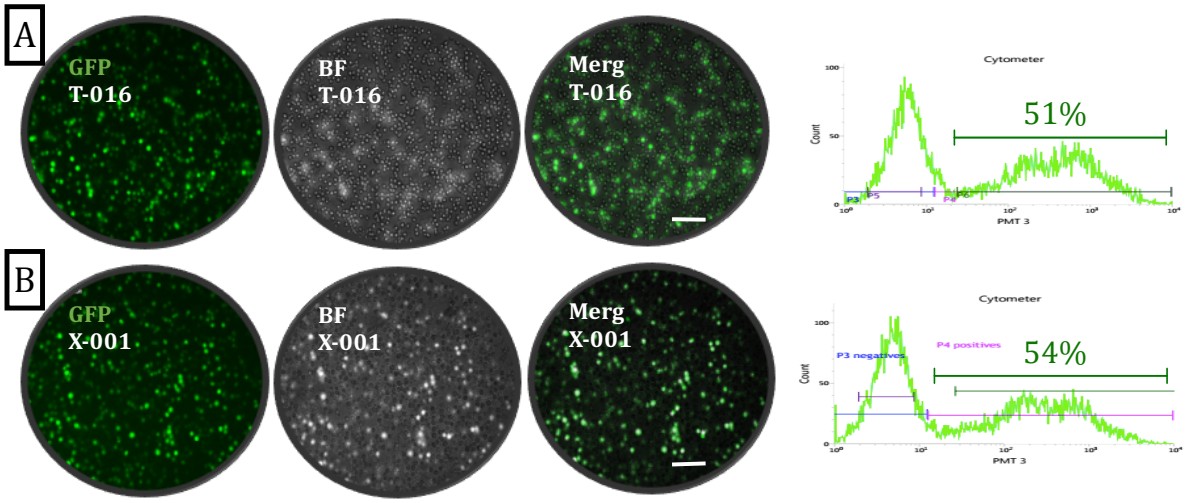


Figure 2.6: Optimization of Amaxa nucleotransfection in BMMC.

BMMCs were transfected with maxGFP-labeled plasmid at a concentration of 2 µg using program T-016 (**A**) or X-001 (**B**). 48 hours after transfection, the cells were washed and analyzed visually by fluorescence microscopy. Representative images of GFP (left), brightfield (BF) (middle) and merged (right). Scale bar represents 30 µm. Also, transfection was quantified by FACS. Histograms show measured cell count (y axis) against level of GFP florescence (x axis). GFP-transfected cells are expressed as percentage of the total cells.

2.7.2 Amaxa nucleofection for BMMC (optimized protocol)

On brief, 4-5×10^6 BMMCs / reaction were washed twice with IMDM and spun at 380xg for 5 min. After discarding the supernatant, cell pellet was resuspended with 82µl and 18µl of the cell specific Nucleofector solution and supplement (provided with kit), respectively. 2µg of CADM1 ShRNA plasmid was added and mixed. cell suspension was transferred into the Nucleocuvette (provided with kit) and ran in the Amaxa

Nucleofector II (Amaxa) using the optimized Nucleofector program (X-001). Transfected cells were pipetted out into 6-well plates. Cells were flooded with 2-ml of complete culture media with cytokines and incubated for 6 hours at 37^{o}C and 7.5% CO^{2} to allow cell recovery. Then, cells were washed with complete media to totally get rid of the transfection reagents. After 48 hours, cells were sorted using fluorescence activated cell sorting (FACS). Only the GFP expressing cells were carried out for subsequent experiments.

3 unique constructs of CADM1 ShRNA plasmids and scramble control were tested separately to identify the most efficiently transfected construct. 48 hours post transfection, cells were sorted by FACS and lysed. Knockdown efficacy was judged by western blot (Method was described in section 2.5.3). ShRNA constructs number 2 and 3 showed 100% knockdown (Figure 2.7). Thus, 1 µg of each constructs was used simultaneously for subsequent knockdown experiments, as recommended from the company.

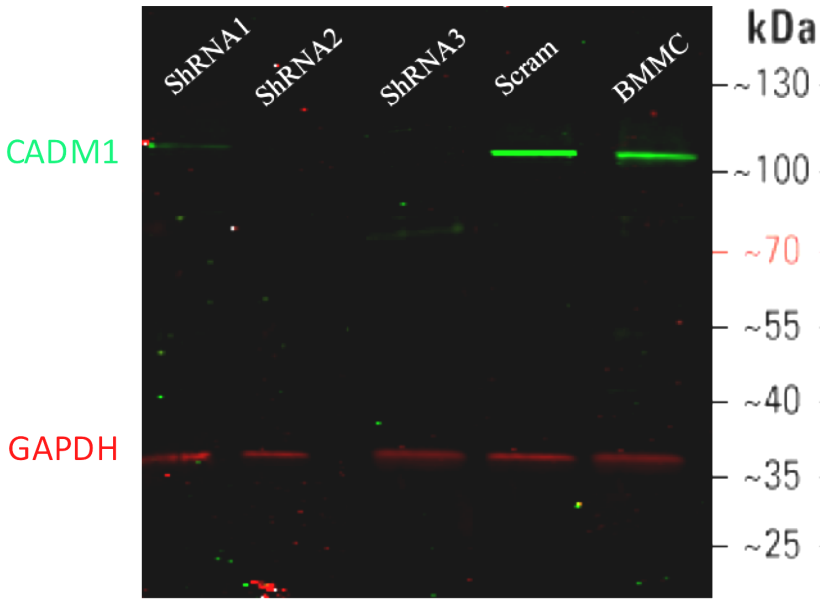


Figure. 2.7 CADM1 ShRNA constructs knockdown efficacy.

BMMCs cells were transfected with 3 ShRNA constructs using the Amaxa nucleotransfection. 48 hours post transfection cells were lysed and CADM1 expression determined by western blot. GAPDH was used as loading control. Representative examples of three experiments performed

2.8 Statistical analysis

The results are expressed in the figures as the means ± standard error of the mean (SEM) of at least 3 independent experiments; unless otherwise stated. Statistical comparisons with the appropriate controls of data from adhesion assay experiment with blocking peptide and all knockdown experiments were performed using one-way repeated measures (ANOVA) followed by Tukry's post-test. Data from other experiments were analyzed using paired t-test . Probability values (*P)* < 0.05 were considered statistically significant. All data handling, statistical analysis and graphs were performed using Graphpad Prism (version 6.0h).

Chapter 3: Characterization of bone-marrow mast cells (BMMC) culture

3.1 Introduction

Unlike other hematopoietically-derived cells, MCp originate from pluripotent bone marrow-derived stem cells (Kirshenbaum et al., 1992) (Chen et al., 2005), and circulate immature MCp in the blood (Metcalfe et al., 1997). MCp then migrate to target tissues where they differentiate into their mature phenotype under the control of tissue-specific micro-environmental factors (Galli, 1990). The major factors found to support mast cell differentiation and maturation are IL-3 (Razin et al., 1984) and SCF (Tsai et al., 1991) (Irani et al., 1992) (Mitsui et al., 1993). Mature mast cells are recognized by the expression of high levels of surface FcεRI and Kit, and functional response to IgE-mediated crosslinking and activation. Because mature mast cells are usually found only as a minor population within healthy tissues, it is problematic to isolate sufficient numbers of primary mast cells for research (as mentioned in section 1.1.6). Therefore, *in vitro* differentiation of bone marrow stem cells to mast cells is an alternative approach for generating a large number of mast cells (Razin et al., 1982b), and BMMC have been extensively used to study mast cell activation and signaling (Bischoff, 2007).

In order to use BMMCs and DRG co-culture as a model to investigate the bidirectional communications between the immune and sensory nervous systems, it was first necessary to optimize BMMC culture conditions. One of the important issues regarding BMMC culture is whether to culture BMMC with or without SCF. SCF is a cytokine which plays a major role in hematopoiesis by binding to c-kit receptors (receptor tyrosine

kinase or CD117). All hematopoietic progenitor cells express c-kit receptors, but with the notable exception of mast cells, they then downregulate c-kit expression as they mature. Mast cells are the only mature hematopoietic cells that express c-kit receptors (Okayama and Kawakami, 2006). Although mice with c-kit ($Kit^{W/Wv}$)or SCF ($Kit^{Sl/Sld}$) mutation show profoundly decrease in tissue mast cells numbers (Kitamura et al., 1978) (Kitamura and Go, 1979), mouse BMMC can be successfully differentiated and survive till maturation with IL-3 alone in the absence of SCF *in vitro* (Razin et al., 1984).

In addition to acting as a growth factor for mast cells, SCF is also reported to modulate IgE- mediated mast cell activation. While synergistic administration of SCF and Ag enhance IgE mediated activation (Bischoff and Dahinden, 1992) (Hundley et al., 2004) (Tkaczyk et al., 2004), it has been reported that chronic exposure to SCF (for 4-week-culturing duration) attenuates IgE- mediated activation in BMMC (Ito et al., 2012). Given the complexity of SCF signaling in BMMCs, I first investigated in depth the role of SCF in growth and function of BMMCs compared their properties when cultured in the presence of IL-3, or a combination of IL-3 and SCF.

3.2 Results

3.2.1 Effects of culturing BMMC with SCF on cell growth

The effect of SCF on BMMC growth was evaluated by comparing the total cell number of BMMCs in cultures with and without addition of SCF. Each BMMC preparation from one mouse was divided equally into two groups. One was cultured with IL-3 (5ng/ml) in the presence of SCF (10ng/ml) for 6 weeks termed chronic SCF-cultured (cSCF-BMMCs). The other was cultured with IL-3 alone for the same period (BMMC). Live cells were counted once per week by hemocytometer with trypan-blue staining. After 4 weeks of culture, the cell suspension of both (cSCF-BMMCs) and (BMMC) showed identical cell morphological characteristics, consisting of non-adherent, uniformly sized spherical cells. However, BMMC cultured with a combination of IL-3 and SCF showed higher cell count at every time point (Figure 3.1). Cell counts from both BMMCs culture conditions reached their maximum point at week 4 and the total cell number of BMMC cultured in the presence of SCF was significantly (P=0.04) higher (25.6 ± 3.8 x10^6 BMMC/ prep) than the total cell number of BMMC cultured with IL-3 alone (15.6 ±1.8 x10^6 BMMC/ prep). After reaching their maximum point at week 4, BMMCs from (cSCF-BMMCs) culture decreased slightly in number around week 5 (22.55 ± 1.6 x10^6 BMMC/ prep) before apparently reaching a steady state level (22 ± 1.6 x10^6 BMMC/ prep) at week 6 (end point). In contrast, BMMCs from (BMMC) cultures gradually diminished in number from week 4 until returning almost to their starting level (9.15 ± 0.15 x10^6 BMMC/ prep) after 6 weeks.

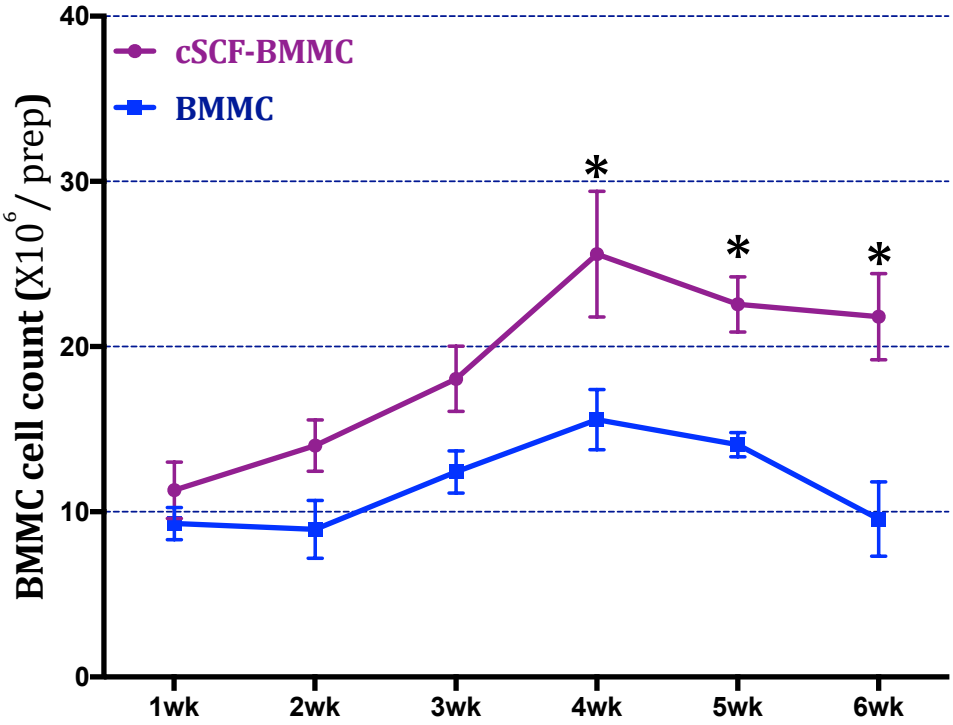


Figure.3.1 SCF enhances and prolongs BMMC growth

BMMCs were cultured with IL-3 (5ng/ml) in the presence (cSCF-BMMCs) or absence (BMMC) of 10ng/ml SCF for 6 weeks. The data shown are the mean ± SEM of 3 independent experiments, each performed in duplicate. Data were analyzed using multiple t-test followed by Holm-Sidak method. *p<0.05

3.2.2 Effects of culturing BMMC with SCF on maturation and purity

In order to investigate the role of SCF in the maturation of BMMCs, cells from each preparation were analyzed for surface expression of the high-affinity IgE receptor (FcεRI) and SCF receptor (c-kit), the classic mast cell markers, by flow cytometry. BMMCs were cultured with IL-3 with the presence (cSCF-BMMCs) or absence (BMMC) of SCF for 4 weeks. Then, BMMCs were stained with antibodies against FcεRI, and c-kit. Forward scatter and side scatter of the cells were used to determine viable cells for subsequent analysis of fluorescent-labeled antibodies in the BMMC population (Figure 3.2). (cSCF-BMMCs) and (BMMC) cultures showed the same high expression level of c-kit and FcεRI (99.3% and 99.1%, respectively). In agreement with previous reports, both preparations successfully generate a homogeneous population of typical phenotypic characteristics of mast cells (Ito et al., 2012).

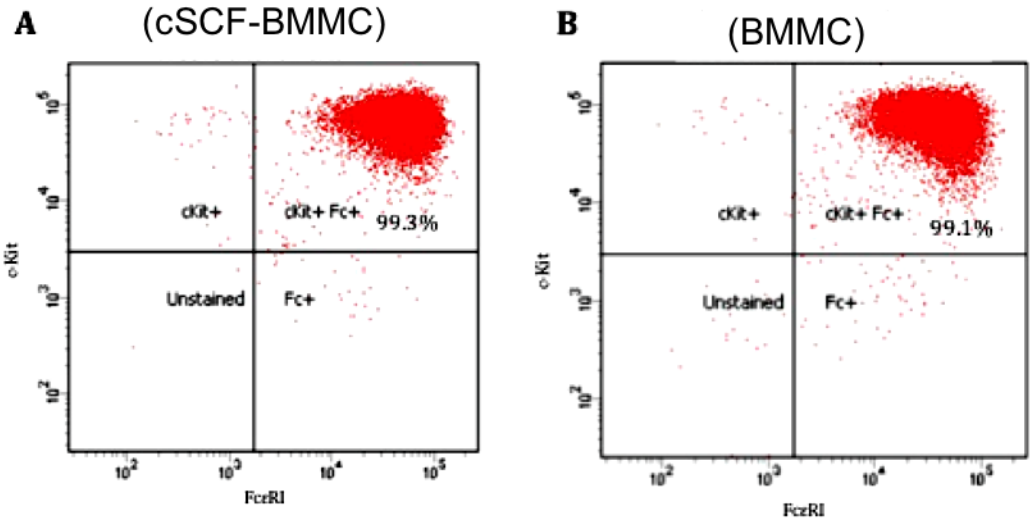


Figure 3.2 The maturation and purity of BMMCs were confirmed by flow cytometry.

Flow cytometric analysis of cell surface expression of FCεRI versus c-Kit in BMMCs. 1X10^6 BMMCs per test were cultured for 4 weeks in the presence of SCF (10ng/ml) and IL-3 (5ng/ml) (cSCF-BMMCs) (**A**) or IL-3 alone (BMMC) (**B**) Numbers in dot plots indicate the percentage of cells in the indicated gate. Representative examples of 3 independent experiments.

3.2.3 Effects of SCF on IgE-mediated mast cell degranulation

To investigate the role of chronic culturing BMMC with SCF on IgE-mediated responses, BMMC degranulation from (cSCF-BMMCs) or (BMMC) cultures were compared using β-hexaminidase (β-Hex) assay (see method, section 2.3.3) . Pre-sensitized BMMCs were stimulated with varying concentration of the antigen DNP, 0-600 ng/ml, for 30 minutes (see method, section 2.3.1.1). Both culture conditions showed concentration-dependent degranulation by DNP (Figure 3.3). There was no significant difference in the percentage of BMMC degranulation between both cultures at all concentrations of DNP tested. The highest DNP concentration (600ng/ml) induced the maximum degranulation, measured as 43.2 ± 0.93% and 42.5 ± 1.1 % from (cSCF-BMMCs) and (BMMC) cultures, respectively. These data indicate that chronic exposure of BMMCs to SCF (10ng/ml) doesn't affect IgE-mediated BMMC degranulation within the time frame and culture conditions used in the present study.

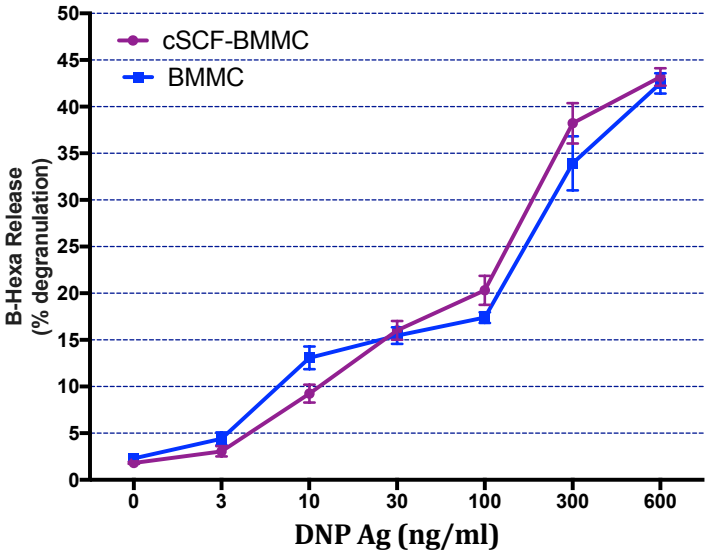


Figure 3.3. In the short term, culturing BMMCs with SCF does not alter antigen-induced degranulation.

BMMCs were cultured in IL-3 in the presence (cSCF-BMMC) or absence of SCF (BMMC) for 4 weeks. The cells were then sensitized with IgE overnight, before being challenged with the indicated concentrations of Ag (DNP) for 30 minutes and β-hex released into the extracellular medium determined and expressed as a percentage of the total cellular content of β-hex. Data shown are mean ± SEM of 3 independent experiments, each performed in duplicate. Data were analyzed using two-tailed paired *t*-test.

Next, I examined the acute effect of SCF on IgE-mediated BMMC degranulation. BMMCs were cultured in medium supplemented with IL-3 in the presence of SCF for 4 weeks (cSCF-BMMCs). Then, BMMCs were synergistically stimulated with SCF (10 and 100ng/ml) and DNP (100ng/ml) for 30 minutes. Results showed that there were no noticeable differences in percentage of β-Hex release in the absence (19.8 ± 1.2 %) or presence (20.3 ± 0.83%) of 10 ng/ml of SCF (Figure 3.4). However, at the higher concentration of SCF tested (100ng/ml), a significant (*P*= 0.024) enhancement of IgE-mediated β-Hex release (25.4 ± 1.8 %) was observed. This data demonstrates that co-stimulation of SCF enhances IgE receptor-dependent mediator release in BMMCs.

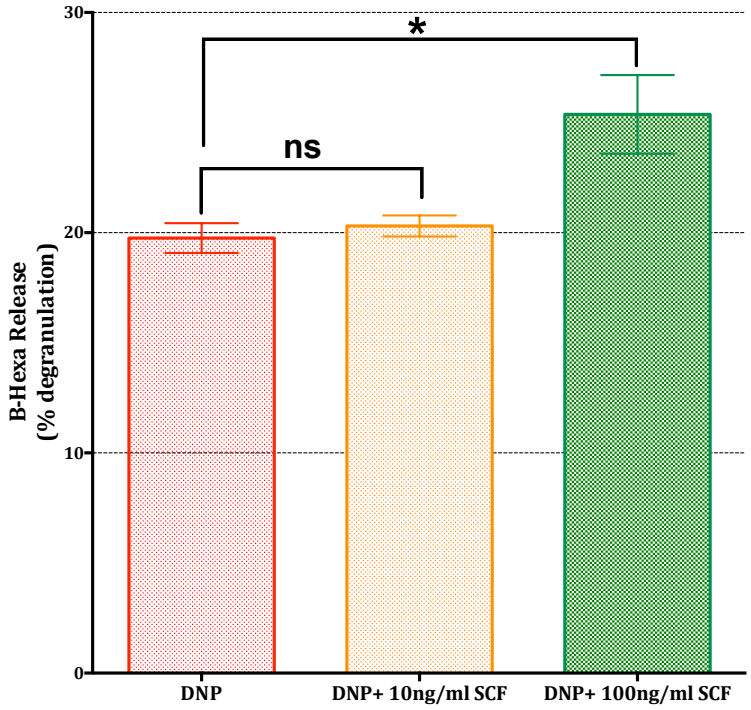


Figure. 3.4. Co-stimulation of BMMCs with SCF enhances IgE-mediated degranulation.

BMMCs were cultured in IL-3 in the presence of SCF (cSCF-BMMC) for 4 weeks. The cells were then sensitized with IgE overnight before being stimulated with Ag (100ng/ml DNP) and SCF (10 and 100ng/ml) for 30 minutes, and degranulation (β-hex) determined. Data shown are mean ± SEM of 3 independent experiments, each performed in duplicate. * indicates $p<0.05$ compared to DNP-only stimulated BMMCs. ns= non-significant. Data were analyzed using one-way ANOVA test with Turkey's multiple comparison post-test.

3.3 Discussion

Before going on to establish an *in vitro* model for studying mast cell-sensory neuron interactions, it was necessary to find the optimum protocol for generating mature, functional mast cells. Mast cells develop different phenotypical and pharmacological characteristics depending on local micro-environmental factors. Those factors include, but are not limited to, various cytokines, and inflammatory mediators (Galli, 1990) (Moon et al., 2010). These factors, in combination or alone, influence gene expression in mast cells and phenotypic alteration and tissue-specific mast cell heterogeneity. As mentioned in section 1.1.4, mast cell heterogeneity determines and regulates mediator production to different stimuli (Metcalfe et al., 1997). Moreover, mast cell heterogeneity is observed not only between species but also between organs and even within the same organ (Moon et al., 2010). This makes it difficult to isolate large numbers of mature mast cells with identical characteristics directly from tissue. As an alternative, *in vitro* culture systems from mast cell progenitors, such as BMMC, have been established to produce large numbers of homogenous mast cells (Moon et al., 2010). BMMC can be successfully differentiated and survive for a limited period with IL-3 alone without SCF (Razin et al., 1984). However, SCF/c-Kit interaction has been identified as a necessary factor for mast cell production *in vivo. Because of these conflicting observation, it was crucial to study the effect of SCF on BMMC proliferation, maturity, and pharmacological responsiveness.*

I compared two different *in vitro* BMMC culture conditions. Although IL-3 alone supported BMMC growth, addition of SCF significantly enhanced mast cells proliferation and prolonged their survival. These results are in the line with previous reports (Haig et al., 1994) (Ito et al., 2012). Similar synergistic effects of SCF with IL-3 have also been observed with peritoneal mast cells (Haig et al., 1994). The SCF enhancement effect is thought to result from its ability to prevent apoptosis (Iemura et al., 1994). Removing SCF from BMMC cultures for 6 hours, already induces a decrease in cell numbers, concomitant with condensation of nuclear chromatin and nuclear pyknosis which are characteristic morphological features associated with apoptosis.

In addition to supporting mast cell growth, SCF may also impact on mast cell maturity and FcεIR expression. Consistent with previous studies (Ito et al., 2012), I found that the presence or absence of SCF didn't change BMMC purity in term of c-kit and FcεRI expression, nor did chronic exposure to low levels of SCF significantly alter mast cell degranulation in response to Ag. This observation contrasts with that of Tomonobu Ito (Ito et al., 2012) who argue that chronic exposure to SCF results in a hypo-responsiveness phenotype to Ag caused by cytoskeletal reorganization. The time of exposure to SCF may be important for observing this hypo-responsiveness as another study reported there is no difference in BMMC response to IgE stimulation when cultured with 100ng/ml of SCF for 12 days, if the exposure is extended beyond 12 days then a significant decrease in degranulation is observed (Takano et al., 2008). SCF concentrations used in these previous studies (Ito et al., 2012) (Takano et al., 2008) were notably 10-times higher than used here . I decided to use the physiological concentration of SCF in tissue. The normal level of SCF in serum of 257 healthy subjects measured using ELISA was 3-8 ng/mL (Langley et al., 1993). In peritoneal fluid of 32 healthy females, the average SCF concentration was reported to be 2.84 ng/ml (Osuga et al., 2000). At these low concentrations, SCF may support mast cells proliferation and chemotactic migration but do not cause activation.

In contrast to the effect of chronic SCF on IgE-mediated degranulation, acute stimulation of BMMCs with SCF concomitantly with antigen enhanced IgE-mediated degranulation. This observation is reported by many authors and is a common finding with mast cells isolated from different tissues and species (Bischoff and Dahinden, 1992, Hundley et al., 2004) (Tkaczyk et al., 2004) (Hill et al., 1996). While SCF by itself does not induce degranulation or cytokine production, it synergizes with Ag to enhance B-hex in rat BMMC (Hill et al., 1996) and human mast cells (Hundley et al., 2004) (Tkaczyk et al., 2004) and histamine release and cytokine productions (Bischoff and Dahinden, 1992).

SCF enhances IgE- mediated activation by interact with its signaling pathway (Figure 3.5). As discussed in (section 1.1.2.1), IgE crosslinking results in a sequence of

phosphorylation events. IgE and c-kit share many downstream signaling molecules such as PLCγ, PI3K and cytosolic adapter molecules Grb2 (Tkaczyk et al., 2004). In addition, both pathways activate MAPK/STAT and NFκB pathways that is important for some of cytokine production such as IL-6 (Akira, 1997) (Kalesnikoff et al., 2002). However, unlike IgE that activates Src kinase to phosphorylate the transmembrane adapter molecules LAT1 and LAT2, c-Kit directly phosphorylates only LAT2 by its intrinsic tyrosine kinase (Tkaczyk et al., 2004). Inability of c-kit to activate LAT1 and NFAT pathways could explain its inability to mediate degranulation and some of cytokine production, respectively, in the absence of Ag (Gilfillan et al., 2009) (Klein et al., 2006).

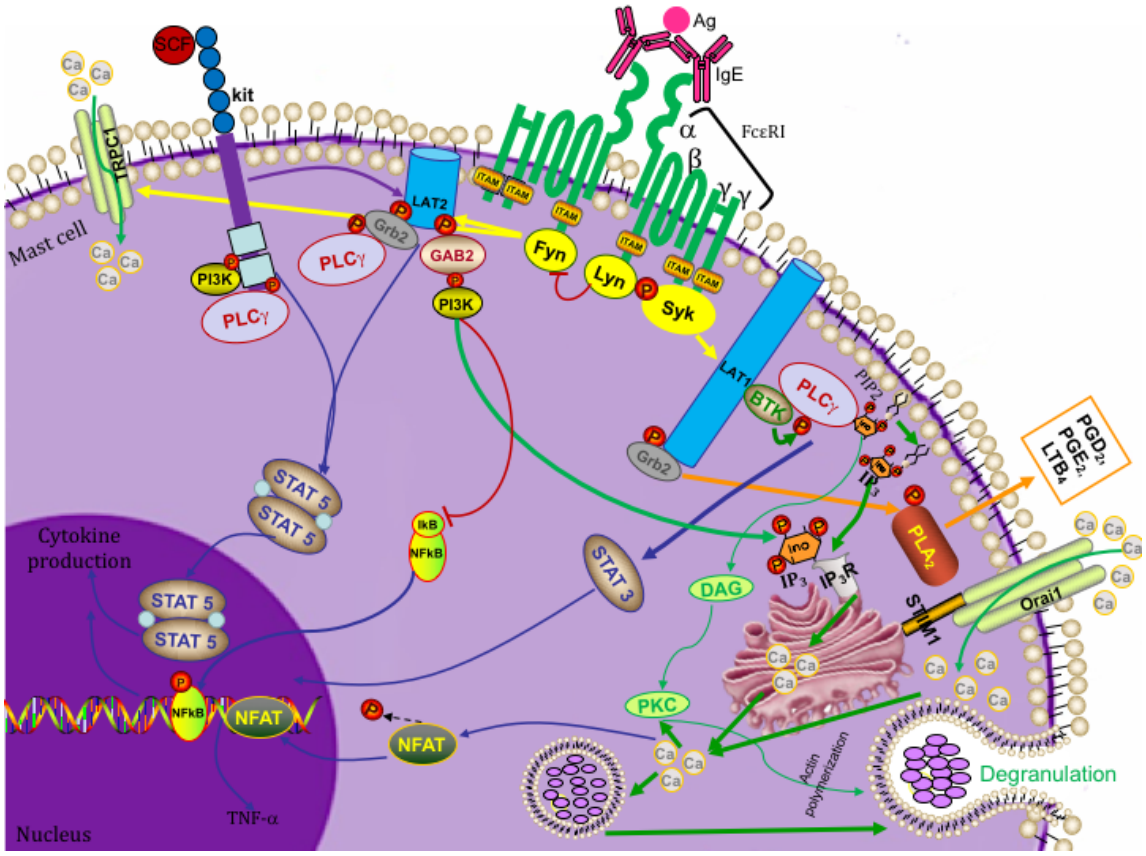


Figure. 3.5 Integration of mast cells signalling pathways mediated by FcεRI and c-kit.

Upon cross linking of FcεRI by IgE and SCF binding to c-kit, a series of phosphorylation and recruitment of intracellular molecules, leads to a large increases in intracellular calcium ultimately results in degranulation and cytokine production. The Figure is modified from Figure 1.1

My data showed in general that, except for cell growth, chronic exposure of BMMCs to low concentrations of SCF has no significant effect on maturation or IgE-mediated activation. Indeed, BMMC can be cultured and survive with IL-3 alone *in vitro*. However, there is one crucial reason why supplementing the culture media with SCF is desirable, and that is the fact that mast cells can't survive *in vivo* without SCF. Mutations at the W locus that encodes c-kit or at the S1 locus that encodes SCF result in mast cell deficiency in mice (Kitamura et al., 1978) (Kitamura et al., 1987). Even *in vivo*, mature mast cells within tissues are continuously exposed to soluble or transmembrane SCF from neighboring cells such as fibroblasts, smooth muscle cells and endothelial cell (Metcalfe et al., 1997). Indeed, IgE-mediated mast cell activation, *in vivo*, likely occurs in a background of SCF and SCF-c-kit signaling. To mirror this *in vitro*, BMMCs were cultured with 10ng/ml SCF for 4 weeks for all subsequent BMMC preparations till maturation. Then, BMMC were cocultured with DRG without SCF, as well as, all IgE-mediated mast cell activation experiments reported in this thesis were carried out without the presence of SCF to eliminate the possible enhancement effect of SCF on IgE-mediated mast cells activation.

The present study has characterized BMMCs in culture supplemented with IL-3 and SCF for 4 weeks. BMMCs have a phenotypic character of mature mast cells as judged by their morphology, >99% pure FcεRI^{+} Kit^{+} cells, and their degranulation in response to IgE-mediated stimulation. Although mouse mast cells can differentiate and survive in culture supported with IL-3 only, culturing BMMC in the presence of SCF enhances the growth and survival of mast cells. These results emphasize that mast cell phenotypic and functional characteristics are influenced by micro-environmental factors.

Chapter 4. The Impact of sensory neurons on mast cells function

4.1 Introduction

The increased number of mast cells and mast cell-nerve associations have been described in many neurogenic inflammatory disorders like irritable bowel syndrome (Barbara et al., 2004, Park et al., 2003), and fibromyalgia (Blanco et al., 2010). Based on these observations *in vivo*, the functional bi-directional crosstalk between mast cells and neurons was studied *in vitro*. The majority of those *in vitro* studies used co-culture model of mast cells and neurons (Furuno and Nakanishi, 2011). This model is very informative regarding the molecular and structural formation of this neuroimmune synapse. It provides a useful system to study the mechanism of direct mast cell crosstalk to neurons without the influence of other intermediary cells. However, many different protocols have been developed to co-culture mast cell and neurons *in vitro.* The first effort to establish mast cell-neuron co-culture was by using RBL cell line, which are widely used as model mast cells (Passante et al., 2009), and neurons isolated from murine SCG (Blennerhassett and Bienenstock, 1990) (Blennerhassett et al., 1991). But since RBL is a cancerous cell line and transformation can alert normal cell functions (See introduction section 1.1.6.2 and Table 1.3), the co-culture model was improved to use murine bone marrow-derived mast cells (BMMC) as a model for mucosal mast cells instead of RBL. Along the same line, sympathetic SCG is replaced by adult murine DRG. DRG cultures have been widely accepted as standard model for peripheral sensory neurons to study pain mechanism *in vitro* (Melli and Höke, 2009) (See section 1.2.4).

The success of BMMC-DRG co-culture model attracted much interest to study mast cell-nerve interactions *in vitro*. However, most of these studies investigated the crosstalk in

this functional unit by activation of either neurons or mast cells and then look for other cell type response (Section 1.3.1). It has shown that the electrical or chemical stimulation of nerves activate mast cells, and that mast cell-derived mediators can evoke neuronal response (Janiszewski et al., 1990) (Blennerhassett et al., 1991) (Suzuki et al., 1999) (Suzuki et al., 2001) (Mori et al., 2002) (De Jonge et al., 2004) (Furuno et al., 2004) (Nakanishi and Furuno, 2008). However, the effect of close contact between these two cells on mast cells functions, in particular their response to Ag, is still unknown. Here in this chapter, I describe the results of experiments I performed to investigate the effect of sensory neuron interactions on the biological function of mast cells, in particular, how this interaction may modify IgE-mediated activation. I also examined how activation of sensory neurons impacts mast cell activation, with a view to gaining a better understanding of how mast-cell-sensory neuron crosstalk may be altered in pain . Finally, I examined the specific adhesion of mast cells to neurites. But before all that, I characterized my DRG culture. Then, I investigated the effect of co-culturing BMMC on DRG culture morphology.

4.2 Results

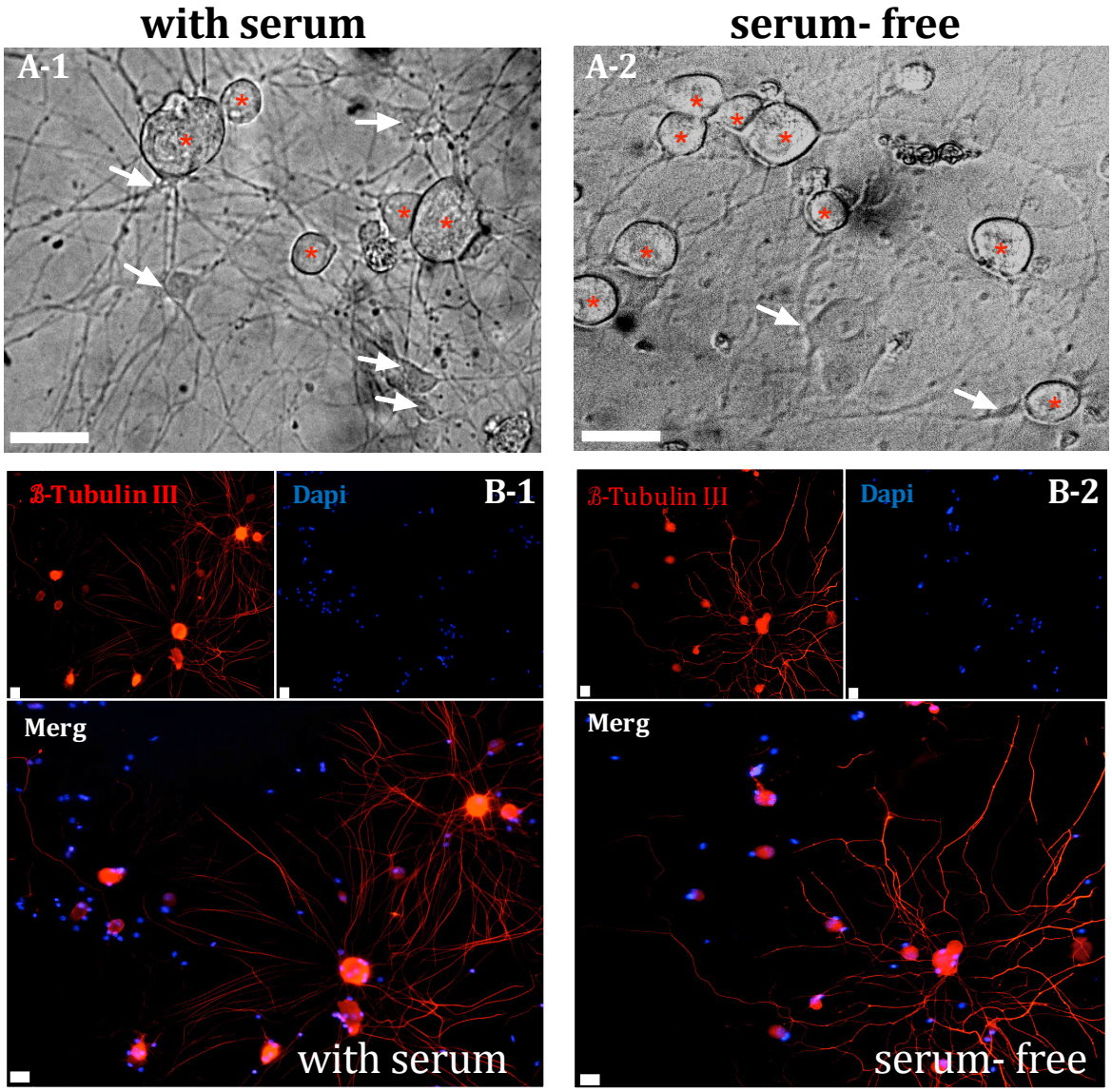
4.2.1 Characterization of primary DRG culture

There are many different protocols to culture primary DRG neurons. Because I aimed to study the direct communication between mast cells and sensory neurons without the influence of other intermediary cells, and because typical dissociated primary DRG culture is heterogeneous with mixture of neurons and non- neuronal cells, the neuronal purity of DRG culture was my primary concern in evaluating the best dissociation and culture conditions to use in my research. As described in methods (Section 2.1.2.2), to limit the proliferation of non-neuronal cells in DRG cultures, I used B-27 supplement instead of serum and anti-mitotic reagent Cytosine β- D -arabinofuranoside (Ara-C) that slows glial cell division.(Liu et al., 2013). Although DRG can be cultured from neonatal or adult animals, I decided to isolate DRG from adult mice (8-12 weeks) for 3 reasons (Melli and Höke, 2009): **1)** cultures prepared from isolated adult DRG offer a model more similar to *in vivo* DRG neurons because they provide completely developed and mature neurons, **2)** survival of adult DRG neurons is not dependent upon serum so they can be cultured in defined media, without serum the density of non-neuronal cells can be more easily controlled. **3)** I am interested in studying BMMC-DRG co-culture as a model of neurogenic inflammatory diseases that are more common in adulthood.

To examine the purity of my neuronal cultures, I first isolated DRG from 8-12 week old male mice, and cultured the cells recovered following enzymatic digestion either in serum-free medium supplemented with 1 μM Ara-C (serum- free culture) or with 10% heat inactivated FBS and without Ara-C (culture with serum) for 72hr (the detailed protocol is in section 2.1.2.2). The purity of neurons in the cultures was quantified by immunocytochemistry and expressed as the percentage of anti-β III Tubulin positively stained cells relative to the total number of DAPI positively stained cells.

Bright-field images of cultures prepared with both protocols (Figure 4.1 A1-2) revealed a heterogeneous population of bright round soma of different sizes with extensive outgrowth of neurites which formed networks (asterisks). In both cultures, a thin layer

of glia growing beneath the neurons was identified as small, dark and spindle-shaped cells (arrows). All these observations were reported in previous studies (Liu et al., 2013). Under bright-field microscopy, neuronal and glial populations can be distinguished by their morphologies. Analysis of fluorescent images (Figure 4.1 B1-2), showed that growing cells in serum free defined media yielded a culture consisting of 30% β III tubulin -positive neurons, this was decreased to 7% in cultures grown with serum (Figure 4.1 C). Thus, the purity of neuronal cells in serum- free culture is significantly higher than culture with serum by 4-fold (P=0.0145) .


with serum
serum- free
A-1
A-2
B-Tubulin III
Dapi
B-1
B-Tubulin III
Dapi
B-2
Merg
Merg
with serum
serum- free

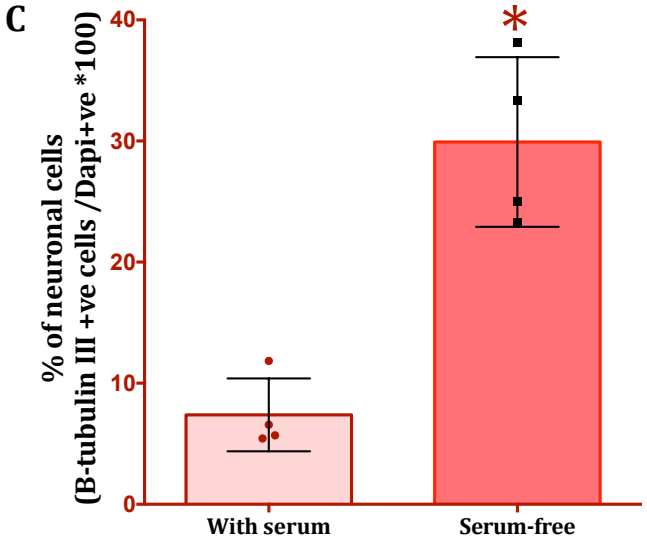
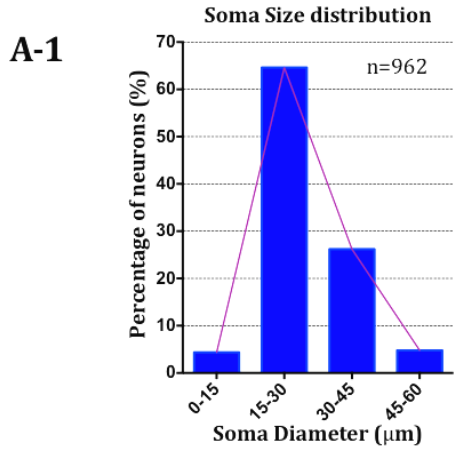

C
% of neuronal cells
(B-tubulin III +ve cells /Dapi+ve *100)
40
30
20
10
0
*
With serum
Serum-free

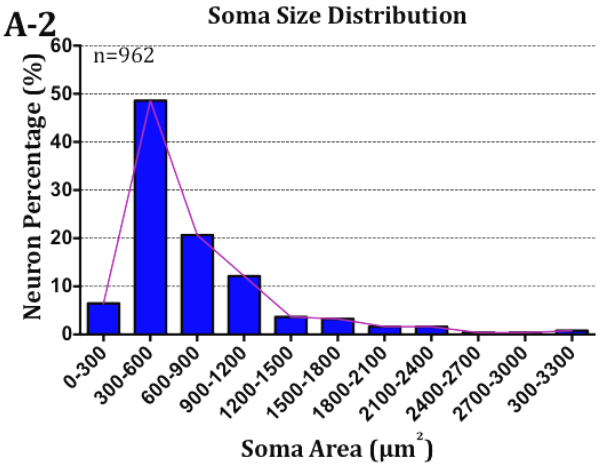
Figure. 4.1. Effect of Ara-C and serum-free medium in controlling proliferation of non-neuronal cells in cultures of primary DRG.

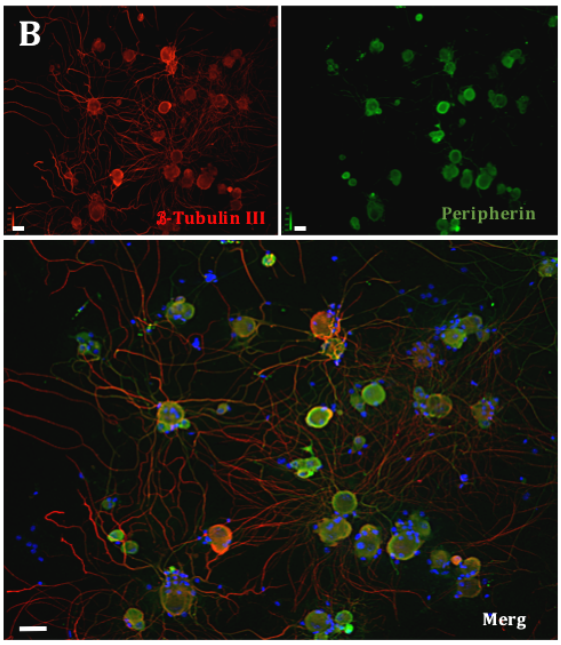
(Figure of the previous page) Isolated DRG cultured with 1 μM Ara-C and serum free medium or with serum for 72hr. Bright-field images from culture with serum (**A-1**) and serum free culture (**A-2**) showing that the primary culture of DRG consists of a heterogeneous population of neurons with different soma size and they exhibit a network of neurites. The arrows indicate non-neuronal cells while asterisks indicate somas. (**B-1**) (**B-2**) Showing β-Tubulin III immunocytochemistry staining for DRG culture from culture with serum and serum free culture, respectively. The purity of DRG culture was expressed as percentage of β-Tubulin III immunoreactive cells to total cells in control culture with serum and serum free cultures (**C**) * denotes $P<0.05$ paired students *t*-test (N=4). Scale bar represents 25μm

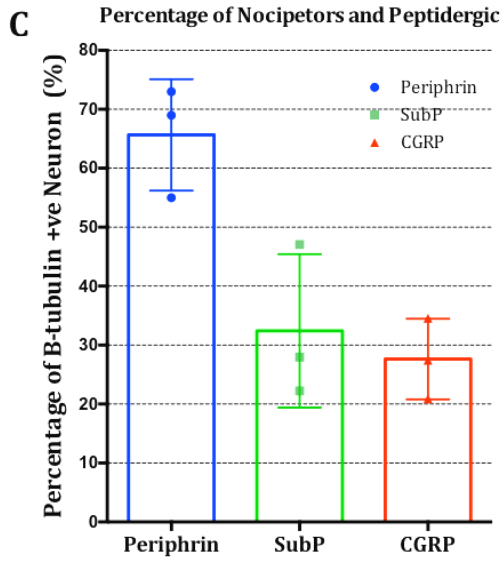
To characterize neurons based on their function, I examined the proportion of nociceptive and mechanoreceptive neurons in my protocol. The simplest method to define neurons to nociceptive and mechanoreceptive is by measuring their soma size (Yoshida and Matsuda, 1979) (Harper and Lawson, 1985b) . Soma size can be expressed as diameter or area. As defined previously by Scoggs and Fox (1992) , small neurons (diameter: < 30 μm and area: <600 μm^2) are considered to be nociceptive while medium (diameter: 30-40 μm and area: 600-1200 μm^2) and large neurons (diameter: > 40 μm and area: >1200 μm^2) are mechanoreceptive (Scroggs and Fox, 1992). The soma size distribution of neurons from my protocol was unimodal with more than 60% of the population were small-sized nociceptive neurons (Figure 4-2A). To confirm this result, cells were stained with peripherin, a neurofilament protein expressed selectively in nociceptive neurons (Goldstein et al., 1991). To provide the total number of neurons in DRG culture, cells were also stained with anti-β III Tubulin (Figure 4-2 B). Data are expressed as the mean ± SEM. The result shows that 66 ± 5 % of β III Tubulin-immunoreactive neurons are positive for peripherin (Figure 4-2 C). Taken together, the data confirms that my protocol generates neuronal cultures enriched for nociceptors.

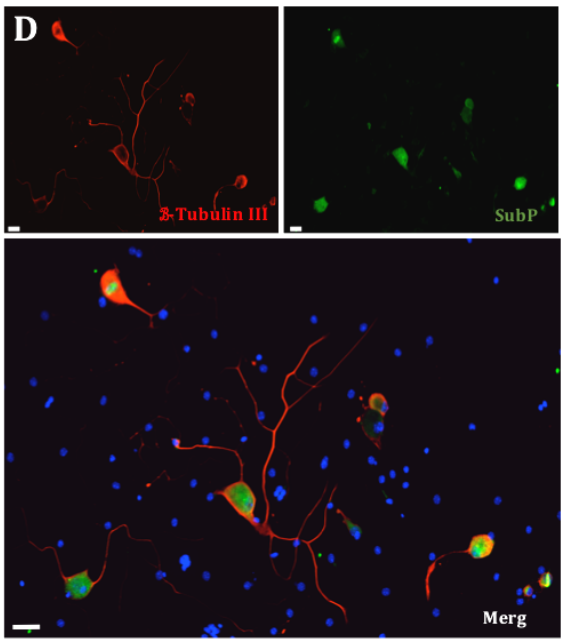
Nociceptors can be further categorized into peptidergic and non-peptidergic subsets. Peptidergic neurons contain subP and CGRP, while non-peptidergic do not. To further complete the picture, neurons were stained with antibodies to subP and CGRP (Figure 4-2 D-E). Then, cells were quantified as the percentage of total β III Tubulin-immunoreactive neurons(Figure 4-2 C). Results showed that 32 ± 8 % and 28 ± 4 %of β III Tubulin-immunoreactive neurons were positive for subP and CGRP, respectively. Collectively, my protocol produces neuronal culture with at least 60% nociceptors and 50% of the nociceptors are peptidergic.


A-1
Soma Size distribution
n=962
Percentage of neurons (%)
0
10
20
30
40
50
60
70
0-15
15-30
30-45
45-60
Soma Diameter (µm)


A-2
Soma Size Distribution
n=962
Neuron Percentage (%)
0
10
20
30
40
50
60
0-300
300-600
600-900
900-1200
1200-1500
1500-1800
1800-2100
2100-2400
2400-2700
2700-3000
300-3300
Soma Area (µm²)


B
β-Tubulin III
Peripherin
Merg


C
Percentage of Nocipetors and Peptidergic
Percentage of B-tubulin +ve Neuron (%)
0
10
20
30
40
50
60
70
80
Periphrin
SubP
CGRP
Periphrin
SubP
CGRP


D
β-Tubulin III
SubP
Merg

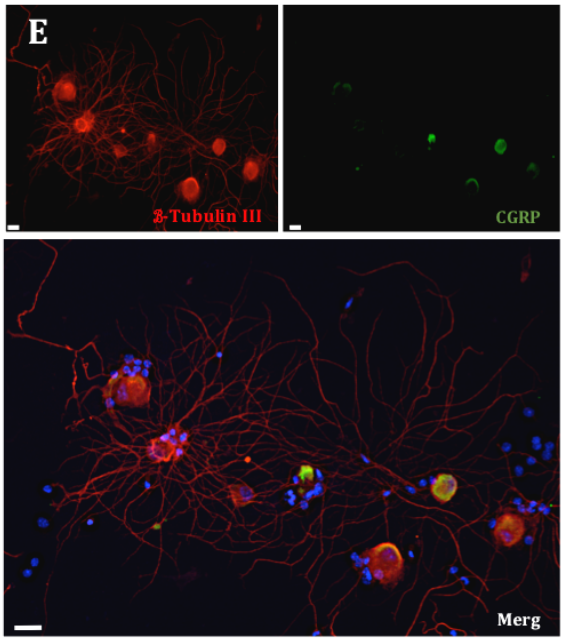

E
β-Tubulin III
CGRP
Merg

Figure. 4.2. Characterization of DRG culture.

(Figure of the previous page) Isolated DRG cultured with 1 μM Ara-C and serum free medium for 72hr. Soma size distribution based on soma diameter (**A-1**) and area (**A-2**) showing that my DRG preparation is enriched for small- nociceptors <30 μm in diameter. (**B**) Immunocytochemistry visualizing β-III tubulin and nociceptor marker peripherin. (**C**) Percentage of cells positive for peipherin, substance P (SubP) and calcitonin gene-related peptide (CGRP) as divided by number of β-III tubulin immunoreactive neurons (N=3). (**D**) Immunocytochemistry is visualizing β-III tubulin and SubP. (**E**) Immunocytochemistry is visualizing β-III tubulin and CGRP. Scale bar represents 25μm

4.2.2 Effect of co-culture of BMMC with DRG on neuronal morphology

It has been reported that the length of SubP- immunoreactive nerve fibers is increased in airway of allergic conditions such as asthma (Ollerenshaw et al., 1991). In addition, activated mast cell produce NGF and TNFα that could enhance neuronal outgrowth (Leon et al., 1994) (Kakurai et al., 2006). Thus, I aim here to study the effect of co-culturing BMMC on neuronal morphology, I measured total neurite length and complexity (number of neurite crossing points of a concentric circle set with radii increasing by 20μm) in BMMC-DRG co-culture (detailed method is in section 2.5.2.4). To control for the effects of BMMC media on neuronal morphology, parallel experiments were performed with DRG alone treated with complete co-culture medium and IL-3 for 24 hours. The neurites and BMMCs were detected by immunocytochemistry staining for βIII-tubulin and c-kit, respectively (Figure 4-3 A-B). Culturing BMMCs with DRG showed no significant difference in total neurite length (Figure 4-3 C) from DRG monoculture (2997μm and 2530μm), respectively (P=0.35). Co-culturing BMMC with DRG showed some enhancement in neurite complexity compared to DRG monoculture (Figure 4-3 D) but this did not achieve statistical significance. In the case of DRG monoculture, the mean number of crossings reached a maximum of 19 around 60 μm from the soma while in co-culture a maximum of 22 crossings were measured at the same distance. Taken together, the data indicate that co-culturing BMMC with DRG does not alter neuronal morphology.

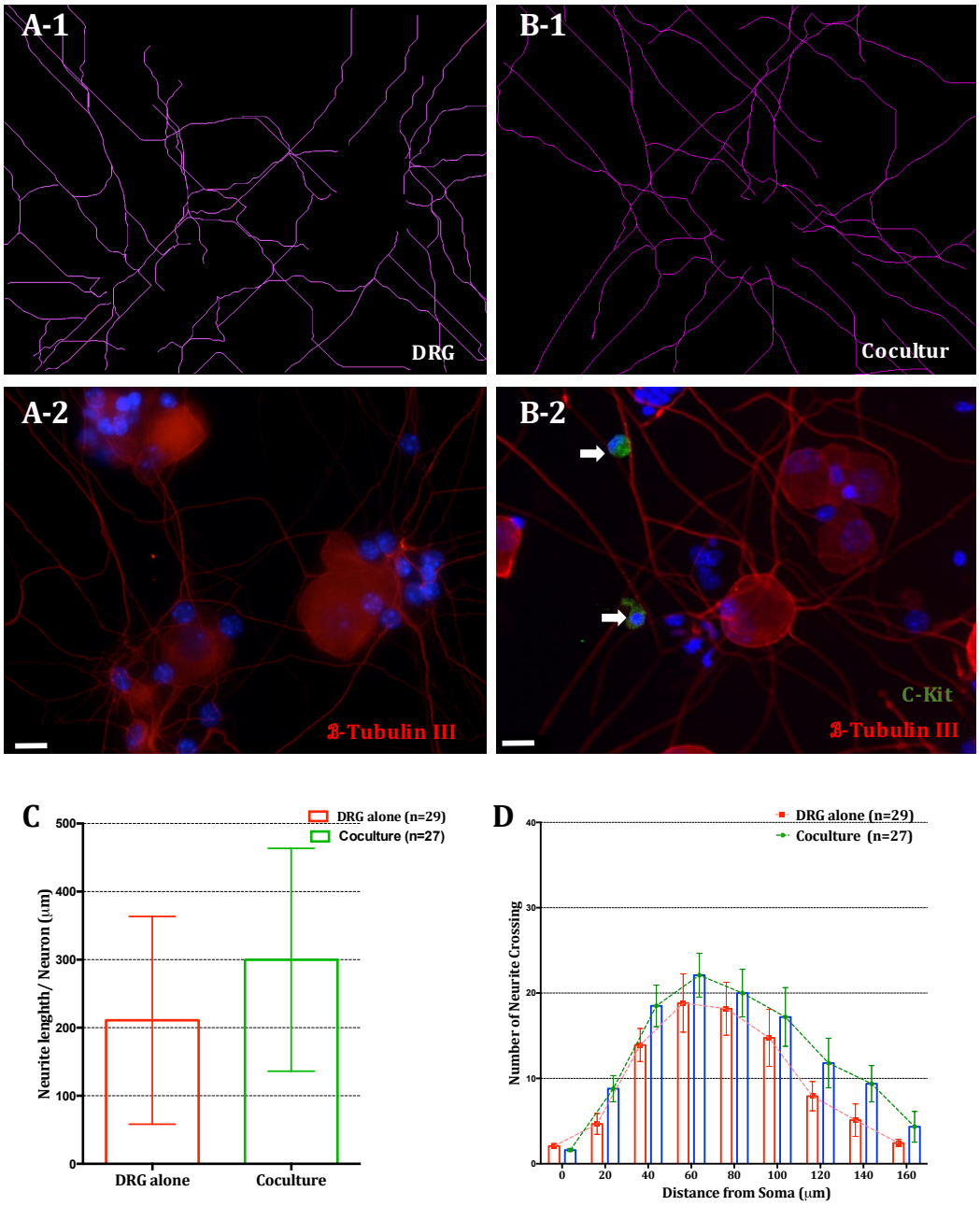


Figure. 4.3. Effect of BMMC co-culture with DRG on neuronal morphology.

(**A1-B1**) Representative schematic tracings of the neurites from DRG culture alone or BMMC-DRG co-culture, respectively. Soma and neurites were visualized with β-III tubulin and c-kit in DRG (**A2**) and co-culture(**B2**). (**C**) Shows quantitative analysis of total neurites length in DRG alone and co-culture using unpaired *t*-test. (**D**) Shows plot of sholl analysis of the neurites. Each bar represents the mean ±SEM of number of crossing neurites found in each given distance from the soma. Statistical analysis using multiple T-test. N=3. Scale bar represents 10μm

4.2.3 Neurons enhance IgE-mediated mast cell degranulation *in vitro*

The observation of significant increases in the number of degranulated mast cells in proximity of mucosal innervation in biopsies from IBS patients with respect to controls (Park et al., 2003) (Barbara et al., 2004) raises the interesting possibility that neurons could influence mast cell degranulation. To investigate this possibility, I co-cultured IgE-sensitized BMMC with DRG for 24 hours. I then stimulated BMMC cultured alone or co-culture with DRG with the antigen (Ag) DNP, and I measured the percentage of β-hexosaminidase (β-hex) release in Ag-independent (Ag-Indep) BMMC and Ag-activated BMMC (the detailed protocol is in section 2.3.1 and 2.3.3) . Initially, the percentage of β-hex release was measured from supernatant collected at several time points (2-6-24 hours) following BMMC co-cultured with DRG. I found that the percentage of β-hex released from BMMC in co-culture increased slightly with time (Figure 4.4 A). In contrast, the percentage of β-hex release from BMMC alone stayed approximately the same across the three time points investigated. The percentage of Ag-activated degranulation from BMMC co-cultured with neurons for 2 hours was 28 ± 1 % (N=3, each performed in duplicate) while co-culture for 24 hours increased the percentage of Ag-activated degranulation up to 33 ± 2 % . Similarly, the percentage of antigen-independent degranulation from BMMC in co-culture increased from 6 ±2% following 2 hours co-culture to 9± 0.6% after 24 hours of co-culture. However, the maximum of Ag-independent and Ag-activated degranulation from BMMC in co-culture at 24 hours was 2.5-fold, and 1.6 fold increased, respectively, compared to degranulation in BMMC alone (Figure 4.4 B). These results indicate that neuronal co-culture with mast cells not only activate mast cell but also enhance their response to Ag. Interestingly, both effects are increased with the time.

Next, I investigated the kinetics of the effect of sensory neuron co-culture on the enhancement of Ag-activated BMMC degranulation. I compared the degranulation from BMMC alone or in co-culture at each time point. At the 2-hour time point, the presence of neurons did not significantly change Ag-independent nor Ag-activated degranulation (P=0.084 and P=0.16, respectively) (Figure 4.4 C1, D1). In sharp contrast, after 6 hours

of co-culture of BMMC with neurons, both Ag-independent and Ag-activated BMMC degranulation was significantly enhanced (P=0.007 and P=0.046, respectively) (Figure 4.4 C2, D2). In the same way, co-culture of neurons with BMMC for 24 hours caused a significant augementation of both Ag-independent and Ag-activated BMMC degranulation (P=0.0091 and P=0.0002, respectively) (Figure 4.4 C3, D3). These findings suggested that the mechanism of neuronal-enhancement of mast cell degranulation is relatively slow with significant onset after 6 hours and increasing over 24 hours of co-culture.

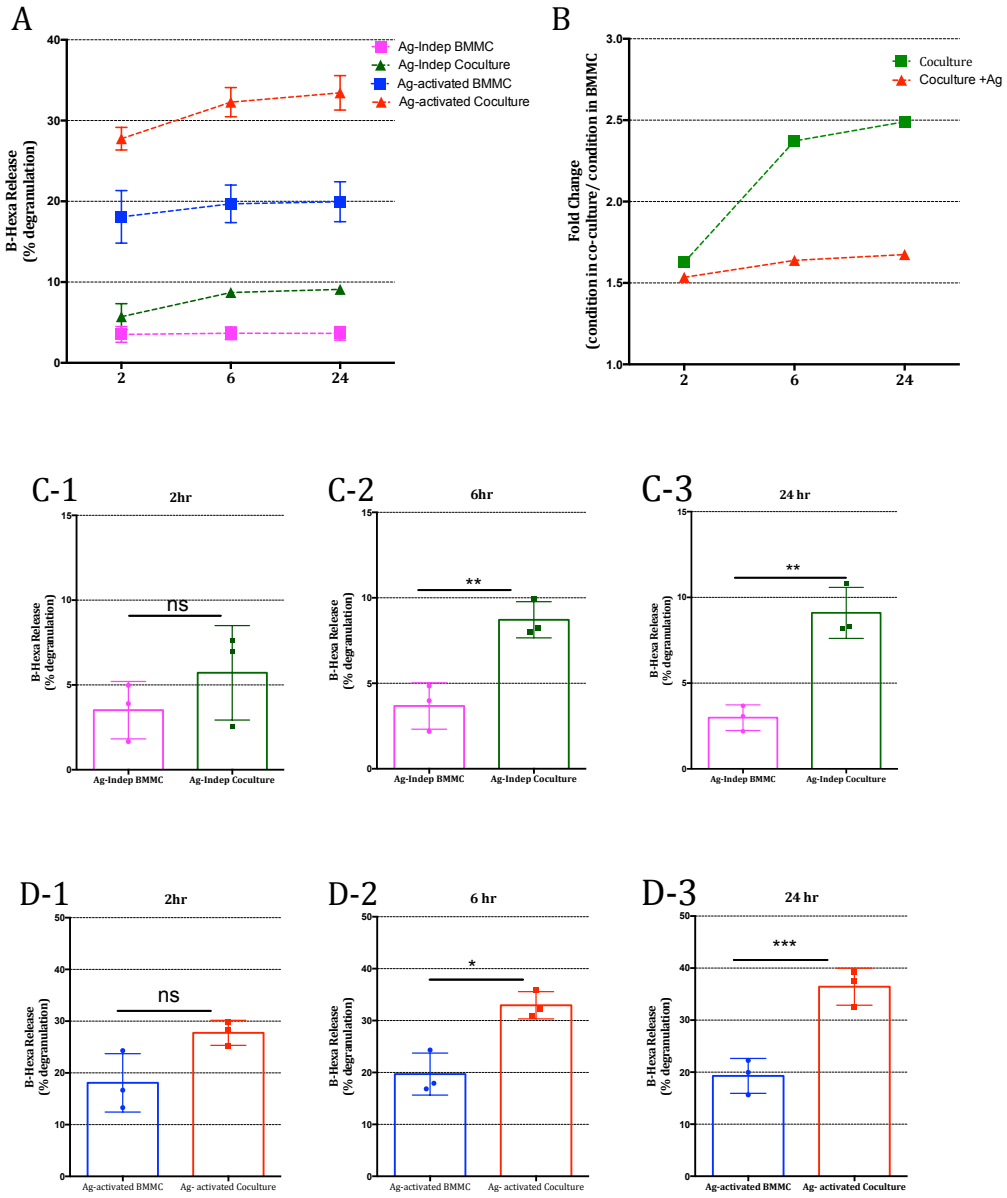


Figure. 4.4. Kinetics of the effect of sensory neuron co-culture on the enhancement of Ag-activated BMMC degranulation.

(**A**) Pre-sensitized BMMCs were cultured alone or with DRG for various times as indicated. Then, β-hexosaminidase (β-hex) release was measured in resting condition or after DNP stimulation. Total β-hex measured from BMMC lysed with 0.5% Triton Xl00. (**B**) Shows the fold-change in the percentage of β-hex release from BMMC in co-culture to BMMC alone at different time points. (**C1-3**) Shows the effect of co-culture of BMMC with DRG on constitutive degranulation at each time point. (**D1-3**) Shows the effect of co-culture of BMMC with DRG on Ag-activated degranulation at each time point. Data shown are mean ± SEM in (A - B) and SD in (C-D) of N=3, each performed in duplicate. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$ compared to BMMC alone. Data were analyzed using two-tailed paired *t*-test.

To examine the possibility that mediators released from neurons in co-culture could be accountable for the enhancement of mast cell degranulation in co-culture, I incubated BMMC for 24 hours with supernatant from BMMC-DRG co-culture. Then, I measured β-hex at resting condition or after DNP stimulation. As shown in (figure 4.5 A), in contrast to BMMC-DRG co-culture, supernatant from co-culture alone had no effect on constitutive (P=0.59) or Ag-activated (P=0.69) mast cell degranulation. Next, I examined whether neuronal enhancement of Ag-activated mast cell degranulation requires cell-to-cell contact or soluble factors only. I cultured BMMCs in the upper chamber of Transwell inserts, 0.4- m pore size (Costar) and DRG in the lower chamber. I found that co-culturing BMMC with DRG using a transwell system did not enhance either constitutive (P=0.73) or Ag-activated (P=0.49) BMMC degranulation (Figure 4.5 B). Collectively, my data indicate that neurons enhance mast cell degranulation in a cell–cell contact-dependent manner.

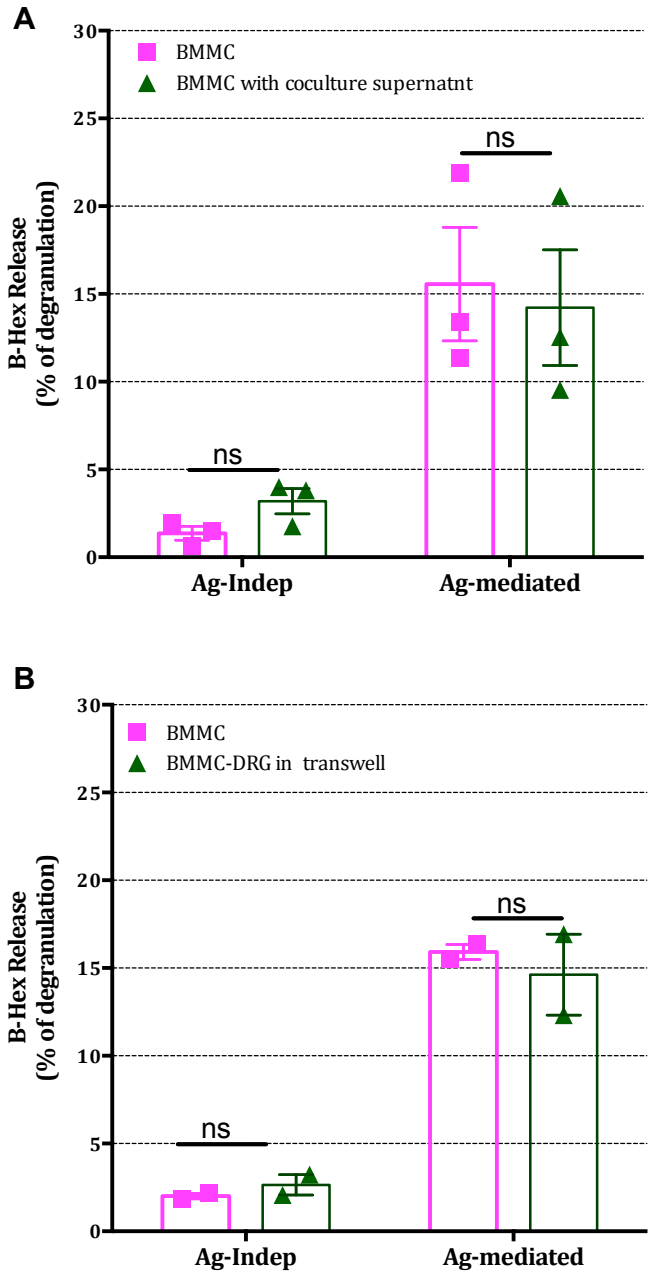


Figure. 4.5. Effect of BMMC-DRG co-culture supernatant on BMMC degranulation.

(**A**) Pre-sensitized BMMCs were cultured alone or with supernatant from BMMC-DRG co-culture for 24 hours. Then, β-hexosaminidase (β-hex) release was measured in resting condition or after DNP stimulation. Total β-hex measured from BMMC lysed with 0.5% Triton Xl00. (**B**) Pre-sensitized BMMCs were cultured alone or on the top of DRG cultured using a transwell system for 24 hours. Data shown are mean ± SEM of N=3 in A an N=2 in B, each performed in duplicate. Data were analyzed using two-tailed paired *t*-test.

4.2.4 Neurons enhance IgE-mediated mast cell IL-6, but not TNFα production.

To further confirm the influence of neurons on mast cells function, I measured Ag-induced production of pro-inflammatory cytokines from mast cells, like IL-6 and TNFα. I co-cultured BMMC with DRG or separately for 24 hours, then I stimulated BMMC with Ag (DNP) for 6 hours in media, collected the supernatant, and measured cytokines by ELISA. I found that upon Ag-induced stimulation, there was a significant increase in IL-6 released from BMMC in co-culture (772 ±69 pg/ml) compared to BMMC cultured alone (309 ± 21pg/ml; equivalent to a 2.5-fold increase, P= 0.035) (Fig 4.6 A). There was also a slight increase, though not significant (P=0.07) in IL-6 release from unstimulated BMMC. In contrast, the amount of TNFα released from BMMCs was not affected by co-culture with DRG (Figure 4.6 B). As a control, I measured the concentration of IL-6 and TNFα cytokines in supernatant collected from DRG monoculture. However, a negligible amount of both cytokines was detected. These results indicated that neurons enhance IL-6 production by mast cells without affecting TNFα production.

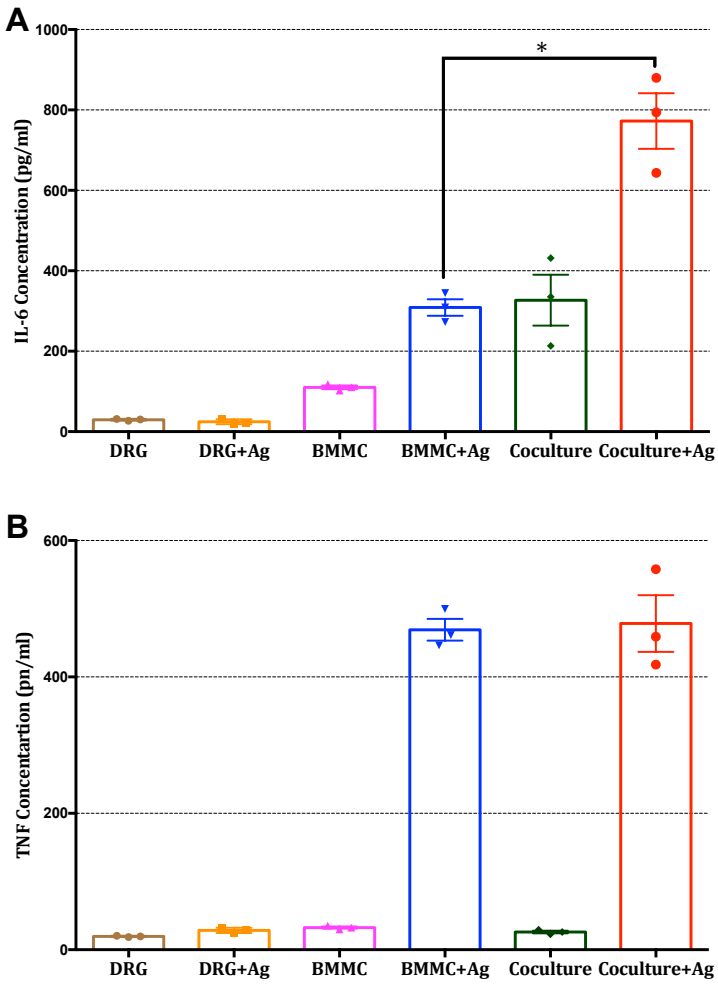


Figure. 4.6. Effect of sensory neurons on IgE-mediated cytokine production from mast cells

Pre-sensitized BMMCs were cultured alone or with DRG for 24 hours. Then, IL-6 (**A**) and TNFα (**B**) production were measured in resting condition or after DNP stimulation for 6 hours. Data shown are mean ± SEM of N=3, each performed in duplicate. * $p< 0.05$ compared to stimulated BMMC alone. Data were analyzed using two-tailed paired *t*-test.

4.2.5 Evaluation of BMMC-DRG crosstalk.

Having shown that neurons promote mast cell degranulation when in co-culture, I next looked for evidence of intercellular communication between mast cells and neurons and whether this communication results in cellular activation. Cell activation could be studied indirectly at a single cell level by monitoring intracellular calcium. To do so, I co-cultured BMMC with DRG for 24 hours. Then I loaded the co-culture with 2.5μM Fura-2AM, a fluorescent calcium indicator, and measured the change in fluorescence in both cell types after activation. First, I activated pre-sensitized BMMCs alone (Figure 4.7A) and in co-culture (Figure 4.7B) with 100ng/ml DNP (Ag). As expected, BMMC in both conditions showed a response within a minute of antigen application. Surprisingly, nearby neurons also showed an increase in fluorescence 10 seconds after the BMMCs (Figure 4.7B). However, applying 100ng/ml DNP to DRG monoculture didn't produce any response in all cells (Figure 4.7C). This indicated that the response shown in neurons in co-culture is a result of Ag-induced BMMC activation. Another important observation, co-culture BMMC with DRG increased the change in fluorescence from 0.041 in monoculture to 0.275 in co-culture. This suggests that neurons modulate FcεRI-stimulated calcium signaling.

Next, 1μM of capsaicin was applied to DRG monoculture, and as expected from mixed DRG culture (figure 4.2), only 2 nociceptor out of 6 neurons responded (Figure 4.7C). 62 mM KCl was applied to test neuronal response in general, all cells except one (non-neuronal cell) responded.

Next, I activated nociceptors in co-culture by 1μM of capsaicin. Nociceptors showed increase in fluorescence within one minute of drug application followed by activation of some of BMMCs, as indicated by increased fluorescence (Figure 4.7 D). Taken together, these results confirm direct crosstalk between mast cells and sensory neurons in co-culture.

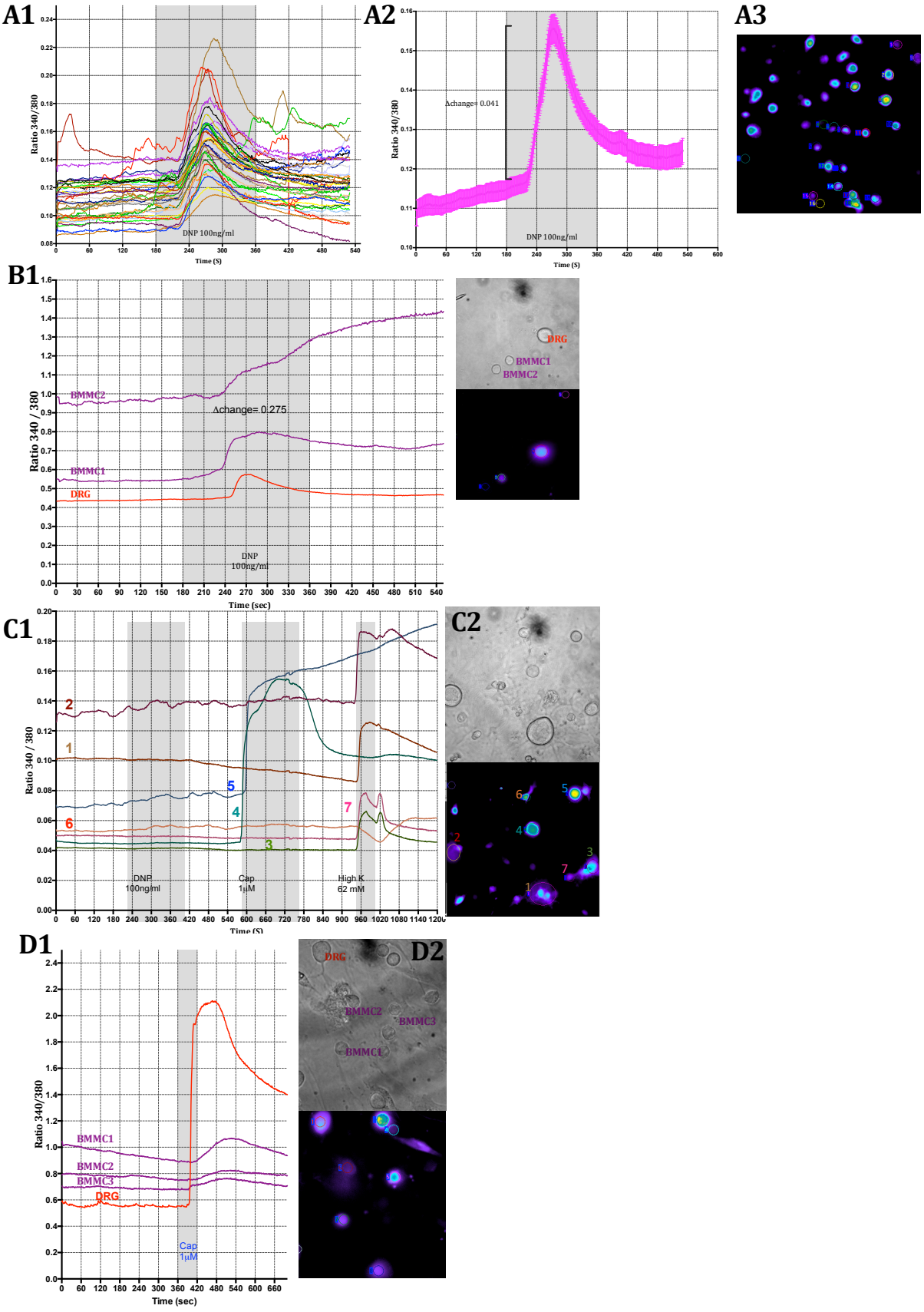

A1
Ratio 340/380
DNP 100ng/ml
Time (S)
A2
Δchange= 0.041
Ratio 340/380
DNP 100ng/ml
Time (S)
A3
B1
Ratio 340 / 380
BMMC2
Δchange= 0.275
BMMC1
DRG
DNP
100ng/ml
Time (sec)
DRG
BMMC1
BMMC2
C1
Ratio 340 / 380
2
1
5
4
7
6
3
DNP
100ng/ml
Cap
1μM
High K
62 mM
Time (S)
C2
D1
Ratio 340/380
BMMC1
BMMC2
BMMC3
DRG
Cap
1μM
Time (sec)
D2
DRG
BMMC2
BMMC3
BMMC1

Figure. 4.7. Bidirectional activation of BMMCs and neurons in co-culture

(Figure of the previous page) **.A1,** change in calcium signaling, as reported by Fura-2, of individual BMMC in response to 100ng/ml DNP (n = 39) and mean response ± SEM (**A2). (A3),** Image of BMMCs loaded with 1μM Fura-2AM. (**B1)**, Example trace of the change in calcium in a neuron following Ag-induced activation of BMMCs kept in co-culture with neurons for 24 hours (**B2)** Brightfield and fluorescence images of Fura-2 loaded co-culture. (**C1)**, sample control trace for DRG after activation with 100ng/ml DNP then 1μM of capsaicin and 62mM KCl (n=7). (**C2)**, Brightfield and fluorescence images of Fura-2AM loaded DRG culture. (**D1),** example trace of the change in calcium in BMMCs kept in co-culture for 24 hours and following stimulation of neurons with capsaicin. (**D2),** Brightfield and fluorescence images of Fura-2AM loaded co-culture.

Since I showed before that co-culturing sensory neurons with mast cells induced constitutive degranulation at rest, I also examined the effects of simply adding BMMC to calcium signals measured in 'resting' neurons. I loaded DRG with Fura-2AM and monitored baseline calcium levels before adding BMMCs and monitoring the fluorescence for a further 10 minutes. Addition of BMMCs did not produce any immediate significant increase in intracellular calcium in neurons. Moreover, activation of BMMCs with antigen after 10 minutes of co-culture didn't produce a measurable effect on neuronal intracellular calcium as seen in established co-cultures (Figure 4.8). This suggests that mast cells may need to settle for some time on DRG before effective communication is established.

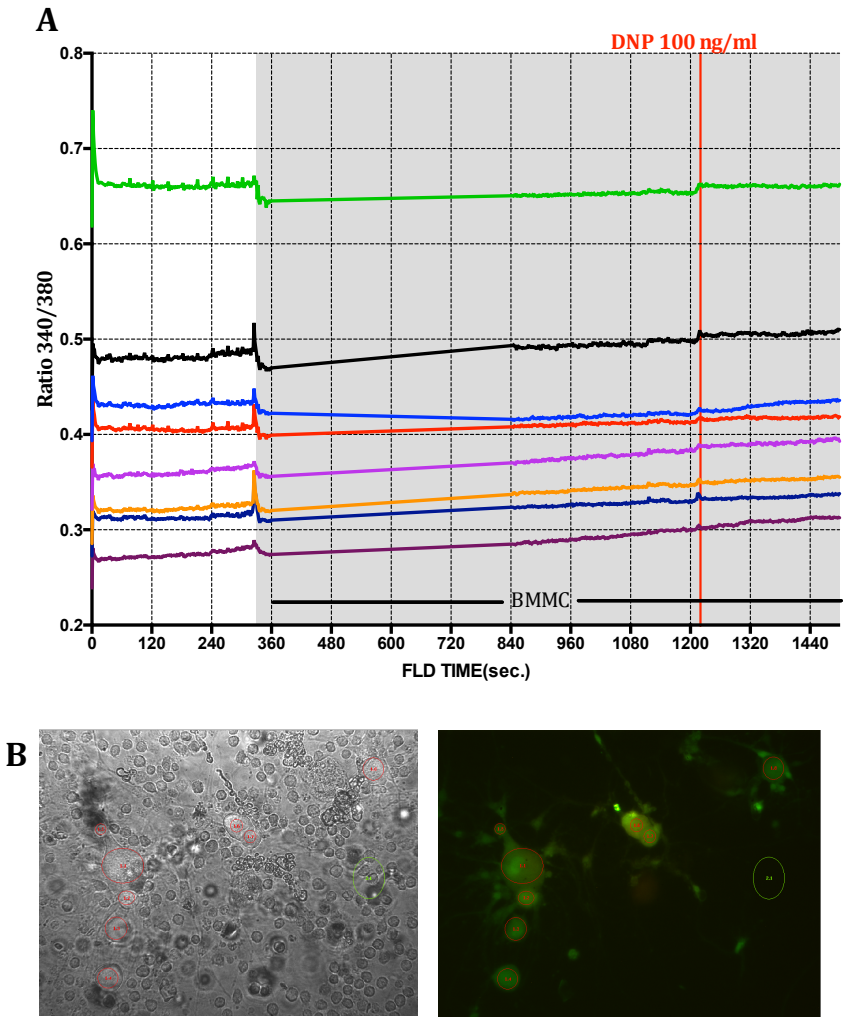


Figure. 4.8. The immediate response of neurons to BMMCs.

(**A),** Example traces of the immediate effect of adding BMMCs on intracellular calcium levels of neurons and following Ag-induced BMMCs activation(n=9). (**B),** brightfield and fluorescence images of 2.5µM Fura-2AM loaded DRG culture after adding BMMCs.

4.2.6 Effect of sensory neurons activation on mast cells degranulation.

As shown in the previous section and reported by others (Suzuki et al., 1999) (Mori et al., 2002) (Furuno et al., 2004) (Suzuki et al., 2007) (Hagiyama et al., 2011), stimulation of sensory neurons induces calcium signal in mast cells in co-culture but classic degranulation is not investigated . Because many of the symptoms of neuroinflammatory disorders are a result of mediators released from activated mast cells, I attempted to complete the picture by examining the effect of activated sensory neurons on mast cell degranulation in co-culture. Sensory neurons in 1-day old co-culture with BMMCs were activated with 1µm of Capsaicin for 20 minutes at 37^{o}C. Then, the percentage of β-Hex release in the supernatant from co-culture was measured. As shown in (Figure 4.9 A), the percentage of β -hex release from BMMC after capsaicin- mediated sensory neuron activation was significantly higher than β-hex release in co-culture in rest (17 ±0.73% and 10.1 ±0.54%, respectively P=0.02). As a negative control, β-hex release was also measured from DRG alone and capsaicin- activated DRG to rule out the possibility of having β-hex release from DRG culture. Moreover, β-hex release was also measured from BMMC alone and capsaicin- activated BMMC to rule out the possibility of non-specific activation of BMMC by capsaicin. All together the results from these experiments indicate that sensory neuron activation results in mast cells degranulation and release of mediators.

To examine the possibility that the mediator released from activated sensory neurons could be responsible for the increased mast cell degranulation in co-culture, I incubated BMMCs for 20 minutes with supernatant from capsaicin-activated DRG culture. The percentage of β-hex release in both groups was almost the same 5.4 ±0.78% (figure 4.9 B). This suggests that the soluble factors from activated sensory neurons alone are not enough to cause mast cell degranulation, and cell-cell contact is required for this phenomenon.

Because I showed before that sensory neurons enhance Ag-activated mast cell degranulation (section 4.2.3), I aimed to investigate if the mediators released from activated sensory neurons are responsible for this enhancement. For this, I cultured BMMC with supernatant from capsaicin-activated DRG for 20 minutes then I activated BMMC with DNP (Ag) for 30 minutes. Unexpectedly, the percentage of β-hex released from BMMC with capsaicin-activated DRG supernatant after Ag stimulation was significantly lower than the control (16.5 ±1.9% and 11.7 ± 0.8%, respectively) (P=0.042) (Figure 4.9 C). However, the lack of increase in Ag-activated mast cells degranulation with supernatant from activated sensory neurons confirms again that the crosstalk between mast cells and sensory neurons depends on direct contract between the two cell types.

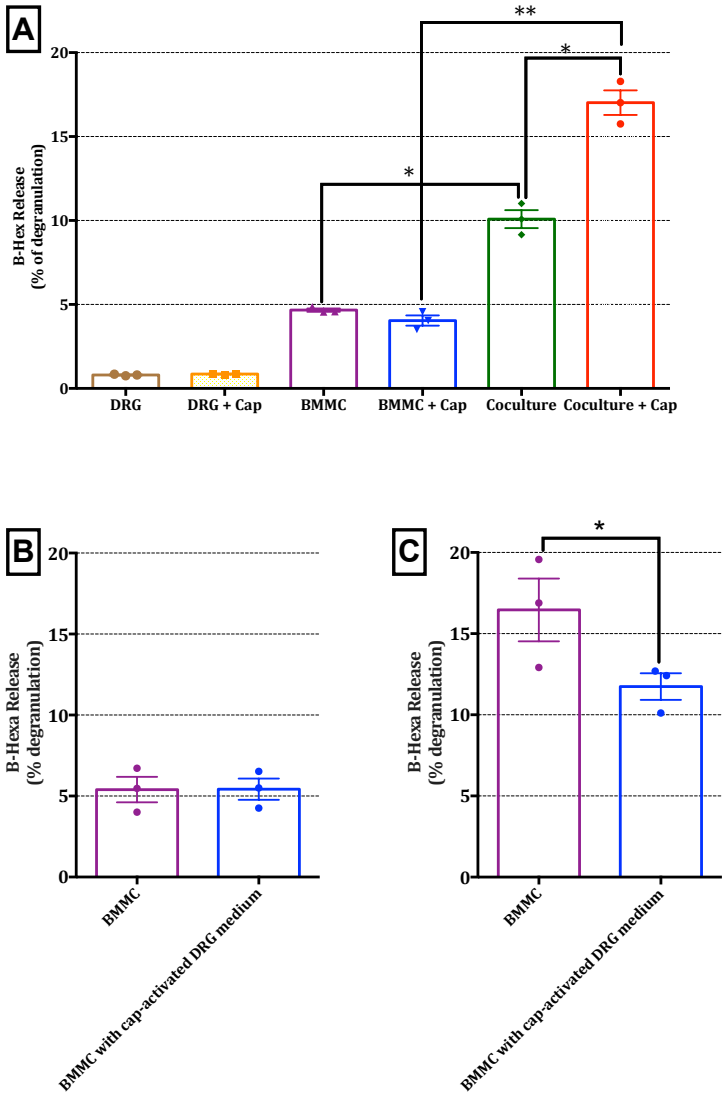


Figure. 4.9. Effect of capsaicin-activated sensory neurons on BMMC degranulation.

(**A**) Sensory neurons maintained for 1-day in co-culture with BMMCs were activated with 1µm of Capsaicin for 20 minutes at 37°C. Then, the percentage of β-hexosaminidase (β-Hex) release was measured. For control, β-Hex release was also measured from DRG, BMMC and capsaicin-activated DRG and BMMC (**B**) BMMCs were cultured alone or with supernatant from capsaicin- activated DRG for 20 minutes. Then, β-hex release was measured. (**C**) Pre-sensitized BMMCs were cultured alone or with supernatant from capsaicin (Cap)- activated DRG for 20 minutes. Then, β-hex release was measured after activation with 100ng/ml DNP for 30 minutes. Total β-hex measured from BMMC lysed with 0.5% Triton X l00. Data shown are mean ± SEM of N=3, each performed in duplicate. For (**A**), Data were analyzed using one-way ANOVA followed by Turkey's multiple comparison post-test, while for (**B** and **C**) two-tailed paired *t*-test. * $p< 0.05$ and ** $p< 0.01$ compared to appropriate controls.

4.2.7 Adhesion of mast cells to neurites in co-culture model.

Because the sensory neurons' enhancement of mast cell activation required cell-cell contact, I next assessed the intercellular adhesion between mast cells and neurons. In order to obtain a quantitative measurement, I used Calcein-AM flurometric adhesion assay. To optimize this assay, I first quantified the lifetime of Calcein in mast cells by measuring the fluorescent signal from Calcein-labeled BMMCs at different time points (30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, and 24 hours). Then, I calculated relative intensity compared to the reading at 0 point. Calcein signals in BMMCs were found to be stable for 3 hours after loading. After 24 hours (end of experiment), the relative intensity of Calcein signal was however decreased to 0.27 (Figure 4.10). Based on this result, I set up the optimum time for adhesion assay to be done after 2 hours of co-culture.

I performed adhesion assay as shown in material and method (Section 2.4); DRG were first seeded on Matrigel-coated wells and cultured for 1 day in order to establish an extensive network of neurites. The next day, Calcein- labeled BMMCs were added to DRG for 2 hours. Non-adherent cells were washed away by spinning the plate upside-down. To control for non-specific adhesion of BMMCs to Matrigel, some Calcein–labeled BMMCs were cultured on 1 day-old Matrigel-coated wells in the same way as the ones in co-cultures. Even after vigorous spinning (Figure 4.10 B), a high proportion of BMMCs remained attached to the neurites compared to wells with BMMC cultures alone (Figure 4.10 C), and moreover the adherent BMMCs appeared to preferably contact to neurites over cell bodies (Figure 4.10 B).

To quantify the adhesion between mast cells and neurons, I measured the fluorescent from Calcein-labeled BMMCs before (for total) and after spinning (for adherent) by plate reader. I expressed the results as percentage of the adherent to the total. Without the presence of DRG, only a few percentages of BMMCs (6±0.7%) adhered to the plate (Figure 4.10 E). In the presence of DRG, the number of adherent BMMCs increased to (28 ±1 %).

These results suggested that mast cells significantly attached to neurons *in vitro* (*P*=0.003). This morphological attachment may influence mast cell signaling.

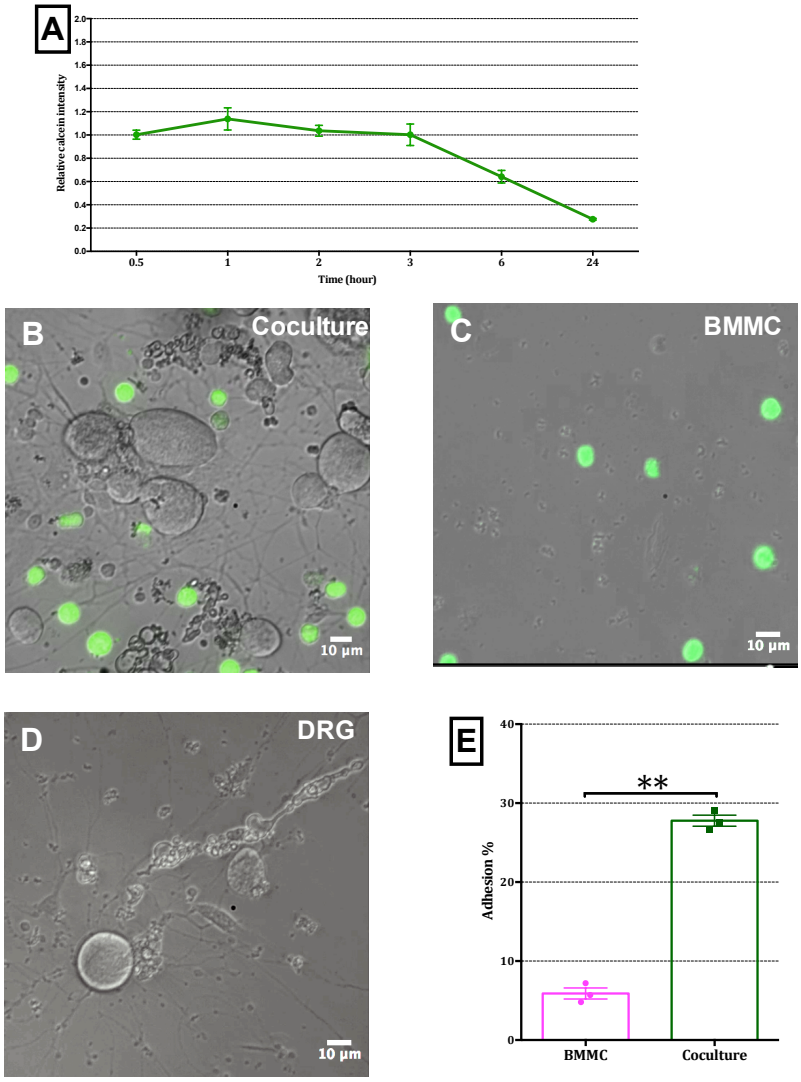


Figure. 4.10. Adhesion of BMMC to neurons.

(A) Kinetics of calcein labeling of BMMCs. **(B)** Superimposed bright-field and fluorescent image showing Calcein-labeled BMMCs (green) were co-cultured with DRG for 2 hours. Non-attached BMMCs were removed. Then, images of live cells were captured by inverted fluorescent microscopy. **(C)** Fluorescent image of non-specific attachment of calcein labeled-BMMCs to a matrigel-coated well. **(D)** DRG culture alone. (E) BMMCs adhesion to DRG quantified as percentage of adherent BMMC to total BMMC before washing. Data are shown as mean ± SEM from N=3. Each done in duplicate. Data were analyzed two-tailed paired *t*-test $^{**}p<0.01$ compared to BMMC culture alone. Scale bar represents 10µm

4.3 Discussion

The close contact and interaction between mast cells and neurons are increased in many neurogenic inflammatory disorders (Barbara et al., 2004) (Park et al., 2003). Indeed, mast cells attached to neurons in IBS biopsies show evidence of degranulation, consistent with the view that neurons may lead to increased mast cell activity (Park et al., 2003) (Barbara et al., 2004). Sensory neurons are a potential source of potent mast cell activators, including subP (Karimi et al., 2000) (Okabe et al., 2006) and CGRP (Forsythe et al., 2000) (Rychter et al., 2011). In neurogenic inflammatory disorders, sensory neurons increase neuropeptide production (Mayer et al., 1988) (Baluk, 1997). The close association of mast cells to sensory neurons may thus expose mast cells to these neuropeptides and may result in altered mast cell function. On the other hand, a growing body of evidence suggests that mast cells and their mediators may also participate in sensory neuronal signaling (van Houwelingen et al., 2002) (Kakurai et al., 2006). Collectively, these studies offered substantial evidence for the functional bi-directional crosstalk between mast cells and sensory neurons. However, the role of cell-cell contact in modulating this crosstalk remains unresolved. In this chapter, I aimed to investigate the effect of direct contact between sensory neurons and mast cells on their biological activity by using BMMC-DRG co-culture methods. The *in vitro* method used in this study has great potentials for conducting detailed investigations on neuro-immune interactions in general and sensory neuron-mast cell interactions in particular.

To characterize functional interactions established in DRG-BMMC co-cultures, I examined neuronal morphology. Addition of BMMCs to DRG culture for 1 day did not affect either total neurite length or neurite complexity. Although it is reported that activated mast cells produce NGF and TNF-α, which enhance neuronal outgrowth and plasticity (Leon et al., 1994) (Kakurai et al., 2006), in my co-culture system, the former, at least was not apparent. Key methodological differences that could account for the lack of morphological changes in my co-culture system could be due to the use of adult versus embryonic DRG culture (Leon et al. 1994) . It is well known that embryonic DRG cultures are dependent on NGF for their survival (Melli and Höke, 2009), while adult

DRG cultures that are not dependent on NGF for their survival despite its receptors expression. Indeed, the role of NGF in adults shifts away from the neurotrophic effect to a pro-inflammatory effect that regulates neuronal function and plasticity (Sofroniew et al., 2001). Kakurai et al., 2006 proposed that mast cell- derived TNFα promotes sensory neuron elongation *in vivo* in a contact hypersensitivity model in mice. The evidence for mast cells being the source of TNF-α relies on comparisons made between wild type and c-kit mutant mice, which are known to be deficient in mature mast cells. However, because the full extent of the loss of c-kit signaling is not yet completely understood, some of these differences may be due to changes in neuronal development and/or function. Interestingly, one study showed that co-culture of activated peritoneal mast cells with 4-day-old myenteric neurons for 2 days significantly reduces neuronal survival (Sand et al., 2009). This neuronal loss was shown to be dependent on mast cell degranulation and release of PG and IL-6. Peritoneal mast cells are classified as CTMC (Chen and Enerbäck, 1999) while myenteric neurons are referred as the motor neuron of the unique enteric nervous plexus (Furness et al., 2014). Mast cell and neuron heterogeneity is an important issue because it may lead to different response to the same stimulus. Moreover, it is well known that *in vitro* some mediators that cause persistent firing of primary DRG can induce neuronal apoptosis (Dodd, 2002); of note is that in my study the period of mast cell activation was limited to 30 minutes and thus any pro-apoptotic effects of mast cell derived mediators were unlikely to have been activated.

Because mast cells mature only within tissues (Galli, 2000) when they are in proximity to other cell types, one can speculate that mast cell interactions with other cells may regulate mast cell function. To test this idea, the influence of DRG neurons on mast cell degranulation was quantified and compared with data from BMMCs alone. Interestingly, I found that co-culture mast cells with neurons enhances not only constitutive (antigen-independent) mast cell degranulation but also Ag-activated mast cell degranulation. In both conditions, the development of this enhancement is time-depended, with a significant effect only measurable after 6 hours of co-culture and then increasing over

the ensuing 24 hours. Besides, I showed that sensory neurons-mediated mast cell degranulation is a contact-dependent as compared to results from co-cultures in a transwell and supernatant from co-culture failed to enhance mast cells degranulation.

The observation that mast cell degranulation may be modulated by communication with other cells, even in the absence of external stimulation, has been reported previously. co-culture HLMC with ASM show increased constitutive histamine release (Hollins et al., 2008) which starts after 16 hours of co-culture (Lewis et al., 2016). Also activated T-cells enhance constitutive histamine release from BMMC when direct co-cultured for 16 hours (Inamura et al., 1998). Co-culture LAD2 with tumor cells, like pancreatic ductal adenocarcinoma for 24 hours also show enhanced constitutive tryptase release (Ma et al., 2013). However, IgE-mediated mast cell degranulation is influenced differently by communication with other cells. ASM (Hollins et al., 2008) and bone-marrow stromal cells (Brown et al., 2011) significantly decrease IgE- mediated histamine and B-hex release, respectively. In contrast, co-culture mast cells with activated T-cells results in enhancing Ag-activated mast cells degranulation (Inamura et al., 1998).

Mast cells, upon activation, not only release stored mediators by degranulation but also synthesize de novo cytokines (See section 1.1.3.3). Different cytokines have different functions in health and diseases. Some of these cytokines are reported to influence pain perception and are likely involved in sensory neuron activation . For example, IL-6 and TNFα are both known to sensitize nociceptors (Hughes et al., 2013). Thus, I measured the production of these cytokines in BMMC-DRG co-culture. My results showed that neurons enhanced mast cells derived-IL-6 secretion in co-culture, but this effect was significant only after Ag stimulation. Conversely, TNFα production by mast cells in co-culture did not change with the presence of neurons even after Ag- stimulation. These data indicate that cross-talk with neurons initiates or facilitates a stimulatory signal in mast cells that enhances degranulation and Ag-induced IL-6 production. This is in agreement with what has been reported *in vivo*, namely that mast cells of IBS patients

secrete greater amounts of the IL-6 (Liebregts et al., 2007) by influences enteric neuronal hyperexcitability (O'Malley et al., 2011). IL-6 has a pivotal role in the pathology of inflammatory neuropathy. *In vivo*, IL-6 has been found to induce mechanical nociceptive plasticity (Melemedjian et al., 2010) (Hughes et al., 2013) that evokes allodynia (Oka et al., 1995) (Dina et al., 2008). Therefore, pain-related disorders may involve an increase in IL-6 levels. Moreover, the role of IL-6 in development of neurogenic inflammation depends also appears to involve its effects on sensory neurons because selectively blocking IL-6 receptors in neurons reduces inflammation in an Ag-induced arthritis model (Ebbinghaus et al., 2015). Indeed, anti-IL-6 receptor antibodies show promising therapeutic potential in controlling pain in rheumatoid arthritis (Nishimoto et al., 2009).

It has been shown that co-culture mast cells with fibroblasts (Fitzgerald et al., 2004), and immune cells like regulatory T-cells (Ganeshan and Bryce, 2012) also lead to enhanced IL-6 production from mast cells. In contrary to my result, enhancement of TNFα production by mast cells has been reported following co-culture mast cells with other cell types such as pancreatic ductal adenocarcinoma (Ma et al., 2013).. TNF-α has also been reported to cause a pronounced sensitization in colonic sensory afferents (Hughes et al., 2013), but, at least in my model, it seems sensory neurons don't influence TNF-α synthesis by mast cells in response to Ag. Remarkably, the selective enhancement in certain mast cells' mediators (but not all mediators) may underlie the mechanism of sensory neuron-mast cell cross talk. The pathways of IgE- mediated IL-6 and TNFα transcription in mast cell are different (Section 1.1.2.1). IgE- mediated TNFα production is dependent on PKC/NFAT signaling pathway, but this pathway doesn't contribute in IL-6 production (Klein et al., 2006) (Figure 1.1). This suggests that the neuronal enhancement of IgE- mediated mast cells function most likely doesn't involve PKC/NFAT pathway.

With respect to neuroimmune and neuronal-mast cell interaction, *my study is the first to show that direct contact with sensory neurons enhances IgE- activated mast cell mediator secretion.* However, the vital role of nociceptive neurons on IgE-mediated activation has been hinted at previous in *in vivo* experiments. Neonatal capsaicin treatment to denervate nociceptors abolishes IgE-mediated dermatitis in mice (Nakano et al., 2008) and IgE-induced inflammation in rats' lungs (Sestini et al., 1989).

A number of different mechanisms may underlie the enhancing role of IgE-mediated mast cell function following co-culture with sensory neurons. It could either reflect an increased proliferation and survival of mast cells or an increase in signaling / activation of mast cells. I tested the first hypothesis because previous reports have found that other cells types induce mast cells proliferation like human umbilical vein endothelial cells (HUVEC) (Mierke et al., 2000), fibroblasts (Sellge et al., 2004), epithelial cells (Hsieh et al., 2005) and airway smooth muscle (Hollins et al., 2008). However, the cell growth rate of BMMCs after maturation doesn't go with this possibility (Figure 2.1) because it is known that mast cells stop proliferation after maturation . Moreover, the standard curve of total B-Hex absorbance from different BMMC cell count (Figure 2.2) shows that more than 300 $x10^3$ BMMCs are needed to get the 20% enhancement of B-Hex absorbance as seen in co-culture. Thus, it seems nonsensible that BMMC could extensively proliferate that much in co-culture to produce this significant high amount of B-Hex. *However, further experiments needs to be done to assess the possibility of neuronal effect on BMMC proliferation in chronic co-culture.* Suggested methods like tetrazolium salts (MTT) with colorimetric detection are recommended.

Another possible enhancement of mast cell function in the presence of neurons is due to chemical intercellular crosstalk. Thus, I reproduced previously published data regarding mast cell-neuron positive feedback loop. Consistent with what has been reported, I confirmed the intercellular communication between mast cells and neurons by monitoring calcium signaling in both cell types (Suzuki et al., 1999) (Suzuki et al., 2001)

(Suzuki et al., 2005). Ag-induced mast cells activation was followed by neuronal activation, while Capsaicin- induced nociceptor activation was followed by mast cell activation. The lag time between activation of the cells , likely arising from the time needed for chemical communication by release mediators such as SubP (Suzuki et al., 1999) to generate signals in the contacted cell. I did not seek to identify the specific mediators involved in this communication as it was beyond the scope of my study and this has already been the topic of intensive investigation (See introduction section 1.3.1). However, it is worth noting that in previous studies of intercellular communication between mast cells and neurons, after stabilizing the co-culture for 2 days (Suzuki et al.), while I found that neurons could induce mast cell degranulation at rest within as minimum as 6 hours, indicating that mast cell attachment to sensory neurons is rapid and capable of activating mast cells independent of external stimulants. To test this hypothesis, I measured calcium signals in neurons immediately after addition of mast cells, however no obvious response from neurons was recorded. Moreover, neurons didn't response even after stimulating mast cells with Ag for 10 minute. This suggests that mast cells may need to establish good structural interaction with neurons before effective communication is established, and that's seems to needs some time (more than 10 minutes). Collectively, these findings support the critical role of cell-cell contact between mast cells and neurons for their effective communication. Thus, my next aim was to examine the adhesion between neurons and mast cells.

The mechanism of the increased mast cell activation in the presence of sensory neurons could be explained due to the mediators released from activated neurons that could induce calcium signaling (Figure 4.7) and, more importantly, degranulation (Figure 4.9) in mast cells in a contact-dependent mechanism. This has been suggested by other authors (Janiszewski et al., 1994) (Mori et al., 2002) (Suzuki et al., 1999) (Suzuki et al.). But, the fact that physical separation of mast cells from neurons and/or incubation of mast cells with supernatant collected from neuronal cultures did no reproduce the enhancement effect of sensory neurons seen in co-culture suggests that this communication may depend on more than just the soluble factors. Indeed, direct cell-cell contact between mast cells and neurons appears vital in this crosstalk. As reported

by other authors using co-culture methods (Ohshiro et al., 2000) (Suzuki et al., 2001) (Suzuki et al., 2004), I subsequently showed that mast cells firmly adhere to neurons. Although the adhesion kinetics were not studied in detail in this study because of the short lifespan of calcein fluorimetric stain, 2 hours of co-culture was sufficient time to get more than 20% of mast cells attached to neurons. This percentage is in agreement with that reported previously using a more subjective method for quantifying mast cell adhesion by counting mast cell in co-culture under light microscope (Furuno et al., 2005). However, the percentage of mast cells adhered to neurons was low compared to that reported for human lung mast cells adherent to fibroblast (Trautmann et al., 1997), ASM (Yang et al., 2006) or bronchial epithelium (Sanmugalingam et al., 2000) when quantified using histamine-based adhesion assay. In this assay, total histamine from co-culture are measured. Knowing that fibroblast ASM and epithelium cells don't contain histamine, the total amount of histamine in co-culture will correlate to number of adherent mat cell. However, the fact that other cells may influence histamine release and content in mast cells could questioning the validation of this method. This difference in adhesion percentages could be contributed to mast cell heterogeneity between BMMC and lung mast cells, different cell type partners, different adhesion molecules involved and/or different methods to quantify the adhesion.

Mast cell tendency to attach to other cells supports the theory that adhesion is required for them to perform their function normally. Indeed, mast cells are not found in blood, but mature within tissues following contact with other cells or matrix. Therefore, *in vivo* mature mast cells' phenotype is regulated by other cells. This has led to the speculation that studying the pharmacological regulation of mediator release from mast cells in suspension does not reflect the mast cell phenotype *in vivo*. Cell adhesion may influence intracellular signaling and the response to different agonists. Thus, I hypothesize that mast cells' attachment to neurons enhances mast cell function. Interestingly, the kinetics of the increase in mast cell degranulation following co-culture with neurons closely paralleled the kinetics of adhesion . Although the exact molecular mechanism of how attachment of mast cells to neurons enhances mast cell function needs further

investigation, a number of mechanisms can be proposed. **(1)** The close apposition of mast cells to nociceptor neurons may form a kind of synapse. This structural arrangement could expose mast cells directly to undiluted neuronal mediators as sub P and CGRP that activate and induce mast cells degranulation (Suzuki et al., 1999) (Forsythe et al., 2000) (De Jonge et al.) (Rychter et al., 2011), and their effects are additive with IgE –mediated activations. **(2)** Adhesion of mast cells to neurons and exposure to neuronal mediators could alter mast cells phenotype and may result in enhancing their IgE-mediated response (Blennerhassett and Bienenstock, 1998). As mentioned in introduction (Section 1.1.4), changing mast cell microenvironment could influence their heterogeneity and response to different stimuli by inducing different gene expression (Metcalfe et al., 1997). **(3)** Mast cell attachment results in cytoskeletal changes and redistribution of secretory granules to the cell periphery and enhanced IgE-mediated degranulation (Hamawy et al., 1994). **(4)** Mast cell adhesion to neurons helps to stabilize FcεRI at the membrane and 'signaling complexes' through anchoring of adaptor proteins. IgE-mediated activation in fibronectin-adherent mast cells markedly enhances FcεRI tyrosine phosphorylation compare to non-adherent cells (Hamawy et al., 1993). In support of the latter, it has previously been shown that co-culture BMMC with fibroblasts or SCG for 3 days results in increased FcεRI surface expression and enhanced Ag-induced calcium signaling (Katz et al., 1992) (Suzuki et al., 2005).

Among other adhesion molecules, recent studies identified CADM1 as an adhesion molecule expressed on mast cells that may mediate homotypic adhesion to neurons (Furuno et al., 2005) (Hagiyama et al., 2011). *However, the contribution of this adhesion molecule in functional crosstalk has not been studied before, and subsequently became the focus of my studies.*

In conclusion, the results reported in this chapter add to existing evidence for a positive feedback loop between mast cells and sensory neurons. Moreover I showed for the first time that sensory neurons have the ability to enhance IgE-mediated mast cell activation.

My data indicates that this effect is dependent on cell-cell contact. *Identifying the adhesion molecules involved in this crosstalk and understanding the signaling pathways behind this are important future goals.*

Chapter 5: Investigating the involvement of CADM1 in mast cell –neuron crosstalk

5.1 Introduction

Cell-cell adhesion is a fundamental process for intercellular communication. It ensures tight contacts between neighbouring cells, which are necessary not for maintaining tissue integrity but also for regulating specific cell-cell signalling. With respect to mast cells, mast cells *in vivo* mature only after they migrate to tissues and adhere to ECM or other cells such as fibroblasts, muscle cells and neurons (Hamawy et al., 1994). In the preceding chapter, I showed that mast cells attach directly to sensory neurons in culture and that there are significant bi-directional consequences of this attachment. I demonstrated that structural contact of mast cells to neurons induces mast cell activation and production of pro-inflammatory mediators. More remarkably, neurons enhanced IgE-mediated mast cell activation in cell–cell contact-dependent manner. *However, the identify and role of adhesion molecules involved in this crosstalk is unknown.*

As mentioned in introduction (section 1.1.1.1), mast cells express a variety of adhesion molecules, including integrins (Sperr et al., 1992), E- and N-cadherins (Nishida et al., 2003) (Suzuki et al., 2004), ICAM1 (Toru et al., 1997), and cell adhesion molecule-1 (CADM1) (Ito et al., 2003) (Yang et al., 2006) (Moiseeva et al., 2013a). Among all these molecules, I chose to focus on CADM1, because as mentioned in Section 1.3.2.1, it had previously been proposed to participates in mast cell adhesion to other types of peripheral neurons (Furuno et al., 2005) (Hagiyama et al., 2011). More importantly, it is suggested that CADM1 enhance neuronal to mast cell crosstalk. This suggestion was based on the observation that calcium signal of mast cell lines like IC-2 that express all other adhesion molecules except CADM1 do not show an increase in response to

scorpion venom-activated superior cervical ganglion neurons in co-culture compared to BMMCs, which express endogenous CADM1 (Furuno et al., 2005). Indeed, transfection of IC-2 cells with CADM1 normalized their response to activated neurons in co-culture. However, the contribution of CADM1 in neuronal enhancement of IgE-signalling in mast cells was not investigated yet.

5.2 Results

5.2.1 CADM1 expression and distribution in mast cells and neurons

First, I investigated CADM1 protein expression in BMMC and DRG cultures. Lysates were prepared from each culture separately then probed for CADM1. GAPDH and α-Tubulin were used as loading controls for chemiluminescent and Li-cor WB techniques, respectively. My results showed that both cell types express CADM1 (Figure 5.1) but of different molecular weights. BMMC expressed CADM1 as a protein with molecular weight of ~100 kDa, while in DRG the protein was ~75 kDa. Notably, the band from DRG lysate was more intense compared to that from BMMCs. As a positive control, CADM1 expression was also investigated in lung tissue (Masuda et al., 2002) where I observed it as a single band of ~75 kDa (Figure 5.1a). Furthermore, because it is reported that mouse fibroblasts don't express CADM1 (Ito et al., 2003), I used NIH/3T3 lysate as a negative control to validate CADM1 antibody specificity. As expected, no bands were detected at any known size of CADM1 isoforms (Figure 5.1a).

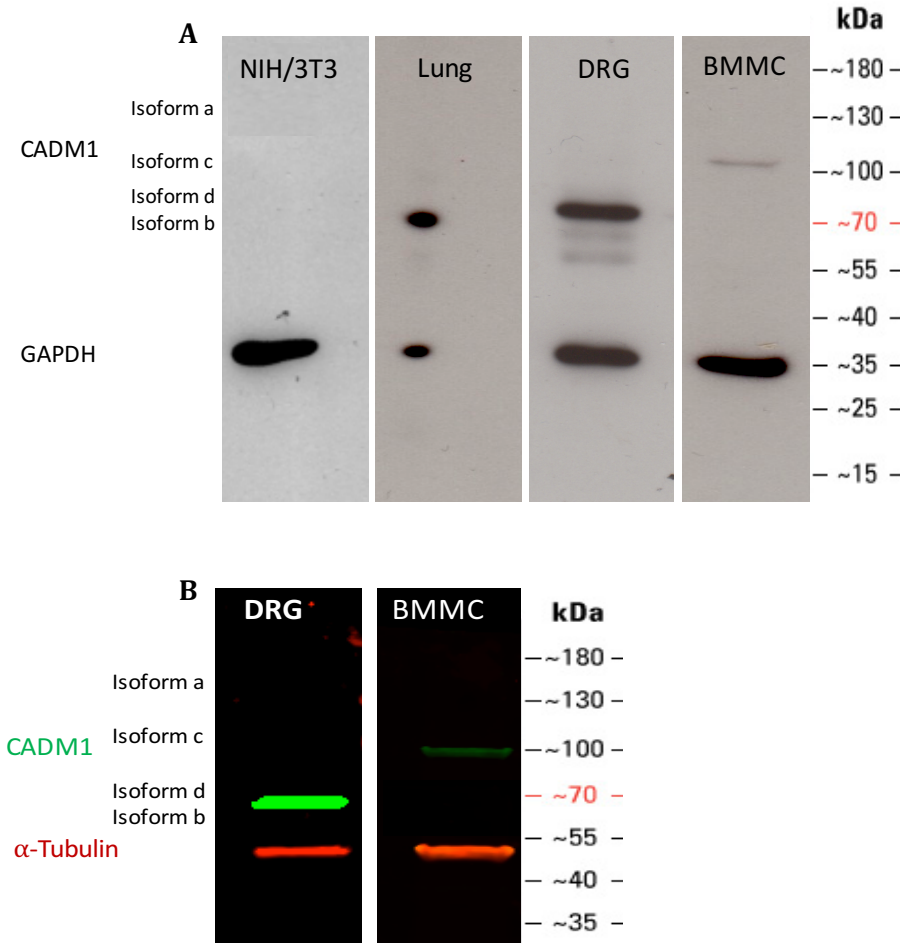
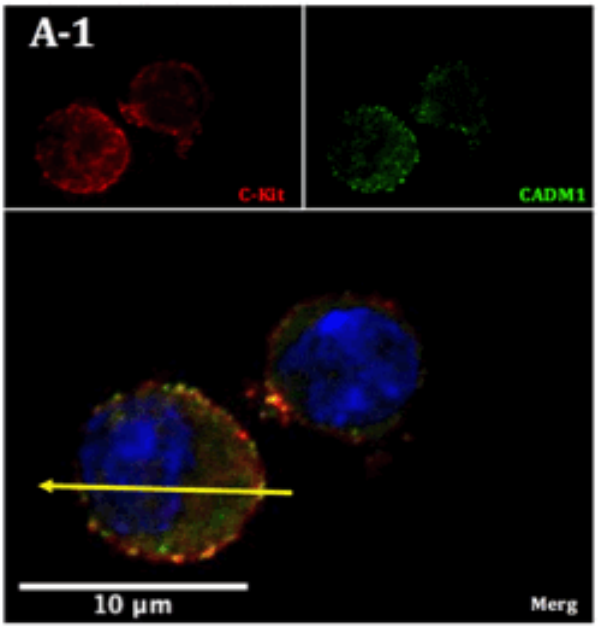
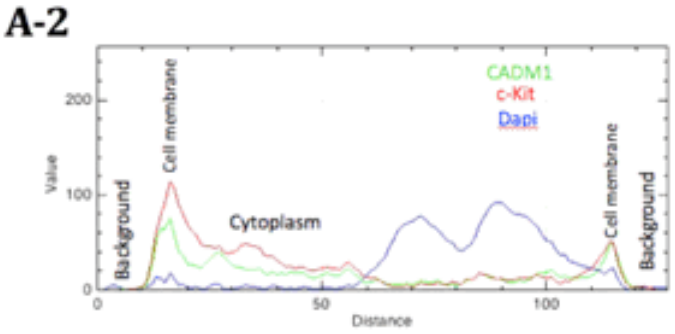


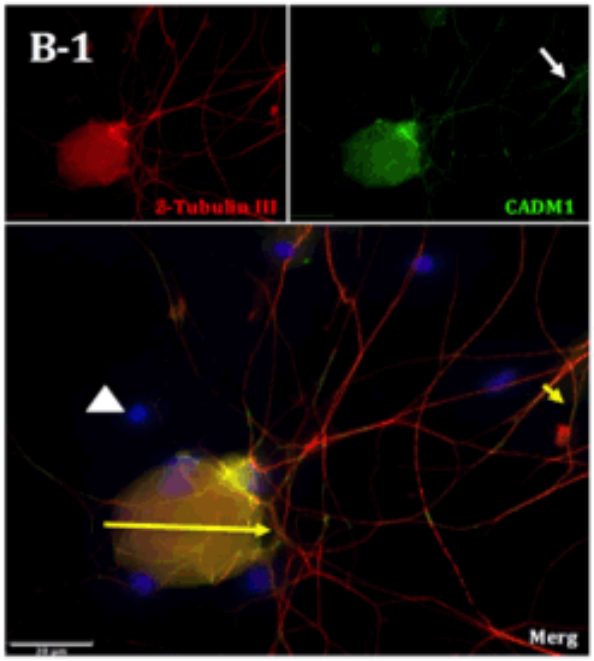
Figure. 5.1. Protein expression of CADM1 in BMMC and DRG.

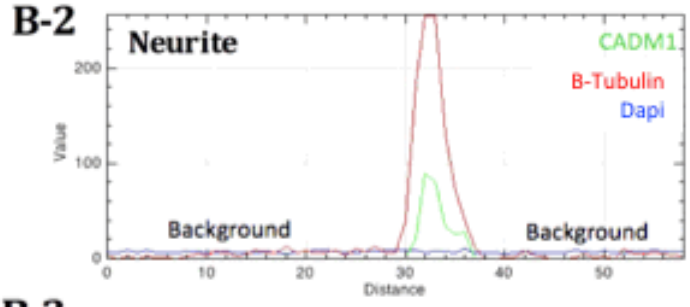
Immunoblot of lysates prepared from NIH/3T3, Lung, DRG and BMMC cultures,. The blots were probed with anti-CADM1. Bands were detected using chemiluminescent **(A)** or Li-cor **(B)** methods. The blots were probed again with anti-GAPDH and anti-α-Tubulin to indicate the total amount of proteins loaded per lane. The m.w. scale is shown to the right of the blot. The expected sizes of the different CADM1 isoforms (a-d) are shown on the left. Lysate from lung tissue and NIH/3T3 were used as positive and negative controls, respectively. Representative examples of three experiments performed on each cell type.

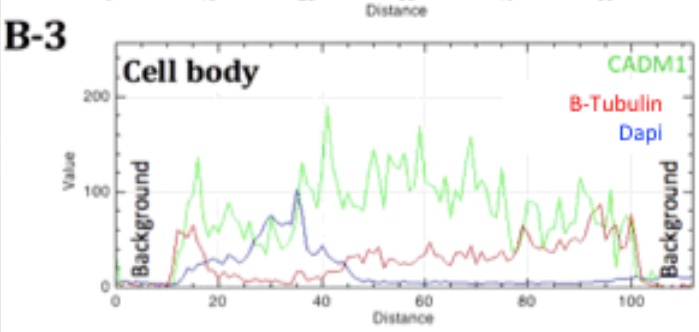
Next, I examined the subcellular distribution of CADM1 in BMMCs and neurons. BMMCs cultures were double stained with Abs against CADM1 and the mast cell marker c-Kit. All BMMCs showed strong expression of CADM1. As shown in (Figure 5.2 A), CADM1 staining was concentrated mainly at the cell membrane of BMMCs as was c-Kit. CADM1 showed a punctate distribution, compared to c-Kit. Fluorescence intensities of CADM1 and c-Kit were plotted across the cell (Figure 5.2 A2), fluorescence intensities of both Abs significantly decreased in cytoplasm compared to the membrane. This confirms that CADM1 and c-Kit are located in the peripheral margin of mast cells. 2-day old DRG cultures were also double stained with Abs against CADM1 and the neuronal marker β-tubulin. All different sizes of soma showed strong expression of CADM1. In contrast to BMMCs, CADM1 distributed homogeneously within the soma (Figure 5.2 B). However, CADM1 stained some of neurites (white arrows), mainly the ones with big diameter (Figure 5.2C) but not along their whole length. Remarkably, CADM1 was expressed only in β-tubulin immunoreactive neurons but not any of the non-neuronal cells (arrowheads). Neurites and soma showed the same pattern of CADM1 distribution as shown in fluorescence intensities plot (Figure 5.2B 2-3); in both structures CADM1 showed diffuse distribution.


A-1
C-Kit
CADM1
10 μm
Merg


A-2
CADM1
c-Kit
Dapi
Cell membrane
Cytoplasm
Cell membrane
Background
Background
Value
Distance


B-1
β-Tubulin III
CADM1
Merg


B-2
Neurite
CADM1
B-Tubulin
Dapi
Background
Background
Value
Distance


B-3
Cell body
CADM1
B-Tubulin
Dapi
Background
Background
Value
Distance

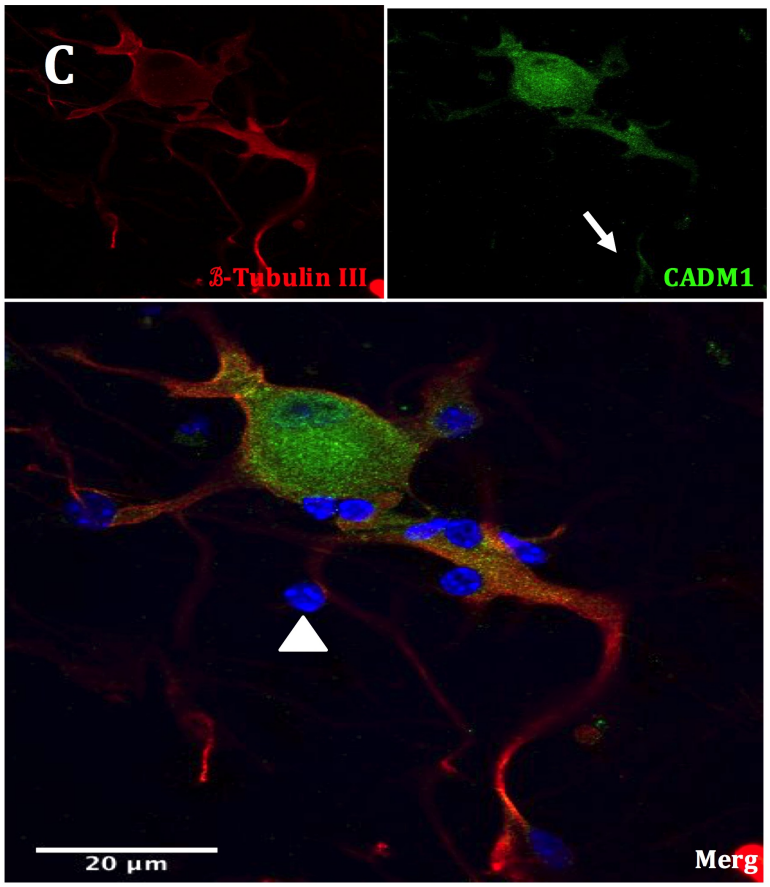


Figure.5.2 Immunocytochemistry expression of CADM1 by BMMCs and neurons.

(A-1), Confocal immunofluorescence images of c-Kit (red) and CADM1(green) in BMMC. Scale bar represents 10µm. **(A-2),** The plot shows the variation in fluorescence intensity along the yellow arrow. Notably, the cell membrane is the most intensely fluorescent. **(B-1)** immunofluorescence images of β-Tubulin (red) and CADM1(green) in DRG culture. White arrows indicate CADM1 immunoreactive neurites. Note that non-neuronal cells are negative for CADM1 (arrowheads). Scale bar represents 20µm. **(B-2)** and **(B-3)** plots show the variation in fluorescence intensity along the yellow arrows in neurite and soma, respectively. **(C),** Confocal immunofluorescence images of β-tubulin (red) and CADM1(green) in DRG cultures (bottom). Scale bar, 20 µm. Representative examples of three experiments performed on each cell type.

Because immunocytochemistry analysis suggested that CADM1 is concentrated at the cell surface of BMMCs, FACS was performed to measure CADM1 surface and total expression in BMMCs. 1X10^6/reaction of non- permeabilized and TritonX100-permeablized BMMCs were double labelled with conjugated c-Kit and CADM1 Abs. Then, CADM1 expression was measured by FACS from both groups. Results from FACS analysis revealed that almost all BMMCs expressed pronounced surface CADM1 at level comparable to total expression (Figure 5.3). However, BMMCs expressed surface CADM1 as two distinct population subsets. The majority (88.3%) of BMMCs expressed surface CADM1 with medium fluorescence intensity and only (9.5%) was with high fluorescence intensity. In contrast, BMMCs expressed total CADM1 as single population with high fluorescence intensity.

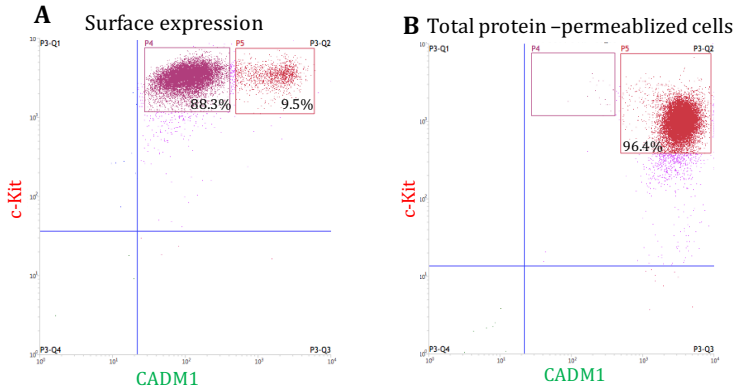
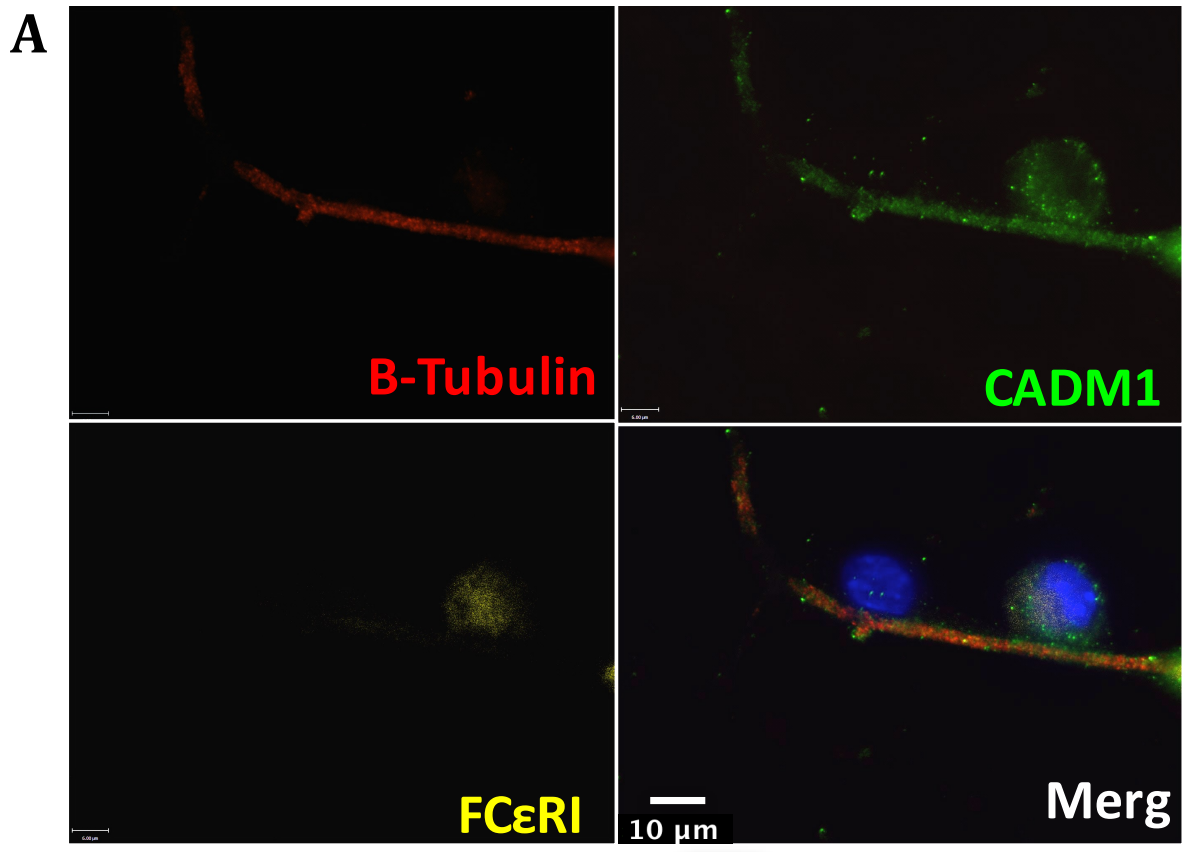


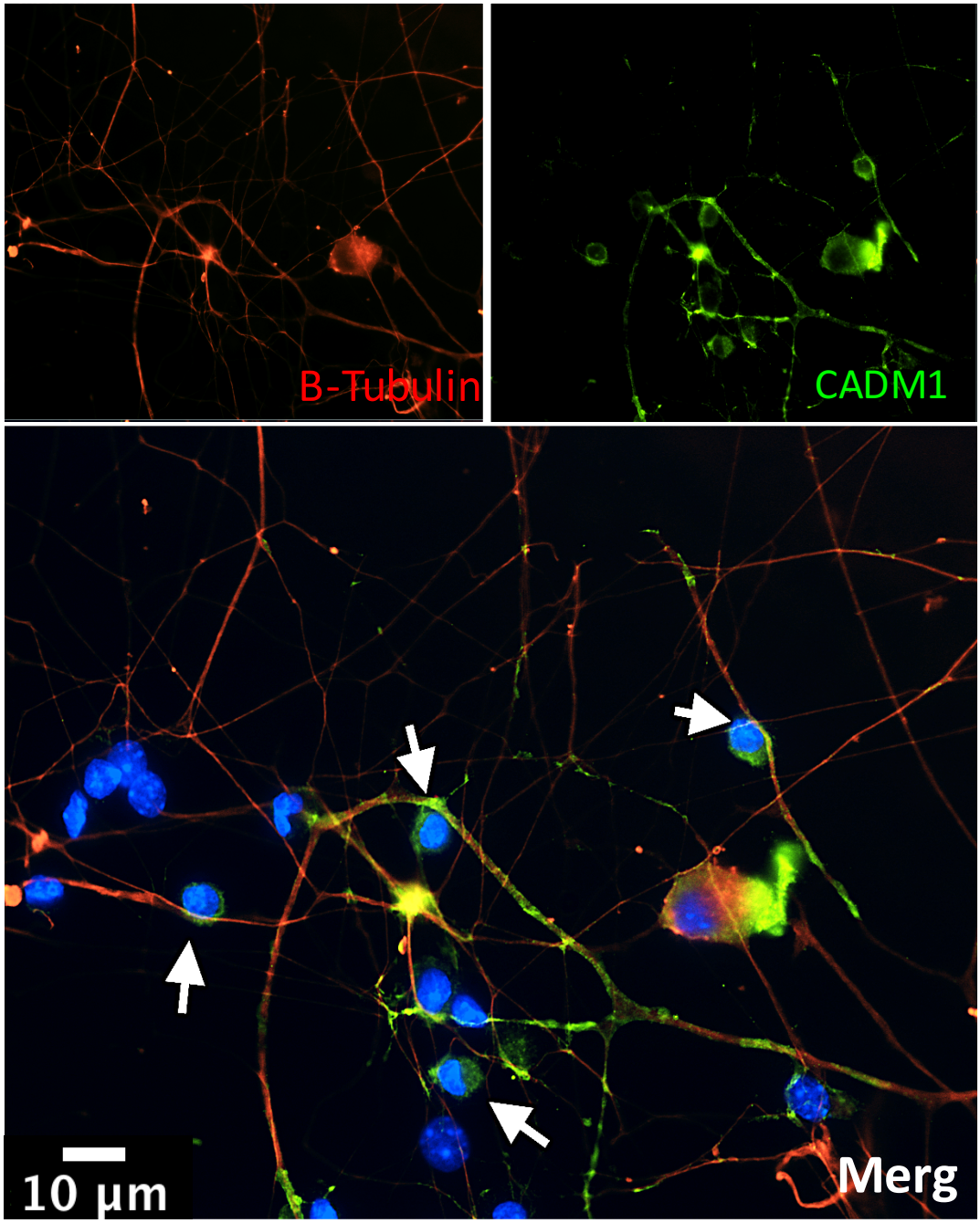
Figure.5.3 Flow cytometric analysis of surface and total CADM1 expression by BMMCs.

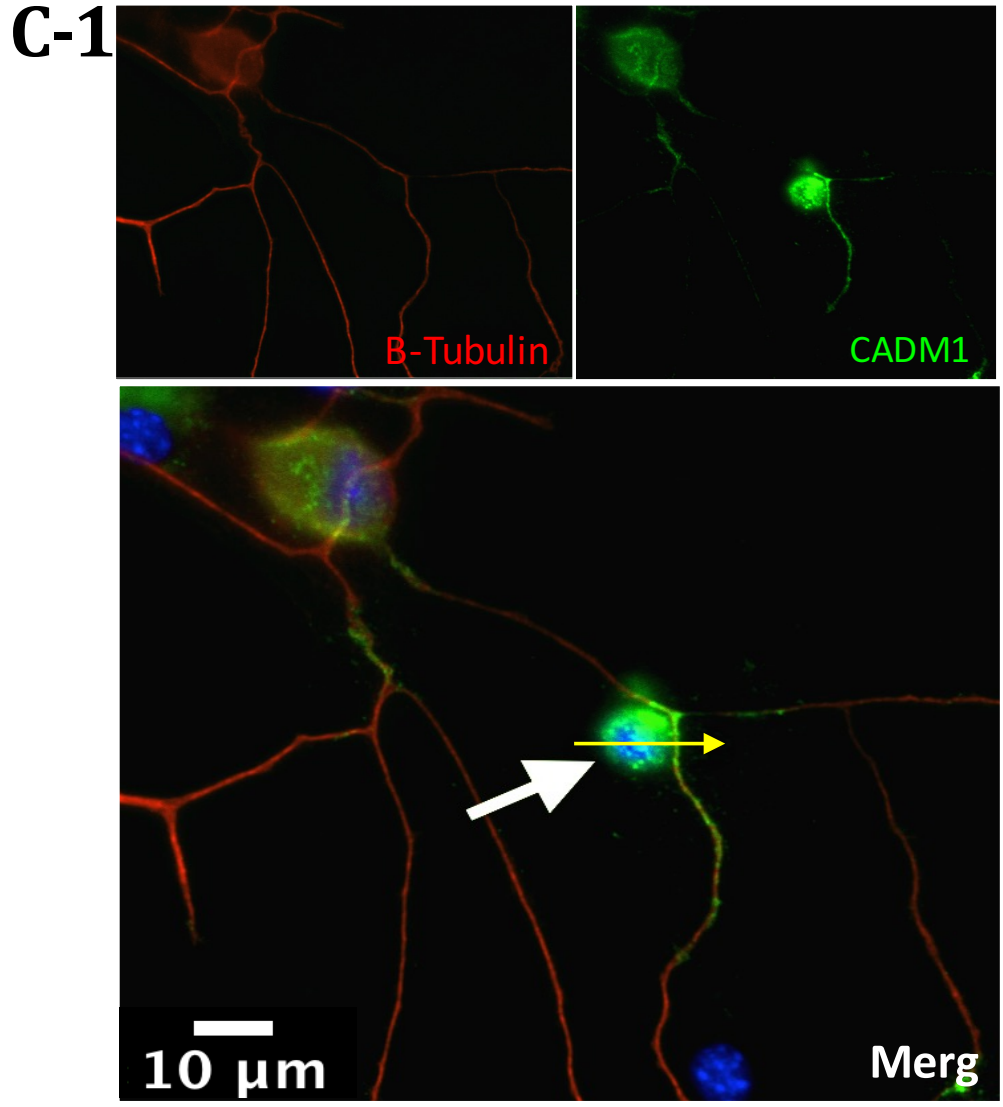
1X10^6 BMMC/reaction of non- permeablized **(A)** and TritonX100-permeablized **(B)** BMMCs were double labelled with conjugated c-Kit and CADM1 Abs and analysed by flow cytometry. BMMCs were gated as c-kit$^+$ CADM1high cell subset and c-kit$^+$ CADM1medium cell subset. Numbers in plots are the percentages of cells in the indicated gate. Representative examples of two experiments.

As I showed above, CADM1 was highly expressed on BMMCs and DRG neurons. Next, I investigated the localization of this adhesion molecule in BMMCs-neuron co-culture by immunocytochemistry. BMMCs were co-cultured with DRG culture for 24hours. After removal of non-adherent BMMCs, co-cultures were stained with the Abs to CADM1 , FcεRI and β-tubulin to identify mast cells and neurons, respectively. Representative fluorescence images are shown in (Figure 5.4). CADM1 clearly stained almost all BMMCs and neuron soma but not the whole length of neurites (Figure 5.4B). Interestingly, CADM1 appeared intense at the site of neurites where BMMCs attached. Fluorescence intensities plot of the site of BMMC-neuron attachment showed that CADM1 intensity increased in the periphery of BMMCs, but, in addition, it extended to area of β-tubulin fluorescence signal with no discontinuation of its signal (Figure 5.4C), consistent with the notion that CADM1 may mediate BMMC attachment to neurons.



B





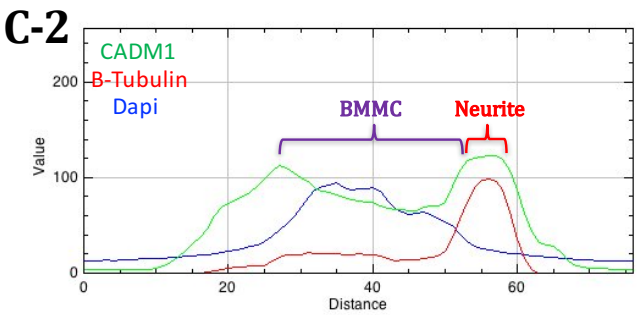


Figure 5.4 A representative fluorescence images of the co-culture of BMMC and DRG neurons.

BMMCs were co-cultured with DRG for 24 hours. After removal of nonadherent BMMCs. Co-cultures were stained with CADM1 and β-Tubulin to label neurons. **(A)** Confocal image for co-culture with BMMC labelled with FcεRI. **(B-C1)** showing that CADM1labeled all BMMCs and part of neurites where BMMCs attached (White Arrow). **(C-2)** Fluorescence intensities plot of the site of BMMC- neurite attachment (yellow arrow) scale bar represents 10 µm. Representative examples of three experiment.

5.2.2 BMMCs adhesion to neurons is mediated mainly by CADM1

Having shown that both BMMCs and neurons strongly express CADM1 when analyzed by both confocal immunofluorescence and western blot, I next wished to determine whether CADM1 is required for BMMC- Neuron adhesion. Calcein-labeled BMMCs were incubated with different concentration of CADM1 peptide (0, 1, 3, 10 and 30 μg/ml) for 30 minutes at 37^{o}C before co-culture them with DRG. After 2 hours, non-adherent BMMCs were removed, and the percentage of adherent BMMC was calculated. My results showed that blocking CADM1 on BMMCs inhibited the adhesion of BMMC to neurons in a concentration-dependent manner (Figure 5.5 A) and was almost abolished (3.1 ± 2 %) at the maximum concentration of blocking peptide tested (30μg/ml) when compared to percentage of BMMC adhered to neurons in the absence of blocking peptide (37.1 ± 1.26%). In contrast, incubating DRG cultures with CADM1 peptide before co-culture did not significantly reduce adhesion of BMMC except at the maximum concentration of blocking peptide (30μg/ml) where some attenuation in percentage of BMMC adhesion to neurons could be seen, but this did not achieve significance (P=0.062) (Figure 5.5 B). Unfortunately, higher concentrations of the blocking peptide could not be tested for technical reasons. None the less, these results indicate that CADM1 on mast cells mediates up to 90% of the adhesion between mast cells and neurons.

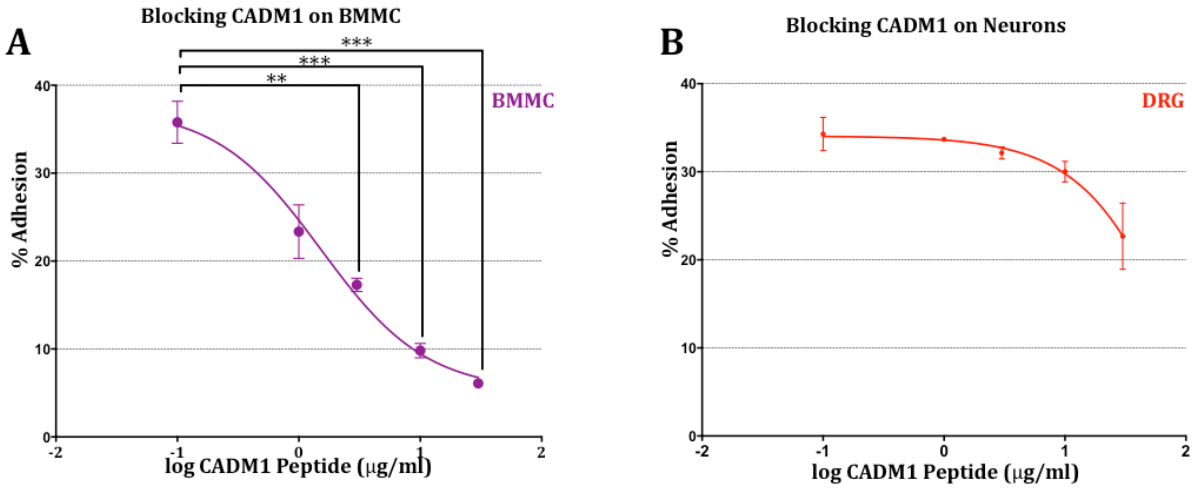


Figure 5.5 Attenuation of BMMC- neurons adhesion by CADM1 blocking peptide.

BMMCs(A) or DRG cultures (B) were incubated with different concentration of CADM1 blocking peptide for 30 minutes at 37°C before co-culture. Percentage of adherent BMMC was calculated using the calcein adhesion assay. Each condition was done in duplication. N=3. Each point represents the mean ±SEM. One-way ANOVA followed by Turkey's multiple comparison post-test was performed. * denotes $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$ compared to the percentage of BMMC adhesion in the absence of CADM1 blocking peptide.

To validate the results from the CADM1 blocking peptide, I also performed CADM1 knockdown experiments in BMMCs (detailed protocol is in section 2.7.2). On brief, 4-5×10^6 BMMCs / reaction were transfected with 2µg of CADM1 ShRNA or scrambled plasmid using Amaxa Nucleofector II (Amaxa). After 48 hours, cells were sorted using FACS. Only the GFP expressing cells were carried out for subsequent experiments. The effectiveness of CADM1 knockdown was ~100% in GFP-sorted BMMC, (see section 2.7.1). Transfected BMMC were incubated with DRG cultures for 2 hours at 37°C prior to performing the adhesion assay (described in section 2.4.2). CADM1 knockdown significantly reduced the adhesion of BMMC to neurons from 37 ± 1.3% in non-transfected cells or 33 ± 0.7 % in scrambled ShRNA transfected cells to 11.6 ± 0.8% in CADM1 ShRNA transfected BMMCs (Figure 5.6). This result confirms the major role of CADM1 in BMMC adhesion to neurons.

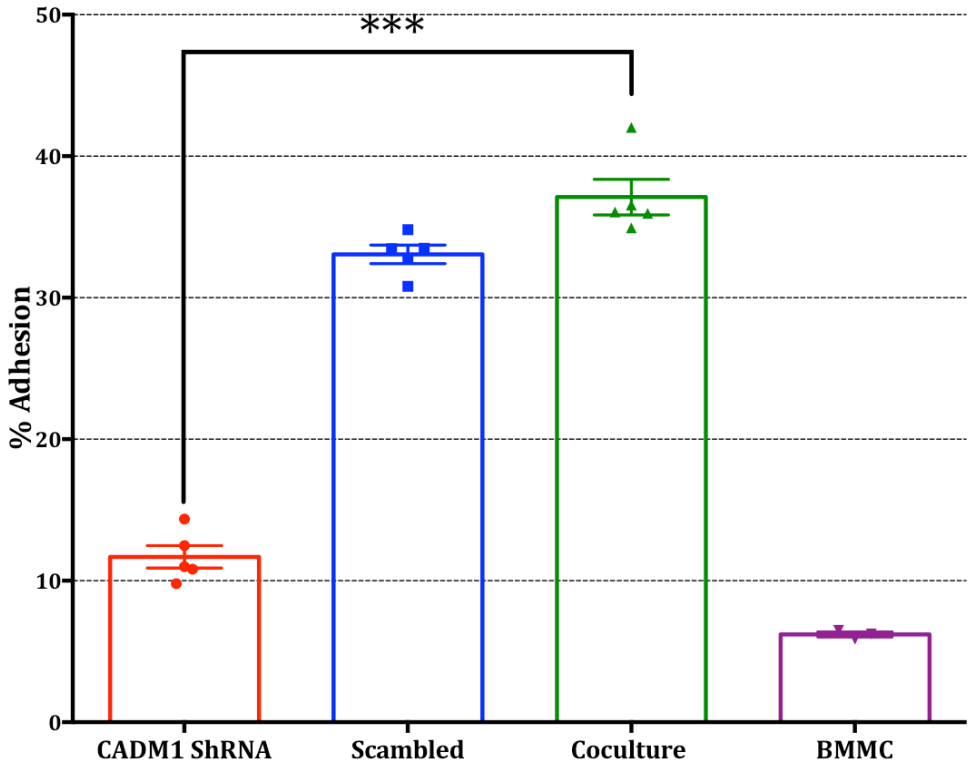


Figure 5.6 CADM1 knockdown attenuates BMMCs adhesion to neurons.

BMMCs were transfected with CADM1 ShRNA or scrambled control ShRNA and then their adherence to neurons tested using a calcein based assay. CADM1 knockdown significantly reduced BMMC adhesion. (N=4). Each bar represents the mean ±SEM. One-way ANOVA followed by Turkey's multiple comparison post-test was performed. *** denotes $p < 0.001$ compared to non-transfected BMMC group.

5.2.3 CADM1 mediates neuronal-enhancement of mast cell degranulation

Neuronal-enhancement of mast cell degranulation is cell-cell contact dependent. Because I showed that BMMCs adhere to neurons to a large extent by CADM1, here I investigated if blocking CADM1-mediated adhesion could affect neuronal-enhancement of mast cell degranulation. 2.5X10^5 BMMCs/ well were co-cultured with DRG for 24 hours in presence or absence of 10µg/ml of CADM1 blocking peptide. Next day, BMMCs in co-cultures were stimulated by DNP for 30 minutes, supernatants collected and assayed for β-Hex. The neuronal-enhancement of BMMC degranulation was inhibited significantly following treatment with the CADM1 blocking peptide. The fold-change of Ag-independent degranulation following CADM1 blocking peptide was also reduced to 1.3 compared to 3.3 in the control group (P=0.0143) (Figure 5.7 A). Moreover, the decrease in fold-change was even more striking (P=0.0019) following Ag-treatment (Figure 5.7 B), (0.99 in blocking peptide treated cells compared to 2.88 in control group.) Thus adhesion via CADM1 is necessary for neuronal-enhancement of BMMC degranulation.

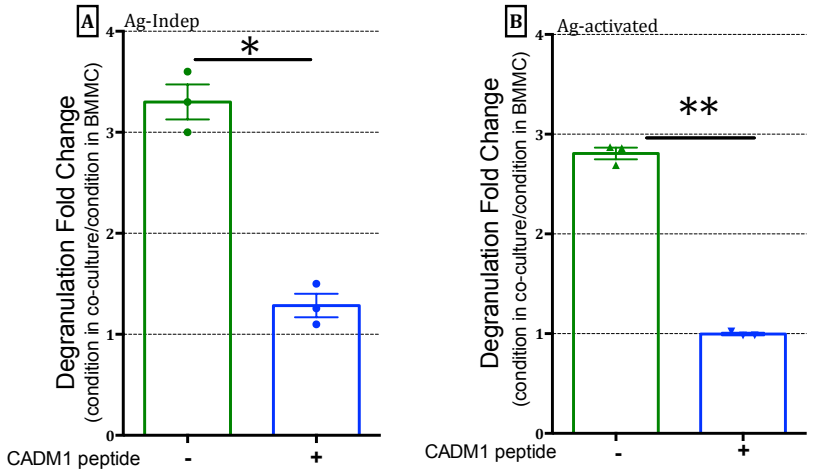


Figure 5.7. CADM1 blocking peptide reduces neuronal enhancement of BMMC degranulation.

BMMCs were co-cultured with DRG for 24 hours in the presence or absence of 10µg/ml CADM1 blocking peptide. Fold change of Ag-Indep degranulation **(A)** or Ag-activated degranulation **(B)** were calculated and compared by two-tailed paired *t*-test, * denotes $p < 0.05$, ** $p < 0.01$. Each bar represents the mean ±SEM. (N=4).

Next, I transfected BMMCs with CADM1 ShRNA or control scramble ShRNA. 48-hour post-transfection, BMMCs from each group were GFP-sorted by FACS then co-cultured with DRG for 24 hour . Next day, BMMCs were stimulated with DNP for 30 minutes. Supernatants were collected and assayed for β-Hex. Both the Ag-Indep and Ag-activated degranulation were significantly reduced in co-culture of CADM1-knockdown BMMCs with DRG (Figure 5.8). These results show that CADM1 not only promotes structural adhesion between mast cells and sensory neurons but also promotes the functional crosstalk.

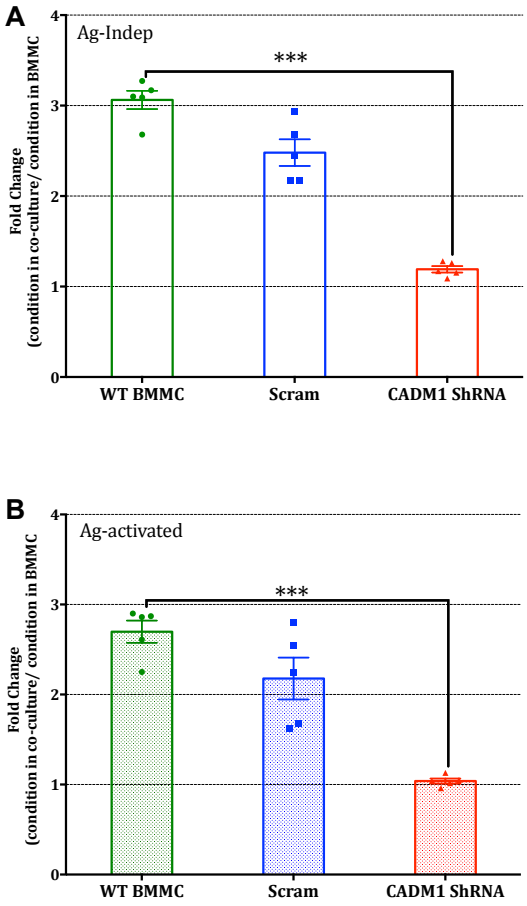


Figure 5.8 CADM1 knockdown reduces neuronal enhancement of BMMC degranulation.

WT, CADM1-knockdown or scramble-transfected BMMCs were co-cultured with DRG for 24 hours. Fold change of Ag-Independent degranulation **(A)** or Ag-activated degranulation **(B)** were calculated and compared by one-way ANOVA followed by Turkey's multiple comparison post-test , ***denotes $p < 0.001$ compared to wt BMMC-DRG co-culture. Each bar represents the mean ±SEM. (N=3).

5.2.4 CADM1 mediates neuronal-enhancement of IgE-mediated IL-6 production from mast cells

Co-culture BMMCs with DRG also induces the enhancement of IgE-mediated IL-6 secretion, so next I examined whether CADM1 is also needed for this functional effect. To do so, CADM1 ShRNA transfected BMMCs were co-cultured with DRG for 24 hours, before being stimulated with DNP for 6 hours. Supernatants were then collected and assayed for IL-6. IL-6 secretion from BMMC monoculture, BMMC-DRG co-culture, and scram-ShRNA transfected BMMC in co-culture were measured in parallel. Again, knockdown of CADM1 in BMMC significantly reduced IL-6 production by 62% compare to control BMMC-DRG co-culture (459 ± 30 pg/ml and 1209 ± 93 pg/ml, respectively) (Figure 5.9). Remarkably, IL-6 levels detected from CADM1 –ShRNA transfected BMMCs in co-culture were as low as those measured from BMMC monocultures, highlighting the importance of adhesion in regulating the signaling pathway leading to cytokine expression and secretion. This result further suggests that CADM1 is critical for neuronal-enhancement of Ag-activated IL-6 production in mast cells.

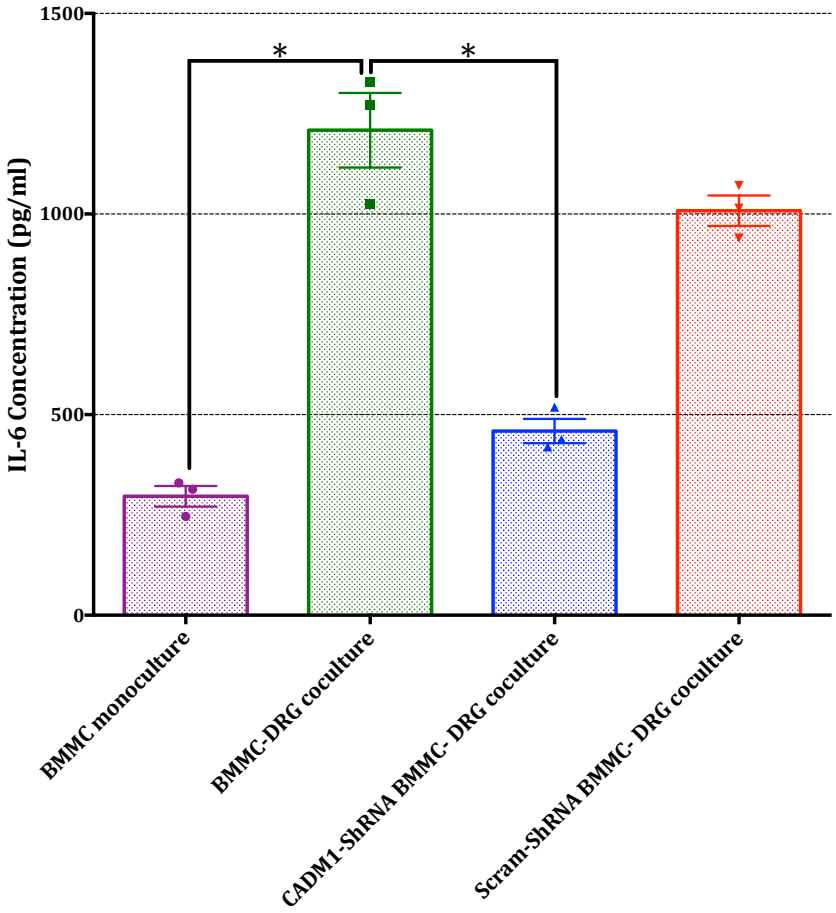


Figure 5.9 CADM1 knockdown reduces neuronal enhancement of IL- production by BMMCs.

BMMC monoculture and WT, CADM1-knockdown or scramble-transfected BMMCs were co-cultured with DRG for 24 hours. Then, BMMCs were stimulated with 10ng/ml DNP for 6 hours. IL-6 was measured from supernatants and compared by one-way ANOVA followed by Turkey's multiple comparison post-test , *denotes $p < 0.05$ compared to wt BMMC-DRG co-culture. Each bar represents the mean ±SEM. (N=3).

5.3 Discussion

As shown in chapter 4, direct physical attachment between mast cells and neurons was found to be essential to activate and enhance mast cell degranulation and IL-6 secretion in co-culture. This indicates that adhesion molecules may play a key role in mediating the cross talk between these two cell types. Among many adhesion molecules, I am interested in CADM1 as it is common adhesion molecule in neurons (Biederer et al., 2002) and mast cells (Ito et al., 2003). Indeed, it has been shown previously that CADM1 mediate attachment of mast cells to SCG (Furuno et al., 2005). *However, the contribution of this adhesion molecule in the DRG enhancement of mast cell function has not been scientifically explored. Thus I aimed in this chapter to study CADM1 expression in BMMCs and DRG, then to investigate its contribution in structural and functional communication between the two types of cells.*

First, I studied the expression of CADM1 in BMMCs and sensory neurons by western blot. Both cell types were found to express CADM1 but as proteins of different molecular weights, indicative of different isoforms. CADM1 has four isoforms resulting from alternative splicing (Section 1.4.2.2). The difference in isoforms reflects cell-type specific function. Consistent with previous reports (Ito et al., 2003) (Furuno et al., 2005) (Hagiyama et al., 2011), BMMCs were found to express CADM1 isoform **c**, a protein with molecular weight of ~100 kDa. Whereas DRG express CADM1 of ~75 kDa protein which corresponds to the expected molecular weight of isoform **d,** the same isoform found in lung (Masuda et al., 2002) (Koma et al., 2008). The abundance of CADM1 in DRG was ~3-fold that found in BMMCs. In contrast to my result and that of others (Hagiyama et al., 2011), (Furuno et al., 2012) reported that DRG only weakly express CADM1. However, they isolated DRG from newborns which may well have different properties than adult DRG (Melli and Höke, 2009). The maturity of neuronal culture could influence CADM1 expression, for example it has been shown previously that the CADM1 isoform type and expression are changed during cerebrum maturation (Hagiyama et al., 2011).

Next, I investigated CADM1 distribution in BMMCs and neurons by immunocytochemistry. Although both cell types were found to express CADM1, BMMCs appeared to express more CADM1 on their surface compared to what appeared to be largely intracellular expression of CADM1 on neurons. Surface expression of CADM1 on BMMC was confirmed by FACS. Interestingly, CADM1 was expressed in only some neurites, but when BMMCs were co-cultured with DRG, BMMCs were observed to attach to those parts of the neurites where CADM1 was expressed. I also observed that very few BMMCs attached to neuronal soma despite the high expression of CADM1. The preference of BMMCs to attach to neurites over soma may be because CADM1 is not expressed on surface of the soma. However, surface expression of CADM1 on neurites can't be confirmed even with confocal due to their small diameter. Altogether, the data suggest that not only the difference in CADM1 isoforms but also the difference in CADM1 distribution could influence CADM1 function in different cell types.

The function of CADM1 in neurons has been studied by Biederer and coworkers. They showed that CADM1 mediates homotypic adhesion between neurons and helps to form synapses in the CNS (Biederer et al., 2002). Although CADM1 is expressed on BMMC surface, *in vitro* in monocultures at least it seems that it doesn't mediate homotypic adhesion as the cells do not adhere to each other, consistent with the view that *trans* homotypic binding is isoform dependent(Hagiyama et al., 2011).

My data demonstrate that CADM1 is a major key adhesion molecule that mediates adhesion of mast cells to neurons. Blocking CADM1 on BMMCs before co-culture with neurons reduced the percentage of BMMCs adhered to neurons. Knockdown CADM1 expression from BMMCs also significantly reduced their adhesion to neurons; although, it also highlights that despite the effectiveness of the ShRNA in ablating expression of CADM1 in BMMCs, the reduction in adhesion achieved is less than that obtained with the blocking peptide. This likely reflects the non-specific blocking effect of the peptide. The peptide is designed against the whole extracellular domain of CADM1, which is comprised of 373 amino acids (Sun et al., 2014). Thus, this large molecular weight

peptide could interfere with other adhesion receptors that may be involved in mast cell adhesion to neurons such as N-cadherin (Suzuki et al., 2004) on mast cells or nectin-3 on neurons (Furuno et al., 2012). Another possibility is that CADM1 knockdown leads to compensatory upregulation of other adhesion molecules, as suggested by (Moiseeva et al., 2014). CADM1 knockdown in the HMC-1 results in markedly enhanced integrin-dependent adhesion of the cells to ECM. Nonetheless, undoubtedly CADM1 is responsible for the majority of BMMCs net adhesion to neurons.

In contrast to blocking CADM1 on BMMCs, incubation of DRGs with CADM1 blocking peptide before co-culture had little effect on BMMC adhesion. The explanations behind this could be one of the following: **(1)** Peptide didn't work on live DRG because DRG express CADM1 mostly intracellularly (Figure 5.2B). **(2)** Higher concentration of blocking peptide is needed to block CADM1 on DRG since DRG express 3-fold higher levels of CADM1 than BMMCs, as shown by western blot (Figure 5.1). That could be the case because the highest concentration of peptide used (30μg/ml) in this experiment showed some reduce in adhesion although not significant. **(3)** DRG express shorter isoform of CADM1(isoform **d**) than BMMCs (isoform **c**), that may have different affinity and binding ability to blocking peptide. **(4)** DRG express another adhesion molecule which forms heterotypic complexes with CADM1 on BMMCs. In addition to CADM1, Nectin-3 has been reported to be highly expressed by DRG and localized at the attachment point of CADM1 expressed by mast cells (Furuno et al., 2012). Blocking nectin-3 on DRG was reported to decrease mast cells attachment to neurons. This suggests the heterotypic binding of CADM1 and nectin-3 in BMMC-DRG co-culture. However, since they used embryonic DRG culture which express low level of CADM1, they may underestimate the major role of CADM1. A recent transcriptome analysis of adult mouse DRG reveal that the expression ratio of nectin-3 is very lower than CADM1 (Usoskin et al., 2015). *Knocking down the adhesion molecules in DRG is a more specific approach to precisely identify which one is the CADM1 partner on mast cells*. Despite all those explanations and the possible partner molecule on DRG, BMMCs obviously appear to attach to neurons largely via their surface expression of CADM1.

I have shown that constitutive degranulation and IgE-mediated degranulation was significantly greater in BMMC-DRG co-culture compared to BMMC monoculture (Figure 4.4) and that this enhancement in degranulation is cell-cell contact depended (figure 4.5). I therefore hypothesized that attenuating mast cells adhesion to neurons by blocking CADM1 would in parallel attenuate neuronal- enhancement of IgE- mediated mast cell function. To test this idea, CADM1 on mast cells was blocked or downregulated before co-culture them with neurons. The constitutive degranulation and IgE- mediated degranulation for co-culture of CADM1- ShRNA BMMCs was almost comparable to the amount of degranulation from BMMC monoculture. This indicates that CADM1-dependent adhesion of mast cells to neurons activates and enhances mast cells function.

The next question is whether CADM1- mediated adhesion also contributes to enhancement of IL-6 synthesis pathway in mast cell- neuron co-culture. Again, I found that knockdown CADM1 from BMMCs before co-culture decreased IL-6 production following IgE- mediated mast cell activation. The interaction between IL-6 and CADM1 and its consequence on mast cell biology has been suggested before (Hollins et al., 2008). Blocking of IL-6 and CADM1 decreased the effect of airway smooth muscle on mast cell proliferation co-culture. Because of their blocking effects were additive, this suggests cooperative interaction of IL-6 and CADM1. *However, further studies to investigate mechanism of IL-6 and CADM1interaction are required.*

It has been shown that CADM1 is critical for efficient communication between mast cells and neurons. Ectopic expression of CADM1 in CADM1- negative mast cells line (IC-2) resulted to increase IC-2 cell calcium signals following scorpion venom -evoked neurite activation (Furuno et al., 2005). Interestingly, the same results have been obtained from an *in vivo* study (Ito et al., 2007). BMMCs from CADM1-knockout mice were transplanted to mast cell-deficient (Kit^{W}/Kit^{Wv}) mice. Electrical stimulation of mesenteric nerves didn't evoke mast cell degranulation, while transfection with mast cells expressing CADM1 normalized the response. Collectively, the data suggest that CADM1 promotes communication between neurons and mast cells. Moreover, my work further indicates a

functional consequence mediated by CADM1-dependent adhesion of mast cells to neurons is enhanced IgE- signaling.

Potential mechanisms that could be proposed to explain effect of CADM1 on neuronal-enhancement of mast cell activation. Simply, CADM1 could enhance mast cell-nerve communication by mediating efficient attachment between those two cells. Efficient attachment facilitates chemical mediator's crosstalk such as subP, tryptase, IL-6, PG, ATP, serotonin and other mediators. All those mediators contribute in feeding back onto the activating this functional unit. This mechanism has been suggested before by Biederer (Biederer et al., 2002). They showed that CADM1 mediates machinery necessary for synapse formation between neurons. More importantly, they demonstrated that synapse was formed at contact site between neurons and non-neuronal cells when transfected with CADM1. However, previous researches failed to observe a synaptic structure formation between mast cells and neurites (Stead et al., 1989) (Blennerhassett et al., 1991).

Another more complicated mechanism that could explain the functional effect of CADM1 is that CADM1 extracellular binding could result in intracellular signaling events in mast cells. Although the partner molecules associated with the cytoplasmic domain of CADM1 is not yet known, but CADM1 has been reported to influence mast cell biology and function (Moiseeva et al., 2012).

As mentioned in introduction (Section 1.4.2.1), it is suggested that CADM1 in mast cell is regulated by microphthalmia transcription factor (MITF) (Ito et al., 2003), because MITF mutant BMMCs don't express CADM1. Interestingly, MITF is critical as well for mast cells to produce many mediators including cytokines and PGD2 (Morii and Oboki, 2004). This lets us speculate that MITF function may be affected by CADM1 Knockdown. That leads to decrease the enhancement of mast cell mediator's production.

Another potential mechanism is that CADM1-mediated mast cell neuronal attachment may influence IgE/FcεRI signaling by either increasing the number of FcεRI receptors on mast cells or contributing in IgE intracellular pathway. However, this speculation was briefly tested in next chapter.

In conclusion, I have defined a novel functional interaction between mast cells and neurons that is mediated, mainly, by cell adhesion molecule CADM1. Indeed, I have shown that CADM1- mediated mast cell- neuron adhesion enhance IgE-signaling and mediator production in mast cells. This supports the key role of CADM1 in regulating mast cell- sensory neuron interaction. Targeting CADM1on mast cells by drugs or antibodies could be new therapeutic approach for many neurogenic inflammation disorders in which atopy plays role. *However, further characterization of CADM1 mechanism in mast cell- neuron interaction will open up a new aspect to understand neuroimmune system.*

Chapter 6: Investigating the mechanism of CADM1- mediated mast cells –neurons crosstalk

6.1 Introduction

The research I presented in the previous chapter, showed that CADM1-dependent adhesion of mast cells to sensory neurons has functional consequences on mast cell biology. Here I present the results from studies initiated to investigate the possible mechanism behind neuronal- enhancement of IgE-dependent mast cell activation in my co-culture system. My primary objectives were to determine whether the enhancement phenomenon is neuron-specific and/ or IgE-activation specific. *The results of these experiments could shed some light on possible neuronal-mast cell interactions accruing in health and disease.*

6.2 Results

6.2.1 neuronal enhancement is unique for IgE-mediated mast cell activation.

Previously I showed that neurons enhance IgE-mediated mast cell activation, but whether this extends to other mast cell activators is unknown. Rather than trawling through and testing all possible stimulatory receptors expressed on BMMCs, I instead investigated the effect of c48/80, a well-known and much used pan-mast cell activator (See section 1.1.2.2). I stimulated BMMC in DRG-BMMC co-cultures with a submaximal concentration of c48/80 (30µg/ml) for 30 minutes and measured degranulation (Method is in section 2.3.1.2). As shown in Figure 6.1, c48/80 induced the same percentage of β-Hex in the presence and absence of DRG (26 ± 2 % and 25.3 ±1 %, respectively)(*P*=0.92), demonstrating that neuronal crosstalk doesn't simply make mast cells hyper-responsive in a non-specific manner but suggests instead that neuronal enhancement is specific for IgE- signaling.

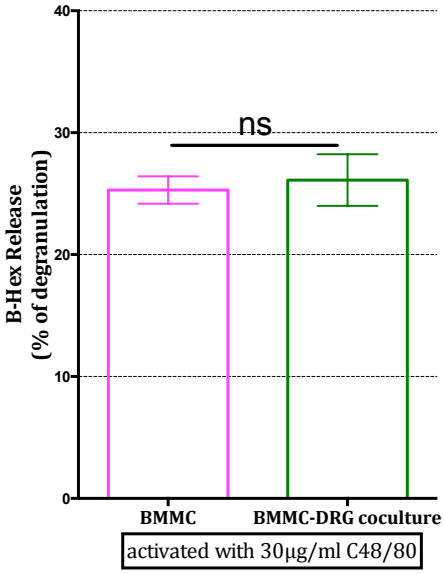


Figure.6.1 Effect of neurons on c48/80-mediated BMMC degranulation.

BMMCs were cultured alone or with DRG for 24 hours. Then, β-hexosaminidase (β-hex) release was measured after stimulation with 30µg/ml of c48/80. Total β-hex measured from BMMC lysed with 0.5% Triton Xl00. Data shown are mean ± SEM of N=3, each performed in duplicate. Data were analyzed using two-tailed paired t-test.

6.2.2 Effect of co-culture BMMC with DRG on FcεRI distribution on BMMC

To explore the mechanisms behind neuronal enhancement of IgE-mediated mast cell activation, I investigated FcεRI expression and distribution on BMMCs and co-culture, as a simple upregulation or stabilization of the receptor at the plasma membrane could account for such an effect. First, BMMCs cultures were double stained with antibodies against CADM1 and high-affinity receptor for IgE (FcεRI). Then, I used Pearson's r correlation coefficient analysis to determine the degree of co-localization of CADM1 with FcεRI (Method is in 2.5.2.4). Signals for FcεRI were well co-localized with CADM1 in merged images (Figure 6.2). Notably, this co-localization was mostly confined to the cell margin. The Pearson's r value was significant (Rr=0.92 ± 0.03, n=70, N=3) consistent with a strong co-localization of FcεRI and CADM1 on BMMCs.

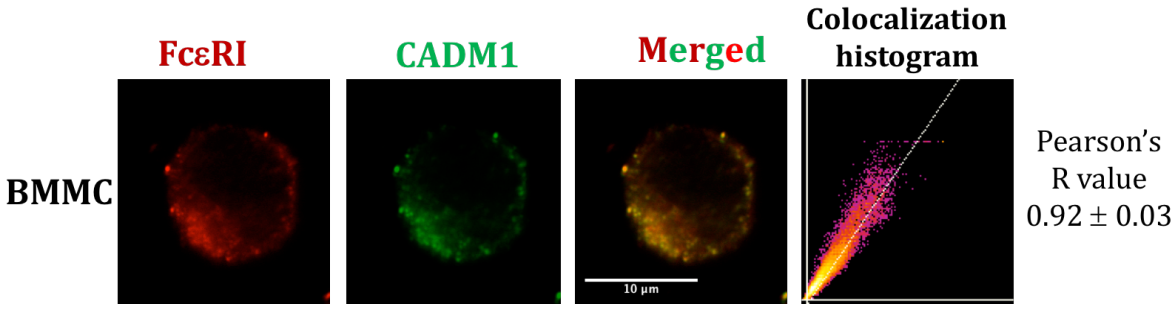


Figure 6.2 A representative fluorescence images of colocalization of FcεRI with CADM1 in BMMCs.

BMMCs were double stained against FcεRI (red) and CADM1 (green). Yellow areas indicate the colocalization in the merged image. Scale bar represents 10 µm. Colocalization was quantified using Pearson's correlation coefficient as R value as shown in scatter plot (right panel). Representative examples of 70 BMMCs from three independent experiment.

To investigate the effect of BMMC attachment to neurons on FcεRI surface expression and distribution, I quantified FcεRI surface expression in attached and unattached BMMCs in co-cultures using immunocytochemistry (Method is in 2.5.2.4). Non-permeablized BMMC-DRG co-cultures were stained against FcεRI to only label surface molecules. Then, mean fluoresce intensities (MFI) were measured by Fiji software (Figure 6.3). There were no differences in MFI of surface FcεRI between unattached and attached BMMCs (15.6 ± 3.2 and 16.23 ± 3.6, respectively) (P=0.54) suggesting that the enhancement of IgE- mediated degranulation in BMMCs in co-culture is not simply caused by up-regulation of FcεRI expression.

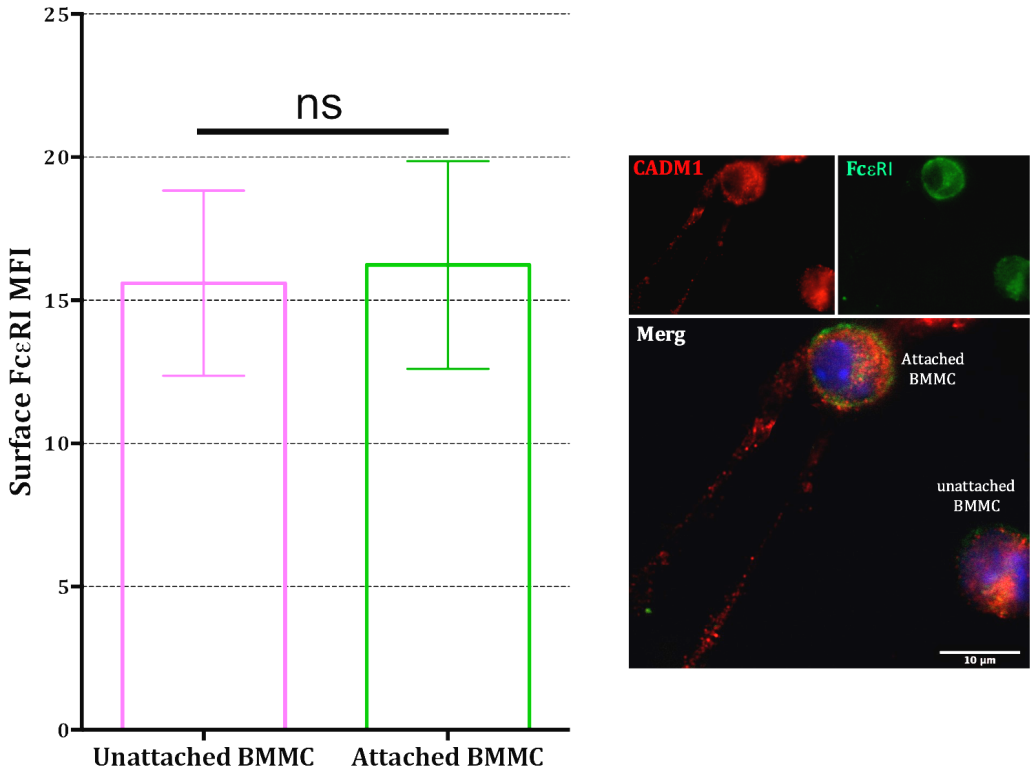


Figure 6.3 BMMCs attachment to neurons doesn't affect on surface FcεRI expression.

Surface FcεRI expression was evaluated for unattached and attached BMMCs in co-cultures by immunocytochemistry. Mean fluorescence intensity (MFI) was calculated from total of 58 unattached BMMCs and 74 attached BMMCs to neurons (N=3). MFI was compared by two-tailed paired t-test.

6.2.3 IgE-mediated BMMC enhancement is unique for sensory neurons

One possible mechanism for CADM1- mediated enhancement of mast cell-neuron interaction is a simple structural mechanism in which CADM1 binding enhances the chemical communication between sensory neurons and mast cells by bringing the two cells in close proximity, and reducing dilution of actively secreted chemical mediators from the neurons onto the apposed mast cells. In such a case, the effect of CADM1 would be dependent on the type of cell the mast cell was adhered to and specific for sensory neuron generated mediators. To test this hypothesis, I reproduced the same system as mast cell-DRG co-culture but I used HEK cells instead of neurons because HEK cells express CADM1. First, I confirmed CADM1 expression in HEK cells by western blotting. GAPDH was used as a loading control. Bands were visualized using Li-cor. As shown in figure 6.4, HEK cells express multiple isoforms of CADM1, viewed as multiple bands at 100, 70 and 60 kDa. As I showed previously (Figure 5.1) , BMMC express only one isoform of CADM1 at ~100 kDa.

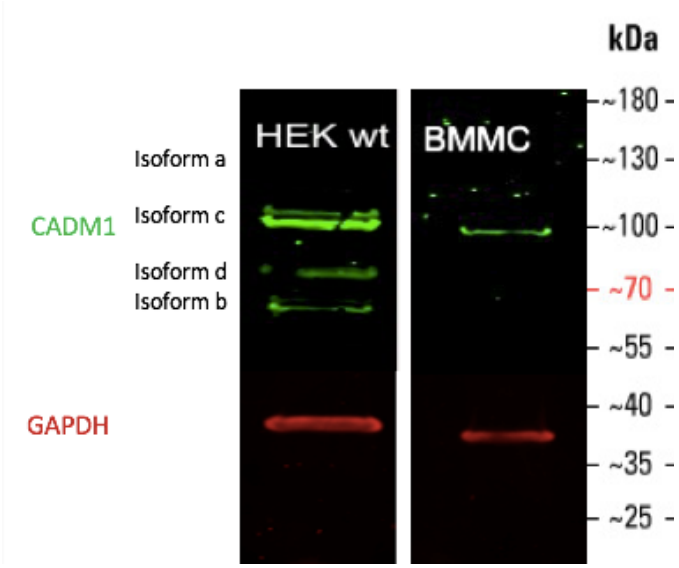


Figure. 6.4. Protein expression of CADM1 in BMMC and HEK cells.

Immunoblot of lysates prepared from HEK and BMMC cultures,. The blots were probed with anti-CADM1 and anti-GAPDH antibodies to indicate the total amount of proteins loaded per lane. The m.w. scale is shown to the right of the blot. Representative examples of two experiments performed on each cell type.

Next, I assessed the in*ter*cellular adhesion between mast cells and HEK cells using the calcein fluorimetric adhesion assay described previously (Section 2.4.3). 10,000 HEK cells/ well were first seeded on 1:5 Matrigel-coated wells. After 1 day, HEK cells reached >75% confluence. 100,000 calcein- labeled BMMCs were then added to the HEK cells and left to adhere for 2 hours. Non-adherent cells were gently removed and washed away by flipping the plate 3 times. To control for BMMC adherence to matrigel, an equal number of calcein–labeled BMMCs were cultured on 1 day-old Matrigel-coated wells in the same way as the ones in co-cultures. To quantify the adhesion between mast cells and HEK cells, I measured the fluorescence from calcein-labeled BMMCs before (for total) and after washing (for adherent) using a plate reader. Results were expressed as percentage of adherent to total. Only a small percentages of BMMCs (6.6 ± 0.35 %) adhered to control matrigel coated wells (Figure 6.5). The presence of HEK cells significantly increased the percentage of adherent BMMCs to (17.2 ±1.5 %) (P=0.017).

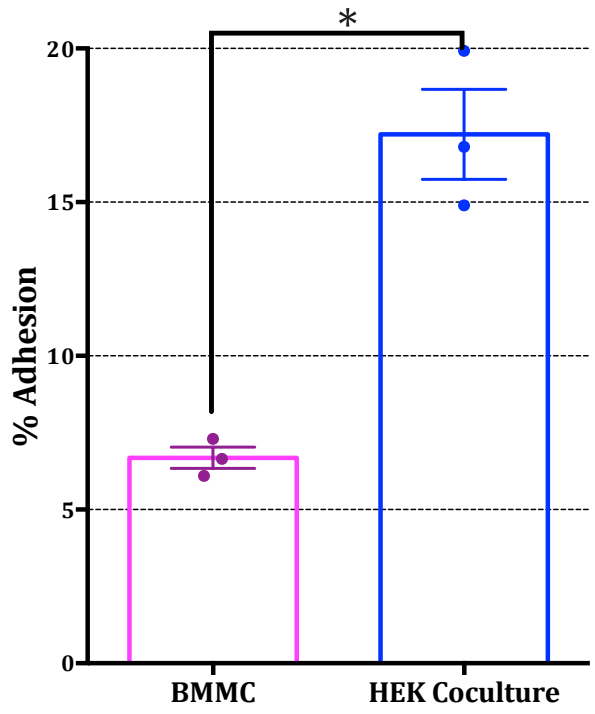


Figure. 6.5. Adhesion of BMMC to HEK cells.

BMMCs adhesion to HEK cells was quantified as percentage of adherent BMMC to total BMMC before washing. Data are shown as mean ± SEM from N=3. Each done in duplicate. Data were analyzed two-tailed paired *t*-test *p<0.05 compared to BMMC culture alone.

To examine whether this adhesion lead to alterations in antigen-stimulated secretion, I investigated the effect of HEK cells on BMMC degranulation. For that, I co-cultured IgE-sensitized BMMCs with 75% confluence HEK cells for one day. I then stimulated BMMC with DNP (Ag), and I measured the percentage of β-hex release in Ag-independent (Ag-Indep) BMMC and Ag-activated BMMC. To control for the effects of adherence, degranulation assays were also performed in parallel with BMMC set up in monoculture. As shown in Figure 6.6, the percentage of ag-independent β-hex released from BMMC-HEK co-culture (5.82 ± 1.6 %) was almost the same as that from BMMC monoculture (6.5 ± 0.74%) (*P*=0.52). Ag-activated degranulation was similarly unaffected by co-culture of BMMCs with HEK cells (17.9 ± 1.8 %) compared with BMMCs kept in monoculture (18.2 ± 1.6 %) (*P*=0.89). HEK cells by themselves secreted negligible amounts of β-hex (2.75 ±0.56 %), and this amount didn't change after Ag stimulation (2.75 ± 0.52%). Thus the data show that although HEK cells express CADM1 and mast cells may adhere to them, this in itself is not sufficient to either activate BMMCs or modulate IgE-mediated degranulation.

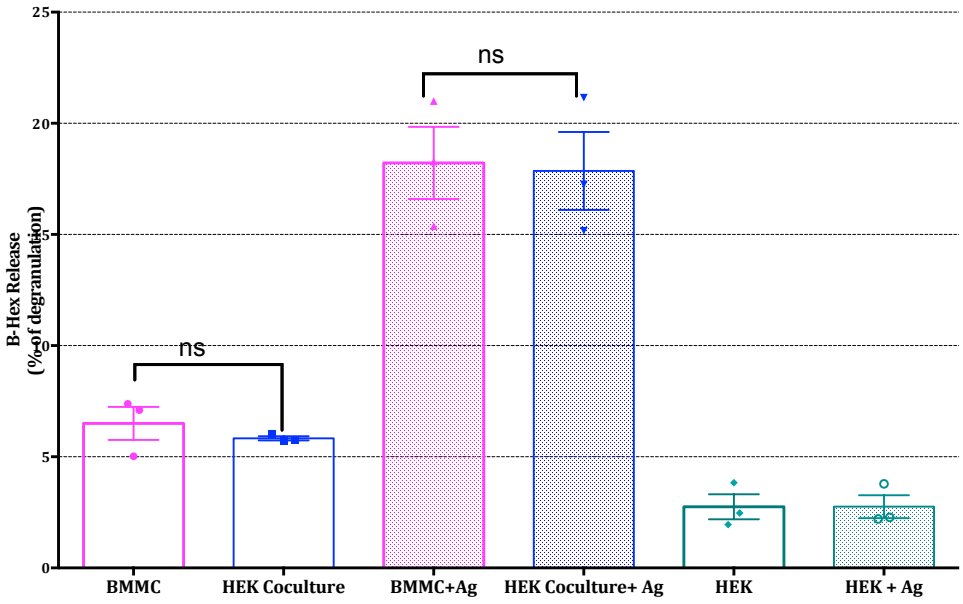


Figure.6.6 Effect of HEK cell on IgE-mediated BMMC degranulation.

Presensitized BMMCs were cultured alone or with HEK cells for 24 hours. Then, β-hexosaminidase (β-hex) release was measured in resting condition or after antigen (Ag) stimulation. Total β-hex measured from BMMC lysed with 0.5% Triton Xl00. Data shown are mean ± SEM of N=3, each performed in duplicate. ns=non-significant compared to BMMC alone. Data were analyzed using two-tailed paired *t*-test.

6.2.4 Silence AP in neurons doesn't block IgE-mediated mast cell enhancement in co-culture

To investigate the specific role of activated neurons in IgE-mediated mast cell enhancement in co-culture, I silenced the AP in neurons by incubation DRG with 1μm from each Tetrodotoxin (TTX) to block TTX-sensitive sodium channels , and ω–conotoxin MVIIC and GVIA to block calcium channels for 10 minutes before BMMC co-culture and during 24-h of co-culture (See material and methods section 2.6.4). First, I tested the cytotoxicity of this toxin cocktail (CTX) on BMMC. BMMCs were incubated with CTX for (2, 6, 12, 24 hours). Then, viability of cells was determined by counting the cells in each condition and calculated the % of viability by using trypan blue (N=3). I found that CTX didn't affect BMMC viability at each time point (Figure 6.7 A).

Next, I examined the effect of CTX in inhibition of KCl- induced neuronal calcium signals. I incubated DRG with CTX for 5 min, Then, I stimulated DRG with KCl in presence of CTX. My preliminary data (N=1, n=29) demonstrated that the calcium signals from neurons showed delay in response to KCl (around 30 sec) compared to neuronal response to KCl after washing for 15 minutes (less than 10 sec) (Figure 6.7 B).

Finally, I tested the effect of silence neurons by CTX on enhancement IgE- mediated BMMC degranulation in co-culture. After 24 hours of co-culturing BMMC with DRG in the presence of CTX, BMMCs were stimulated with 100ng/ml DNP for 30 minutes and percentage of degranulation was measured. I found that CTX slightly decreased, although not significant, the spontaneous (P= 0.26) and Ag-activated BMMC degranulation (P=0.31) in co-culture (Figure 6.7 C). However, CTX didn't affect BMMC degranulation in monoculture (1st and 2nd group in figure 6.7 C).

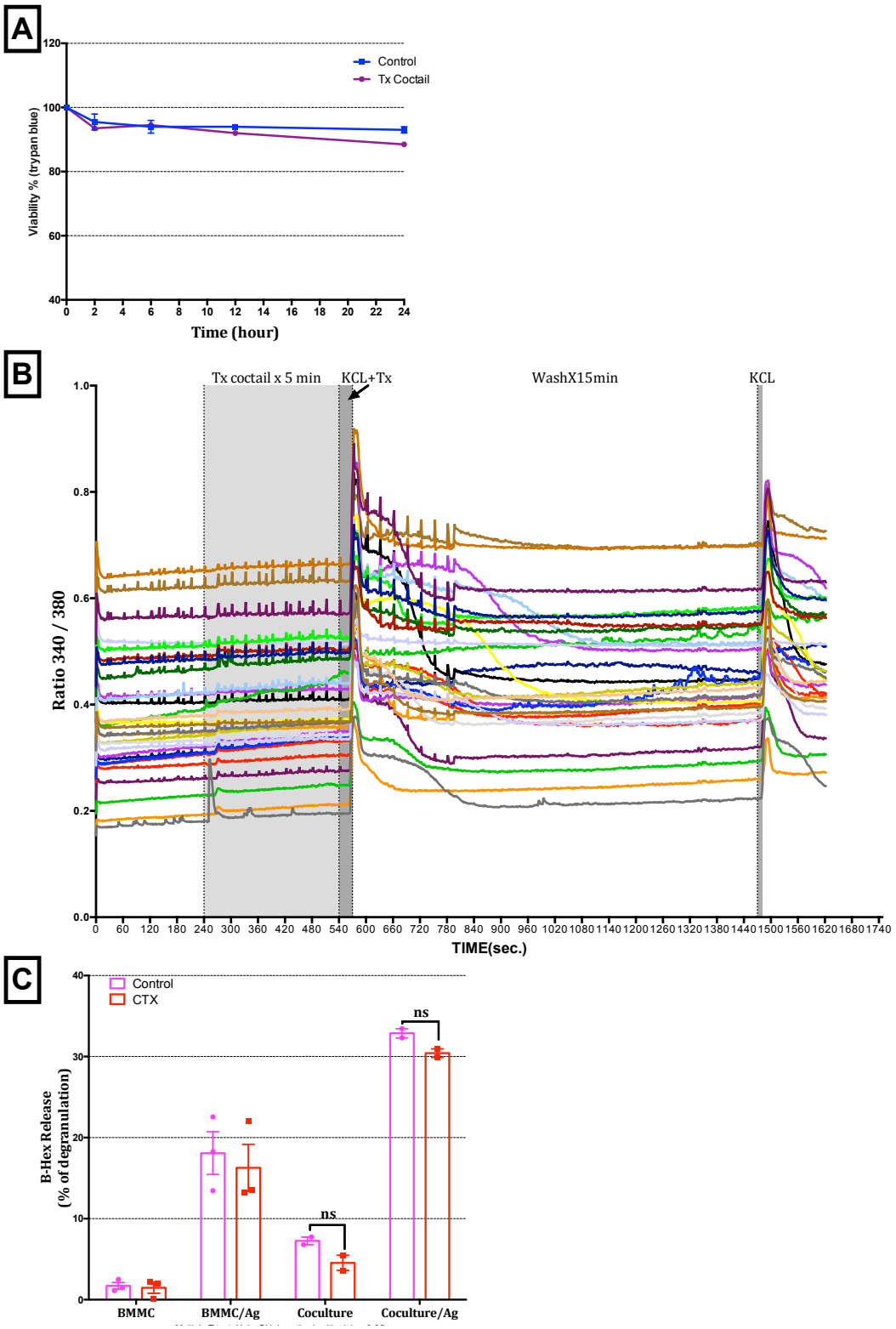


Figure.6.7 Silence AP in neurons doesn't block IgE-mediated mast cell enhancement in co-culture

(**A**) Cytotoxicity of 1 μm from each TTX, ω–conotoxin MVIIC and GVIA (CTX or Tx) on BMMCs for 24 hours. **(B)** The trace of change in calcium signals as reported by Fura-2, of individual neurons in response to 62mM KcL for indicated period (n=29). **(C)** BMMCs were cultured alone or with DRG for 24 hours in the presence or absence of CTX cocktail. Then, β-hexosaminidase (β-hex) release was measured after stimulation with 100ng/ml of DNP. Total β-hex measured from BMMC lysed with 0.5% Triton Xl00. Data shown are mean ± SEM of N=2, each performed in duplicate. Data were analyzed using multiple t-test.

6.3 Discussion

CADM1-mediated attachment of mast cells to sensory neurons is essential for potentiating IgE-mediated activation. The mechanisms by which CADM1 can modulate mast cell-neuron crosstalk however is unknown. To determine whether this crosstalk was specific to IgE- mediated activation or a general change in the responsiveness of BMMCs, I compared the effects of co-culture on antigen stimulation with activation by c48/80, a direct activator of GPCRs in mast cells (Mousli et al., 1990). The results of these experiments showed that co-culture of mast cells with neurons does not enhance c48/80-induced mast cell degranulation, suggesting the effect of neurons on mast cells is specific to the IgE- mediated pathway.

To apprehend how neurons may modulate IgE-mediated mast cell response, it is essential to briefly review what is known about the signaling pathway (more details in introduction section 1.1.2.1 and Figure 1.1). IgE is bound to mast cells via high-affinity receptor FcεRI. Antigen binding to IgE and subsequent crosslinking of FcεRI leads to activation of a cascade of tyrosine kinases and phosphorylation of the receptor itself, adaptor proteins and signaling molecules. This signaling cascade induces an increase in intracellular calcium and subsequent fusion of mast cell granules with the cell membrane. In addition, IgE- mediated signaling activates a variety of transcription factors that ultimate lead to the upregulation and expression of multiple genes including those for coding for a plethora of cytokines, chemokines and growth factors (Metcalfe et al., 1997) (Gilfillan and Rivera, 2009).

To determine at which step neurons influence IgE- mediated mast cells response, first, I investigated FcεRI expression and distribution on BMMCs and co-culture. I found that there is strong co-localization between CADM1 and FcεRI at the plasma membrane. This suggests the possibility of direct interaction between the two molecules at the cell surface although it doesn't explain the enhancement of IgE-mediated response in attached BMMCs. As there was no change in FcεRI surface expression between unattached and attached BMMCs, the data suggest the interaction is more likely to be at

the level of signaling rather than a result of stabilized expression of the receptor at the surface due to alterations in trafficking (Rios and Kalesnikoff, 2015). However, the methods I used for assessing surface expression on BMMCs were admittedly rather crude as it was reliant on antibody staining. Unfortunately, the strong adherence of BMMCs to DRG made it very difficult to isolate these for subsequent more sensitive quantitative methods of surface expression such as FACS.

Although the intracellular pathway of FcεRI trafficking is still not completely understood, the level of FcεRI surface expression is regulated by dynamic processes including: receptor stabilization, recycling and ligand- induced internalization (Rios and Kalesnikoff, 2015). FcεRI receptor stabilization is controlled by intracellular and external factors. Not much is known about the intracellular molecules that influence FcεRI stability, but they include members of Rab family of small GTPase (Kalesnikoff et al., 2007). The essential external factor that influence FcεRI stabilization is IgE binding (Galli et al., 2005). IgE binding prevents the losing of FcεRI from cell surface since the unoccupied FcεRI receptors undergo endocytosis and some of them are recycled back to the surface (MacGlashan, 2007). IgE- bound FcεRI complexes also undergo endocytosis after cross-linking with Ag. This process is known as "Ligand- induce internalization" and is important as a feed-back to regulate the activated receptors (Rios and Kalesnikoff, 2015). Because it was shown previously that co-culture of BMMC with SCG for a longer time (3 days) showed that the expression of FcεRI when quantified by ICC was higher in BMMCs attached to neurites compare to non-attached BMMC in the same co-culture (Suzuki et al., 2005), this indicates the neurons could influence the FcεRI recycling or newly receptor synthesis. *This theory could be tested by using BFA that blocks recycling and receptor synthesis (Borkowski et al., 2001) then measure the surface expression by FACS to test the change in receptor synthesis. Examining the recycling rate could be by selectively labelling FcεRI with (fab) fragment that don't induce receptor aggregation, then measure the surface expression by FACS at different time points (Rios and Kalesnikoff, 2015).*

I have shown enough evidence that neurons influence IgE-mediated mast cells responses. Neurons don't only enhance intracellular calcium signals of IgE- activated mast cells (Section 4.2.5 Figure 4.7 A2 and B1), but their effect is strong enough to enhance IgE- mediated degranulation (Section 4.2.3 Figure 4.4) and IL-6 signaling pathway (Section 4.2.4 Figure 4.6). However, there is a possibility that neurons influence FcεRI –pathway kinase phosphorylation. *A suggested strategy to investigate this possibility is by activating mast cells alone or in presence of neurons with Ag. Then, analyze the level of mast cell protein phosphorylation using Western blot. This would be an important future piece of work to pursue.*

The effect of neurons on enhancing IgE- mediated mast cell response could be linked to mediators such as neuropeptide (Suzuki et al., 1999) or ATP (Suzuki et al., 2007) secreted from activated neurons. As mentioned in the introduction (section 1.1.2.2), extracellular ATP can enhance IgE-mediated degranulation in HLMC but not ionophore-mediated degranulation which is similar to my data (Schulman et al., 1999). Moreover, blocking of P2X didn't affect the enhancement which suggests that this enhancement is through P2Y signaling. Because P2Y influences TRPC channels, it is therefore possible that ATP enhances IgE- mediated degranulation by synergistic modulation of TRPC downstream signaling. Interestingly, ATP is a small, autocrine or paracrine mediator that is rapidly degraded in the extracellular environment. To achieve its intercellular signaling, it requires exceptionally close contact between the cells. This could justify the dependent of the efficiency of mast cell-neuron interaction on adhesion molecules such as CADM1. *Thus, studying the role of ATP and purinergic receptors in mast cell- neuron cross-talk is an exciting potential future project.*

Another potential mediator that may enhance IgE-mediated response in co-culture is SCF (Gilfillan et al., 2009). As discussed in chapter 3, SCF dramatically enhances FcεRI-mediated mast cell degranulation and cytokine release. Interestingly, SCF is also reported to induce IL-6 production by BMMC but not TNFα (Gagari et al., 1997), and to increase IgE-mediated IL-6 mRNA expression (Hundley et al., 2004) but not TNF α (Lin

and Befus, 1997). This is very similar to my data regarding neuronal enhancement of IgE-mediated cytokine production (Section 4.2.4 Figure 4.6). As shown in (Figure 3.5), SCF activates MAPK/STAT and NFκB pathways that is important for IL-6 production (Akira, 1997) (Kalesnikoff et al., 2002). In contrast, c-kit signaling pathway don't involve activation of NFAT and that could explain inability of c-kit to enhance TNFα production (Klein et al., 2006). Furthermore, my data displayed that there is a strong colocalization of CADM1 and Kit molecules on mast cells(Figure 5.2), that may promote their synergistic functions. Therefore, it would be interesting in future to test whether SCF plays a role in neuronal enhancement of mast cell functions. However, I have tried to investigate the role of SCF/c-kit in neuronal enhancement of IgE- mediated mast cell degranulation. Therefore, I incubated mast cells with antibody against c-kit 30 minutes before and during 24- h co-culture. As expected blocking c-kit on mast cells by antibody didn't influence their adhesion to neurons, but it gave an incomprehensible results regarding blocking the enhancement in degranulation in co-culture. Moreover, it surprisingly increased the IgE- dependent degranulation in BMMC monoculture and makes it difficult to interpret. *The question of whether SCF/c-kit plays a role in neuronal enhancement of IgE- mediated mast cell degranulation requires further study with other tools to minimize the possible complicated factors.*

Next, I investigated whether CADM1- mediated enhancement of mast cell function is specific for the crosstalk between mast cells and sensory neurons or this enhancement could be reproduced with other cells expressing CADM1. For that, I used HEK cells since HEK cells express CADM1. Contrasting with the fact that HEK cells express multiple isoforms of CADM1, the percentage of BMMCs attached to HEK cells was less than BMMCs attached to neurons. Indeed, the attachment of BMMC to HEK cells failed to enhance IgE- mediated mast cell response. The differences in HEK experiment results can be contributed to different CADM1 isoforms expressed by neurons and HEK cells. By WB, I showed that neurons express CADM1d isoform. While HEK cells express multiple CADM1 isoforms (c, b, and d). This results suggested that CADM1d isoform on neurons binds firmer and more efficient than other isoforms to CADM1c isoform on BMMCs. This unique ability of CADM1d is because its structural differences from the other three

isoforms (a-c) as discussed by Hagiyama (Hagiyama et al., 2011). He co-cultured BMMCs with Neuro2a neuroblastoma cells that ectopically expressed different CADM1 isoforms. Then, the adhesive strengths were assessed by femtosecond laser-induced impulsive forces. BMMCs attached to Neuro2a-CADM1d neurites firmer than other isoforms. More interesting, mast cells attached to Neuro2a-CADM1d neurites showed more response to activated neurons than the ones attached to other isoforms. He referred this privilege of CADM1d isoform based on the fact that the extracellular juxtamembrane region of all isoforms except CADM1d have O-glycosylation site (See Introduction Figure 1.6). Although the role of O-glycosylation in different CADM1 isoforms has not been studied, it is suggested that O-glycosylation is important for determining association partners for different CADM1 isoforms (Biederer, 2006). The fact that HEK cells express multiple CADM1 isoforms could account for the relatively poor adherence of BMMCs to HEK cells. Since CADM1 acts as an adhesion molecule by assembling itself on the cell membrane as *cis*-homodimer (Masuda et al., 2002) (See introduction 1.4.2.2 Figure 1.5) , it is possible that different isoforms on HEK cells hardly align their different-in-length extracellular domain to form functional *cis*-homodimer on cell surface (Moiseeva et al., 2012). While neurons that express only one type of isoform (CADM1d) form functional CADM1 *cis*-homodimer easier and faster. This could explain the higher percentage of BMMCs attached firmly to neurons.

Although different CADM1 isoforms between HEK cells and neurons could explain the differences in adhesive strength, it doesn't explain the differences in IgE- mediated enhancement in mast cells. This enhancement appears to be cell- type specific for neurons. This raises the possibility of one of two scenarios: one possibility is that the effective adhesion of mast cells to neurons results in exposing the attached mast cells to secreted neurotransmitters which in turn synergize with IgE- mediated activation of mast cells. The second scenario is that different partner molecules associate with CADM1 at BMMC-neuron sites of adhesion than at BMMC-HEK cell sites and that this in turn leads to differential effects on downstream signaling pathways activated through IGE receptors. In either case, it is clear that the functional consequence of CADM1-mediated BMMC adhesion leads to cell specific effects.

Finally, although it is a preliminary data, I found that the safe concentration of CTX cocktail (composed of TTX and ω-conotoxin) on BMMCs that delay KCl-induced neuronal response failed to inhibit the enhancement of IgE- mediated mast cell degranulation in co-culture. Since TTX and conotoxin block only AP-derived dependent synaptic release of neurotransmitter (Brock and Cunnane, 1988), this data indicate that neurons may mediate mast cell enhancement through AP-independent neuropeptide release. Neurotransmitters can be released by alternative pathways of increasing intracellular calcium level (Sharma and Vijayaraghavan, 2003). *A suggested approach to investigate this possibility is by inhibiting the secretory machinery in neurons by using toxin such as botulinum*. The botulinum toxin cleaves the synaptosomal associated protein (SNAP) 25 and syntaxins to prevent the fusion of the vesicles with the neuronal membrane and neurotransmitter exocytosis (Rossetto et al., 2014).

In the same context, I have tried to silence mast cells in co-culture to inhibit the enhancement of IgE-mediated degranulation. Double- blind clinical trials have shown that mast cell stabilizers have a clinical usefulness in managing the pain in IBS and food allergy (Lunardi et al., 1991), (Stefanini et al., 1995). Therefore, it was interesting to test the effectiveness of mast cell stabilizers in inhibition of neuronal enhancement effect on IgE mediated mast cell degranulation. To do so, I incubated BMMC with 100μM of sodium cromoglycat 10 minutes before co-culture and during 24-hour co-culture with DRG . Unexpectedly, it didn't inhibit the neuronal enhancement of IgE- mediated mast cell degranulation. Indeed, it failed to inhibit IgE-mediated BMMC degranulation in monoculture. However, cromoglycate shows a different inhibitory effect depending on mast cell phenotypes and the lack of effect of cromolyn on mouse BMMC degranulation has been confirmed (Oka et al., 2012). On the other hand, nedocromil (another cromones anti-allergic agent) reported to inhibit IgE-mediated degranulation in mouse BMMC (Yazid et al., 2013). *Thus, it worth to repeat this experiment with other mast cell stabilizers as possible therapeutic approach for neurogenic inflammation.*

In conclusion, CADM1-mediated adhesion promotes the development of a microenvironment in which neurons enhance mast cells response to Ag. As the consequence the mast cell-neuron crosstalk gets stronger. This could explain why incidence of neurogenic disorders such as IBS is more in atopic patients (Roussos et al., 2003) (Tobin et al., 2008b). *Further understanding of these signaling pathways is necessary as therapeutic approach.*

Chapter 7: Conclusion and future directions

My data clearly showed that neurons specifically enhance IgE-mediated mast cells degranulation and IL-6 production (chapter 4). This enhancement is CADM1- mediated contact dependent (chapter 5). My study demonstrated for the first time that blocking and/or knockdown CADM1 on mast cells significantly inhibited this enhancement. CADM1 mediates efficient attachment of mast cells to neurons that facilitates the chemical bidirectional communication. The sensitivity of mast cells to IgE-mediated activation with the presence of neurons was not due to modulate the FcεRI expression, but it is presumably as a consequence of intracellular signaling (Chapter 6) (Figure 7.1).

The possibility that cell adhesion molecules (CAM) induce intracellular signaling has been supported in the literature (Cavallaro and Dejana, 2011) (Gibson, 2011) (Du et al., 2014). It has been reported that the adhesion- mediated signaling could be induced by all classes of CAMs. For example, homophilic binding of NCAM, a member of IgSF-CAM, recruits and phosphorylates focal adhesion kinase (FAK). This results in tyrosine phosphorylation of several proteins, including FYN/MAPK which results in the induction of neurite outgrowth (Ditlevsen and Kolkova, 2010). Another IgSF-CAM, Mel-CAM, also recruits FYN that phosphorylates FAK in endothelial cells (Anfosso et al., 1998). In the same way, integrin-mediated mast cell line(RBL) adhesion to fibronectin results in phosphorylation of several proteins, including FAK and PKC that lead to calcium mobilization (Hamawy et al., 1993). Cadherins also have been shown to modulate cell signaling through receptor tyrosine kinases. E-cadherin in epithelial cells can associate through its cytoplasmic domain with receptor tyrosine kinases to induce gene expression through NF-κB (Du et al., 2014)and STAT3 (Raptis et al., 2009). Recently, it has been revealed that NF-κB activation is impaired in CADM1 knockdown human T-cell lines (Pujari et al., 2015). They suggested that CADM1 is required to inhibit IκB-mediated NF-κB negative regulation and, as a result, enhances NF-κB-mediated transcriptional signaling.

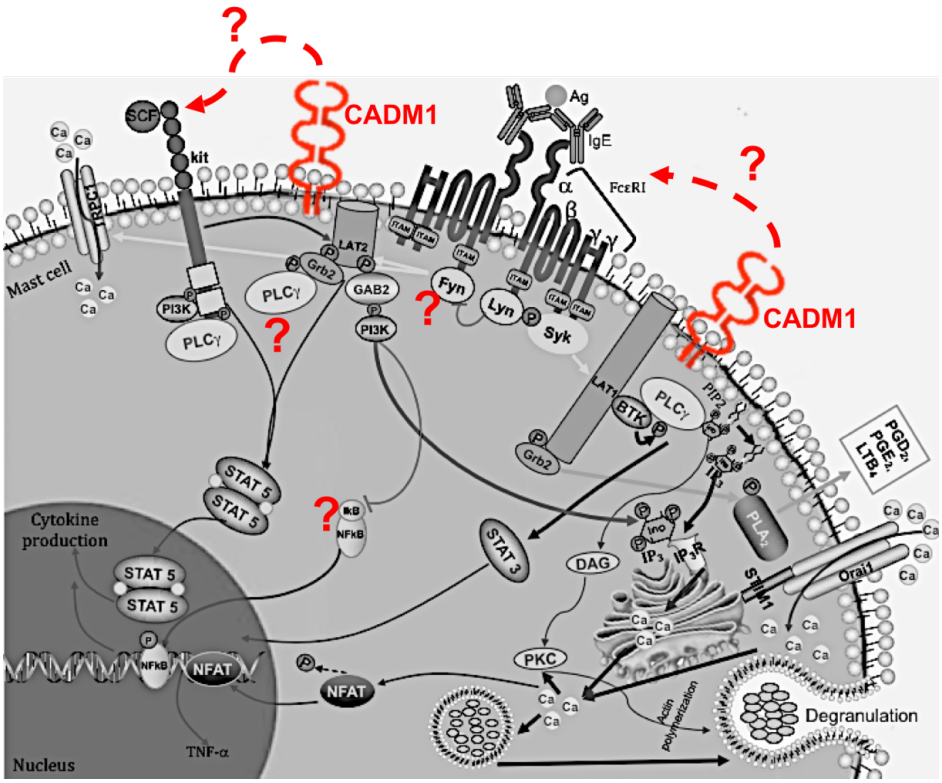


Figure. 7.1 Proposed CADM1- mediated signaling pathways in mast cell.

CADM1 can influence the activity of tyrosine kinase such as FYN/PLC signaling pathway and/ or may interact with other receptor, such as kit, to influence IgE-mediated mast cell activation. The Figure is modified from Figure 3.5

More interestingly, CAMs can also modulate receptor tyrosine kinases signaling within a single cell and without mediate cell–cell adhesion. This can be happened by different mechanisms. It could be by interacting and activating growth factor receptors and other signalling proteins. The prototypical example that is extensively studied is the interaction between NCAM and fibroblast growth factor receptor (FGFR) (Williams et al., 1994) (Francavilla et al., 2007). *NCAM directly binds to FGFR to produce a distinctive signaling cascade from those produced by binding of its ligand* fibroblast growth factor (Francavilla et al., 2009). *Moreover, E-cadherin binds to* vascular endothelial growth factor receptor 2 (VEGFR2) to prevent its internalization (Lampugnani et al., 2006). However, *cis* clustering of CAMs that follow the initiation of trans cell-cell adhesion could amplify other signaling pathways. Clustering is a lateral homotypic binding of

CAMs on cell membrane of the same cell to strength the intercellular adhesion. As a result, signaling membrane receptors come in close contact with their cytoplasmic partners including tyrosine kinase and initiate signaling. Overall, the recognition that CAMs not only maintain cell-cell adhesion but also can induce intercellular communications and signaling supports our proposal that CADM1-mediated adhesion of BMMC to neurons has important functional consequences. Cell signaling driven by CADM1 in BMMC is likely to enhance FcεRI signaling. *Further studies are required to fully understand this signaling process.*

Results of this study would contribute to the available knowledge about mast cell-neuron crosstalk in health and diseases. In fact, studying mast cell- neuron crosstalk in GIT is of great interest because GIT is known to contain the most extensive immune system in the body, as well as, it has a very rich supply of sensory nerves. This anatomy promotes opportunity for nerves that release neuropeptides and other mediators to activate mast cells that nearby (Stead et al., 1989) (Barbara et al., 2004). This bidirectional crosstalk plays a homeostatic unit in maintaining gut physiology (section 1.1.5.1.1), as well as pathogenesis of GI disorders such as IBS.

The contribution of mast cell-nerve interaction in the pathogenesis of IBS is based on the following evidence**: (1)** an increased number of mast cells attached to nerves in IBS patients compare to controls (Park et al., 2003) (Barbara et al., 2004). **(2)** The severity and the frequency of abdominal pain in IBS patients are significantly correlated to the number of degranulated mast cells in direct contact to nerves (Barbara et al., 2004) (Park et al., 2006). **(3)** The severity and the frequency of abdominal pain in IBS patients are also significantly correlated with expression of TRPV1 receptor on sensory neurons in GIT (Akbar et al., 2008). These correlations suggest that as the disease gets worse, the mast cell- nerve interaction got stronger. **(4)** Finally and most important clinically, mast cell stabilizers have been shown to decrease visceral hypersensitivity in patient with IBS (Klooker et al., 2010).

However, the incidence of IBS in atopic patients with known history of conditions such as asthma, eczema, or atopic rhinoconjunctivitis is higher than non-atopic (Powell et al., 2007) (Tobin et al., 2008b) (Lillestøl et al., 2010) (Walker et al., 2014). Indeed, a cross-sectional study on IBS patients showed a significant correlation between serum IgE levels, that a biomarker for allergies, and number of IgE- positive mast cells in intestinal biopsies (Lillestøl et al., 2010). Interestingly, IBS symptoms were positively associated with elevated serum IgE levels (Vara et al., 2016). Thus, *atopic IBS* has been defined as development of IBS in atopic patients (Tobin et al., 2008a).

These literatures indicate that the more severe atopic disease the stronger mast cell-neuron crosstalk. In the light of my data, I propose an explanation for these observations. In atopic patients with high level of serum IgE, the exposure to Ag, likely from ingested food, activates IgE-positive mast cells in the gut. The attachment of mucosal mast cells to neurons, presumably mediated by CADM1, enhances the IgE – mediated mast cells mediators release that induce visceral hypersensitivity. Consequently, symptoms of IBS would get worse.

This explanation could be completely applicable on all mast cell- neuron interactions in different tissue. In fact, it has been suggested that allergen-induced neuromodulation resulted in hyperactivity of nervous system in atopic patients (Undem and Taylor-Clark, 2014). As capsaicin induces strong parasympathatic reflex and sneezing when applied to nasal mucosa of atopic rhinitis patients, an effect that is not seen in healthy subjects (Sarin et al., 2006). All these data emphasize the principal role of IgE-mediated mast cell activation on neuronal activity. An interesting question is whether manipulation of this pathway lead to a substantial improvement in neuronal- related symptoms. Different potential agents targeting mast cell biology has been proposed (Zhang et al., 2016). Among them, *Omalizumab*. Omalizumab stabilizes mast cell functions by neutralizing soluble IgE as well as downregulating surface FcεRI on mast cells. It is known for it is high efficacy in controlling moderate to severe asthma (Society and Network, 2014). In addition, it has shown its effectiveness in treatment of other allergic diseases as rhinitis

(Casale et al., 2001), atopic dermatitis (Lane et al., 2006) and urticaria (Maurer et al., 2013). It was reported in case study that it showed a promising effect in complete resolution of IBS symptoms in patient on Omalizumab for other atopic diseases (Pearson et al., 2015). *Possible future experiments using co-culture model in the same way of this thesis could be used to test the effect of different mast cell-targeting approach on attenuate mast cell-neuron communication.*

Another interesting avenue for future investigation would be detailed exploring the underling signaling of CADM1-dependent neuronal enhancement on IgE-mediated activation in mast cells. Structurally, CADM1 resembles NCAM, both belong to the immunoglobulin superfamily. As I discussed above, NCAM has been showed to induce intracellular signaling and interact with growth factor receptors. Therefore, there is a possibility that CADM1 share the same FYN/MAPK signaling pathway and may interact with other receptor to enhance IgE-mediated mast cell activation. Analysis of FcεRI signaling in co-cultures from CADM1 knockdown BMMCs could shed some light onto potential regulatory mechanism of CADM1 in mast cell activity. A basic technique to study the activity of different kinase that may involve in this signal transduction could be measured by kinase assay. In addition, protein-protein interactions within the pathway could be studied by co-immunoprecipitation technique (Kawakami and Kawakami, 2015).

This study addressed the regulatory role of CADM1 in mast cells activation *in vitro*, however, these results need to be verified *in vivo* in order to determine the role of CADM1 in health and diseases. To do so, mast cell- deficient mice that has been engrafted with CADM1-knockdown BMMCs could be used. Although mast cell- deficient kit^{W}/kit^{Wv} and $kit^{W\text{-}sh}/kit^{W\text{-}sh}$ mice has been used extensively for this purpose, the kit-mutation can confound the results from these mouse models(Kakurai et al., 2006). Therefore, a new mast cell- deficient mouse model has been introduced. The mast cell-deficient in these mice is as a result of ablation of myeloid cell leukemia 1 (Mcl-1) which known as anti-apoptotic factor. Consequently, C57BL/6-Cpa3-Cre^{+}-Mcl-$1^{fl/fl}$ mice with normal kit are produced which are called informally “Hello Kitty” mice (Lilla et al.,

2011). Such models have been extremely useful in assessing the role of CADM1 in mast cell-related disorders in general, and in neurogenic inflammatory disorders in specific. Furthermore, it can be useful to assess whether the absence of CADM1- mediated mast cell adhesion to neurons has adverse effects on the whole organism health. However, before translate these results to human, it is important to compare this data to other mast cells phenotype because of mast cell heterogeneity is a critical issue in mast cell biology.

Another issues that could limit my data and remain to be addressed are: **(1)** Among wide range of mast cell cytokines, only IL-6 and TNFα were measured. Furthermore, a single granule component, β-hexosaminidase was measured for assessment of the level of degranulation. However, other mast cell and neuronal mediators such as neuropeptides, ATP, serotonin and PGD2 are known to intimately involved in the crosstalk. Therefore, these mediators must be investigated in depth in order to final understand the extend of mast cell- neuron interactions in health and the diseases. **(2)** Although I have shown that neurons express CADM1, future studies should carry out to comprehensive investigate CADM1 distribution along the neurites and weather it is in random fashion or its at particular spots, presumably, in co-localization with receptors that involve in crosstalk. Moreover, I didn't test the contribution of CADM1 expressed by DRG on the enhancement of mast cell function in co-culture. The difficulty of transfection primary neurons is the major cause. However, this could represent the objective of future studies.

In summary, my data support the concept that mast cells responsiveness could be influenced by their interaction with other cells such as neurons. Cell signaling mediated by CADM1 is a fundamental process to regulate mast cell- neuron crosstalk that is believed to be important in the progression of many disorders related to neurogenic inflammation.

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