JIHEON SONG

M.SC. THESIS

FUNCTION AND DISTRIBUTION OF NEURONAL
HIGH-AFFINITY IgE RECEPTORS (FcεRI)

By JIHEON SONG, B.Sc.

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TITLE: Function and Distribution of Neuronal High-Affinity IgE Receptors (FcεRI)

AUTHOR: Jiheon Song, B.Sc. (University of Toronto)

SUPERVISOR: John Bienenstock, M.D.

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ABSTRACT

Background

IgE antibodies have high antigen specificity and are the hallmark biomarkers of allergy. IgE binds to high-affinity IgE receptors, known as FcεRI, which are expressed especially on mast cells and basophils. In allergic individuals, antigen binding to IgE that is associated with FcεRI leads to crosslinking of adjacent receptors and subsequently to cell activation, degranulation and/or secretion of bioactive molecules. These molecules together cause minor local tissue reactions such as oedema or itch, but also can cause major systemic reactions such as hypotension, cardiac and respiratory distress or even laryngeal swelling and death. The role of the nervous system in these reactions is usually thought of as secondary. However, in recent years there have been a number of studies suggesting the expression of FcεRI on neurons, opening the possibility that nerves are directly involved in antigen-specific responses and making a previously unrecognized contribution to allergic disease.

Based on these previous observations regarding neuronal FcεRI, the current study employed both *in vivo* and *in vitro* approaches with the following objectives:

1. To confirm the presence of FcεRI on peripheral nerves and demonstrate that they are functionally active under different conditions of IgE sensitization.

2. To examine the pathways involved in neuronal activation by IgE bound to FcεRI and compare and contrast these to those already established for mast cells and basophils.

Methods and Results

A potential role of neuronal FcεRI in the IgE-dependent allergen avoidance behaviour of sensitized mice presented with antigen in sucrose solution was assessed based on published evidence for the involvement of peripheral nerves in this response. Chimeric mice with a wild-type nervous system but lacking FcεRI on hematopoietic cells including mast cells and basophils, failed to exhibit aversive behaviour, whereas mice with FcεRI-bearing hematopoietic cells demonstrated the normal aversive response confirming that FcεRI expression on mast cells is necessary for development of allergen avoidance. While immunohistochemical staining could detect IgE bound to mast cells in tissue samples, no IgE was detected on nerves where the nerves were identified by using a pan-neuronal marker, PGP 9.5, in the intestine of either normal or passively sensitized C57BL/6 and BALB/c mice. Similarly, traditional FACS analysis clearly identified FcεRI on cultured mast cells, but these methods provided no evidence for expression of FcεRI or IgE binding on superior cervical ganglion (SCG) and dorsal root ganglion (DRG) neurons in culture.

To determine evidence for functional FcεRI on neurons *in vitro*, intracellular calcium increase was assessed as a measure of cell activation following sensitization and antigen challenge. Using both microscopy and FACS analysis, calcium fluorophore (Fluo-3, AM) increase could be detected in SCG or DRG that were activated with the calcium ionophore A23187 but not following antigen challenge.

Summary: The current study found no evidence for the presence of the FcεRI on neurons *in situ* or their sensitization by IgE actively or passively using several different approaches both in tissues and cultured SCG or DRG neurons. Possible explanations for the resultant discrepancy with previously published works are discussed.

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LIST OF ABBREVIATIONS

4-MUG	4-methylumbelliferyl-β-D-glucuronide
AraC	cytosine β-D-arabinoside
BMMC	bone marrow-derived mast cell
BSA	bovine serum albumin
CMF	calcium-magnesium-free
DAPI	4'-6-diamidino-2-phenylindole
DC	dendritic cell
dH$_2$O	distilled water
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenol
DRG	dorsal root ganglia
FACS	fluorescence-activated cell sorting
FcεRI	high-affinity IgE receptor
FcεRII	low-affinity IgE receptor
FITC	fluorescein isothiocyanate
GF	germ-free
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI FBS	heat-inactivated fetal bovine serum
HSA	human serum albumin
IgE	immunoglobulin E
i.p.	intraperitoneal
ITAM	immunoreceptor tyrosine-based activation motif
MNV	murine norovirus
NGF	nerve growth factor
NGS	normal goat serum
ns	not significant
OCT	optimal cutting temperature
OVA	ovalbumin
PBS	phosphate buffer saline
PE	phycoerythrin
Pen-Strep	penicillin-streptomycin
PGP9.5	protein gene product 9.5 (ubiquitin carboxy-terminal hydrolase L1)
PMA	12-o-tetradecanoylphorbol-13-acetate (phorbol 12-myristate 13-acetate)
RBC	red blood cell
SCG	superior cervical ganglia
SFB	segmented filamentous bacteria
SPF	specific pathogen free
Syk	spleen tyrosine kinase

INTRODUCTION

From hayfever to insect bite and peanut allergies, allergy is one of the most common and prevalent immune disorders across the world (Viegi 2010, Warner 1999). Normally, our body is equipped with an immune system that acts as a host defense mechanism second to the physical barrier such as skin and epithelium, to guard our body against harmful pathogens. However, when the immune system becomes hypersensitive to allergens from the outside environment, one develops allergic symptoms. Allergic symptoms range from mild, as in pollen allergy or dust mite allergy in households, to extreme and often fatal, as in anaphylactic shock from food allergy. The aetiological mechanisms involved have been studied in great detail over many decades with only relatively little progress made towards treatment, let alone cure, compared to the ever-increasing prevalence across the world (Upton et al. 2000). One interesting point to note is that, in contrast to many other common disorders, a higher percentage of the general population suffers from allergic disorders in the economically affluent parts of the world than in the less affluent counterpart (ISAAC 1998, Weinmayr et al. 2007), or a higher prevalence in areas with higher socioeconomic status than the lower counterpart within one country (Mullins et al. 2010) or region (Samoliński et al. 2012). So-called disease of the rich and affluent, the positive correlation between prevalence and economic wealth has been a hotly debated subject of study in recent years in the fields of immunology and epidemiology, and many explanations have been proposed that may provide ways to control and contain the illnesses.

The fact that it has been only in the last few decades that allergy has become "endemic" across the world suggests that a genetic component has little influence on the aetiology, while a relatively sudden change in environmental surroundings during the last few decades seems to provide a more supportive explanation (Bloomfield et al. 2006). A good example is the Hygiene Hypothesis, which claims cleaner surroundings and less microbial exposure during childhood would lead to a higher susceptibility to allergy in later stages of life (Okada et al. 2010, von Mutius 2010). Although both genetic and environmental factors play a role in allergic symptom development (Yaari et al. 2009), recent clinical studies such as cross-sectional and cohort studies provide ample evidence of less genetic and more environmental component in aetiology of allergic symptoms across global populations (Bloomfield et al. 2006).

Not only does allergy interfere with daily life of those who suffer from allergic symptoms, but it also puts a socioeconomic burden on society as a whole. For instance, an estimated annual cost of asthma care in Canada was found to exceed $500 million annually over a decade ago, and of this $124 million was spent on drugs alone (Krahn et al. 1996). A new approach to prevent allergic disorders is critically needed at this point, as it may promise a huge reduction in healthcare expenditure on allergy-related disorders.

Allergy is a type I hypersensitivity (Ishizaka and Ishizaka 2006), marked by elevations of immunoglobulin E (IgE). Although serum IgE concentration is found to be the lowest among immunoglobulin isoforms, IgE can trigger a powerful response. Upon exposure to a specific type of antigen (allergen) in people prone to develop allergy, the allergen is processed by antigen presenting cells (APCs), such as dendritic cells or

macrophages, and drives the immune response towards specific IgE antibody generation by B cells and an allergic type of T cell response (T_H2) in which allergic types of cytokines such as interleukin (IL)-4 and IL-13 are produced (Venarske and deShazo 2003).

There are two distinct receptors for IgE: the low-affinity receptor (FcεRII; CD23) and high-affinity receptor (FcεRI), and the activation of FcεRI is what ultimately leads to degranulation of mast cells and basophils, and FcεRII has an antibody feedback regulatory role. When IgE released by B cells binds to FcεRI on mast cells and basophils, cells become sensitized. The level of circulating IgE is directly linked to the upregulation of FcεRI and the upregulation of FcεRI expression results in a lower threshold for mast cell activation upon IgE binding (Yamaguchi et al. 1997, Yamaguchi et al. 1999). When the sensitized cells are exposed to the same allergen that caused IgE sensitization, it results in degranulation and secretion of active mediators, which cause physiological responses like vasodilation and smooth muscle contraction (Boesiger et al. 1998).

A wide array of types of immune cells, such as mast cells, basophils, activated eosinophils, Langerhans cells and monocytes, expresses FcεRI (Prussin et al. 2003, Stone et al. 2010). However, subunit composition and the level of impact it has on the cell upon activation differ significantly depending on the cell type in which it is expressed. For example, FcεRI exists in two isoforms: tetramer and trimer. In mast cells and basophils only, all three subunits are expressed as in the form of tetramer ($\alpha\beta\gamma_2$), but the β subunit is absent in all FcεRI-bearing cells other than mast cells and basophils and the receptor is expressed as a trimer isoform ($\alpha\gamma_2$) (Maurer et al. 1994). Although the β subunit is

dispensable in activation and degranulation of mast cells (Alber et al. 1991), it was observed that the β subunit is responsible for signal amplification up to 5- to 7-fold across the plasma membrane upon antigen binding (Kraft et al. 2004, Nadler et al. 2000). Also, it has been suggested that the variation of β subunit expression level among different individuals might be responsible for how an individual may show different responses to immunologic reactions (Lin et al. 1996). It was noted that carboxy terminus of both β and γ subunits participate in phosphorylation signalling pathways, but it is not yet known if the multiple sites of phosphorylation have a synergistic or separate, non-synergistic effect on signal amplification (Weiss 1993, Weiss and Littman 1994).

Mast cell activation via FcεRI has been studied in great detail in the past (Gilfillan and Rivera 2009, Rivera and Olivera 2008). Responsible for innate immunity against invading pathogens, mast cells are also responsible for hypersensitivity immune reactions directed against allergens posing relatively little danger, as manifested in allergic disorders such as anaphylaxis, atopy and rhinitis (Galli et al. 2008). Upon binding of antigen-specific immunoglobulin E (IgE) to an α subunit of the receptor and subsequent binding of antigens to IgE, which crosslink IgE molecules, clustering of FcεRI occurs and multiple signalling cascades are initiated via phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) of β and γ subunits on the cytosol side of the plasma membrane (Li et al. 1992). This transphosphorylation of ITAM regions on β and γ subunits by protein kinases, such as Lyn kinase, signals the initiation of a cascade of phosphorylation events that are about to take place in other key proteins in the downstream region of the signalling pathway (Pribluda et al. 1994). In turn, phospho-

ITAMs attract various key protein kinases such as Src family tyrosine kinases, Lyn and

Fyn that play an important role in mast cell degranulation (Alvarez-Errico et al. 2010,

Parravicini et al. 2002). However, it is interesting to note that activation of Lyn may lead

to positive or negative regulatory response in mast cells depending on various factors,

such as lipid environment surrounding FcεRI (Field et al. 1997, Kawakami et al. 2000).

Further, functional loss of Lyn does not necessarily translate into loss of mast cell

degranulation or cytokine responses (Iwaki et al. 2005, Xiao et al. 2005). A

phosphorylation of ITAM regions results in recruitment of both positive and negative

regulatory proteins including Src homology 2 (SH2) domain-containing signalling

molecules, such as spleen tyrosine kinase (Syk), that ultimately form an aggregation of an

enzyme complex around the initial site of phosphorylation on FcεRI (Kihara and

Siraganian 1994).

Syk activation results in a favourable condition in which more substrates are

phosphorylated. Upon substrate phosphorylation, other lipid enzymes become activated

and lipid messengers released such as diacylglycerol (DAG) and inositol triphosphate

(IP_3). The release of such lipid messengers as an end product elicit calcium release from

the endoplasmic reticulum (ER) calcium store, which further promotes a larger influx of

calcium ions from the extracellular environment via calcium channels like Orai1/CRACM

(Prakriya et al. 2006). Following the rise in intracellular calcium levels, mast cell

degranulation occurs in which many types of messengers are released like prostaglandins,

histamine, leukotrienes and various types of cytokines (Prussin and Metcalfe 2003).

Protein kinase Syk is of a particular importance in mast cell degranulation due to

its close proximity to FcεRI where it is located upstream of the FcεRI/IgE signalling pathway map. With a high affinity for phospho-ITAM region of FcεRI γ chain (Benhamou et al. 1993), Syk tyrosine kinase amplifies and propagates positive signals via transphosphorylation of other Syk molecules and a large number of enzyme substrates, and is an essential component in the signalling transduction as indicated by absence of mast cell degranulation in Syk-deficient mast cells (Costello et al. 2006, El-Hillal et al. 1997). As such, Syk has been a subject of study since its inhibition would lead to downregulation of cytokine release upon FcεRI activation (Kovarova and Rivera 2004), and studies have shown some efficacy of Syk inhibition on mast cell degranulation (Braselmann et al. 2006, Moriya et al. 1997). Among possible candidates for a Syk inhibitor is piceatannol (3,4,3',5'-tetrahydroxy-trans-stilbene), a natural plant secondary phenolic compound found in Norway spruce roots (*Picea abies*) (Münzenberger et al. 1990). Piceatannol has been shown to preferentially inhibit Syk in *in vitro* kinase assays (Oliver et al. 1994). I therefore proposed to initially study the role of Syk in neuronal IgE activation.

The recent discovery of FcεRI expression on dorsal root ganglia (Andoh and Kuraishi 2004b, Rijnierse et al. 2009) and superior cervical ganglia (van der Kleij et al. 2010) highlights the significance of neuroimmune communication. Responsible for allergy and other allergy-related immune disorders, activation of the receptor causes phosphorylation of key proteins in the signalling pathway in immune cells that ultimately leads to allergic symptoms. Previously thought to be restricted to certain types of immune cells such as mast cells and basophils, the expression of FcεRI on sensory neurons

suggests a novel physiological pathway that may be involved in allergy, and further information about this will contribute to the understanding of allergic mechanisms. The proposed study was to investigate the distribution of neuronal FcεRI and the consequence of activation of the receptor. Also proposed was investigation of the key molecules that are involved in signalling cascade downstream of the receptor on neurons in comparison to the well-established processes in mast cells and basophils. In addition, an *in vivo* behaviour study was proposed involving mice chimeric for the presence of FcεRI on neurons but not hematopoietic cells, to identify their functional role and identify if their presence on neurons alone would give rise to classic allergic responses.

MATERIALS AND METHODS

Animals

6- to 8-week old male or female C57BL/6 or BALB/c mice were obtained from Harlan Laboratories or Charles River and housed one to five per cage in specific pathogen free (SPF) conditions (25°C, 12h light cycle) with free access to chow and water. Female C57BL/6 chimeric mice were obtained from Dr. Kelly McNagny at the University of British Columbia, Vancouver, but were much older than employed in other studies (see below). Mice were allowed one week to acclimatize to the surroundings before experiments began. All animals were obtained through Central Animal Facility (CAF) at McMaster University, Hamilton, ON, and, unless otherwise stated, were housed at St. Joseph's Healthcare Hamilton (Hamilton, ON). All animal experiments and experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and approved by the McMaster University Animal Research Ethics Board with the requirements of the Animal Care Committee of McMaster University, Hamilton, ON.

Sucrose Preference Behaviour Study

Mice prefer to drink a sweetened sucrose solution to water under normal circumstances (Inoue et al. 2007, Ninomiya and Imoto 1995). If they are offered a choice, they will drink more sweetened solution than unsweetened water and this can be measured over a defined time period. However, when an unpleasant sensation or effect is associated with the normal preferred sweetened solution, the mouse naturally avoids

drinking sweetened solution in order to avoid the unpleasant sensation (Cara et al. 1997)—this avoidance is referred to as "aversive behaviour." In such paradigm, the sucrose preference behaviour study investigates the aversive behaviour in a defined model where sensitized mice develop avoidance behaviour towards allergen (ovalbumin)-containing sucrose solution. We hoped to use the sucrose preference study by Mirotti et al. (2010) where mice's behavioral response to ovalbumin (OVA)-containing solution with different sucrose concentrations was investigated (**Figure 1**). In the model of food aversion, OVA-sensitized or non-sensitized mice are given choices, water or sucrose-sweetened but egg white-containing water. It was hypothesized that a conflicting situation would arise in which mice experienced an aversive reaction to contact with allergen if sensitized. This is a model of food allergy and takes advantages of the acute unpleasant symptoms associated with consumption of an allergen by a mouse with a high level of IgE antibody directed against that allergen. This reaction depends on the presence of mast cells in the gut sensitized already with allergen-specific IgE interacting with absorbed allergen (Wang and Sampson 2011, Sicherer and Sampson 2010). The consequent unpleasant sensations arising from mast cell degranulation consist amongst other symptoms of intestinal muscle cramps (Skripak and Sampson 2008). According to Mirotti et al. (2010), mouse strain BALB/c showed a larger difference of allergen-containing sucrose solution consumption between non-sensitized and OVA-sensitized groups. However, because C57BL/6 strain was used to construct chimeric mice, the study on non-chimeric mice was conducted with C57BL/6 to keep the strain constant for both studies. Female C57BL/6 mice were sensitized successively on day 0 and 14 with OVA by i.p.

injection of 4μg OVA (grade V albumin from chicken egg white, Sigma-Aldrich) adsorbed to 1.6mg aluminum hydroxide gel (Sigma-Aldrich) suspended in 0.4mL PBS (n=10). Negative control mice (n=10) were injected with PBS only. On day 18, each individual cage was supplied with two identical water bottles and on day 20 individual mouse weights were measured and mice were separated into one mouse per cage. In order to prepare 20% egg white/water solution, egg white was obtained by carefully separating the egg white from the yolk and dissolving the egg white with sucrose in water. The egg white/sucrose solution was prepared on day 20 and stored at 4°C on a stirring plate.

On the day of the test (day 21), one bottle was filled with 20% (v/v) egg white/water with 1% or 2% sucrose (Sigma-Aldrich) and the second bottle remained filled with water (**Table 1**). Sucrose concentrations of 1% and 2% were chosen because it was observed by Mirotti et al. (2010) that 1% showed the greatest sucrose solution intake difference between OVA-sensitized and PBS control groups in C57BL/6 strain. Bottle position was switched every 4h to avoid position preference and bottle weight measured before and 24h after the test. Sucrose solution intake was divided by total intake (sucrose solution and water) and animal weight in order to normalize the effects of body weight. The results were expressed in % sucrose solution intake / total intake / animal weight (g).

Day -7   0   14   18   20   21   22
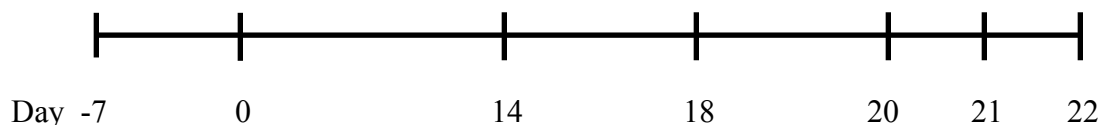
| Acclimatization | Sensitization | | Sensitization | Two water bottles per cage | Weight measurement | Begin | End |

Figure 1. Sucrose preference behaviour study timeline

1% sucrose solution OVA-sensitized	2% sucrose solution OVA-sensitized
1% sucrose solution PBS control	2% sucrose solution PBS control

n=5 per group

Table 1. Groups for sucrose preference study to test consumption of sucrose solution of different sucrose concentrations among non-sensitized and sensitized mice

Chimeric Mice

Experiments were designed to take advantage of the sucrose preference test in allergen-sensitized mice to test whether the aversive reaction could occur in the absence of hematopoietic cells (i.e. mast cells and basophils) bearing FcεRI. If an aversive reaction occurred in the absence of hematopoietic FcεRI but in the presence of sensitized neurons, proof would be obtained that not only were FcεRI present on neurons, but also that they were functionally active. Chimeras needed to be generated in which mice were available for testing that had mast cells without the receptor (FcεRI) for IgE but neurons that had the putative receptor.

Female, engineered chimeric mice were obtained from Dr. McNagny from University of British Columbia (Vancouver, BC). In brief, bone marrow was obtained

from dissected femurs of wildtype ($Fc\varepsilon RI\alpha^{+/+}$) or transgenic ($Fc\varepsilon RI\alpha^{-/-}$) C57BL/6-Ly5.2 (CD45.2 isotype) by flushing femurs with phosphate buffer saline (PBS) under the sterile condition. Red blood cells (RBCs) were lysed with NH_4Cl-containing RBC lysis buffer and the remaining cells were washed and resuspended in 200μL PBS. 1×10^7 donor cells were transplanted into lethally (1100 rads) irradiated C57BL/6-Ly5.1 (CD45.1 isotype) mouse by tail intravenous injection. Recipient mice were then rested for 12-16 weeks and checked for chimerism by examining the frequency of Ly5.1 and Ly5.2 in peripheral blood cells. Only those with over 90% chimerism (i.e. >90% Ly5.2) were selected for the experiment. Chimerism was further evaluated by examining the expression of $Fc\varepsilon RI$ on mast cells *in situ* via immunohistochemical staining and counterstaining with acidic Toluidine Blue. As a result, chimeric mice were hematopoietically either wildtype or $Fc\varepsilon RI\alpha^{-/-}$, but retained expression of $Fc\varepsilon RI\alpha$ on neurons, if present. Upon delayed arrival from University of British Columbia and an MNV outbreak at Central Animal Facility, McMaster University, all mice were transported to the Juravinski Hospital (Hamilton, ON) and the following sucrose preference behaviour study was conducted at the site. By the time the first experiment was conducted with the mice, they had reached the age of 10 months.

Three groups of mice were used: Non-irradiated female control mice (MC $Fc\varepsilon RI\alpha^{+/+}$, n=6), irradiated female chimeric mice (MC $Fc\varepsilon RI\alpha^{+/+}$, n=13) and another group of irradiated female chimeric mice (MC $Fc\varepsilon RI\alpha^{-/-}$, n=10). It was hypothesized that even in the absence of hematopoietic $Fc\varepsilon RI\alpha$ in chimeric mice (MC $Fc\varepsilon RI\alpha^{-/-}$), neuronal $Fc\varepsilon RI\alpha$

would be able to compensate for the role of allergen-avoidance behaviour and still show the same response as in MC FcεRIα $^{+/+}$ groups.

For this study, the same protocol was followed as with the previous study with non-chimeric mice, except that only 1% sucrose solution was used, as opposed to 1% or 2% sucrose solutions. Care had to be taken to obtain consistent results and avoid spillage while ensuring that the bottle stoppers were secure.

Tissue Sections

Jejunum and tongue tissues were obtained from normal or OVA-sensitized C57BL/6 female mice. As in sucrose preference behaviour study, those that were sensitized were done so with OVA in order to obtain high serum IgE levels.

Immunohistochemical staining procedures were followed according to the protocol adapted from Ratcliffe et al. (2010) and Watkins (2011). 1-inch long pieces of jejunum were surgically removed and content removed by flushing with PBS. Segments of jejunum were cut open along the mesenteric border and laid flat on a cut piece of weighing paper (Fisher Scientific). Whole tongue tissue for a positive control was also obtained from OVA-sensitized mice. Both tissues were encased in an embedding cassette (VWR) and were either not fixed or fixed with 1% or 4% formaldehyde for 1 or 3h at room temp. This was freshly prepared on each experiment from paraformaldehyde (Caledon Labs) dissolved in PBS with a few drops of NaOH to speed up the reaction (depolymerization of paraformaldehyde into formaldehyde, von Bohlen und Halbach and Kiernan 1999) and filtered through a filter paper (Whatman) (pH 7.4). Tissues were washed 3× with PBS for 5min on a shaker (VWR) and then cryoprotected with 30%

sucrose PBS solution overnight to prevent damage from the cryostat microtome. Tissues were frozen in optimal cutting temperature (OCT) compound (Electron Microscopy Science) with liquid nitrogen by removing the tissues from the plastic cassette, submerging them in a 12mm×12mm×20mm disposable embedding mold (VWR) filled with OCT compound, and placing the mold 1 inch above the liquid nitrogen for 5min, or until the OCT block became completely frozen. Extra care was taken to avoid cracks in the frozen OCT blocks, as cracks from immediate freezing with sudden temperature change often resulted in tissue damage. OCT tissue blocks were cut with microtome blade (Leica Biosystems) on microtome cryostat (Microm HM550, Thermo Scientific) at -22°C at thickness of 10μm and two cut sections were mounted on each electrostatically charged glass slide (Fisherbrand). Then, OCT compound was washed off the slides with two 5min washes at room temp in PBS in a shaking coplin jar and slides were air-dried. Tissues on the slide were marked by hydrophobic pen (Vector) circle, and sections were blocked with 4% normal goat serum (NGS) (Invitrogen) in PBS for 30min at room temp to prevent non-specific antibody binding.

Tissue sections prepared from OVA-sensitized mice were stained for PGP9.5 and IgE heavy chain by overnight co-incubation with two primary antibodies [PGP9.5: rabbit anti-human PGP9.5 polyclonal IgG (AbD Serotec); IgE: rat anti-mouse IgE monoclonal IgG2a-κ (Invitrogen)] at room temp and 3h co-incubation with two secondary antibodies [PGP9.5: Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Invitrogen); IgE: Alexa Fluor 594-conjugated goat anti-rat IgG (H+L) (Invitrogen)] (**Table 2**) in dark at room temp. Between antibody incubations, slides were washed three times with PBS for

5min in a shaking coplin jar. During the incubations, slides were placed in a humidified

dark chamber to avoid tissue drying and photobleaching. Different dilutions of primary or

secondary antibodies were tested (1:100, 1:200 or 1:500) to obtain the optimal result.

In addition, tissue sections were prepared from non-sensitized mice and incubated

with 1μg/mL anti-DNP IgE (Sigma-Aldrich, monoclonal clone SPE-7) for 3h at 37°C, 5%

CO_2 and stained for PGP9.5 and IgE as with tissues from sensitized mice. Also, tissue

sections obtained from non-sensitized mice were stained for FcεRIα with primary

antibody [hamster anti-mouse FcεRIα monoclonal (MAR-1) (LifeSpan BioSciences)] and

secondary antibody [Alexa Fluor 594-conjugated goat anti-hamster IgG (H+L)

(Invitrogen)].

Slides were nucleus-stained with 1μg/mL bisbenzimide (Sigma-Aldrich) in PBS

for 4min at room temp, rinsed twice with PBS and once with distilled water in a shaking

coplin jar and coverslipped with mounting medium Vectrashield (Vector) and a coverslip

(VWR).

	OVA sensitization *in vivo*	anti-DNP IgE sensitization *in vitro*	1° antibody
1	Yes	No	anti-IgE
2	No	Yes	anti-IgE
3	No	No	anti-FcεRIα

Table 2. OVA sensitization and different immunohistochemical staining procedures
for jejunum tissue sections. All tissues were fixed before staining.

Tissue Culture

Superior Cervical Ganglia Culture

We sought to study the presence and functional role of neuronal FcεRI from superior cervical ganglia (SCG) culture. In order to obtain SCG from 1- to 3-day old pups, 14-day pregnant timed-pregnancy or 6- to 8-week old breeding pairs of BALB/c mice were obtained from Harlan Laboratories or Charles River and housed in the same conditions as other animals. From breeding pairs of BALB/c mice, sperm (copulatory) plug visualization was used as an indication of successful mating and fertilization on day 0. Females were separated from males into individual cages on day 14 and pups were delivered on day 19 to 21.

SCG cultures were prepared according to the protocol adapted from Coughlin et al. (1981). Briefly, on day 1, 35mm×10mm polystyrene dish (BD Falcon), 0.17mm-thick Delta culture optical dish (Bioptech), or 24-well polystyrene plate (BD Falcon) was not coated, coated with rat tail collagen (BD Biosciences) only, or coated with rat tail collagen and poly-L-ornithine (Sigma-Aldrich). Where rat tail collagen and poly-L-ornithine coating were applied, dish or plate was rinsed with 3mg rat-tail collagen diluted in 1mL sterile dH$_2$O, air-dried under sterile condition for 30min, and incubated overnight with 1mL 0.01% poly-L-ornithine at 4°C. On the next day, dish or plate was rinsed twice with dH$_2$O and let dry for 30min. SCG were harvested under the dissection microscope from 1- to 3-day old BALB/c pups by decapitation and collected in Puck's saline G [NaCl (8,000mg/L), KCl (400.0mg/L), MgSO$_4$·7H$_2$O (154.0mg/L), CaCl$_2$·2H$_2$O (16.0mg/L), KH$_2$PO$_4$ (150.0mg/L), Na$_2$HPO$_4$·7H$_2$O (290.0mg/L) and D-glucose (1100mg/L); pH 7.4,

filter sterilized with 0.22μm bottle top filter (Millipore)]. Obtained ganglia were washed with Ca-Mg-free (CMF) Puck's saline G [NaCl (8,000mg/L), KCl (400.0mg/L), KH_2PO_4 (150.0mg/L), $Na_2HPO_4 \cdot 7H_2O$ (290.0mg/L) and D-glucose (1100mg/L); pH 7.4, filter sterilized with 0.22μm bottle top filter (Millipore)] and treated with 0.25% trypsin phenol red(+) (Invitrogen, Cat. No.: 15050065) or 0.25% trypsin phenol red(-) diluted from 2.5% trypsin (Invitrogen, Cat. No.: 15090046) in CMF Puck's saline G, for 30min at 37°C with occasional agitations. Then ganglia were incubated with Type IA 0.2% collagenase (Sigma-Aldrich, Cat. No.: C9891) or Type I 0.2% collagenase (Sigma-Aldrich, Cat. No.: C0130), for 10min at 37°C for enzymatic dissociation. Ganglia were washed twice with 10% HI FBS (GIBCO, US origin, certified, Cat. No.: 10082147) or 10% HI FBS (GIBCO, Canada origin, qualified, Cat. No.: 12484028) and triturated with 5mL serological pipette (BD Falcon) capped with P1000 (200-1000μL) pipette tip (Eppendorf) for physical dissociation. Dissociated neurons were seeded on dish or plate in SCG media (2mL in non-optical dish, 1mL in optical dish, 0.5mL in 24-well plate) [DMEM/F (GIBCO) with 1% L-glutamine (GIBCO), 0.5% pen-strep (GIBCO) and 10% HI FBS (GIBCO)] with 0, 100 or 500ng/mL nerve growth factor (NGF) (2.5S β-subunit, Cedarlane) and 0, 10, or 50μM cytosine β-D-arabinoside (AraC) (Sigma-Aldrich). AraC was used to kill non-neuronal cells including glial cells (Andoh and Kuraishi 2004b). Cultures were incubated at 37°C, 5% CO_2 humidified chamber until next procedure began.

Dorsal Root Ganglia Culture

Dorsal root ganglia (DRG) cultures were prepared according to the protocol adapted from Hall (2006). In brief, mice were euthanized with CO_2 and cervically dislocated. Then, viscera were removed and vertebrae exposed. A small snip was made with a pair of scissors between T1 and T2 vertebrae and spinal cord beneath was exposed by carefully removing caudally the covering vertebrae without damaging the spinal cord. Bilateral DRG from T1 to L6 levels were obtained from each side of the spinal cord beneath the ribs under the dissection microscope and collected in Puck's saline G. Approximately 30 ganglia were obtained from one mouse. Obtained DRG were prepared for culture in the same way as in SCG culture.

Bone Marrow-Derived Mast Cell (BMMC) Culture

Unless otherwise specified, BMMCs were obtained according to the following protocol. Bone marrow was flushed from dissected femurs with BMMC media [RPMI (GIBCO) with 6% BMMC supplement, 1% penicillin-streptomycin (pen-strep) (GIBCO), 0.1% fungizone (Invitrogen) and 10% heat-inactivated fetal bovine serum (HI FBS) (GIBCO)] [BMMC supplement: 4mM L-glutamine (GIBCO), 1mM sodium pyruvate (GIBCO), 50μM 2-mercaptoethanol (BioRad), 0.1mM MEM non-essential amino acid (GIBCO)] under sterile condition. Then, live cells were counted by trypan blue exclusion and 4×10^6 cells were incubated in 20mL BMMC media with 5mL spleen cell-conditioned medium (see below) in a 75cm^2 tissue culture flask (Sarstedt). Cells were resuspended in 20mL of fresh BMMC media and 5mL spleen cell-conditioned medium every 6-7 days at concentration of 0.1-0.2$\times10^6$/mL until cells became mature in 6 weeks. At this stage

greater than 99% of the cells were mast cells as determined by Toluidine Blue staining (Diaz et al. 2006) or 97% of cells were mast cells as determined by FACS analysis (Karimi 1999). In all experiments involving BMMCs, cells were always derived from 6-8 week old BMMC cultures.

Spleen Cell-Conditioned Medium

Spleen cell-conditioned medium was prepared according to the protocol adapted from Ohmori (1994). Spleen cell-conditioned medium was used to maintain mast cell culture because it contains interleukin 3 (IL-3), a cytokine necessary for cell proliferation, and this was obtained from spleens from the same mice BMMCs were obtained from. Briefly, cells were gently dispersed from a dissected spleen with back of a 1mL syringe (BD Falcon) plunger and filtered through a 70μm cell strainer (BD Falcon). 2×10^6 cells were resuspended in 50mL BMMC media and incubated in $75cm^2$ tissue culture flask (Sarstedt) with 0.5mL of 1mg/mL pokeweed-mitogen (Sigma-Aldrich, Lectin from *Phytolacca Americana*). After 7 days, supernatants were then collected by centrifugation at 1500rpm for 20min, filtered through 0.45μm bottle top filters (Millipore), and stored at -20°C for future use.

Intracellular Calcium Measurement and Flow Cytometry

Microscopy

Immunohistochemistry

Tissue sections were visualized with a fluorescence microscope (Zeiss AxioImager Z1, Carl Zeiss Canada) under FITC (Alexa Fluor 488), Texas Red (Alexa Fluor 594) and DAPI (bisbenzimide, emission wavelength: 461nm).

Calcium Imaging: Cultured Neurons and Bone Marrow-Derived Mast Cells

Intracellular calcium levels were visualized according to the protocol adapted from van der Kleij et al. (2010).

Neurons from 2- or 3-day old SCG culture were sensitized with 0.1, 1, 5, or 10μg/mL anti-2,4-dinitrophenol (DNP) IgE (Sigma-Aldrich, monoclonal clone SPE-7) for 3h or overnight at 37°C, 5% CO_2. Then, unbound IgE was washed off with 37°C SCG media and cells were loaded with 0.1μM, 1μM, 3μM, or 10μM calcium-sensitive dye Fluo-3, AM dissolved in dimethyl sulfoxide (DMSO) (Invitrogen) or 1μM Fluo-3, AM (Invitrogen), a different batch provided by Dr. Luke Janssen (McMaster University, Hamilton, ON, Canada), for 20min, 30min, 1h or 3h at 37°C, 5% CO_2.

As a positive control, 0.2×10^6 or 0.1×10^6 BMMCs were seeded on pre-coated dishes or plates respectively, in the same way as with neurons and incubated at 37°C in BMMC media with 20% v/v spleen cell-conditioned medium for 2 days. Then, cells were sensitized with anti-DNP IgE and loaded with Fluo-3 AM in the same way as with neurons.

Unbound Fluo-3, AM was washed off from neurons or BMMCs with SCG media, Puck's saline G or HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10mM), NaCl (140mM), KCl (5mM), $CaCl_2$ (1mM), $MgCl_2$ (0.6mM), bovine serum albumin (Sigma-Aldrich, 1mg/mL), D-glucose (1mg/mL), sulfinpyrazone (Sigma-Aldrich, 0.1mg/mL); pH 7.4, filter sterilized with 0.22μm bottle top filter (Millipore)]. Intracellular calcium levels of sensitized neurons were measured upon stimulation with 1, 10, 100, 500, 1000ng/mL, or 10μg/mL DNP-human serum albumin (HSA) (Sigma-Aldrich) diluted with the same media in which cells were washed previously (SCG media, Puck's saline G, or HEPES buffer) by introducing 100μL of antigen or calcium ionophore (A23187) into 400μL (24-well plate), 200μL of stimulant into 1.8mL (optical and non-optical dish), or 50μL of stimulant into 150μL (non-optical dish). As a positive control, non-sensitized neurons or BMMCs were stimulated with 1μM calcium ionophore (A23187, Sigma-Aldrich), and fluorescence measured in the same way as with DNP-HSA stimulation. As soon as the stimulants were introduced, images from time-lapse fluorescence imaging were taken every 0.5s and visualized under FITC with upright fluorescence (Zeiss AxioImager Z1, Carl Zeiss Canada), inverted fluorescence (Nikon TE2000U, Nikon Canada; Nikon TE2000E, Nikon Canada) or confocal (LSM510, Carl Zeiss Canada at Electron Microscopy, Faculty of Health Sciences, McMaster University, Hamilton, ON) microscope. Images were processed by AxioVision 8.2 (Carl Zeiss Canada), LSM Image Browser (Carl Zeiss Canada) or NIS element viewer (version 1.0, 3, Nikon Canada), and analyzed by AxioVision 8.2 (Carl Zeiss Canada).

Intracellular calcium level in DRG was also measured in the same way as described above with SCG culture, except DRG culture was prepared in 0.17mm-thick Delta culture optical dish (Bioptech), and time-lapse fluorescence imaging was visualized with confocal microscope (LSM510, Carl Zeiss Canada) only.

Plate Reader

Use of the plate reader was attempted to assess fluorescence and visualize intracellular calcium levels. It was expected that this system and ability to examine multiple wells would have speeded up testings. However, the baseline absorbance value was observed to be too high when the fluorescence was measured with the plate reader, and thus changes in fluorescence were too small to be measureable. This method could not be used for further studies.

Flow Cytometry

Superior Cervical Ganglia Neurons

Because no change in intracellular calcium level was detected microscopically from IgE-sensitized neurons upon DNP-HSA stimulation, another approach was taken that did not depend on optical visualization of neurons bound to the dish or plate surface, and fluorescence was measured quantitatively using flow cytometry.

Flow cytometry is a laser-based, biophysical technology that is a very consistent and highly sensitive methodology used generally to examine surface and intracellular aspects of cells. However, it was previously unknown if neurons from SCG culture could be characterized using flow cytometry due to their different characteristics, such as shape,

size, adherence to dish, etc., from other cell types in cell suspension, such as mast cells, T cells or macrophages. Therefore, attempts were made to prepare neurons so that final product resembled other cell types in cell suspension. Briefly, neurons from 2-day old SCG cultures in 24-well plates were sensitized with anti-DNP IgE and loaded with Fluo-3, AM in the same way as with the previous study with microscopy. Cells were washed twice with warm PBS at room temp, harvested from the plate with ice-cold PBS wash, as cold temperature detaches cells from dish surface, and resuspended in Puck's saline G into single cell suspensions by gentle pipetting for 30s.

Also, uncultured neurons prepared immediately after enzymatic and physical dissociation were sensitized with anti-DNP IgE and loaded with Fluo-3, AM in the same way as neurons from 2-day old cultures, and resuspended in Puck's saline G.

The percentage of neurons with increase in intracellular calcium level, as indicated by increase in FITC level, was measured every 30s with FACSCanto (Becton Dikinson) upon stimulation with 1μM calcium ionophore (A23187, Sigma-Aldrich), or 10 or 100ng/mL DNP-HSA (Sigma-Aldrich) by introducing 100μL of 5× concentrated stimulant into 400μL cell suspension. When DNP-HSA stimulation failed to elicit FITC level increase, cells were stimulated with additional 100μL 5uM A23187. Data were analyzed with FlowJo (version 8.7, TreeStar).

After failed attempts to detect fluorescence from IgE-sensitized neurons with DNP-HSA stimulation with flow cytometry, attempts were made to directly stain for FcεRIα on non-sensitized neurons and IgE on IgE-sensitized neurons. For FcεRIα staining, neurons were harvested from 2-day old SCG culture in 24-well plates with ice-

cold PBS and resuspended in Puck's saline G. Then, neurons were stained with 1:200 FITC-conjugated anti-FcεRIα antibody (eBioscience) with 30min incubation at 4°C. Cells were washed twice with Puck's saline G and percentage of FcεRIα$^+$ cells was determined with FACSCanto (Becton Dikinson). Data were analyzed with FlowJo (version 8.7, TreeStar).

For IgE staining, neurons from 2-day old SCG culture in 24-well plate were sensitized with 1μg/mL anti-DNP IgE (Sigma-Aldrich, monoclonal clone SPE-7) for 3h at 37°C, 5% CO$_2$. Then, cells were harvested with ice-cold PBS and resuspended in Puck's saline G. Cells were stained for IgE with 1:200 phycoerythrin (PE)-conjugated anti-mouse IgE antibody (Southern Biotech) with 30min incubation at 4°C and washed twice with Puck's saline G. Percentage of IgE$^+$ cells was determined with FACSCanto (Becton Dikinson) and data analyzed with FlowJo (version 8.7, TreeStar).

Bone Marrow-Derived Mast Cells

As a positive control and similar to SCG neurons, BMMCs were sensitized with different concentrations of anti-DNP IgE and loaded with Fluo-3, AM in the same way as neurons and resuspended in Puck's saline G. BMMCs were also stimulated with 1μM A23187 to check that Fluo-3, AM was working in this cell type. Additionally, in order to study the effect of enzymatic digestion of ganglia with trypsin on the ability of sensitized BMMCs to still respond to DNP-HSA stimulation after sensitization with anti-DNP IgE, BMMCs were incubated with trypsin and collagenase in the same way as neurons were incubated, and the same methods used for of the intracellular calcium measurement with flow cytometry procedures.

Similarly, BMMCs were stained for FcεRIα or IgE and the percentage of FcεRIα$^+$ or IgE$^+$ cells were determined in the same way as with neurons.

Bone Marrow-Derived Mast Cell β-Hexosaminidase Assay

β-hexosaminidase is an enzyme that is a granule constituent and its secretion is used to quantitate degranulation of mast cells. The β-hexosaminidase assay was done according to the protocol from Blank and Rivera (2006). The assay has been carried out in order to test the ability of BMMCs to be sensitized with IgE and to degranulate upon antigen challenge with DNP-HSA, thus acting as a positive control for the functionality of the reagents used in the experiments with SCG neurons. Also, the calcium-sensitive fluorescence dye, Fluo-3, AM, was tested in the system to confirm that it did not interfere with degranulation of the mast cells. Therefore, by testing out this system with BMMCs, we hoped to confirm IgE and DNP-HSA together were able to elicit a response in a well-established model of mast cell degranulation. At the same time, we hoped to optimize the reagent conditions for subsequent experiments.

Unless otherwise stated, in the β-hexosaminidase assays, monoclonal clone SPE-7 anti-DNP IgE for sensitization, DNP-HSA for stimulation (both from Sigma-Aldrich) and Fluo-3, AM for calcium-sensitive fluorescent dye from Invitrogen, were used. BMMCs were prepared in U-bottom 96-well plate (BD Falcon) suspended in 100μL BMMC media at concentration of 10^6 cells/mL and sensitized with 1μg/mL anti-DNP IgE for 2h at 37°C with occasional agitations. Cells were washed in HEPES-Tyrode buffer [NaCl (8,000mg/L), D-glucose (500mg/L), bovine serum albumin (BSA) (Sigma-Aldrich, 500mg/L), HEPES (1,430mg/L), KCl (100.5mg/L), CaCl$_2$·H$_2$O (73.5mg/L) and

$NaH_2PO_4 \cdot H_2O$; pH 7.4, filter sterilized with 0.22μm bottle top filter (Millipore)] and 100μL cell suspension was challenged with 0, 0.1, 1, 10, 25, 100 or 1000ng/mL DNP-HSA for β-hexosaminidase release. Cells were incubated for 1h at 37°C except for positive control where 10% Triton-x 100 (Sigma-Aldrich) was introduced 10min before the end of incubation to obtain total β-hexosaminidase release. 50μL supernatant was transferred to flat-bottom 96-well plate (BD Falcon) and 50μL assay solution [5mL of citrate buffer: 185mL sodium citrate (0.1M) and 215mL citric acid (0.1M); pH 4.5 and 80μL 4-methylumbelliferyl-β-D-glucuronide (4-MUG) (10mM, Sigma-Aldrich)] was added. After 1h-incubation at 37°C, reaction was stopped by adding 100μL glycine buffer [glycine (15.0g/L) and NaCl (11.7g/L); pH 10.7]. Fluorescence was measured with a fluorescence plate reader (excitation: 360nm, emission: 452nm; slit width: 5-10; LS 50B, PerkinElmer).

Testing Different Concentrations of IgE and DNP-HSA from Different Batches

Different concentrations of anti-DNP IgE and DNP-HSA were used to obtain the best degranulation response of mast cells. Previous study was repeated with 0.01, 0.1 or 1μg/mL anti-DNP IgE and 0, 0.1, 1, 10, 25, 100 or 1000ng/mL DNP-HSA from two different batches.

A23187-Induced β-Hexosaminidase Release

In order to study β-hexosaminidase release induced by A23187 stimulation, the previous study was repeated except cells were stimulated with calcium ionophore (A23187, 0.1, 1, 2.5 or 10uM, Sigma-Aldrich) or DNP-HSA (0, 10 or 25ng/mL).

Effect of Fluo-3, AM on BMMC β-Hexosaminidase Release by DNP-HSA

In order to study the effects of Fluo-3, AM on BMMC's β-hexosaminidase release, observations from two experiments were made: the effects of Fluo-3, AM loading on DNP-HSA stimulation and Fluo-3, AM stimulation on β-hexosaminidase release. In the first study, β-hexosaminidase release was measured in the same way as in the previous study, except cells were loaded with 1μM Fluo-3, AM with 30min incubation at 37°C between IgE sensitization and DNP-HSA stimulation. In the second study, DNP-HSA was replaced with Fluo-3, AM and β-hexosaminidase release was measured in the same way as before.

Statistical Analysis

All statistical calculations were done using Prism 5 for Mac OS X (Version 5.0b, GraphPad Software, Inc.). For the sucrose preference behaviour study with non-chimeric mice (**Figure 2**), statistical significance was calculated using Student's *t*-test, which compared control and sensitized group means within each sucrose concentration (1% and 2%). Two-way ANOVA followed by a Bonferroni correction was used to study the effect of OVA-sensitization or sucrose concentration on sucrose solution intake. For the sucrose preference behaviour study with chimeric mice (**Figure 3**), two-way ANOVA was used to calculate statistical significance between two group means (non-irradiated MC FcεRIα $^{+/+}$ and irradiated MC FcεRIα $^{+/+}$) with two independent variables (PBS control and OVA-sensitized) followed by a Bonferroni correction to study the effect of irradiation on sucrose solution intake. Then, non-irradiated MC FcεRIα $^{+/+}$ and irradiated MC FcεRIα $^{+/+}$ groups were combined and the means from the two groups were compared to that of MC

FcεRIα $^{-/-}$ group means using two-way ANOVA followed by a Bonferroni correction. One-way ANOVA followed by a Bonferroni correction was used to compare % mast cell degranulation response in β-hexosaminidase assay across different concentrations of DNP-HSA stimulation (**Figure 17B**). Two-way ANOVA followed by a Bonferroni correction was used to compare the effect of different combinations of anti-DNP IgE and DNP-HSA concentrations on mast cell degranulation (**Figure 18**). Also, one-way ANOVA was used to compare mast cell degranulation across different stimulator (**Figure 19,20B**). Student's *t*-test was used to compare the effect of Fluo-3, AM loading on mast cell degranulation (**Figure 20A**). Differences between groups were considered statistically significant when p-value was ≤0.05 (*: p<0.05 and **: p<0.01). Statistics were presented as the t-value or F-statistic with degrees of freedom written in parenthesis, followed by the p-value.

RESULTS

Sucrose Preference Behaviour Study

Non-Chimeric Mice

There was a significant difference between OVA-sensitized and PBS control groups: using two-way ANOVA, it was observed that sensitization with OVA affected sucrose solution intake ($F_{(1,19)}$=5.46, $p<0.05$). Similarly, using two-way ANOVA, it was observed that sucrose concentration affected sucrose solution intake ($F_{(1,19)}$=12.61, $p<0.01$) (**Figure 2**) as there was a significant difference between 1% and 2% groups. When sucrose solution intake was compared between OVA-sensitized and PBS control groups within each sucrose solution concentration using Student's *t*-test, there was a larger significant difference in 1% sucrose solution group (t_8=2.44, $p<0.05$) than in 2% sucrose solution group (t_8=0.91, ns), and 1% was chosen as the sucrose solution concentration for subsequent experiments with chimeric mice. The result goes in line with a previous study where intake of 1% sucrose concentration provided a larger sucrose solution intake difference between OVA-sensitized and PBS control groups than 4% or 8% sucrose concentrations (Mirotti et al. 2010).

Chimeric Mice

In order to study the effect of irradiation and bone marrow transplantation in the generation of chimeric mice (irradiation and bone marrow transplantation) on sucrose solution intake, irradiated MC FcεRIα $^{+/+}$ and non-irradiated MC FcεRIα $^{+/+}$ groups were

compared using two-way ANOVA. There was no significant difference observed between the two groups ($F_{(1,11)}$=0.4252, ns) (data not shown).

Then, the two groups (irradiated MC FcεRIα $^{+/+}$ and non-irradiated MC FcεRIα $^{+/+}$) were combined as MC FcεRIα $^{+/+}$ and compared with MC FcεRIα $^{-/-}$ to study the effect of neuronal FcεRIα, if any, on sucrose solution intake in chimeric mice. It was hypothesized that if neuronal FcεRIα were present, it would compensate for absence of hematopoietic FcεRIα and OVA-sensitized mice would still show aversive behaviour towards allergen-containing sucrose solution. However, using two-way ANOVA, no significant difference between PBS control and OVA-sensitized groups was observed in MC FcεRIα $^{-/-}$ group (t_{24}=0.8293, ns), but a significant difference between PBS control and OVA-sensitized groups was observed in MC FcεRIα $^{+/+}$ group (t_{24}=3.383, $p<0.01$) (**Figure 3**). It was concluded that the absence of hematopoietic FcεRIα resulted in obliteration of avoidance behaviour towards allergen in OVA-sensitized mice regardless of putative neuronal FcεRIα expression.

Immunohistochemistry

Unfixed tissue sections were easily damaged after sectioning with cryostat microtome as visualized with light microscope prior to the antibody staining (data not shown). Jejunum villi lost morphology and different measures were taken to avoid these artefacts. It was later observed that formaldehyde solution needed to be prepared fresh for every experiment to avoid inconsistent results. This is probably due to a high methanol concentration as formaldehyde is converted into methanol through time (Helander 2000), and this does not act optimally to fix tissues.

Tissue fixation with formaldehyde for 3h was necessary for obtaining good tissue morphology after cryostat sectioning, as no fixation or 1h fixation resulted in tissue damage upon cryostat sectioning. Also, fixation with 4% formaldehyde resulted in a better tissue morphology than that with 1% formaldehyde (data not shown). In addition, it was observed that 24h cryoprotection with 30% sucrose PBS following formaldehyde fixation was necessary. Without cryoprotection, tissues were prone to physical damage upon freezing with liquid nitrogen and cryostat sectioning (data not shown).

Optimal results were obtained with primary antibody dilution of 1:500 and secondary antibody dilution of 1:200 for anti-PGP9.5. For anti-IgE, the same dilutions for primary and secondary antibodies were optimal as determined on tongue tissue. However, dilutions for anti-FcεRIα antibodies could not be optimized since positive results were never obtained in tissue sections (data not shown).

Jejunum

In tissue sections obtained from OVA-sensitized mice, PGP9.5 staining (FITC) was visible at 20× magnification especially between the smooth muscle (longitudinal and circular) layers and in villi of the jejunum, as indicated by thin lines in multiple layers (axons) and sporadic large oval bodies (soma) between the muscle layers (**Figure 4**). This staining was consistent with the location and characteristics of the myenteric plexus (Sternini et al. 1999). Abundant nuclear staining (DAPI) was visible, but no IgE staining (Texas Red) was visible at 20× magnification. However, at 40× magnification some IgE staining along the villous epithelium was observed, but only occasional faintly stained

cells were visible in the villi, but never in or between the smooth muscle layers. No co-localization of the PGP9.5 (FITC) and IgE (Texas Red) was observed (**Figure 5**).

In order to minimize the possible role anti-PGP9.5 antibody might play on interference with IgE staining, tissues were stained with anti-IgE antibody and DAPI only (no anti-PGP9.5 antibody). However, still no IgE staining was observed in the absence of PGP9.5 staining (data not shown).

Also, immunohistochemical staining of the jejunal tissue from non-sensitized mice incubated with anti-DNP IgE showed no IgE staining (data not shown). Attempts were made to stain for FcεRIα in non-sensitized mouse jejunum tissue, but this showed no staining either (**Figure 6**).

Tongue

Due to the abundance of mast cells and their expression of FcεRIα and therefore IgE on the mast cells in tongue (Gersch et al. 2002), tongue tissue was used as a positive control for IgE staining. In tongue tissue sections obtained from OVA-sensitized mice, when stained with anti-IgE antibody only (no anti-PGP9.5 or DAPI), staining resembling cell membrane, ring-like structures were visible throughout the tissue sections (**Figure 7**), indicative of mast cells in tongue.

Superior Cervical Ganglia Culture: Effects of NGF and AraC

Dissociated neurons in SCG culture from 4-day old or older pups showed no axonal growth and consequently apoptosis within 48h, so only 2- to 3-day old pups were included in the experiments. NGF was necessary for healthy neuron culture, and optimal

NGF concentration was found to be 100ng/mL in accordance with a previous study (Coughlin et al. 1981, Mobley et al. 1976). However, different concentrations of AraC had no effect on physical appearance or numbers of neurons in SCG culture (data not shown). Although 3-day old cultures had more cell growth than 2-day old cultures, 2-day old cultures were always used for intracellular calcium measurement (microscopy) because neurons in 3-day old culture were easily detached from the dish bottom with washes and therefore could not be used for microscope visualization.

Microscopy

Intracellular Calcium Levels

Superior Cervical Ganglia Neurons

Upright fluorescence microscope (Zeiss AxioImager Z1, Carl Zeiss Canada) was used for most experiments. However this was not ideal for time-lapse imaging as sudden changes in liquid volume in dish or plate upon stimulator introduction occasionally pushed images out of focus (data not shown). In an attempt to improve the situation, the inverted fluorescence microscopes (Nikon TE2000U, Nikon TE2000E, both Nikon Canada) were also tested, but they showed inconsistent basal fluorescence intensity before and upon stimulation. Experimental results were eventually confirmed in confocal microscopy (LSM510, Carl Zeiss Canada). However, it has been suggested in a previous study that the temporary increase in fluorescence upon stimulant introduction might have been caused by non-specific binding of the stimulant (Rijnierse et al. 2009).

Attempts to reproduce data from the previous study (van der Kleij et al. 2010), where IgE-sensitized neurons in SCG culture responded to DNP-HSA with increase in intracellular calcium concentration, were all unsuccessful. Neurons failed to respond to DNP-HSA stimulation at any concentration, and fluorescence stayed at the base line (**Figure 8**). Different alterations in procedure had no effect on failure to respond to DNP-HSA stimulation. These included dish-coating (no coating, rat-tail collagen only or rat-tail collagen with poly-L-ornithine), trypsin [phenol red(+) or phenol red(-)], collagenase (type I or type IA), HI FBS (US origin certified or Canada origin qualified), NGF concentration (0, 100 or 500ng/mL), AraC concentration (0, 10 or 50μM) and anti-DNP IgE concentration (0.1, 1, 5 or 10μg/mL). Also tested were anti-DNP IgE incubation duration (3h or 24h), Fluo-3, AM concentration (0.1, 1, 3 or 10μM), Fluo-3, AM source (Invitrogen or Dr. Luke Janssen), Fluo-3, AM incubation duration (20min, 30min, 1h or 3h), wash medium (Puck's saline G or HEPES buffer) and the images obtained from microscopes (upright optical, inverted optical or confocal). However, when additional 1μM calcium ionophore (A23187) was introduced as a positive control to the neurons that failed to respond to DNP-HSA stimulation, there was always a significant increase in intracellular calcium concentration indicated by increase in fluorescence in all parts of the neurons including the neurites (**Figure 9A,B**). Also, when 1μM calcium ionophore (A23187) was introduced to neurons that were not previously sensitized with IgE or challenged with DNP-HSA, there was a significant increase in intracellular calcium level, similar to what was observed in the previous experiment with A23187 stimulation (**Figure 9C,D**).

When A23187 was used as a stimulator, fluorescence was observed within 5s of stimulation and this fluorescence continued for 30s after which fluorescence gradually diminished (data not shown).

In order to establish that the IgE/DNP-HSA was functionally active, BMMCs were used in the following experiment. As another positive control, IgE-sensitized BMMCs showed significant increases in fluorescence when cells were challenged with 10ng/mL DNP-HSA (**Figure 10**) or 100ng/mL (data not shown). In contrast to A23187 stimulation (data not shown) where fluorescence was observed 5s after stimulation, there was about 30s delay in fluorescence after DNP-HSA stimulation on BMMCs. The delayed response to antigen in sensitized mast cells has been described in previous studies (Scharenberg and Kinet 1994, Xu et al. 1998). This fluorescence continued for another 3-5min after which fluorescence gradually diminished (data not shown).

Dorsal Root Ganglia Neurons

Since experiments with SCG neurons were not showing positive results, DRG neurons were cultured in case these ganglia expressed functional IgE receptors (FcεRI) and similar experiments were carried out as with SCG neurons. However, no fluorescence was observed when IgE-sensitized DRG neurons were stimulated with DNP-HSA at any concentration (data not shown). Also, similar to SCG neurons, cells responded to A23187 stimulation, as indicated by higher percentage of fluorescent cells (data not shown).

Flow Cytometry

Intracellular Calcium Level

Superior Cervical Ganglia Neurons

When non-sensitized neurons were stimulated with 1μM A23187, approximately 50% population responded to the stimulation within 15s indicated by higher FITC intensity (**Figure 11**). The percentage of cells with higher fluorescence intensity decreased and returned to the basal level within 1min. However, when the IgE-sensitized neurons were stimulated with 10 (**Figure 12A**) or 100ng/mL DNP-HSA (data not shown), cells failed to respond to the stimulation, as indicated by constant percentage of cells with fluorescence. When the unresponding cells were stimulated with additional A23187, approximately 50% population again responded to the stimulation within 5s (**Figure 12B**), similar to the previous result where approximately 50% of neurons responded to A23187 stimulation with no previous DNP-HSA stimulation.

Similar results were observed with uncultured neurons after enzymatic dissociation as with neurons from 2-day old SCG culture: no response was observed with DNP-HSA stimulation at any concentration, but showed fluorescence with A23187 stimulation (data not shown).

Bone Marrow-Derived Mast Cells

In contrast, BMMCs responded to both A23187 stimulation and IgE sensitization/DNP-HSA stimulation. When non-sensitized BMMCs were stimulated with 1μM A23187, >80% population responded to the stimulation and the gradual decrease in

fluorescence was much slower than that observed with neurons. The fluorescence remained above basal level even after 60min (**Figure 13**). Sensitized BMMCs responded to 10 (**Figure 14**) or 100ng/mL DNP-HSA stimulation (data not shown) with approximately 66% population showing higher fluorescence intensity after the stimulation. In line with the result from intracellular calcium measurement with microscope imaging, BMMCs responded to DNP-HSA with a greater delay where the highest percentage of cells with higher fluorescence was achieved at 30s time point, then gradually decreased in fluorescence until the fluorescence returned towards the basal level after 60s.

Stemming from the idea that enzymatic digestion with trypsin and collagenase during SCG culture preparation might damage FcεRIα and hence no response to DNP-HSA with IgE sensitization, BMMCs were incubated with trypsin and collagenase in the same way as with neurons, and the intracellular calcium measurement with flow cytometry was performed. Trypsin or collagenase digestion had no effect on IgE sensitization nor DNP-HSA stimulation as indicated by fluorescence with IgE-sensitized BMMCs upon 10ng/mL DNP-HSA stimulation even after trypsin and collagenase digestions (data not shown).

Direct FcεRIα and IgE Staining

When non-sensitized neurons were stained for FcεRIα with a primary conjugated antibody, there was no significant difference between non-stained and stained samples (4.9% and 6.5%, respectively) (**Figure 15A,B**). As a positive control, when non-

sensitized BMMCs were stained for FcεRIα, there was a significant difference between non-stained and stained samples (1.4% and 98.1%, respectively) (**Figure 15C,D**).

Also, when neurons were stained for FcεRIα-bound IgE with a primary conjugated antibody, there was no difference between non-stained, stained (both with sensitization) and stained (without sensitization) samples (2.7%, 4.7% and 7.8%, respectively) (**Figure 16A-C**). However, when BMMCs were stained for FcεRIα-bound IgE, there was a significant difference between non-stained and stained (both with IgE sensitization, 3.7% and 97.4%, respectively), and but no difference was observed between non-stained (without sensitization) and stained (with sensitization) samples (3.7% and 4.4%, respectively) (**Figure 16D-F**).

It was also observed that trypsin and collagenase incubations had no effect on subsequent IgE binding to FcεRIα and its staining (data not shown).

β-Hexosaminidase Assay

Testing Different Concentrations of IgE and DNP-HSA from Different Batches

We set out to determine the ability of anti-DNP IgE and DNP-HSA to sensitize and challenge mast cells and cause degranulation of β-hexosaminidase through high-affinity IgE receptor-mediated activation of BMMCs. We used a relatively easy and simple method to quantitatively measure mast cell degranulation by testing for the release of a cellular constituent (Karimi et al. 2004).

When anti-DNP IgE and DNP-HSA from two different batches were used to sensitize and challenge BMMCs, the combination of anti-DNP IgE from the first batch and DNP-HSA from the second batch were observed to result in the most ideal β-hexosaminidase degranulation response pattern from mast cells (**Figure 17A**). Higher DNP-HSA concentrations than 10ng/mL caused less β-hexosaminidase release (**Figure 17B**); 10ng/mL was chosen as the concentration used for all subsequent experiments. This combination was also tested in all experiments with SCG cultured neurons but without success.

Also, when different concentrations of anti-DNP IgE were used to sensitize mast cells, 1μg/mL was observed to elicit the greatest degranulation response upon stimulation with DNP-HSA (**Figure 18**) and was chosen as the concentration for all subsequent experiments.

A23187-Induced β-Hexosaminidase Release

1μM A23187 caused degranulation response in mast cells similar to that obtained when stimulated with 10 or 25ng/mL DNP-HSA (**Figure 19**) and was chosen as a positive control stimulator for other intracellular calcium measurement experiments.

Effect of Fluo-3, AM on BMMC β-Hexosaminidase Release by DNP-HSA

When cells were previously loaded with Fluo-3, AM, there was no difference in IgE-sensitized mast cell degranulation between loaded and unloaded cells after stimulation with 10 or 25ng/mL DNP-HSA (**Figure 20A**). Also, when DNP-HSA was replaced with Fluo-3, AM as a stimulant of IgE-sensitized mast cells, Fluo-3, AM did not

cause any degranulation (**Figure 20B**). As a result, it was observed that Fluo-3, AM as a

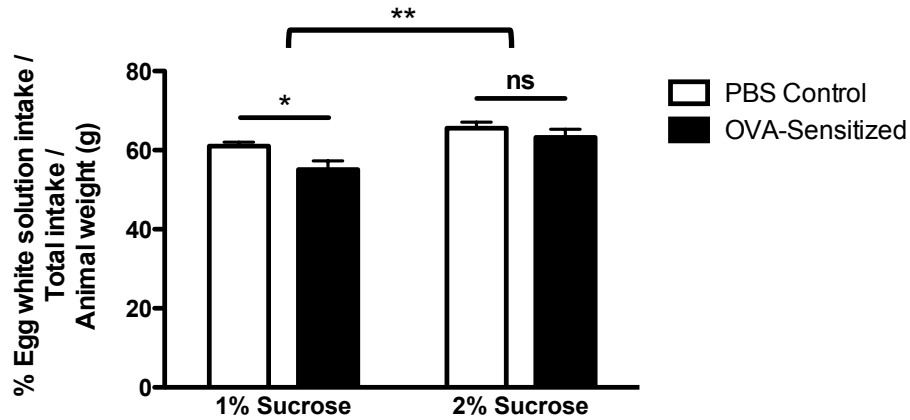loading dye or stimulant did not affect degranulation of mast cells.

Figure 2. Effect of OVA sensitization and sucrose concentration on sucrose solution intake in non-chimeric mice
There was a significant difference in sucrose solution intake between 1% and 2% sucrose groups (p<0.01). Also, there was a significant difference in sucrose solution intake between PBS control and OVA-sensitized groups in 1% sucrose group (p<0.05), but no significant difference was observed in PBS control and OVA-sensitized groups in 2% sucrose group. Error bars represent SEM; *: p<0.05, **: p<0.01, ns: not significant; n=5 per group.
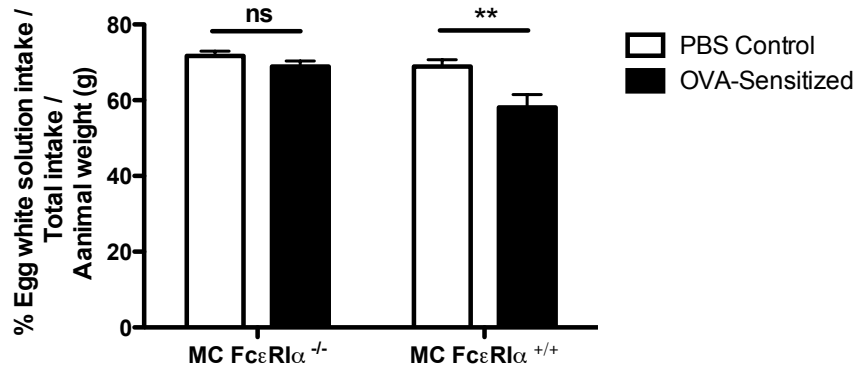
Figure 3. Effect of OVA sensitization and hematopoietic FcεRIα chimerism on 1% 1% sucrose solution intake

There was no significant difference in 1% 1% sucrose solution intake between PBS control and OVA-sensitized groups in MC FcεRIα $^{-/-}$, but there was a significant difference between PBS control and OVA-sensitized groups in MC FcεRIα $^{+/+}$ (p<0.01). Error bars represent SEM; **: p<0.01, ns: not significant; n=13 (PBS control) and n=11 (OVA-sensitized) per group.
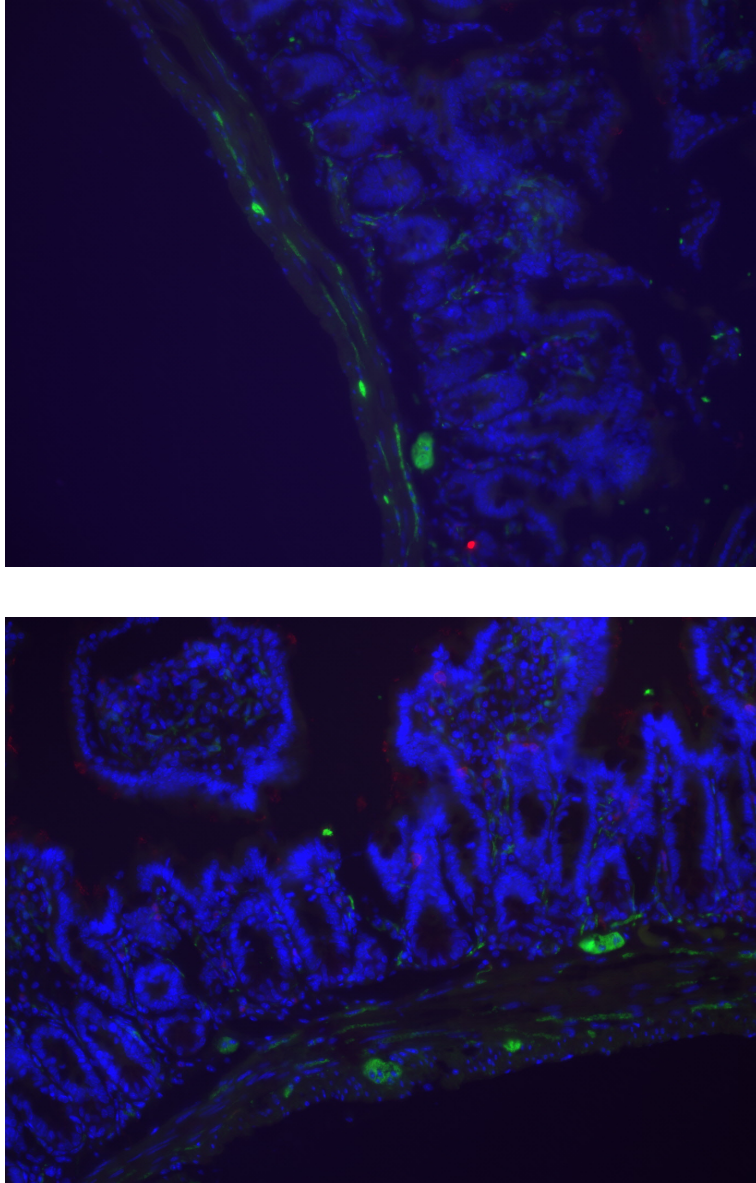
Figure 4. Immunohistochemical images of PGP9.5 and IgE co-staining on jejunal tissue from OVA-sensitized mouse (20×, duplicates)
Tissues were stained for PGP9.5 (FITC) and IgE (Texas Red). No specific IgE staining was observed. Also, no co-localization of PGP9.5 and IgE staining was observed. Green: FITC, Red: Texas Red, Blue: DAPI.
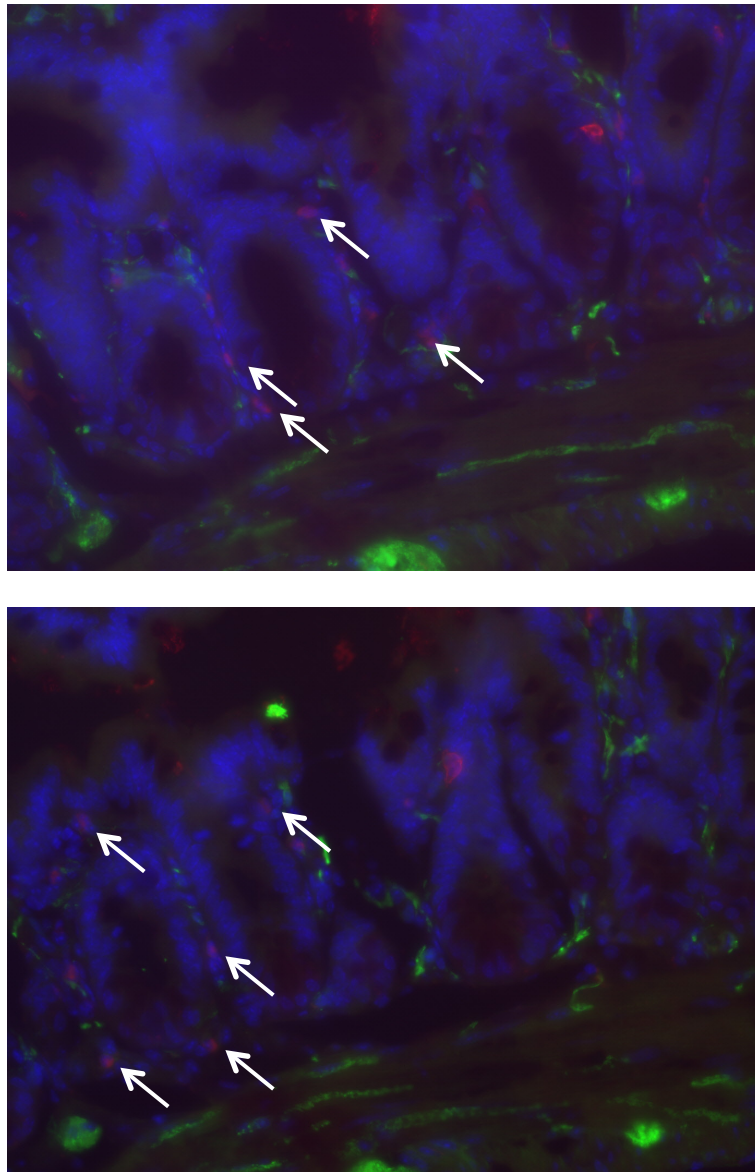
Figure 5. Immunohistochemical images of PGP9.5 and IgE co-staining on jejunum tissue from OVA-sensitized mouse (40×, duplicates)
Tissues were stained for PGP9.5 (FITC) and IgE (Texas Red). No co-localization of PGP9.5 and IgE staining was observed. However, occasional red staining cells were seen in the villous structures (arrow), which probably represent mast cells. Green: FITC, Red: Texas Red, Blue: DAPI.
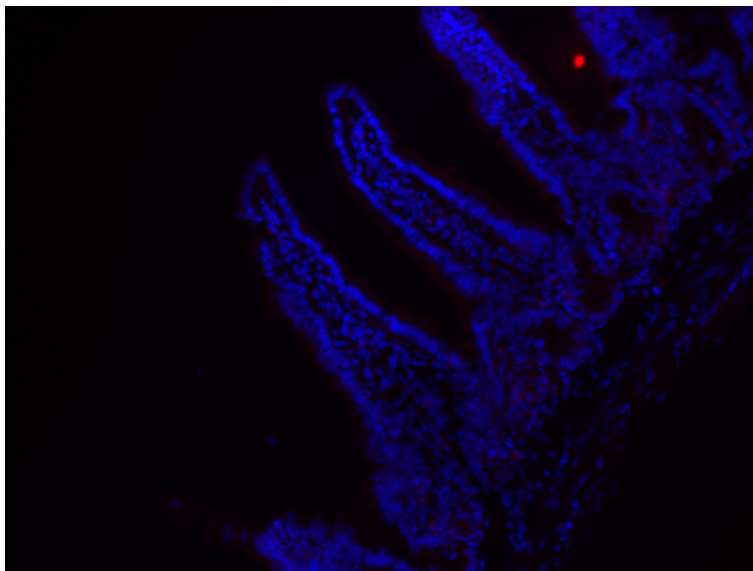
Figure 6. Immunohistochemical image of FcεRIα staining on jejunum tissue from non-sensitized mouse (20×)
Tissues were stained with anti-FcεRIα and DAPI only. No FcεRIα staining was observed. Red: Texas Red, Blue: DAPI.
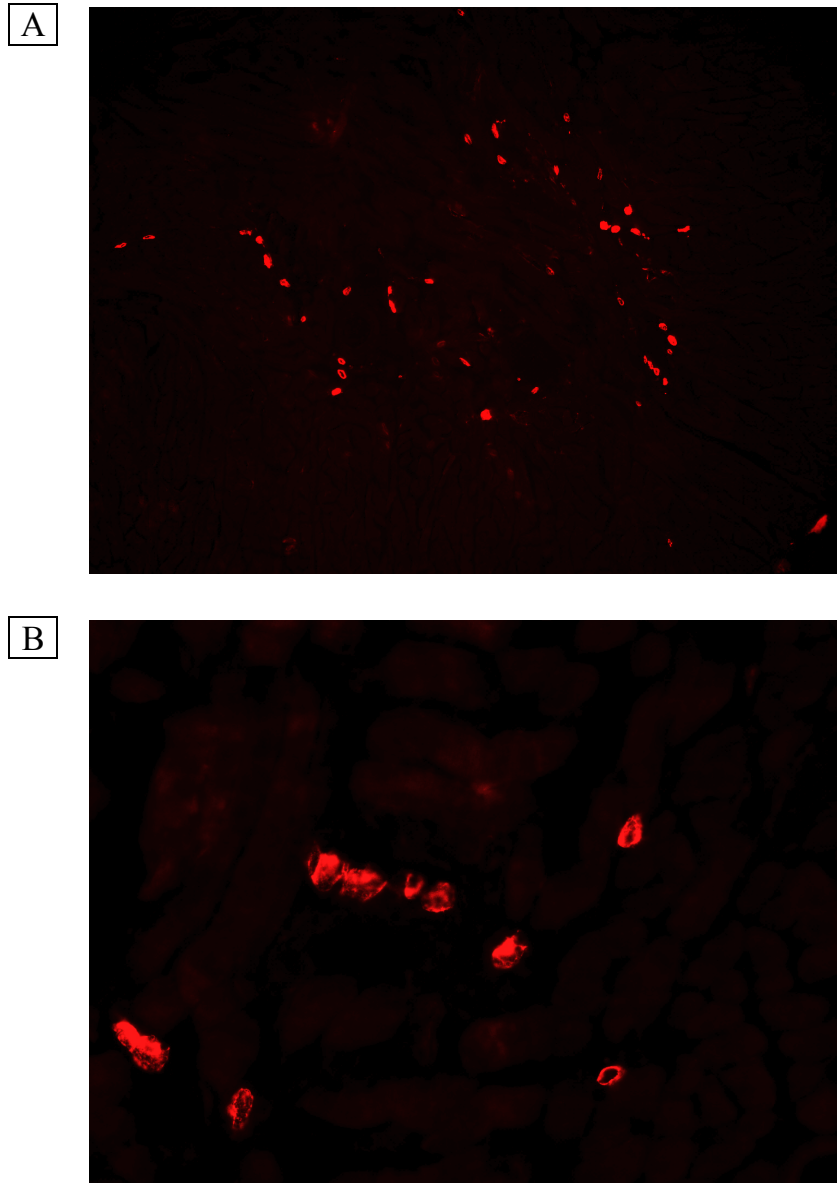
Figure 7. Immunohistochemical images of IgE staining on tongue tissue from OVA-sensitized mouse
Tissues were stained with anti-IgE only. Staining was observed that resemble cell membrane structure as indicated by ring-shaped morphology, which represent mast cells. Red: Texas Red; A: 10×, B: 40×.

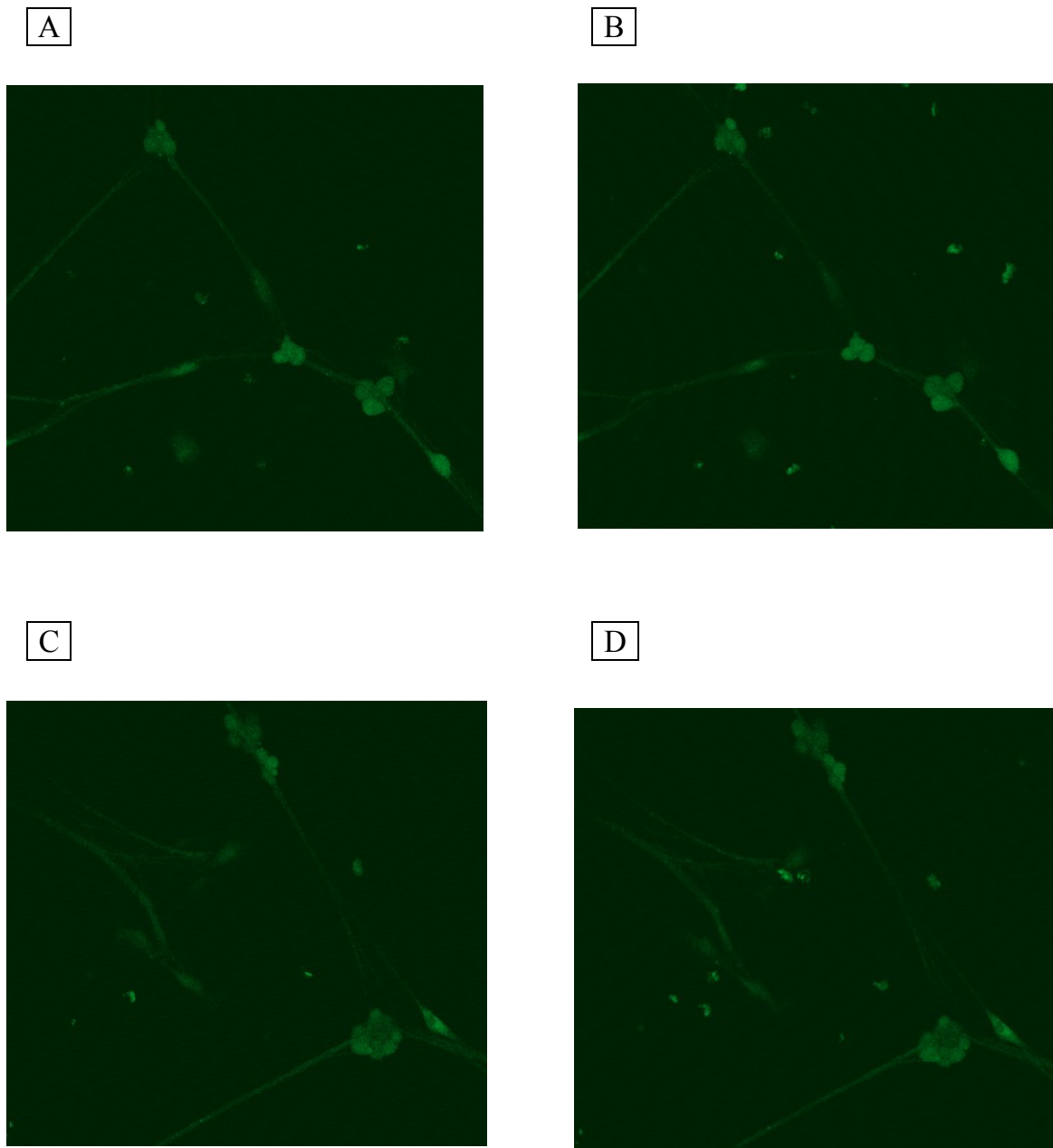**Figure 8. Confocal images of intracellular calcium measurement of IgE-sensitized neuron before (A,C) and 60 sec after (B,D) 10ng/mL or 100ng/mL DNP-HSA stimulation. (A,B: 10ng/mL; C,D: 100ng/mL DNP-HSA stimulation)**
No significant difference in fluorescence intensity was observed between before and for 60 sec after 10ng/mL or 100ng/mL DNP-HSA stimulations. B and D images shown at 60 sec after stimulation.
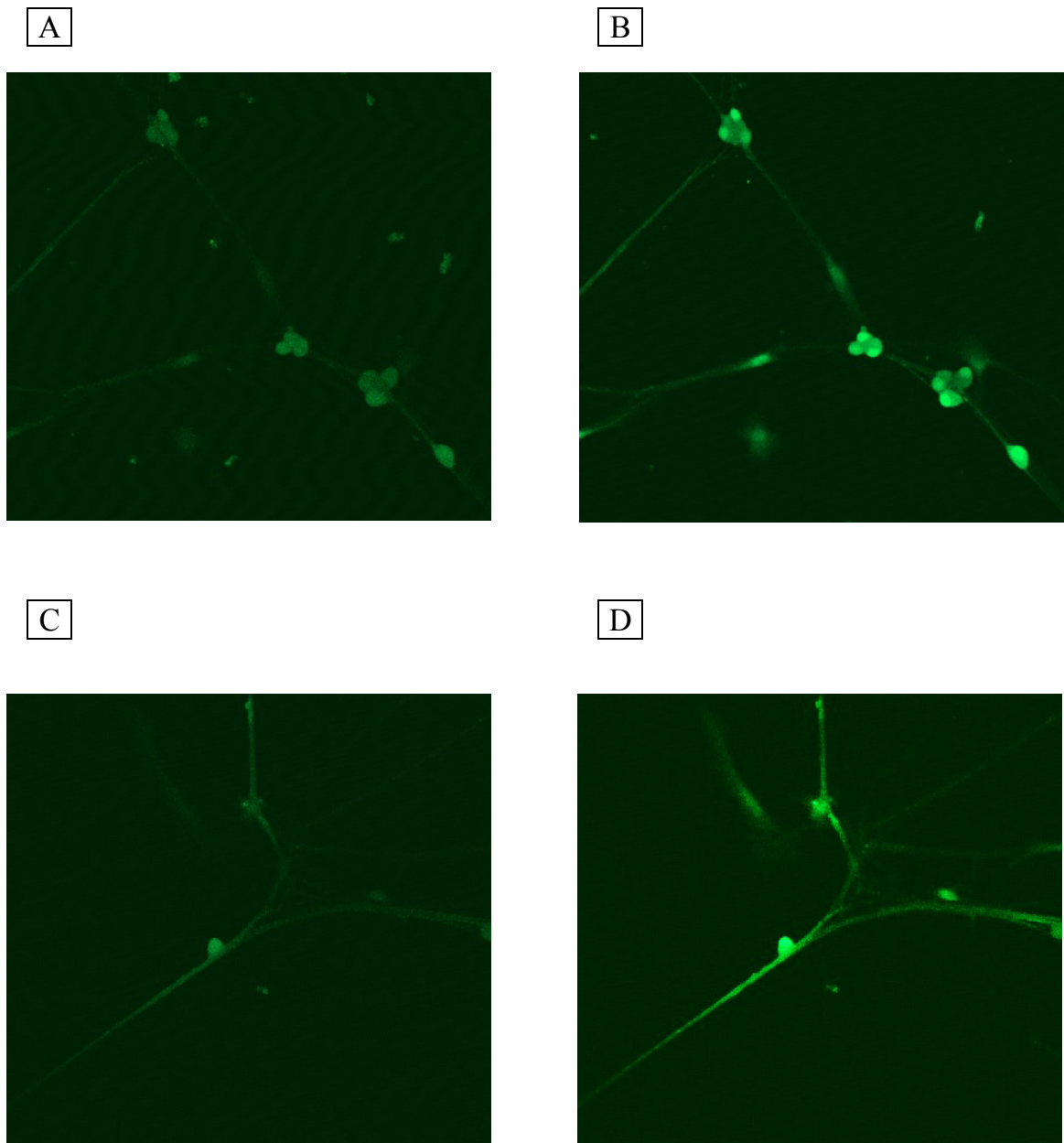
Figure 9. (A,B) Confocal images of intracellular calcium measurement of IgE-sensitized neuron previously challenged with 10ng/mL DNP-HSA (See Figure 8A,B). (C,D) Confocal images of intracellular calcium measurement of IgE-sensitized neuron previously unchallenged. Before (A,C) and after (B,D) 1μM A23187 stimulation. There was a significant difference in fluorescence intensity before and after 1μM A23187 stimulation in both neurite and soma. B and D images shown at 5 sec after stimulation.
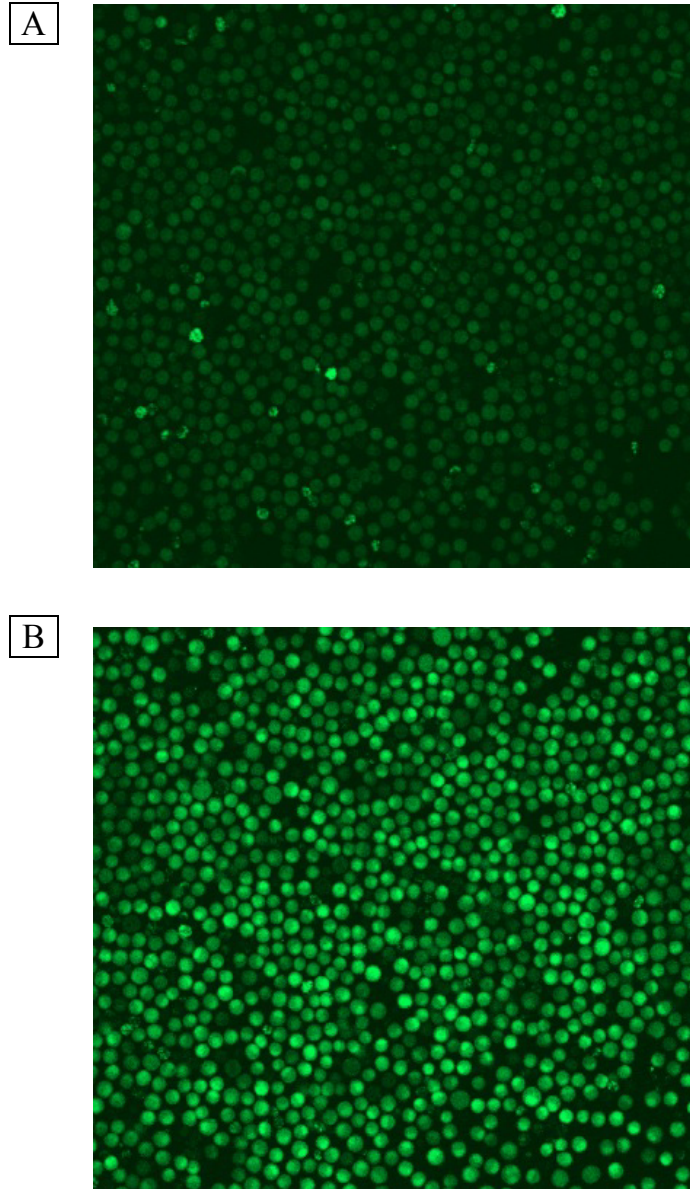
Figure 10. Confocal images of intracellular calcium measurement of IgE-sensitized mast cell before (A) and after (B) 10ng/mL DNP-HSA stimulation
There was a significant difference in fluorescence intensity before and after 10ng/mL DNP-HSA stimulation. Time interval: 30s.

C

Red: 0s
Blue: 15s

Red: 0s
Blue: 60s
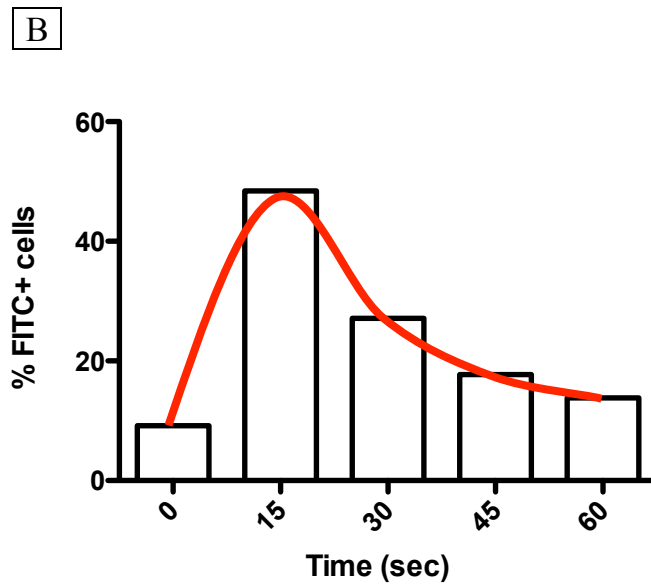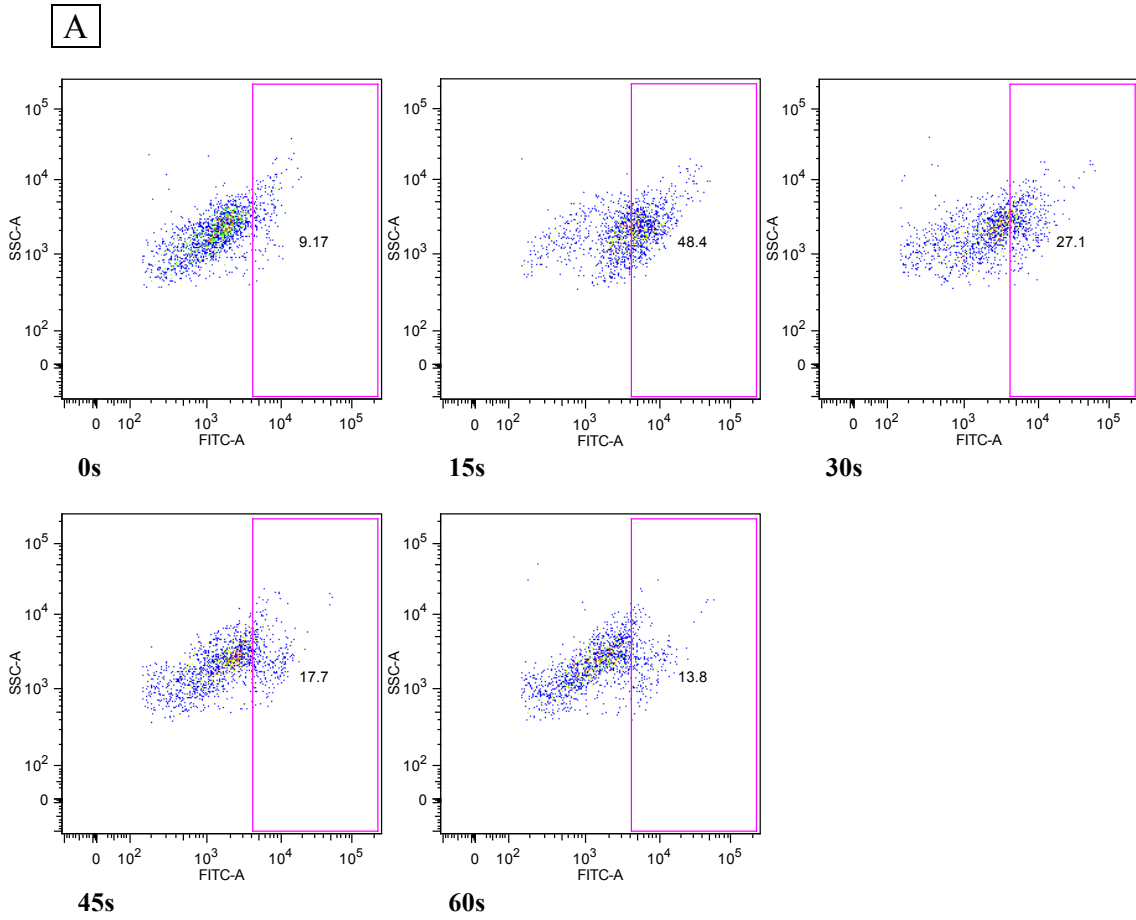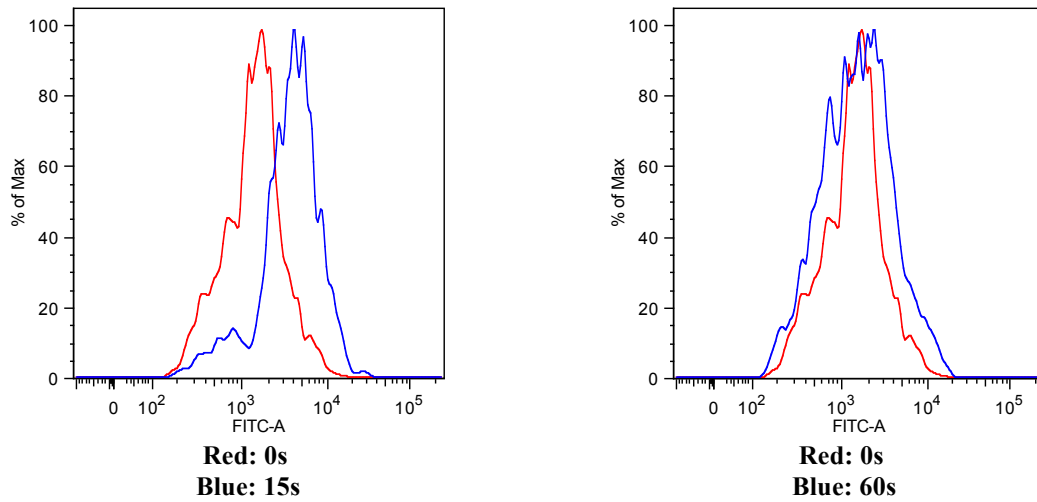
Figure 11. Flow cytometric analysis of Fluo-3, AM (FITC)-stained SCG neurons stimulated with 1μM A23187

FACS dot plots (A), % of fluorescent cells over time (B) and histogram comparison of fluorescent cell populations at two different time points (C). Neurons responded to 1μM A23187 stimulation with increase in fluorescence. The percentage of cells with fluorescence reached its peak at 48.4% <15s after stimulation from 9.17% before stimulation. After it reached its peak, less cells showed fluorescence with gradual decrease to 13.8% after 60s. Time: seconds after stimulation; Number inside dot plot: % cell population with fluorescence.

0s **10s** **20s** **30s** **40s** **50s** **60s**

5s after A23187 stimulation

C

D

Red: 0s
Blue: 10s

Red: 0s
Blue: 60s

Red: 0s
Blue: 5s after A23187 stimulation
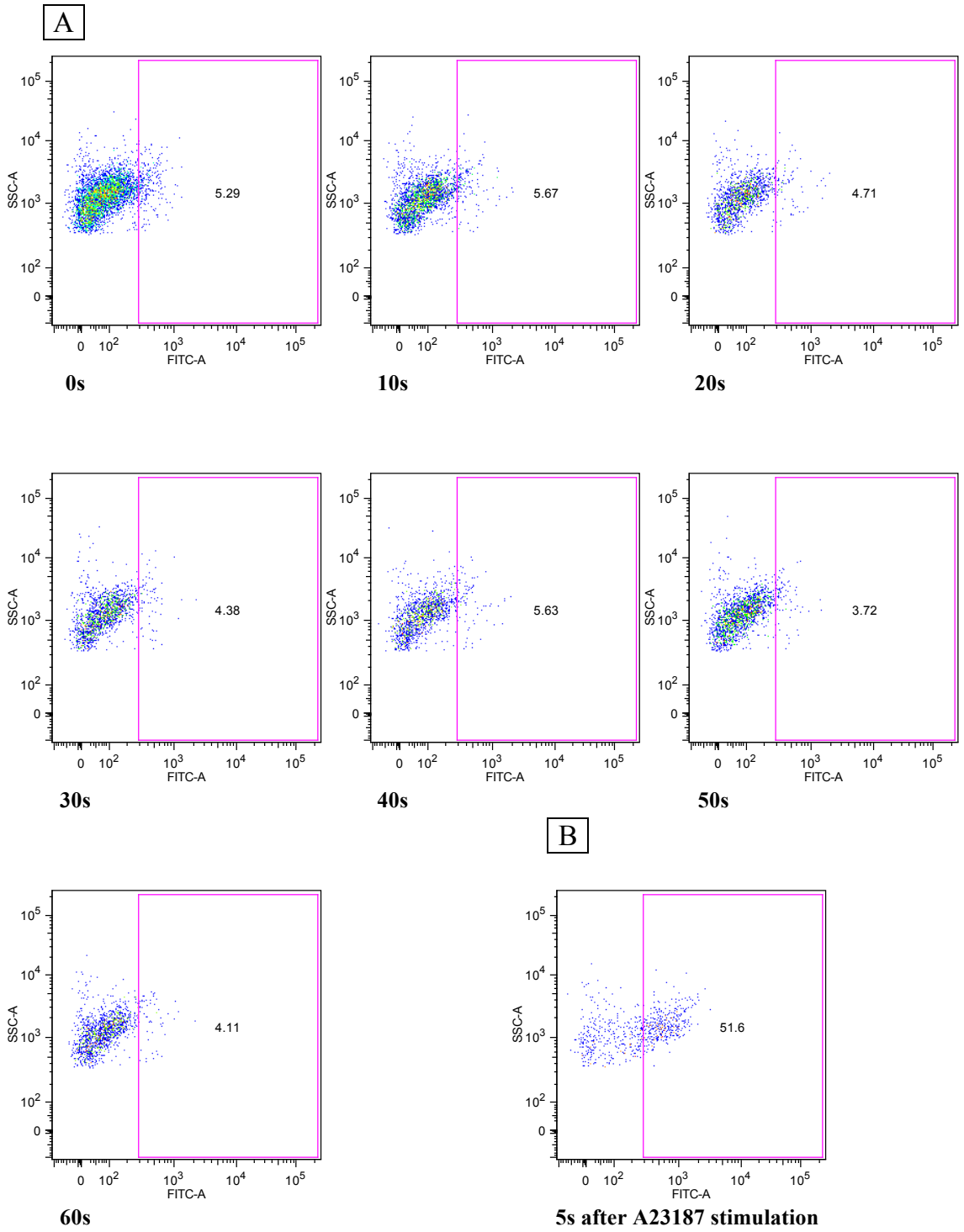
Figure 12. Flow cytometric analysis of IgE-sensitized, Fluo-3, AM (FITC)-stained SCG neurons stimulated with DNP-HSA.
FACS dot plots of neurons stimulated with 10ng/mL DNP-HSA (A) and additional 1μM A23187 (B). % of fluorescent cells over time (C) and histogram comparison of fluorescent cell populations at two different time points (D). There was no significant difference in fluorescence intensity before and after 10ng/mL DNP-HSA stimulation, but when the neurons were stimulated with additional 1μM A23187, cells (51.6%) responded with greater fluorescence 5s after stimulation. Time: seconds after stimulation; Number inside dot plot: % cell population with fluorescence.
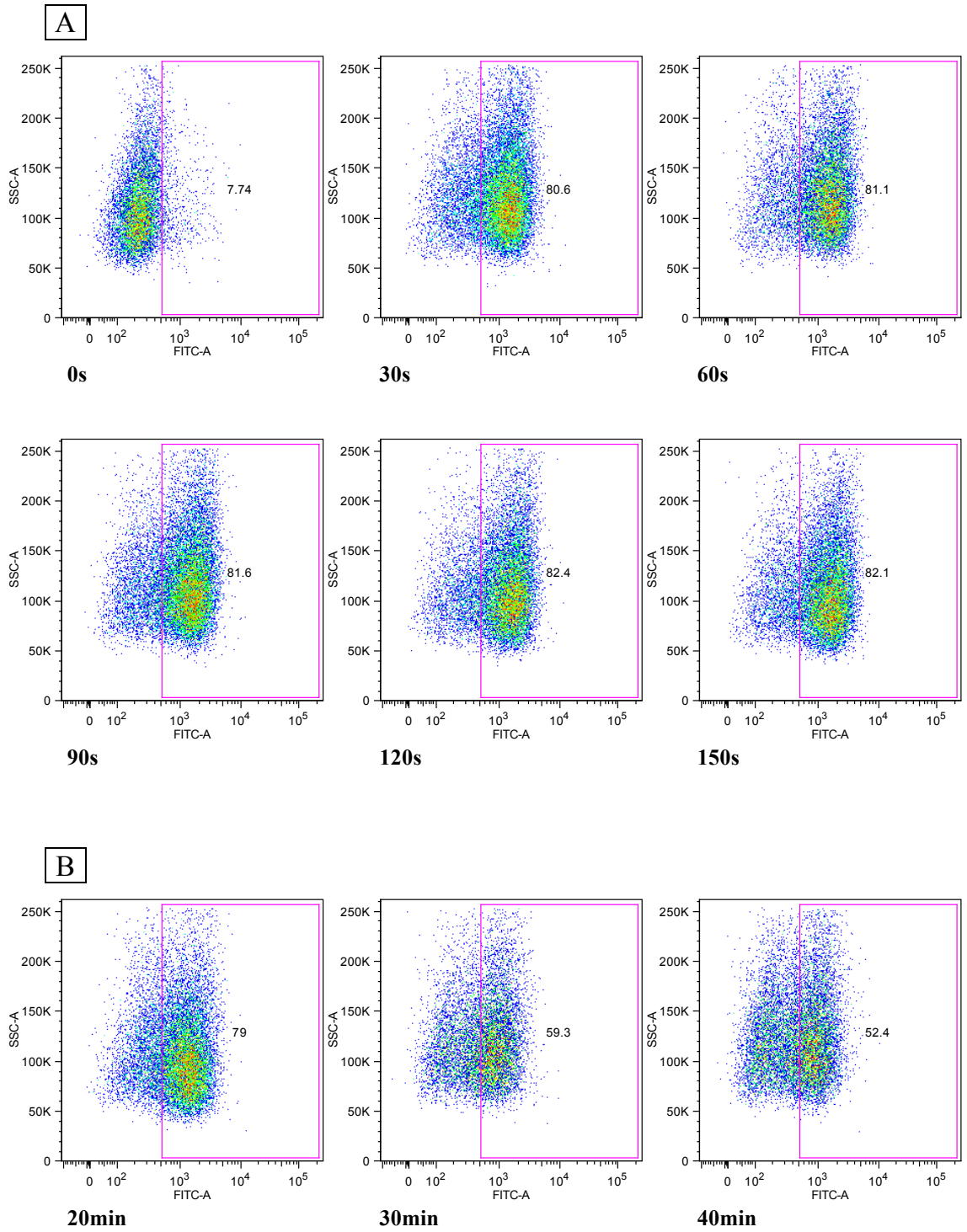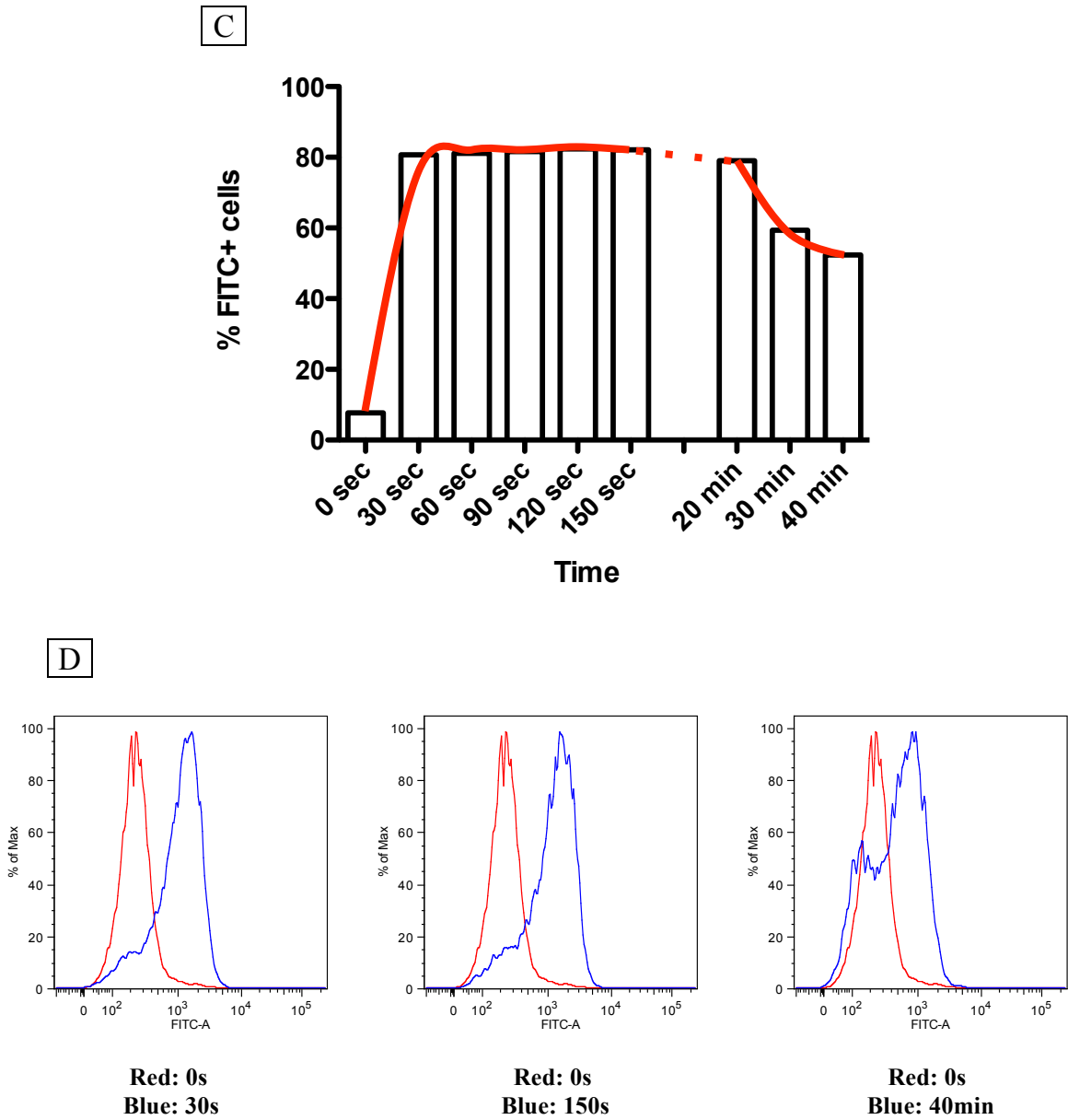
Figure 13. Flow cytometric analysis of Fluo-3, AM (FITC)-stained BMMCs stimulated with 1μM A23187
<30s after A23187 stimulation, greater % cells showed fluorescence (80.6%) than before stimulation (7.74%) and the fluorescence intensity remained constant for over 3min period (A). Cells gradually lost fluorescence and by 40min, approximately half (47.6%) of cell population lost fluorescence (B). Time: seconds or minutes after stimulation; Number inside dot plot: % cell population with fluorescence. % of fluorescent cells over time (C) and histogram comparison of fluorescent cell populations at two different time points (D).

B

C

Red: 0s
Blue: 10s

Red: 0s
Blue: 30s

Red: 0s
Blue: 60s
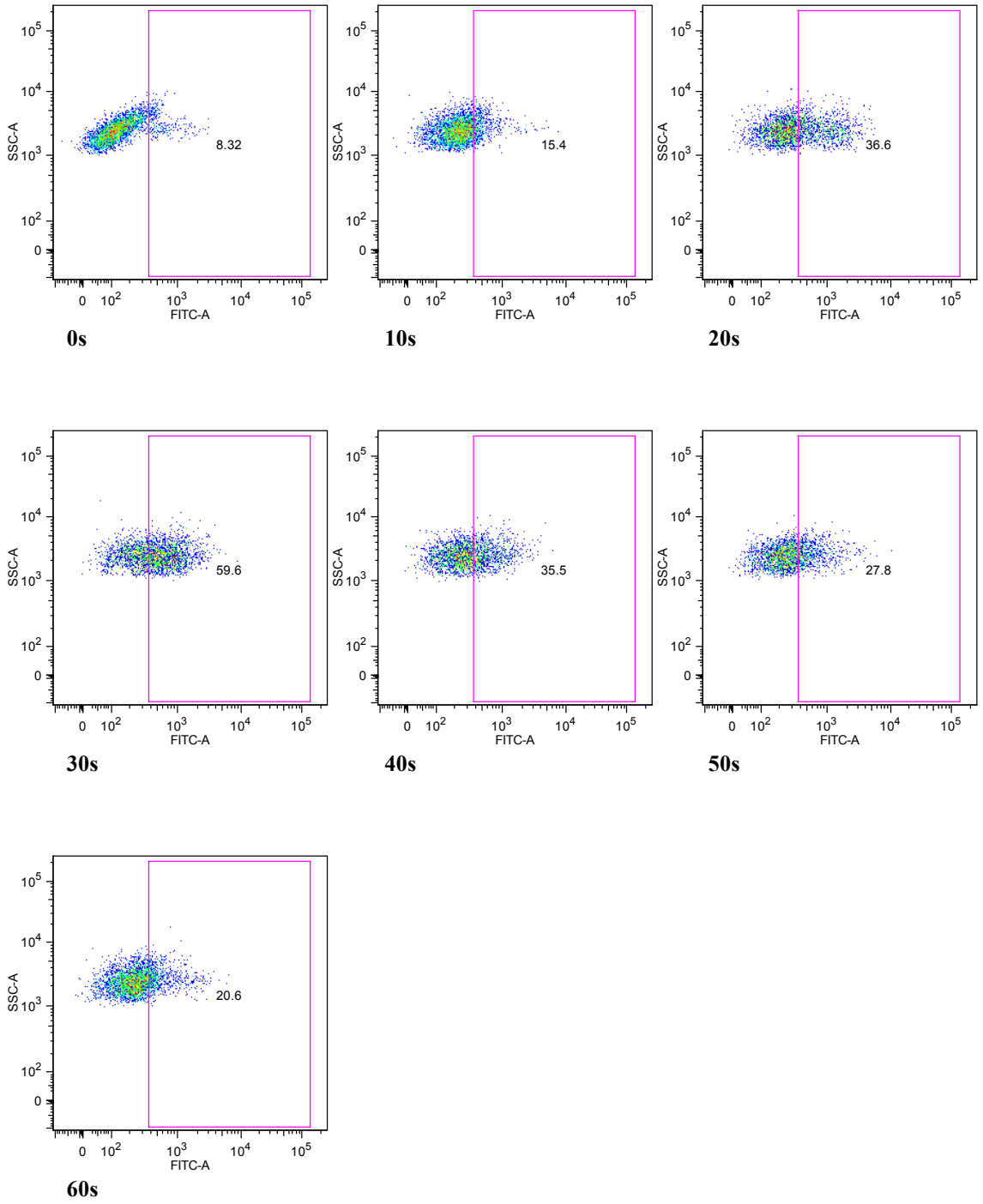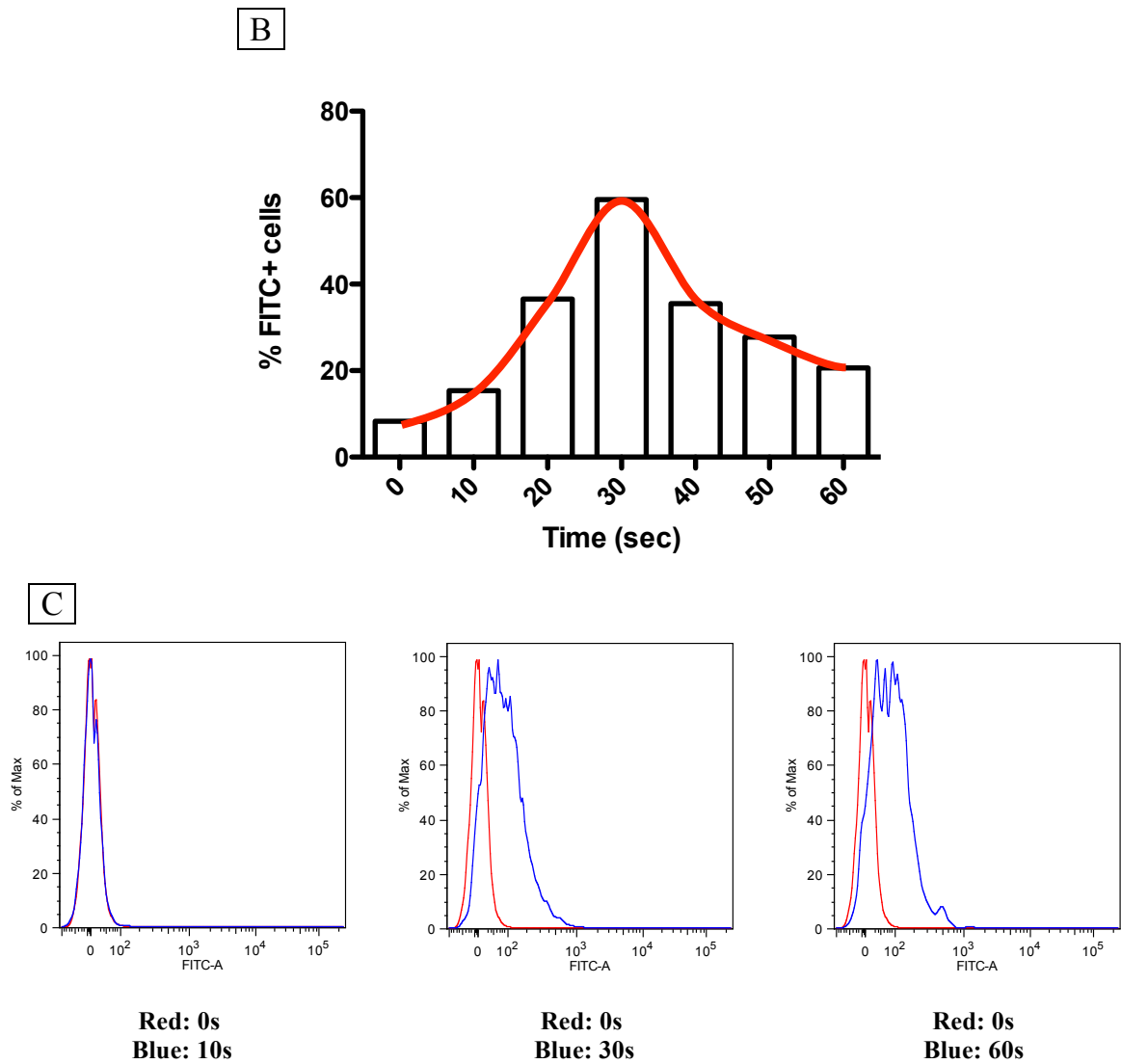
Figure 14. Flow cytometric analysis of IgE-sensitized, Fluo-3, AM (FITC)-stained BMMCs stimulated with 10ng/mL DNP-HSA
FACS dot plots (A), % of fluorescent cells over time (B) and histogram comparison of fluorescent cell populations at two different time points (C). Sensitized cells responded to 10ng/mL DNP-HSA stimulation and percentage of cell population with fluorescence reached its peak (59.6%) 30s after stimulation, after which fluorescence gradually decreased to 20.6% at 60s. Time: seconds after stimulation; Number inside dot plot: % cell population with fluorescence.

E

F
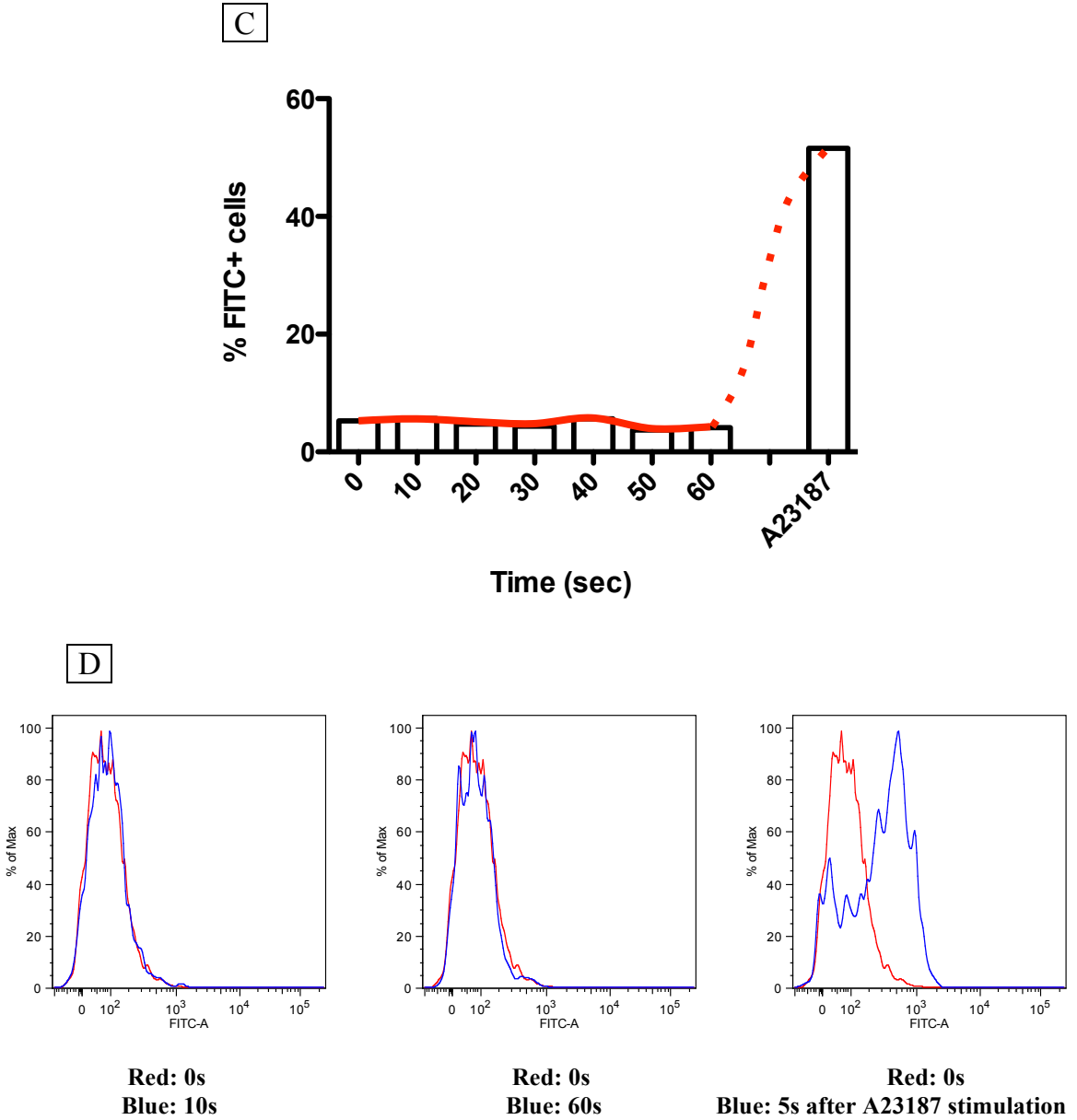
Neuron

BMMC

Red: unstained
Blue: stained

Red: unstained
Blue: stained
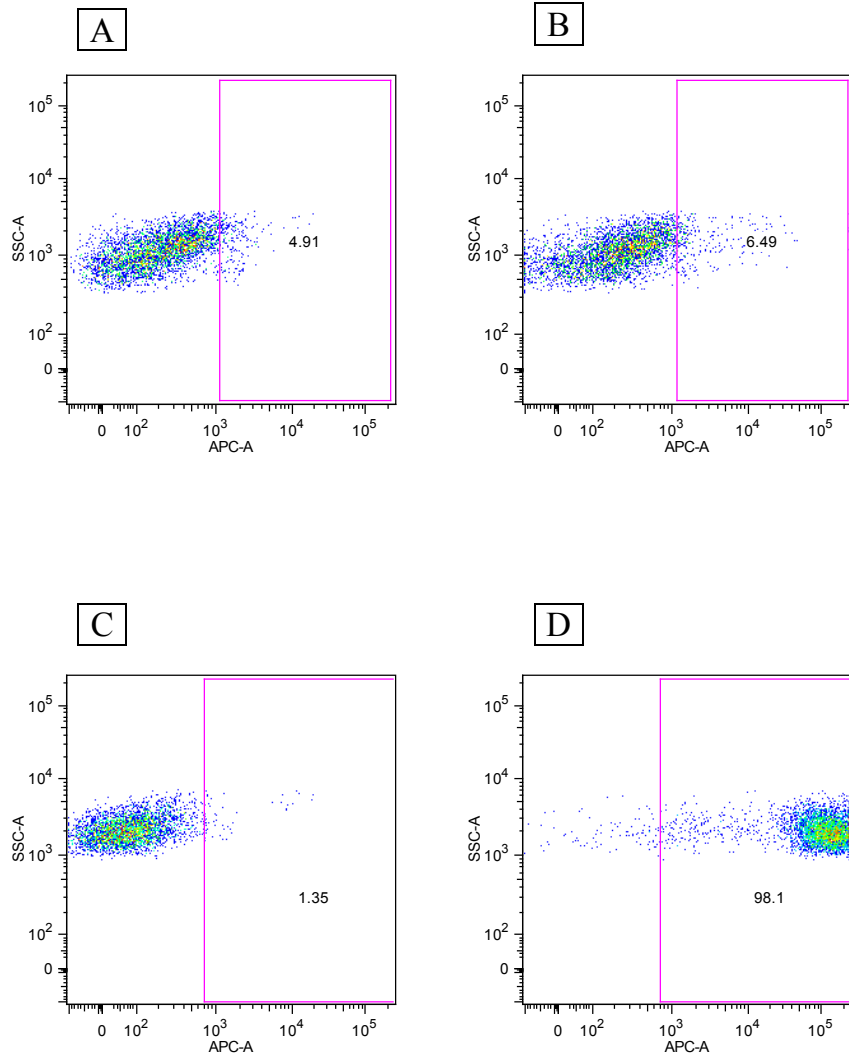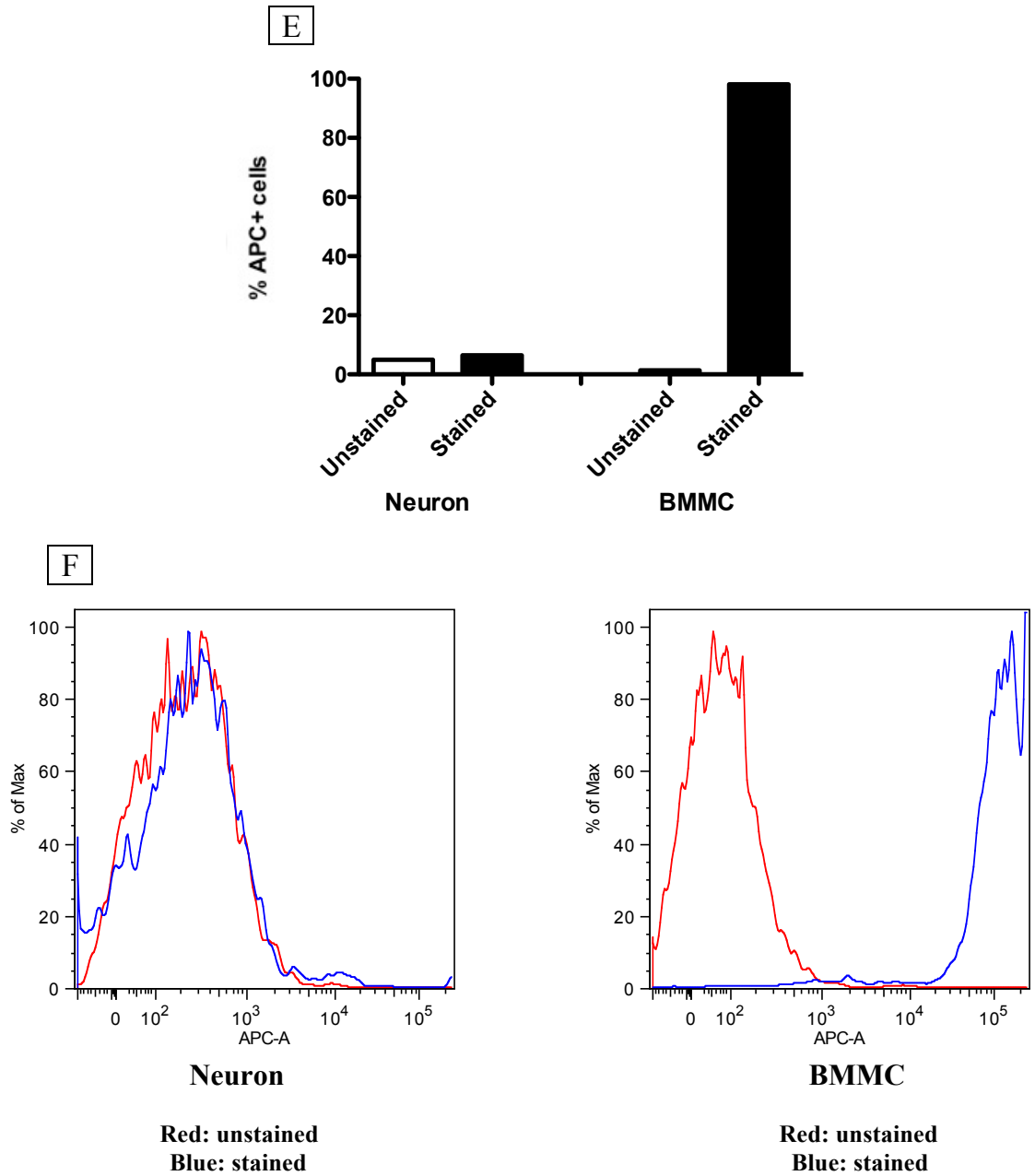
Figure 15. Direct FcεRIα staining with APC-conjugated anti-FcεRIα on non-sensitized SCG neurons and BMMCs
FACS dot plots (A-D), % of fluorescent cells (E) and histogram comparison between unstained and stained cell populations (F). No significant difference was observed for neurons between unstained (A, 4.91%) and stained (B, 6.49%), but a significant difference was observed for BMMCs between unstained (C, 1.35%) and stained (D, 98.1%). Number inside dot plot: % cell population with fluorescence.

G

H

Neuron

Red: unstained
Blue: stained without sensitization
Green: stained with sensitization

BMMC

Red: unstained
Blue: stained without sensitization
Green: stained with sensitization
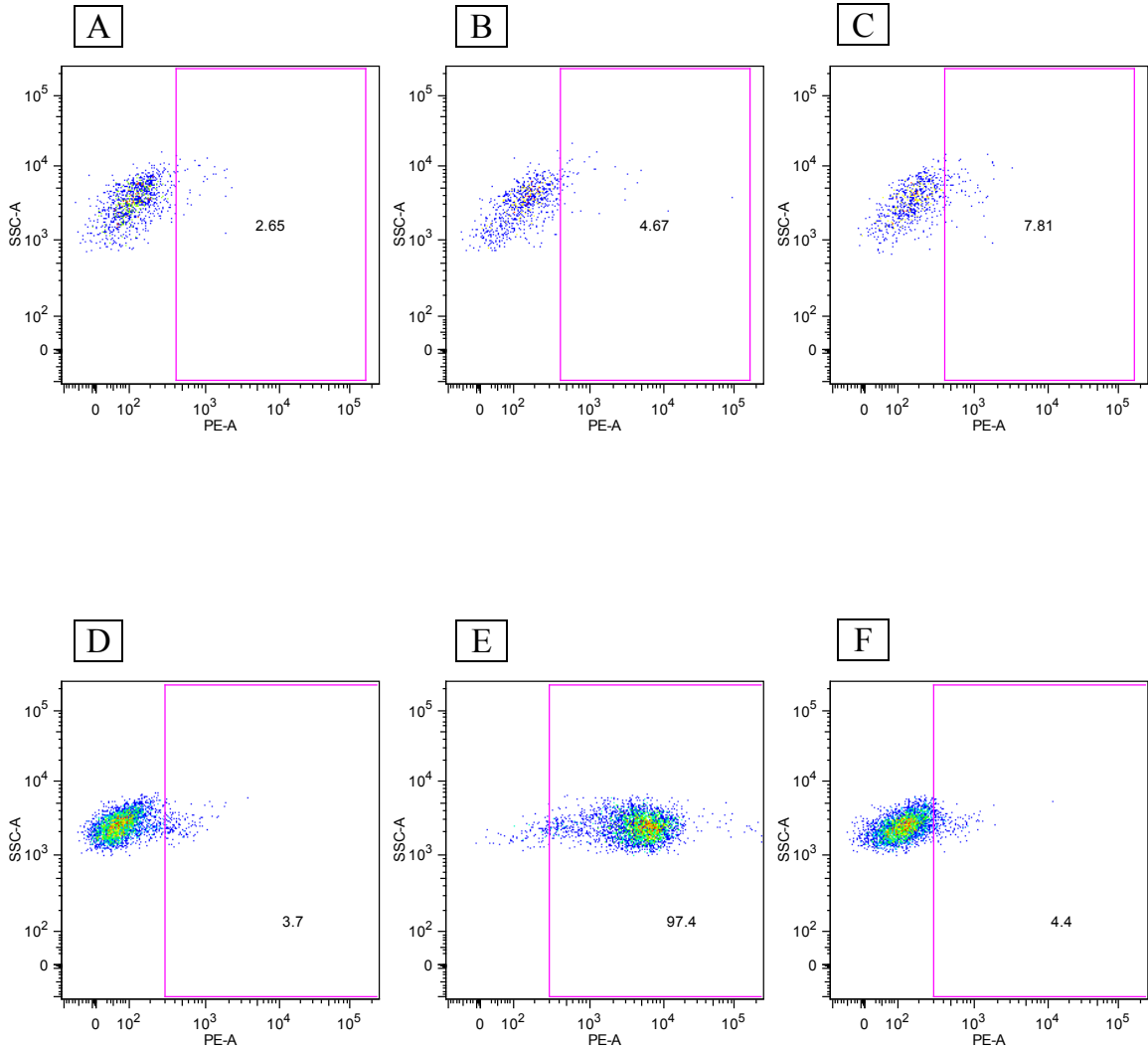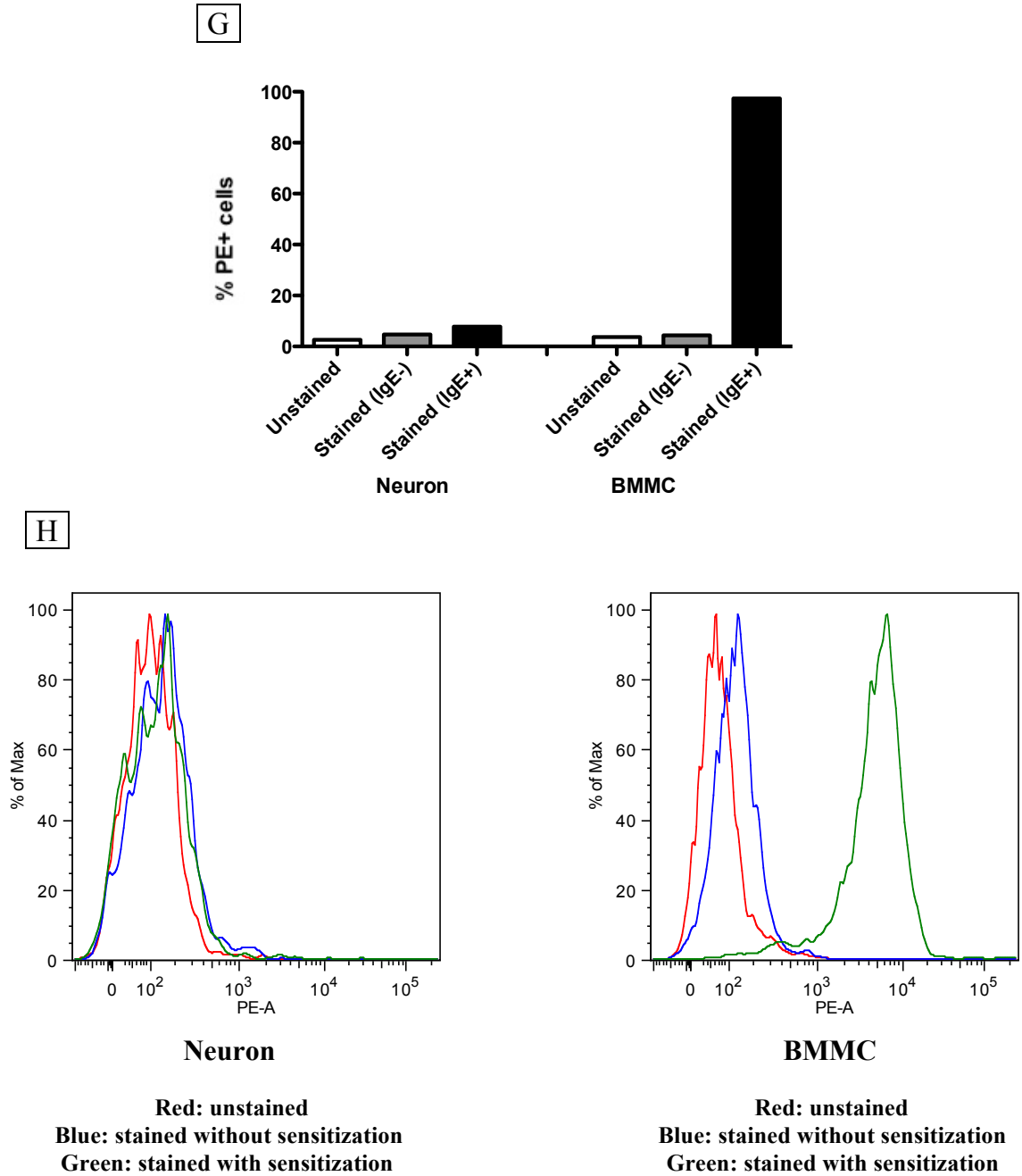
Figure 16. Direct IgE staining with PE-conjugated anti-IgE on SCG neurons and BMMCs
FACS dot plots (A-F), % of fluorescent cells (G) and histogram comparison between unstained and stained (with or without sensitization) cell populations (H). No significant difference was observed between unstained neurons (A, 2.65%), those stained with IgE sensitization (B, 4.67%) or stained without IgE sensitization (C, 7.81%). However, a significant difference was observed between unstained BMMCs (D, 3.7%) and those stained with IgE sensitization (E, 97.4%), but no significant difference was observed between unstained BMMCs (D, 3.7%) and those stained without IgE sensitization (F, 4.4%). Number inside dot plot: % cell population with fluorescence.
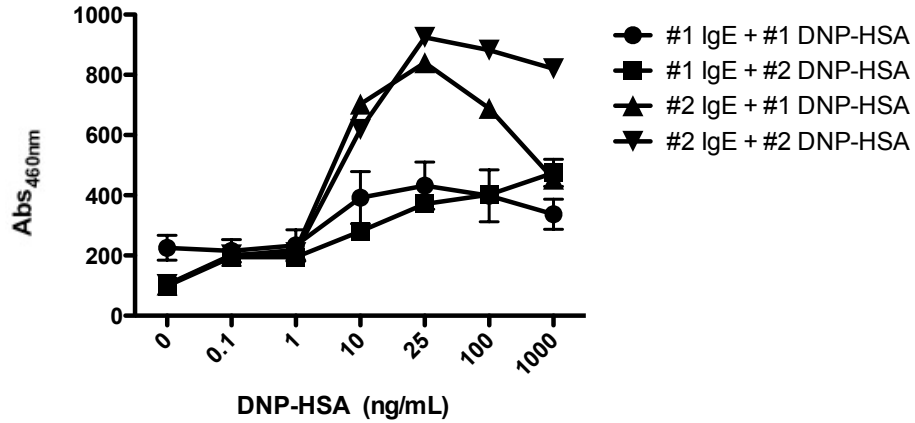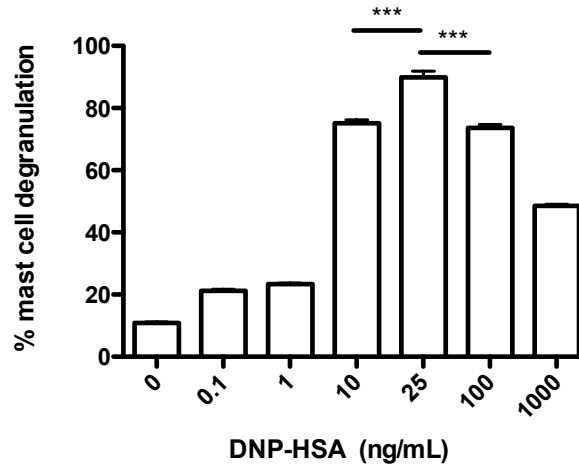
Figure 17. Concentration- and batch-dependent anti-DNP IgE sensitization DNP-HSA stimulation of BMMCs on β-hexosaminidase assay
(A) Anti-DNP IgE from the second batch and DNP-HSA from the first batch were observed to be ideal choice for mast cell degranulation. (B) Upon sensitization with anti-DNP IgE from the second batch and stimulation with DNP-HSA from the first bath, 25ng/mL DNP-HSA concentration showed the highest degranulation percentage (90%) from mast cell and less degranulation was observed in lower and higher DNP-HSA concentrations (10 and 100ng/mL). Error bars represent SEM; ***: $p < 0.001$; n=3 per group.
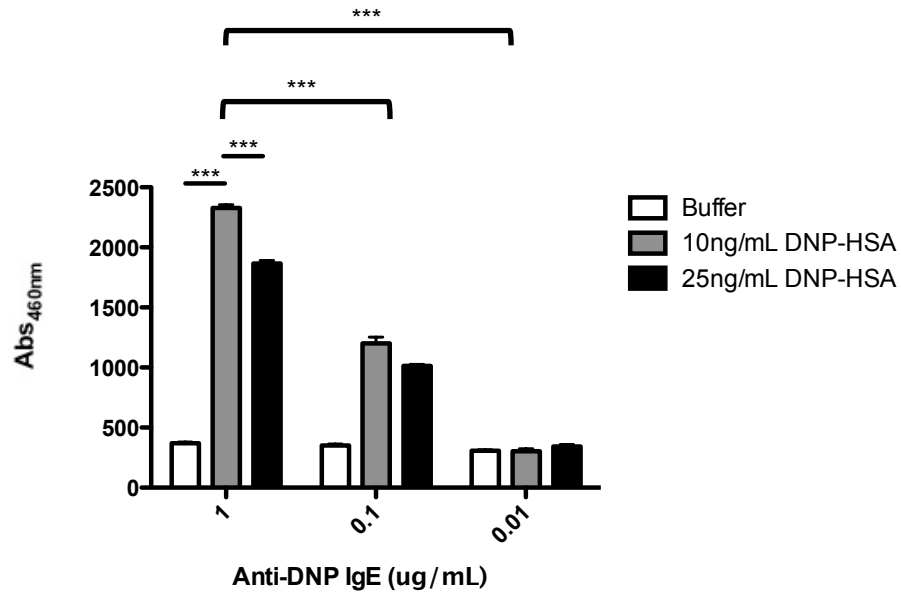
Figure 18. Concentration-dependent anti-DNP IgE sensitization of BMMCs on β-hexosaminidase assay
1μg/mL anti-DNP IgE sensitization caused the greatest mast cell degranulation response when stimulated with 10ng/mL DNP-HSA and the effect of anti-DNP IgE was diminished as the concentration decreased to 0.1μg/mL and completely abolished at 0.01μg/mL. Error bars represent SEM; ***: p<0.001; n=2 per group.
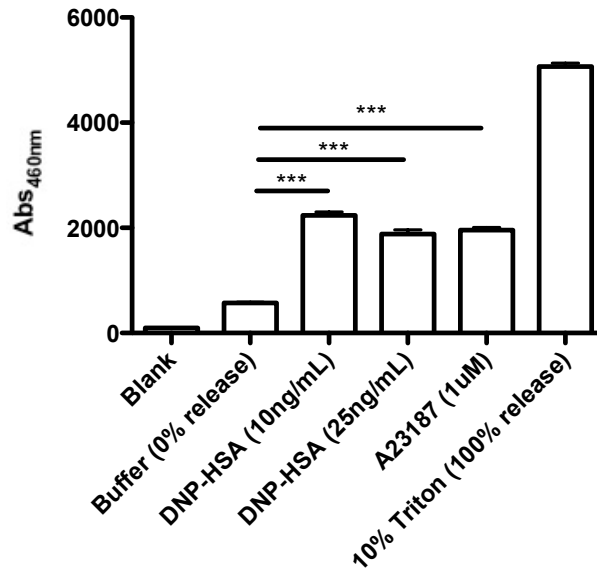
Figure 19. A23187 as a positive control stimulator for β-hexosaminidase assay
Stimulation with 1μM A23187 caused a significant mast cell degranulation comparable to that with DNP-HSA (10 and 25ng/mL) and was chosen as the positive control for subsequent experiments. Error bars represent SEM; ***: $p<0.001$; n=2 per group.
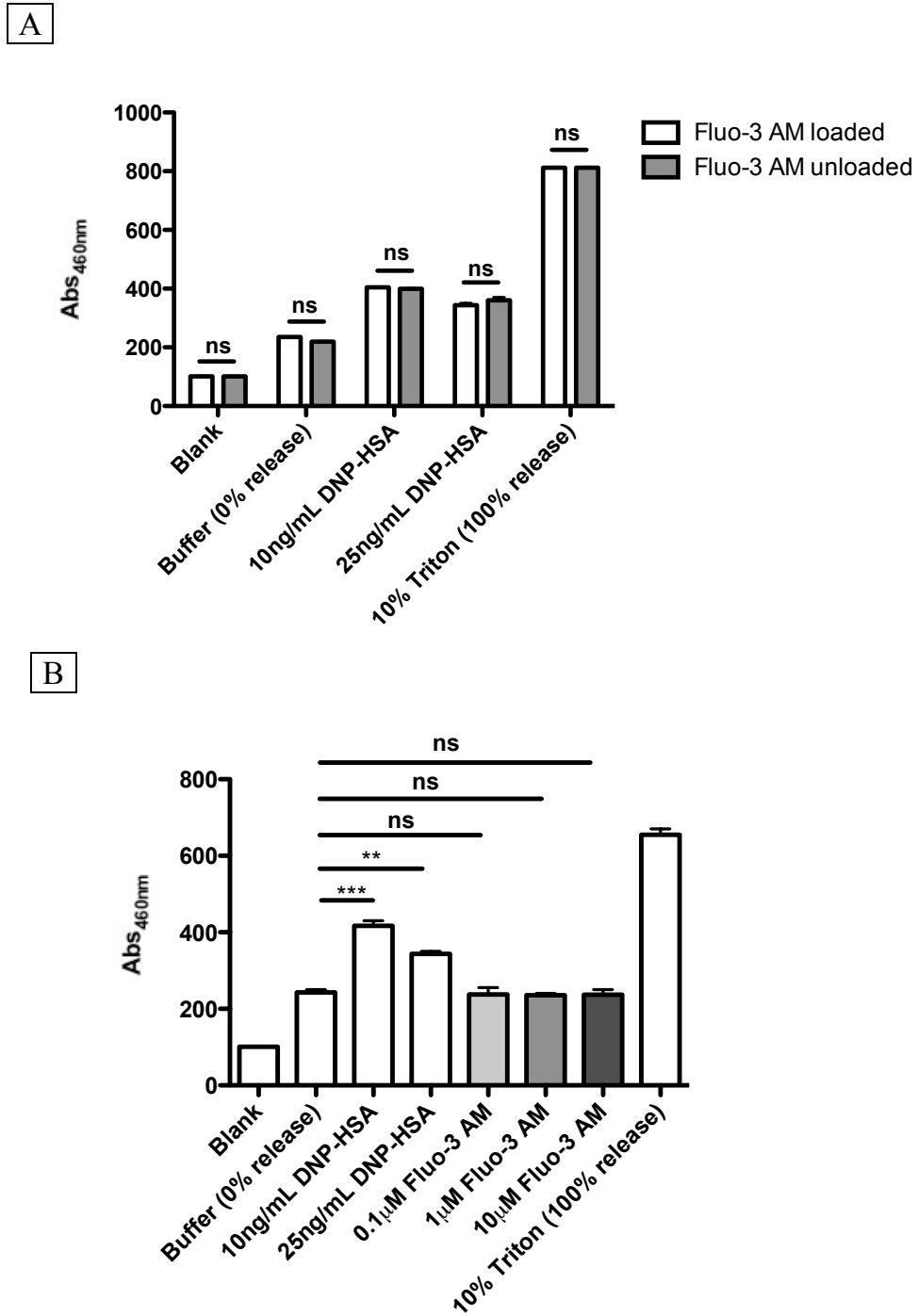
Figure 20. Effect of Fluo-3, AM loading and as a stimulator on mast cell degranulation on β-hexosaminidase assay

(A) There was no significant difference in mast cell degranulation between cells loaded and not loaded with Fluo-3, AM. (B) Fluo-3, AM as a stimulator did not cause any significant degranulation in mast cells. Error bars represent SEM; **: $p<0.01$; ***: $p<0.001$; ns: not significant; n=2 per group.

DISCUSSION

A previous publication from our lab (van der Kleij et al. 2010) showed evidence that SCG neurons express high-affinity IgE receptors (FcεRI) that are functionally active, while others have published data using DRG neurons (Andoh and Kuraishi 2004b). The aims of the present study were to repeat the previous studies and examine the distribution of the FcεRI on neurons in tissues such as the intestine. We then wished to determine if activation of these receptors utilized the same molecular pathways that have been described for mast cells and basophils (Rivera and Gilfillan 2006).

Van der Kleij et al. (2010) showed that all three subunits (α, β and γ) of FcεRI are present on primary sensory SCG neurons, from evidence of immunohistochemical staining of primary SCG cultures with PGP9.5 (pan-neuronal marker) co-localization. They also showed that they were functionally active when sensitized with IgE and challenged with antigen since this led to an intracellular calcium increase that was visualized microscopically (van der Kleij et al. 2010). The finding was supported by presence of mRNA transcripts of all three receptor subunits in SCG neuronal cultures measured by RT-PCR. In addition, sensitized neurons that were stimulated with antigen were able to activate interconnecting neurites, confirming functionality of the receptor-bearing neurons. In accordance with a previous study (Karimi et al. 2004), where mast cells with FcεRI showed a dose-dependent response to antigen stimulation, neurons also showed a similar response pattern where 10ng/mL antigen stimulation elicited the greatest percentage of responsive neurons and less response with lower or higher concentrations of antigen. Not only did the findings confirm the presence and

functionality of the receptors on neurons, but also they suggested similar characteristics to its counterpart in mast cells. Other cell types express IgE receptors and the receptors may consist of high or low affinity types. FcεRII (CD23) is the low-affinity IgE receptor and it is expressed on many non-immune and non-hematopoietic cells in tissues, such as human epithelial cells (Tu et al. 2005), where their function may be associated with antigen uptake. The three subunits that together make up the high-affinity counterpart FcεRI (α, β and γ) are expressed as a tetramer (αβγ$_2$) only on two related hematopoietic cell types, mast cells and basophils. The trimeric IgE receptor (αγ$_2$) is also found on Langerhans cells in the skin, dendritic cells, eosinophils (especially when activated) and platelets (Prussin and Metcalfe 2003) where they bind IgE and perform different functions than when on mast cells and basophils (Bieber 2007, Reich et al. 2001).

Prior to the study with neuronal FcεRI on SCG cultures, it was also shown by Andoh and Kuraishi (2004b) that FcεRI is expressed in adult DRG neurons from primary cultures. In contrast to SCG neurons, where FcεRI expression was observed on all structural aspects of neurons, including both soma and neurites, in DRG cultures, a smaller percentage of cells were positively stained for FcεRI on the soma only. In addition, among cultured DRG neurons, less than half (44%) of the cells were stained positive for IgE after sensitization with IgE. Andoh and Kuraishi (2004b) went one step further and identified positive FcεRI immunohistochemical staining on neurons in the dermis in sections of the skin in which FcεRI expression was co-localized with PGP9.5. This finding was supported by Rijnierse et al. (2009) where, in addition to FcεRI-bound IgE immunohistochemical staining of DRG in primary culture, intracellular calcium

levels were increased upon sensitization and antigen challenge, similar to the study by van der Kleij et al. (2010).

Since we anticipated that our studies would show IgE bound to sensitized neurons and mast cells *in vivo*, we designed initial studies to test this. The symptoms of mast cell and basophil degranulation depend on where and to what extent this happens (Mekori and Metcalfe 2000). In people who are food allergic, the presence of the specific allergen in the mouth may be enough to cause local mast cell release of cellular contents ranging from mild symptoms such as tingling of the tongue to massive swelling of the lips and extending even to the larynx (Amlot et al. 1987, Ortolani et al. 1988), which may obstruct breathing and cause death by asphyxiation (Ewan 1996).

Mirotti and his associates (2010) recently have utilized this aspect of food allergy in their model. When mice are given two choices, water and sweetened sucrose water, they have a tendency to drink more sweetened solution than the unsweetened counterpart (Inoue et al. 2007, Ninomiya and Imoto 1995). However, when the sweetened solution contains a substance that elicits an immune response and allergic symptoms, mice would show aversive behaviour towards the sweetened solution (Cara et al. 1994) and drink less than when no allergic substance was present. Mirotti et al. (2010) have described the phenomenon in their study where mice that were previously sensitized with OVA showed this aversive behaviour towards OVA-containing water at different sucrose concentrations. Such behaviour arises from IgE binding to FcεRI and subsequent challenge with allergen on mast cells that are present in the digestive tract, where contact with allergen occurs. Following the model, a study was proposed using chimeric mice

that would allow us to see if FcεRI on neurons but not mast cells would demonstrate the same behavioural function *in vivo*. FcεRI expression would be absent on hematopoietic cells, including mast cells, in chimeras but would still remain, if present, on neurons. Even in the absence of mast cell FcεRI, it was hypothesized that chimeras would still show the aversive behaviour towards OVA-containing sucrose water due to the functional FcεRI on neurons. This is important, as while we know that bidirectional communication between mast cells and nerves occurs (Furuno et al. 2004, Suzuki et al. 2001), for example in the intestines, we do not know whether and how neuronal sensitization itself would play a role in allergy.

However, the results from our study indicate that the aversive behaviour towards OVA-containing sucrose solution was absent in the absence of hematopoietic FcεRI. While these data were obtained from a study conducted with animals older than 10 months, they still showed that Mirotti and his associates were correct, and that mast cells appeared to be essential in this model of aversive behaviour in sensitized mice. Our results further supported that putatively sensitized neurons may not cause allergic symptoms *in vivo*.

When the behaviour study was conducted, mice reached the age of 10 months and this is due to two reasons: first, there was a delay with receiving the chimeric mice from Dr. Kelly McNagny in University of British Columbia (Vancouver, BC). In addition, there was a virus (MNV) infection in the Central Animal Facility at McMaster University (Hamilton, ON) and mice from an external untested source were not being accepted. Eventually they were received, relocated and transferred to the Juravinski Hospital

(Hamilton, ON) where the *in vivo* study was done, which further delayed the testing protocols.

In the clinical setting, an individual's age and sex are the two most important determinants in incidence of different types of allergy (Almqvist et al. 2008, Katial and Zheng 2007, Weng 2006) in addition to a genetic propensity. Furthermore, the severity of symptoms of some forms of allergy in the aged is decreased, and this is probably due to decrease in serum IgE levels (Mediaty and Neuber 2005, Prussin and Metcalfe 2003). In turn, lower serum IgE levels may have led to downregulation of FcεRI expression on neurons to an undetectable level, as high serum IgE has been shown to induce FcεRI expression (Yamaguchi et al. 1997, Yamaguchi et al. 1999). Therefore, it is possible that the age of the mice might have affected OVA-induced serum IgE levels and the neuronal receptor expression to account for the lack of ability to detect it in the study with chimeric mice. However, as pointed out, mast cells in these aged animals were clearly sensitized, albeit possibly at a lower level than if younger animals had been used.

While aversive behaviour was demonstrated by these mice, thus showing that there was an adequate IgE sensitization of mast cells, we could not conclude that the IgE sensitization of neurons had occurred as we had anticipated at the beginning of this study. This led to another study of immunohistochemistry. The study was first conducted with jejunum since van der Kleij et al. (2010) had shown *in vivo* sensitization of the myenteric plexus. Fixed tissues from the tests of aversive behaviour were previously stored but they first needed to be optimized in order to test systems. Our initial efforts were directed to the immunohistochemical demonstration of a universal neuronal membrane marker,

PGP9.5, in the myenteric plexus of jejunal tissue. In this help was received from Dr. Elyanne Ratcliffe (McMaster University, Hamilton, ON) and we learned that of the several monoclonal antibody preparations tested, only one gave good results (**Figure 5**).

We then moved on to looking for the presence of IgE and its high-affinity receptor on neurons in normal, unsensitized tissue. Our initial negative results necessitated the testing of all parameters involved since even jejunal tissues from sensitized mice failed to show positive staining either for the FcεRI or IgE. We tested the methods of fixation including percentage of paraformaldehyde, time of fixation, and also the temperature at which this was conducted. Also tested were the functionality of the reagents used, different sources of these reagents, and optimized these in tongue tissue as well, and in later experiments, on bone marrow-derived mast cells.

In the course of jejunal and tongue tissue preparation for immunohistochemistry, a great deal of time was spent on optimization of methodology. One of the most important steps in tissue preparation for immunohistochemical staining is tissue fixation. There are many tissue fixatives available and care must be taken for the choice of fixative depending on the method of analysis. One of the most commonly used for histology is formaldehyde solution (formalin) (Jamur and Oliver 2010), which shows a broad range of specificity for many different cell types. Formaldehyde reversibly (with short fixation duration) or irreversibly (with long fixation duration) fixes cells or tissues upon introduction by crosslinking primary amino groups (NH_2) in proteins to the neighbouring amino groups of either another protein or DNA and forming $-CH_2-$ linkage between them.

Commercially available formaldehyde solution is supplemented with methanol that acts as a stabilizing agent (Helander 1999). However, it is currently unknown what role methanol plays on formaldehyde fixation and thus formaldehyde solution with a lower methanol concentration is preferred by many. This is often prepared by dissolving paraformaldehyde in alkaline water before each use (Robards and Wilson 1993), and therefore the solution was prepared fresh before use in this study as well. It was observed with jejunal and tongue fixation in this study that fixation with 4% formaldehyde solution was necessary for a good morphology of the tissue sections after cryostat sectioning. The problem of tissue damage or lack of preservation of intact villous structure was mitigated with 4% formaldehyde fixation. In addition, fixation duration played an important role, as underfixation (<3h) or overfixation (>3h) resulted in tissue damage and poor immunostaining. It is important to note that fixation duration had to be determined empirically as it depends on the tissue type and target protein of interest (Randolph-Habecker 2012). Another important factor for good tissue morphology is fixation temperature. Fixation is most commonly carried out at room temperature, as too hot or cold temperature may result in not only too fast or slow fixation rate and poor tissue morphology, but also tissue autolysis especially if a too slow rate of fixation is used. Room temperature was observed to be the ideal choice for this study as well.

Preservation of tissue in frozen sections, if not fixed, was inconsistent and therefore did not yield good results. It was not possible to determine whether 4% formaldehyde fixation, which was necessary to preserve tissue integrity, was detrimental to the preservation of FcεRI and IgE. However, it was later found with tongue tissue that

4% formaldehyde fixation had no effect on IgE staining (see below), and possibly FcεRI as well.

It was also noted that fixation overnight followed by cryoprotection with 30% sucrose solution in PBS was an indispensable step in preserving tissue morphology and preventing artefacts. This goes in accordance with the study by Palmer et al. (2012), where 30% sucrose solution in PBS was used as a cryoprotection agent to reduce freezing artefacts.

Another critical factor in obtaining good tissue staining is antibody dilution, whose optimal values were obtained empirically after many attempts. Antibody dilution for specific protein targets was suggested by Dr. Elyanne Ratcliffe (McMaster University, Hamilton, ON). In accordance with her suggestion, 1:200 and 1:500 observed in jejunal immunohistochemical staining were found to be ideal for anti-PGP9.5 primary and secondary antibody staining, respectively. It was also observed from tongue immunohistochemical staining that the same dilutions were ideal for anti-IgE primary and secondary antibody staining (**Figure 7**).

In order to determine the presence of FcεRI and IgE bound to FcεRI, immunohistochemistry was carried out with anti-FcεRIα or anti-IgE, and co-localization with PGP9.5 was visualized. Protein gene product 9.5, also known as ubiquitin carboxy-terminal hydrolase L1, is an enzyme whose expression is very highly specific to neurons and is often used as a pan-neuronal marker (Doran et al. 1983). It was anticipated that if FcεRI were present on myenteric plexus neurons, expression of FcεRI staining would be visualized, co-localized with PGP9.5. However, due to the receptor's high affinity for IgE

and the steric hindrance upon IgE binding (Keown et al. 1995), it was predicted that staining directed against FcεRI-bound IgE would have a more likely outcome. Therefore, mice were sensitized with OVA to increase serum anti-OVA IgE level (Mirotti et al. 2010), and hence likelihood of FcεRI-bearing cells with consequent IgE binding.

As a result, no IgE staining and thus no co-localization with PGP9.5 on myenteric plexus was observed. However, faint IgE staining on the surface of villous epithelium of jejunal tissue was seen (**Figure 5**). Also, staining that resembles ring-like structures were also observed in the lamina propria of villi. This was not seen in all tissues and only in occasional villous structures. The size and distribution of mast cells in mouse jejunum reported in a previous study (Yamate et al. 2011) shows similarity to the IgE staining obtained from this study, in contrast to mast cells of rat (Vicario et al. 2010) and human (Guilarte et al. 2012) origins, where the number of mast cells is much more abundant. Another attempt was made with IgE immunohistochemical staining by *in vitro* direct IgE sensitization of non-sensitized mouse jejunal tissue sections with 1μg/mL anti-DNP IgE. However, still no IgE staining with PGP9.5 co-localization was observed.

Lastly, FcεRI on jejunal tissue from non-sensitized mice was directly examined with anti-FcεRI antibody, but no staining was again observed at any condition (**Figure 6**). We also tested the stored fixed tissue from the mice and their controls we had used for the behavioural experiments. We observed no positive neuronal associated staining in these experiments either.

After failing to visualize IgE co-staining with PGP9.5 in the myenteric plexus of jejunal tissues, a control system was necessary to establish the functionality of the

materials and methodology used. Mast cells are especially abundant in the tongue (Scandiuzzi et al. 2010), and its easy access made the tongue an optimal choice for the study. We therefore tested direct staining with only anti-IgE antibody to avoid possible interference of the staining with two other primary antibodies (anti-PGP9.5 and DAPI). The distribution and concentration of positive staining that we observed in this study resembled mast cell Toluidine blue staining described in a previous study (Gersch et al. 2002), of oval-shaped cells, evenly distributed in between skeletal muscle fibres of the tongue. As a result, the observation of IgE staining in tongue from my study is a strong indication that materials and methodology of IgE staining used in both jejunal and tongue tissues were appropriate. However, attempts to show FcεRI in the tongue of even non-sensitized mice were unsuccessful even with indirect staining methods. Presumably this is due to the steric hindrance of IgE already bound to FcεRI in non-sensitized mice.

Since our immunohistochemical experiments were unsuccessful, an attempt was made to replicate previous data (van der Kleij et al. 2010) with primary SCG culture, where calcium mobilization was visualized with the calcium-sensitive dye, Fluo-3, AM upon sensitization with anti-DNP IgE and antigen (DNP-HSA) challenge.

In SCG neuron culture, it was observed that NGF at a concentration of 100ng/mL was optimal for healthy neuronal growth, in accordance with a previous finding (Coughlin et al. 1981). In addition, AraC, which stops glial cell growth in neuron culture (Delivopoulos and Murray 2011, Andoh and Kuraishi 2004b), had no effect on the physical appearance of SCG neurons at any concentration. However, the same concentration (10μM) was used in all SCG cultures to keep the culture environment

constant. Also, in accordance with Coughlin et al. (1981), SCG neurons showed higher

confluence at day 3 and showed apoptosis after day 7. In all experiments with SCG

culture, 2-day old SCG culture was used except when neurons were harvested for flow

cytometry due to the fact that neurons were easily detached from the dish bottom after

day 2 where strong adhesion was needed for intracellular calcium microscopy. As for

microscopy, optical microscopes, both upright and inverted, were shown to give

inconsistent results and were later substituted with confocal microscopy, which showed

much more stable fluorescence with much higher resolution.

The lack of any fluorescence change in IgE-sensitized SCG neurons upon DNP-

HSA stimulation at any concentration was an unanticipated outcome as it was expected

that IgE-sensitized neurons would respond to DNP-HSA stimulation as shown before

(van der Kleij et al. 2010). Much effort was spent to carry out the study in an

experimental setting that resembled the previous study as closely as possible. After failed

attempts to elicit intracellular calcium response in SCG neurons, a control system needed

to be established in order to confirm the materials used in the study were functionally

active.

Calcium ionophore (A23187) was used to elicit a positive intracellular calcium

response. Also known as calcimycin, it is a byproduct of the fermentation of

actinobacteria Streptomyces Chartreusensis (Abbott et al. 1979). A23187 forms an ion

channel in the cell membrane that allows a free passage of divalent cations, with a higher

permissibility for Ca^{2+} than others, and by introducing a direct passageway between intra-

and extracellular environments. Attempts were made to bypass the need for FcεRI activation and induce an increase in intracellular calcium levels in neurons.

Another positive control used in this study was bone marrow-derived mast cells. FcεRI expression and its activation upon IgE sensitization and antigen challenge in mast cells are responsible for degranulation of mast cells and release of pro-inflammatory mediators, such as prostaglandins, histamine, leukotriene and cytokines (IL-4, -5, -13 and TNF-α), which lead to a broad range of clinical allergic symptoms such as bronchoconstriction, oedema, itchiness and anaphylactic shock (Gilfillan and Rivera. 2009, Rivera and Olivera. 2008). Using a well-established model of mast cells in terms of FcεRI expression and its activation, it was expected that IgE/DNP-HSA-induced FcεRI activation would elicit intracellular calcium response on flow cytometry. This method is generally more sensitive than use of optical or confocal microscopy. It also provides additional information to that obtained by microscopy such as cellular size. However, in this case we were using it to show that the reagents were not the cause themselves of the failure to obtain positive results with immunohistochemistry.

Mast cells showed a large fluorescence change upon IgE sensitization and DNP-HSA stimulation. In accordance with a previous study (Karimi et al. 2004), 10ng/mL DNP-HSA concentration elicited a large calcium response. What was interesting to note was that there was approximately 30s delay in calcium response when stimulated with DNP-HSA, whereas only 5s delay was observed with A23187 stimulation. This is probably due to the different signalling pathways: IgE-dependent pathways require

phosphorylation of key proteins (Gilfillan and Rivera 2009) while calcium ionophore-induced calcium level change does not.

Mast cells were also tested for their expression of FcεRI using the same source of conjugated primary antibodies and were shown to stain 98% of BMMC (**Figure 15**). The results indicate that the reagents used for intracellular calcium measurement were functional and suggest that the problem lies within SCG neurons, either neurons themselves or the ways in which the cultures were prepared.

While the experiments discussed above show that the reagents themselves were functionally active, they were not performed with SCG or DRG neurons but BMMC. Therefore, we thought it necessary to use another method to test for the presence or absence of FcεRI on neurons and whether IgE binding to its putative presence would elicit intracellular calcium increase as shown with BMMC.

Flow cytometrical analysis is an often-used methodology for the examination of cellular surfaces and changes such as the presence of intracellular molecules. On the whole, determination of these depends on the quality and specificity of antibodies used. We decided to try and use this methodology to examine SCG neurons for the presence of FcεRIα on neuronal cell surface and also for the functional consequence of IgE binding. However, to do this, we first had to establish that SCG neurons could be successfully detached from the culture plate on which they were grown and that their shape after dissociation would still allow flow cytometry determination. For example, the presence of neurites would have possibly resulted in false readings of cells, which are assumed to have somewhat spherical shape in flowing through narrow capillaries while the analysis

proceeds. To our surprise, the dissociation from culture plates using the methods we established allows very good analysis of membrane and intracellular aspects of neurons. This suggests that axons have retracted causing only neuronal soma for investigation. This methodology has not been previously used for this type of analysis and may be of great value to others in this field.

With flow cytometry, SCG neurons were shown not to possess FcεRI, and were not capable of binding IgE in contrast to similar positive experiments under similar conditions performed with BMMC. They did not increase their intracellular calcium content after passive sensitization with DNP-specific IgE and subsequent challenge with the antigen. However, A23187 as before greatly increased the fluorescence.

We have examined SCG and DRG from BALB/c mice and it is possible that the negative results nevertheless resulted from this choice of mouse strain. However, van der Kleij et al. (2010) also used BALB/c, as did Rijnierse et al. (2009). We also have looked at C57BL/6 mice with similar negative results. Andoh and Kuraishi (2004b) used ICR mice and we are left without explanations based on mouse strains used. Similarly I have tested both females as in the behavioural test and the animals used for it, and males in all other experiments. In the positive experiments, for example in the tongue, gender differences were not encountered.

The conditions under which an animal is housed exert great influence on their immune responses. An extreme example of this is mice in a germ-free (GF) environment where their immune responses in general are at an extremely low level (Hooper et al. 2012) and it is established that the bacterial environment in their intestines is a major

factor in determining the capacity of the immune system to respond to new antigens

(Round and Mazmanian 2009). Talham et al. (1999) showed that the presence of common

commensal bacteria, the segmented filamentous bacteria (SFB), were sufficient to fully

expand the immunological repertoire of a GF mouse. Recent observations suggest that the

presence or absence of specific component of the intestinal microbiota can account for the

completely different immune responses of a mouse strain obtained from two different

commercial sources (Ivanov et al. 2008). Our lab (Bravo et al. 2011) and others (Diaz

Heijtz et al. 2011) have recently shown that bacterial differences may account for

behavioural effects in normal mice and gut-neuronal effects (McVey Neufeld 2012) in GF

environment. Therefore, it is possible that the mice we are using today in our conditions

may have different microbiomes from those present when van der Kleij et al. (2010)

carried out their experiments. We are aware that the Central Animal Facility, like others

around the world, has suffered from outbreaks of viral infection, as occurred at the start of

my project with behavioural experiments. Some viral infections are known to promote

IgE responses (Grayson et al. 2007, Cheung et al. 2010), but others seem to suppress

them (Morimoto et al. 1999). Whether an unnoticed or undetectable viral infection has

changed the ecology in our animal facility and caused the failure to find IgE or its high-

affinity receptor on neurons is not known, but might be worth investigating in the future.

In summary, it is currently unknown as to why the experiments of others showing

IgE sensitization of neurons could not be repeated. It would be valuable to repeat these

studies using mice housed at the Central Animal Facility in case that environment was

different from that at St. Joseph's Healthcare Hamilton. In this respect, it was shown that

attempts to induce a chemical colitis with dextran sulphate, a classic model, has failed on a number of occasions, even when using 6% in the drinking water. Normally 3-4% is very effective. This suggests that animals housed at St. Joseph's under similar circumstances differ in reactions to those housed at the Central Animal Facility, possibly accounting for the discrepancies in results in the time intervening between my experiments and those of Dr. van der Kleij.

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