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Neuropeptide-Induced Mast Cell Degranulation and Characterization of Signaling Modulation in Response To IgE Conditioning

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ABSTRACT:

As tissue-resident immune cells, mast cells are frequently found in close proximity to afferent neurons and are subjected to immunoactive mediators secreted by these neurons, including substance P (SP) and calcitonin gene-related peptide (CGRP). Neurogenic inflammation is thought to play an important role in the pathophysiology of many diseases. Unraveling the cellular mechanisms at the interface between the immune response and the peripheral nervous system is important for understanding how these diseases arise and progress. In this work, mast cell degranulation following direct exposure to CGRP and SP was studied both at the bulk and single-cell levels to characterize the mouse peritoneal mast cell response to neuropeptides and compare this response to well-studied mast cell activation pathways. Results show that mast cells secrete fewer chemical messenger-filled granules with increased IgE pre-incubation concentrations. The biophysical characteristics of mast cell degranulation in response to SP and CGRP is in many ways similar to calcium ionophore-induced mast cell degranulation; however, neuropeptide-stimulated mast cells secrete reduced chemical messenger content per secretion event, resulting in an overall relative decrease in secreted chemical messengers.

INTRODUCTION

Mast cells, often implicated for their role in allergy and inflammation, contain a large number of cytoplasmic granules with preformed mediators including, but not limited to, histamine, serotonin, tumor necrosis factor α (TNF- α), and beta hexosaminidase, which are secreted in response to a diverse set of environmental signals. Additionally, chemokines, growth factors, prostaglandins, leukotrienes and TNF- α can be synthesized *de novo* and secreted into the extracellular environment upon mast cell activation. These secreted mediators enhance inflammation, pain, and vasodilatation at the site of activation.^{1, 2} This process has been extensively described in the context of allergy wherein mast cells are activated via multivalent antigen-mediated crosslinking of immunoglobulin E (IgE) bound to the high affinity IgE receptor Fc ϵ RI or the low affinity IgE receptor, Fc ϵ RII (also known as CD23).^{3, 4}

Although the central role of mast cells in allergic (IgE-mediated) processes of the immune system is well established, their role in many other immune functions, including neurogenic inflammation, is not as

clearly defined. The localization of mast cells in connective tissue and mucosal sites throughout the body facilitates contact with a wide range of complex and diverse microenvironments. This diversity is magnified by an equally broad set of functions that mast cells perform within their environment, including activation in response to immune signals such as chemoattractants and neuropeptides.^{1, 5-8} Activation by neuropeptides can initiate and sustain feedback signaling between mast cells and the surrounding tissue microenvironment.

Of particular interest to this work is the secretion of substance P (SP) and calcitonin gene-related peptide (CGRP) from peripheral neurons. SP is a member of the tachykinin class of peptides which act through the neurokinin family of receptors (NKRs).^{9, 10} CGRP, as its name suggests, is an alternative splicing product of the calcitonin gene and acts through the calcitonin receptor-like receptor (CRL).^{10, 11} These neuropeptides are widely recognized for their role in many inflammatory diseases including atopic dermatitis, asthma, and arthritis.¹²⁻¹⁷ Both substance P and CGRP are secreted from the same subset of capsaicin-sensitive primary afferent neurons and are co-localized in, and co-secreted from, dense-core secretory granules and a variety of other cells including glia cells.^{9, 10, 18} Similarly, both substance P and CGRP have been shown to induce mast cell degranulation as part of the pro-inflammatory functionality of these neuropeptides, propagating the inflammatory response through the secretion of histamine and TNF- α , which act on the afferent peripheral neurons.^{9, 14, 19-21} Although substance P-induced mast cell degranulation has been established in certain mast cell subtypes, the data exploring the effects of CGRP on mast cell degranulation are conflicting.^{8, 14, 22-24} Furthermore, despite mast cell expression of both CRL and NKRs, it also has been suggested that substance P- and CGRP-induced mast cell degranulation may result from direct activation of GTP-binding (G) proteins rather than receptor-mediated signaling.^{2, 8, 9}

This intricate system is critical to neurogenic inflammation, and it is important to clearly understand the individual roles of each cell type and molecular signaling species at the single cell level. However, beyond the limited use of whole-cell patch clamp techniques and fluorescence spectroscopy,²⁵ neuropeptide-induced mast cell degranulation has not been extensively studied at the single cell level. This work demonstrates the use of carbon-fiber microelectrode amperometry (CFMA) to characterize CGRP- and SP-induced mouse peritoneal mast cell degranulation from single cells. CFMA provides sub-millisecond resolution and sufficient sensitivity to detect individual cytoplasmic granule secretion events. Here, CFMA was used to explore the biophysical and kinetic properties of mast cell degranulation in response to neurogenic peptide stimulation.^{6, 7, 26}

Several studies by other groups have described IgE-induced upregulation of neuropeptide receptors including the NK 1, 2, and 3 receptors for SP.^{8, 24} This work describes IgE concentration effects on neuropeptide-induced degranulation of mouse peritoneal mast cells in both bulk assays, utilizing high performance liquid chromatography (HPLC) with electrochemical detection, and single cell CFMA. To

the best of our knowledge, IgE-dependent modulation of neuropeptide-induced mast cell degranulation has not been reported and may have significant implications for neurogenic inflammatory diseases.

Our results indicate that exposure to IgE decreases mast cell sensitivity to neuropeptide-induced degranulation. Additionally, CFMA data reveal individual mast cells stimulated by either SP or CGRP demonstrate remarkably similar biophysical degranulation kinetics. At the single-cell level, both neuropeptides induced the secretion of similar levels of serotonin from mast cells relative to the ionophore-stimulated control. The similar kinetics and number of granules secretion events during mast cell degranulation detected by CFMA suggests both neuropeptides act through a similar mechanism.

RESULTS AND DISCUSSION:

The complexity underlying cellular signaling during neurogenic inflammation makes it difficult to decipher the individual contributions of separate cell types. Using bulk and single cell approaches in combination can aid in differentiating the specific role mast cells play within the inflammatory environment. CFMA is used to detect and characterize the real-time secretion of serotonin molecules from individual granule secretion events by single cells, providing detailed information pertaining to the biophysical mechanisms of SP- or CGRP-induced mast cell degranulation.^{16,17,26} HPLC with electrochemical detection, on the other hand, allows us to understand how cell response varies in more complex environments taking into account cell-cell interactions. While serotonin itself is not directly implicated in the inflammatory response, it is packaged with other small molecule mediators (in dense granules) that are secreted simultaneously, and thus can be measured as a proxy for small molecule inflammatory mediators. Mouse peritoneal mast cells were used in this work as a source of primary culture cells that provided accessibility, abundance, and compatibility with the measurement techniques employed herein.

Bulk Concentration and Initial IgE Incubation Studies

Studies using bulk assays of mast cell degranulation were conducted to optimize conditions for characterization of neuropeptide-induced mast cell degranulation and to assess the effect of IgE pre-incubation concentration on neuropeptide-induced mast cell secretion. Mast cells were stimulated with both CGRP and SP, at two concentrations each, selected based on reported values in the literature^{8, 24, 25, 27} and bulk HPLC electrochemical methods in preliminary studies (Supplementary Figure 1). Concentrations for stimulation were selected to be 1 and 10 μM for CGRP and 10 and 100 μM for SP. The 10 and 100 μM concentrations are near the high end of the concentrations used in the previous papers. However, the amount of serotonin that a mast cell may experience, can be higher due to its' close proximity to the neurons. Mast cells were stimulated for 30 minutes with or without 0.5 $\mu\text{g mL}^{-1}$ IgE pre-

incubation overnight (Fig. 1). Both positive and negative controls were included (TNP-Ova with and without anti-TNP-Ova IgE pre-incubation, respectively). A significant, but small, increase in secreted serotonin was detected from CGRP-stimulated mast cells at only the higher concentration (10 μ M CGRP) in the absence of IgE pre-incubation ($p < 0.05$) (Fig. 1A). Despite this minimal response, the data suggest MCs are indeed activated directly by CGRP and more sensitive single-cell experiments were pursued to explore this response further. For SP stimulation (Fig. 1B), enhanced degranulation was observed in mast cells at concentrations of 10 μ M SP without IgE and at 100 μ M SP both with and without IgE. Again, these bulk data suggest direct mast cell degranulation in response to both SP and CGRP. A trend between the exposed concentration and degranulation was also noted, however, more concentration points will be required to confirm this concentration dependence. Furthermore, the observed neuropeptide-induced mast cell degranulation was IgE-dependent. Only the 10 μ M CGRP stimulation without IgE preincubation induced significantly increased secretion relative to the negative control (Fig. 1A). However, due to minimal secretion and lack of significance between IgE and non IgE conditions at 10 μ M CGRP, to examine the IgE effect of CGRP, higher concentrations of CGRP are needed. The effects of IgE were more pronounced for SP-stimulated MCs. SP-induced mast cell degranulation diminished in the presence of IgE at both 10 and 100 μ M concentrations (Fig. 1B). In the absence of IgE pre-incubation, the total amount of serotonin secretion from mast cells increased by 153% and 96% following stimulation with 10 μ M and 100 μ M SP, respectively. One possible explanation for this response could be the modulation of SP receptors with the addition of IgE as previously reported.^{8, 24} Conversely, downregulation of Fc ϵ RI proteins and mRNA has also been demonstrated in mast cells following stimulation with SP.²⁷ Our data further reinforces the relationship between IgE signaling and the mast cell response to neuropeptides.

IgE Concentration Variation

To further explore the effect of IgE conditioning on neuropeptide-induced mast cell degranulation, mouse peritoneal mast cells were stimulated with either 100 μ M SP or CGRP with and without IgE pre-incubation. This high concentration was used in order to distinguish differences seen between the IgE concentration which may be obscured in our bulk cell measurements at lower concentrations. Stimulation conditions were also extended to 2 hours in order to ensure that IgE was actually inhibiting release rather than slowing the degranulation process down. Pre-incubation concentrations of IgE were selected using the reported dissociation constants of the IgE receptors Fc ϵ RI ($k_d = 10^{-9}$ - 10^{-10}) and Fc ϵ RII ($k_d = 10^{-7}$).^{3, 4} Three IgE concentrations were selected - a high concentration to ensure both Fc ϵ RI and Fc ϵ RII (8×10^{-8} M) have IgE bound to them, a moderate IgE concentration between the respective K_d values of the two receptors (3×10^{-9} M), and a low concentration just below the Fc ϵ RI K_d (3×10^{-11} M). Mast cells without

IgE pre-incubation were also prepared. Mast cells were incubated overnight with their corresponding IgE concentration, and washed before exposure to neuropeptide stimulation. TNP-Ova stimulated mast cells were incubated with 8×10^{-8} M IgE overnight to act as the positive control. Mast cells incubated in tris buffer was used as a negative control. In all conditions, mast cell supernatants were collected, and total serotonin content was analyzed using HPLC with electrochemical detection (Fig. 2).

At high incubation concentrations of IgE, neither CGRP nor SP induced significant mast cell degranulation relative to the negative control (Fig. 2). With decreasing pre-incubation concentrations of IgE, both CGRP (Fig. 2A) and SP (Fig. 2B) induced an increased amounts of mast cell serotonin secretion. SP stimulated mast cells at moderate IgE pre-incubation levels, whereas CGRP only induced mast cell degranulation at the lowest IgE concentration. In addition, SP-stimulated serotonin secretion did not continue to increase with decreasing concentrations of IgE beyond the 3×10^{-9} M IgE concentration, unlike the CGRP stimulation. This suggests that mast cells are more responsive to SP at normal IgE levels, while CGRP stimulation has a greater dependence on the IgE concentration.

These variations in response to the concentration of IgE may explain conflicting findings in the literature. Previous research has extensively reported mast cell stimulation in response to SP, while contradictory results have been reported for CGRP.^{8, 14, 22-24} Most often, mast cells are primed with IgE at concentrations of $0.5 \mu\text{g mL}^{-1}$ or $\sim 3 \times 10^{-9}$ M (assuming a molecular weight of ~ 180 KDa overnight.^{5, 6, 8, 27} This concentration is above the K_d of Fc ϵ RI but below the K_d of Fc ϵ R2. Our findings suggest that this concentration of IgE incubation would significantly limit the degree of CGRP-induced mast cell degranulation, while permitting SP-induced serotonin secretion as seen in Fig. 1B.

Carbon-Fiber Microelectrode Amperometry

At the low, more biologically relevant concentrations for SP and CGRP, the bulk mast cell secretion increase was minimal and not consistent at $0.1 \mu\text{M}$ and $1 \mu\text{M}$ CGRP or SP (Data not shown). Therefore, we believe at these relevant concentrations, it is important to do single cell analysis, to gain a more detailed and mechanistic understanding of how mast cell degranulation is impacted by SP and CGRP.

Mast cell CFMA is a technique where a sub-10- μm -diameter carbon-fiber microelectrode is set to a fixed, oxidizing potential and placed in direct contact with a single mast cell (Fig. 3A).²⁶ The selected mast cell is then stimulated using a micropipette to deliver a local dose of a stimulating substance. The subsequent secretion of granular contents is monitored as discrete spikes in current, each corresponding to the exocytosis of a single cytoplasmic granule. Recently, it has been discovered that granular release events are often partial release events.²⁷ Using CFMA alone, we are not able to detect whether the release

event is full or partial release, and therefore, will not be distinguishing between the two types in this work.

For CFMA experiments in this work, a 3-second delivery of 1 μM substance P, 1 μM CGRP, or 10 μM A23187 (a calcium ionophore) were used to stimulate mouse peritoneal mast cell degranulation. The secreted serotonin from each granule is oxidized and monitored as current over time as the selected cell undergoes exocytosis (Fig. 3B). The sub-millisecond time resolution of these measurements facilitates detailed insight into the granule/membrane behavior. Within the collected current trace, individual granule secretion events (individual spikes) are analyzed for several kinetic parameters including spike rise-time (T_{rise}), full-width at half maximum ($T_{1/2}$), and total charge underneath the spike (Q) which is used to quantify the amount of serotonin secreted from each granule using Faraday's law (Fig. 3C). T_{rise} is a measure of the time between 10 to 90% of the full spike height during the initial rise in current and reflects the fast secretion of serotonin unassociated with the intragranular matrix immediately following fusion pore formation between the cytoplasmic and cell membranes. $T_{1/2}$ measures the length of time the granule fusion pore stays open for chemical messenger secretion. This is also associated with the rate at which the biopolymer matrix expands and unfolds to secrete a partial amount of its serotonin content. In addition to these kinetic parameters, the number of spikes (N) can be counted to determine the number of exocytotic events the individual mast cell experiences upon activation, taking into account that the microelectrode contacts $\sim 10\%$ of the total cell surface area.²⁶ Together, these parameters provide a detailed picture of the biophysical mechanics that underlie specific changes in mast cell secretory function. In particular, observed changes in the pattern of these parameters are often very useful when comparing different modes of mast cell stimulation that result in modulation of exocytosis.

To understand if the IgE concentration affects the secretion from isolated mast cells on a single cell level, the mast cells were incubated overnight with or without 0.5 $\mu\text{g mL}^{-1}$ IgE. After washing, individual cells were stimulated with SP or CGRP. Granule secretion events were not detected with CFMA for mast cells in either condition incubated with IgE. Therefore, data for IgE incubated platelets cannot be analyzed. Mast cells not incubated overnight with IgE showed degranulation when stimulated with SP, CGRP or the calcium ionophore positive control (10 μM A23187). The positive control was changed to A23187 from TNP-Ova so that all mast cells will undergo the same conditions until the point of stimulation. This insures that the granules, lipids and proteins are similar and the changes in degranulation are due to the stimulant. Representative amperometric traces can be seen in Fig. 3B. In addition to demonstrating the effects of IgE on mast cells, the biophysical characteristics following stimulation were quantified using the parameters discussed above (Fig. 4 and 5).

CFMA Analysis

Analysis of the CFMA results revealed that individual mast cells stimulated with either SP or CGRP each yielded in less total serotonin than A23187-stimulated controls (SP- and CGRP-stimulated mast cells both secreted 39% less serotonin per cell than the A23187-stimulated cells, Fig. 4A). Further analysis of the CFMA data reveals this marked difference in the degree of mast cell degranulation is largely accounted for by a lower amount of serotonin secretion per granule, with no measureable difference in the number of secretion events per cell (Fig. 4B and 4C). SP- and CGRP-stimulated mast cells secreted 29% and 34% less serotonin per granule, respectively, relative to A23187-stimulated controls, with negligible changes in the number of granule fusion events. The stimulation-dependent alterations in spike area observed in the absence of different cell culture conditions suggests regulated control of the amount of chemical messenger secreted per granule rather than altered granule loading effects. The absence of spike number effects also suggests that the granule trafficking and/or docking machinery can be regulated independently of the processes that regulate the percentage of granular contents secreted during degranulation.

Decreased secretion of serotonin per granule could be a result of shorter granule fusion times if diffusion played the primary role in releasing serotonin upon granule fusion with the cell membrane. However, analysis of the kinetic parameters of mast cell degranulation following CGRP- and SP-stimulation reveal that the substantially smaller spike area values observed in the substance P- and CGRP-stimulated mast cells, compared to A23187 stimulation, were not associated with measureable differences in either spike half-width or spike rise-time (Fig. 5). Together, these findings indicate a remarkably simple mechanism of modulated mast cell degranulation that is largely characterized by a decreased fraction of the granule cargo secretion during exocytosis in the setting of neuropeptide stimulation compared to IgE-mediated mast cell degranulation.

Bulk and Single Cell Comparison

Previous findings have indicated decreased degranulation for TNP-Ova compared to ionophore-based stimulation, with anti-TNP-Ova inducing secretion of 79% of the serotonin secreted during A23187-induced degranulation on a single cell level.⁶ For the current study, CGRP and SP each induced mast cell secretion equal to 61% of A23187 degranulation, therefore showing similar trends to the bulk cell level with CGRP/SP releasing less total serotonin than TNP-Ova stimulation. However, with similar stimulation concentrations (1 μ M SP or 1 μ M CGRP), the bulk mast cells did not secrete measurable serotonin, whereas TNP-Ova stimulation induced significant chemical messenger secretion. The unexpected lack of degranulation is most likely due to the decreased amount of serotonin secretion per granule and that only a fraction of total mast cells degranulate following bulk exposure to CGRP and SP,

1 which is not shown in the single cell data analysis. The fraction of mast cells able to degranulate in
2 response to CGRP and SP may also be different, which may be why SP secretes more serotonin than
3 CGRP. A final hypothesis for the differences can be due to the mast cells sensing their environment and
4 trying to maintain a certain concentration of granule releasate for each type of stimulation. Further studies
5 are needed to confirm these hypotheses.
6

7 **CONCLUSIONS:**

8 The complex relationship between mast cells and peripheral nerve fibers is fundamental to the
9 pathophysiology of many neurogenic inflammatory processes.¹²⁻¹⁵ This work characterizes the direct
10 stimulation of mast cell degranulation in response to both SP and CGRP, critical neurogenic
11 inflammatory mediators, using both bulk assays and single-cell electrochemical approaches. Our findings
12 indicate that SP and CGRP induce mast cell degranulation. The degranulation is also influenced by the
13 amount of IgE present. The observed IgE effects suggest that mast cells may respond to neuropeptides
14 more effectively in the absence of high levels of circulating IgE which correlates with the number of
15 FCεRI on the mast cell surface, and thus the amount of attached IgE.²⁸ Further work will be necessary to
16 understand the signaling mechanisms that drive the decreased sensitivity to neuropeptide stimulation.
17 Single cell analysis of SP- and CGRP-induced mast cell degranulation show that both neuropeptides
18 induce a similar pattern of mast cell degranulation. Mast cell degranulation in response to SP or CGRP
19 stimulation resulted in a similar granule secretion times and number of secretion events relative to
20 A23187-stimulated control cells. This lack of variation in granule kinetics is consistent with the
21 hypothesis that SP and CGRP activate mast cells through a similar mechanism.

22 Finally, when compared to previously reported findings,⁶ the amount of serotonin secretion following
23 both TNP-Ova and A23187 stimulation is greater than the amount secreted due to either SP or CGRP
24 stimulation for both bulk and single cell degranulation. The percentage of TNP-Ova serotonin secretion
25 caused by stimulation from SP and CGRP on a bulk cell level is significantly less than expected when
26 compared to the releasate on a single cell level, likely due to the fact that single cell data only takes into
27 account cells that have granule secretion, whereas bulk cell studies include the averaged behavior of all
28 cells. Bulk assays may also be influenced by additional mechanisms such as serotonin re-uptake, giving
29 the cells more control over their surroundings.⁵ In conclusion, neurogenic inflammation is a highly
30 complex process involving activation of mast cells by CGRP and SP, which can in return stimulate
31 additional neurons. However, secretion in the bulk cell environment is decreased compared to single cell
32 results and is dependent upon the IgE concentration the mast cells are exposed to.
33

METHODS:

Reagents

Cell culture media materials were obtained from HyClone. Calcitonin gene related peptide (CGRP) (Rat, Mouse) was purchased from Phoenix Pharmaceuticals, Inc. [Tyr8]-Substance P and A23187 were purchased from Sigma Aldrich. Anti-trinitrophenol (TNP) IgE and TNP-ovalbumin (TNP-Ova) were obtained from BD Biosciences and Fisher Scientific, respectively. TNP-Ova, A23187, SP and CGRP were diluted in Tris buffer (12.5 mM Trizma-HCl, 150 mM NaCl, 4.2 mM KCl, 5.6 mM glucose, 1.5 mM CaCl₂, 1.4 mM MgCl₂) on the day of the experiment.

Mast Cell Isolation and Exposure to Chemokines

Peritoneal mast cells were isolated as previously described in the following references.^{5, 7} Briefly, male C57BL/6J mice from The Jackson Laboratories were euthanized via CO₂ asphyxiation according to IACUC approved protocol # 1403-31383A. 8 mL of high glucose DMEM supplemented with 10% bovine calf serum and 1% penicillin streptomycin (media) was injected into the peritoneal cavity followed by a 30 second abdominal massage. Approximately 6-7 mL of the peritoneal lavage media was collected from each mouse. The collected mast cells were pelleted at 450 xg for 10 minutes and resuspended in fresh media before being plated onto a confluent layer of 3T3-Swiss albino mouse embryo fibroblasts (purchased from ATCC). Mast cells were primed with IgE at the specified concentration from 0 to 1 x 10⁻⁷ M, as indicated in the experimental procedure, and incubated overnight at 37 °C in 5% CO₂. For single cell experiments, the mast cells were plated in 35 x 10 mm² Petri dishes while 24 well plates were used for bulk experiments. After overnight incubation, the cells were washed three times with tris buffer and prepared for bulk or single cell experiments.

Carbon-Fiber Microelectrode and Micropipette Fabrication

Microelectrodes were fabricated as previously described.^{6, 7} Briefly, individual carbon fibers were aspirated into glass capillaries which were then halved using a pipette puller. The excess fiber was cut using a surgical blade underneath a microscope. The electrodes were epoxied (Epoxy Technology) and cured at 100°C for 24 hours. On the day of the experiment, the electrodes were beveled at 45 degrees with a diamond-coated polishing wheel and placed into isopropyl alcohol until needed. For the stimulating micropipette, a glass capillary was halved using the pipette puller and trimmed to leave a 10-50 μm-diameter opening for the stimulant release driven by a Parker Picospritzer III.

Carbon-Fiber Microelectrode Experiments

The washed mast cells were placed into a 37°C heated petri dish holder on an inverted microscope. The carbon-fiber microelectrode and micropipette were placed on top and near the mast cell, respectively (Fig. 3A). A potential of 700 mV vs. Ag/AgCl reference electrode (BASi) was set using an Axopatch 200B potentiostat, and a 3 second bolus of stimulant was puffed onto the cell. The stimulants used were 10 μ M A23187, 1 μ M SP, or 1 μ M CGRP. The change in current over time for 90 seconds was recorded using Tar Heel CV software (National Instruments), written by Michael L.A.V. Heien. The data collected were filtered with a 200 Hz low-pass Bessel filter and converted to an .abf file format in the data analysis software (MiniAnalysis). The individual spikes in each current trace were analyzed for rise time from 10 to 90% of the spike amplitude (T_{rise}), full width at half max time ($T_{1/2}$), and total area; in addition, the total number of current spikes was counted. After the data were analyzed, outliers (any trace with one of more experimental parameters that was greater than 2 log standard deviations from the log mean for that condition) were removed. Data significance was determined using one way ANOVA and error is given in SEM. During the experiment, the secretion of 17, 18, and 14 individual cells was measured for A23187, CGRP, and SP conditions, respectively.

Bulk Mast Cell Experiments

Mast cells were stimulated with 250 μ L of the selected neuropeptide at 1, 10, or 100 μ M based on the experiment, 100 ng mL⁻¹ TNP-Ova, or Tris and incubated at 37°C for 30 minutes for the initial experiments (Fig. 1) and 2 hours for the IgE variation experiments (Fig. 2) before terminating degranulation stimulation by placing cells on ice. The resulting supernatant was placed in a 96 well Millipore Multi Screen 0.45 μ m filter plate and filtered by centrifuge at 3000 xg for 5 min. 180 μ L supernatant and 20 μ L of 5 μ M dopamine internal standard in 0.5 μ M perchloric acid were combined and placed into an HPLC vial and run using an Agilent 1200 HPLC with a Waters 2465 electrochemical detector as previously described.⁵ Briefly, the solution was separated using a 5 μ m, 4.6 mm $\times$ 150 mm Eclipse XDB C18, and the serotonin and dopamine were detected using a Waters glassy carbon electrode set at a potential of 700 mV vs. Ag/AgCl. The mobile phase, consisting of 11.6 mg L⁻¹ of the surfactant sodium octyl sulfate, 170 μ L L⁻¹ dibutylamine, 55.8 mg L⁻¹ Na₂EDTA, 10% methanol, 203 mg L⁻¹ sodium acetate anhydrous, 0.1 M citric acid, and 120 mg L⁻¹ sodium chloride, was run at 2 mL min⁻¹. In addition, a 5 point calibration curve was prepared using a 500 nM dopamine internal standard and serotonin concentrations ranging from 62.25-1000 nM serotonin dissolved in 0.5 M HClO₄. All spikes in the resulting chromatogram were analyzed by dividing the area of the serotonin spike by the dopamine spike area (5-HT/dopamine internal standard). For all experiments, the R² of the calibration curve was over 0.9993 with a minimum of 3 instrumental replicates for each calibration point. All mast cell

conditions had 4 biological replicates and were q tested for outliers with 95% confidence. Significance between conditions was determined using one way ANOVA. Error bars represent standard deviation.

SUPPORTING INFORMATION

Supporting Information Available: This material is available free of charge via the Internet

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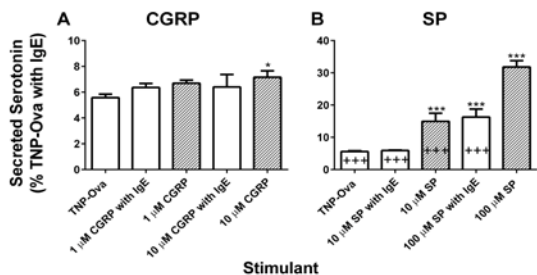


Figure 1 Bulk mast cell secretion in response to a 30 minute calcitonin gene related peptide (CGRP) (A) or substance P (SP) (B) stimulation at various concentrations. Results are shown as percent of the natural allergic (IgE-mediated) reaction pathway secretion. IgE concentrations were at 6.66×10^{-9} M. The negative control (TNP-Ova without IgE) was significantly different than 10 μ M CGRP ($p < 0.05$) and for all SP ($p < 0.001$) in all conditions except 10 μ M SP with IgE. * $P \leq 0.05$ vs. TNP-Ova *** $P \leq 0.001$ vs. 10 μ M SP with IgE and TNP-Ova +++ $P \leq 0.001$ Vs. 100 μ M SP

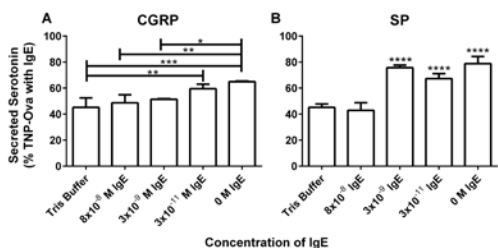


Figure 2 IgE concentration effects after a two hour mast cell stimulation with 100 μ M calcitonin gene related peptide (CGRP) (A) or 100 μ M substance P (SP)(B). Tris buffer was used as a negative control * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ vs indicated positions. **** $P \leq 0.0001$ vs tris and 1×10^{-7} M IgE with SP stimulation

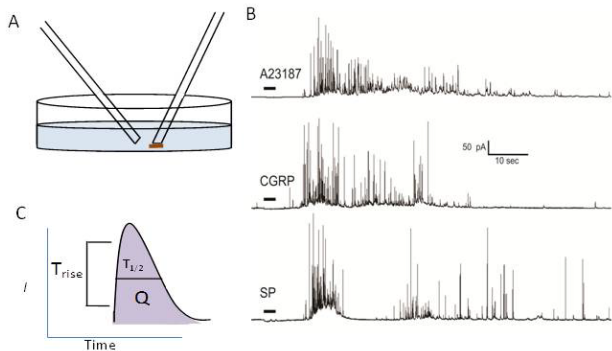


Figure 3 Schematic carbon-fiber microelectrode amperometry (CFMA) setup and example traces. A) CFMA setup (Ag/AgCl reference electrode not shown) B) Representative traces of single cell mast cell secretion C) Schematic current spike resulting from mast cell secretion of a single dense-body granule showing T_{rise} (the time from 10 to 90% of the spike height), $T_{1/2}$ (length of time for full width at half maximum), and Q (amount of serotonin secretion in units of charge).

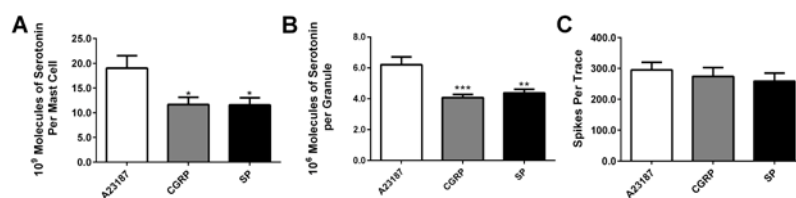


Figure 4 Carbon-fiber microelectrode amperometry (CFMA) serotonin secretion and granule trafficking in response to calcitonin gene related peptide (CGRP), substance P (SP), and A23187. (A) Total amount of serotonin secreted per mast cell, (B) Amount of serotonin secreted per granule, as measured using CFMA, for each stimulation condition (C) Number of granules secreted per stimulation event, as measured using CFMA accounting for 10% coverage of cell by the electrode. * $P \leq 0.05$ *** $P \leq 0.001$ and ** $P \leq 0.01$ vs. A23187

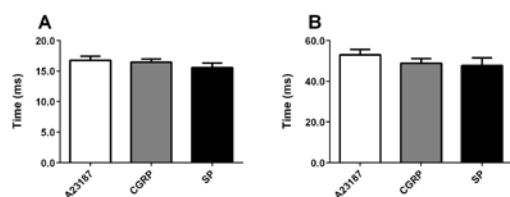
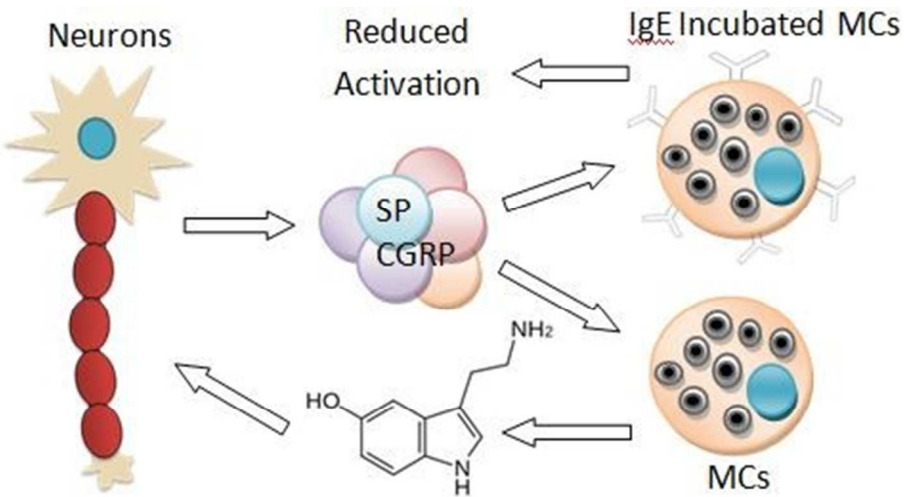


Figure 5 Carbon-fiber microelectrode amperometry granule fusion pore kinetics in response to calcitonin gene related peptide (CGRP), substance P (SP), and A23187. T_{rise} (A) and $T_{1/2}$ (B) of the granule secretion events, as measured using CFMA, for each stimulation type. There is no statistical difference among the presented conditions.



TOC figure

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