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Predicted functionally relevant residue C79 in linker region of Q-SNARE SNAP-23 and it's role in regulating the Kinetics of exocytosis in Mast cells

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SNARE proteins play crucial roles in cellular processes like membrane fusion, protein trafficking, and organelle formation. While the basic mechanism of SNARE-mediated fusion is consistent across organisms, the speed of fusion events varies between different cell types. For example, mast cells release inflammatory substances more slowly than neurons' release of neurotransmitters. This diversity in exocytosis kinetics may arise from variances in motifs or residues within SNARE proteins, or the presence of different *t*-SNARE proteins like SNAP-23 in mast cells and SNAP-25 in neurons. A bioinformatics approach was used to investigate differences in exocytosis kinetics by analyzing motifs and residues within Q_{bc} family of SNARE proteins. Unique functional motifs specific to SNAP-23/25 and conserved motifs within the linker region of Q_{bc} SNAREs were identified. These signatures were then utilized to engineer native SNARE proteins, allowing manipulation of exocytosis speed. Targeting a specific conserved Cys residue in SNAP-23 linker region and introducing analogous mutations found in SNAP-25 helped assess the impact of these alterations on exocytosis kinetics.Transfecting engineered SNAP-23 mutants into mast cells confirmed alterations in exocytosis rate, underscoring the importance of this residue in regulating secretion kinetics. These findings suggest the potential for manipulating mast cell exocytosis and identifying therapeutic targets for immunological disorders linked to abnormal release kinetics of mediators.

Key words: soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE); exocytosis; mast cells; SNAP-23; RE-CRE; kinetics

Abbreviations: SNAP-23/25, synaptosomal-associated protein of 23/25 kDa; RBL-2H3, rat basophilic leukemia; hGH, human growth hormone; SNAREs, soluble N-ethylmaleimide-sensitive-factor attachment protein receptors; VAMP, vesicle-associated membrane protein; UniprotKB, Universal protein resource Knowledgebase; EGFP, enhanced green fluorescent protein.

1. Introduction

Specialized secretory cells, such as neurons, cells of the endocrine system, exocrine system and immune systems, such as mast cells, undergo regulated exocytosis, where secretory granules and vesicles are prevented from fusing with the plasma membrane unless a signal is provided by an external ligand (Sagi-Eisenberg, 2007). For a while, there was a belief that the mechanisms driving regulated exocytosis varied significantly across eukaryotic organisms and even within different cell types, particularly in synapses, where exocytosis occurs rapidly ("Secretory Granule Exocytosis | Physiological Reviews," 2003). However, at the molecular level, exocytosis is regulated by specialized protein machinery that is conserved in all eukaryotes, ranging from yeast to humans (Li and Chin, 2003). This conserved protein machinery includes the *v*-SNAREs and *t*-SNAREs, membrane-associated proteins that play central roles in eukaryotic membrane fusion (Jahn and Südhof, 1999). Even some SNARE-like proteins are present on the membranes of microorganisms and viruses and promote their fusion with the host cell membrane (Barocchi et al., 2005). SNAREs can be categorized functionally as vesicle-associated (synaptobrevin/VAMP family as *v*-SNARE consisting of an arginine-containing motif), and "target" membrane-associated *t*-SNAREs belonging to the SNAP-25/23 and syntaxin families (Rothman, 1994). A *v*-SNARE binds with its matching *t*-SNAREs to create a trimolecular trans-complex, with one unit of syntaxin and either SNAP-23 or SNAP-25 on the opposite membrane (Stow et al., 2006).

Similar protein machinery governs exocytosis from yeast to humans, as we know the kinetics of exocytosis is fastest in neurons (time constant in milliseconds) (Südhof, 2013), whereas slower release occurs in mast cells, which are involved in the release of various proinflammatory mediators during an allergic response. In 2008, Keating *et al.* reported, that changes in vesicle fusion kinetics have direct implications in diseases, such as Alzheimer's and Down syndrome (Keating et al., 2008). Additionally, faster mast cell exocytosis due to advanced glycation end products and aging may cause the generation of Reactive oxygen species (ROS) and lead to inflammation that is typical of chronic diseases such as diabetes, cancer, cardiovascular and neurodegeneration diseases (Sick et al., 2010). However, the evolutionary significance and molecular basis (the major factors, signaling proteins and exact molecular mechanisms responsible) for rapid *v/s* slow kinetics in lower to higher organisms have not been determined.

The variation in exocytosis rate observed between mast cells and neurons could stem from qualitative disparities in the primary *t*-SNAREs SNAP-23 (ubiquitously present in non-neuronal cells, including mast cells) and SNAP-25 (specifically expressed in neurons) like amphiphilic nature of the amino acids located within the linker area, the number of cysteine residues and the degree of palmitoylation in the this regions

(Nagy et al., 2008).Studies carried out in our laboratory have underscored the importance of residues in between N- and C- terminal domain of SNAP-23 which gets phosphorylated on two conserved Ser at positions 95 and 120 upon stimulation of mast cells facilitating exocytosis(Hepp et al., 2005; Naskar and Puri, 2017); however, the specific molecular mechanisms involved are still being investigated. Furthermore, our recent work provided evidence that the highly conserved cysteines in SNAP-23 linker facilitate it's association with membranes (Agarwal et al., 2019). However, the specific motifs and regulatory mechanisms of SNAREs that may play a role in governing the kinetics of exocytosis are not well determined. The objectives of this study were to compare the kinetics of exocytosis in neurons and mast cells and to explore the molecular basis of rapid and slow exocytosis by studying the importance of role of predicted residues of SNAP-23 and SNAP-25. Comparing and modifying the kinetics of exocytosis in mast cells and neurons will reveal how SNARE proteins regulate differential exocytosis in these cells and has various implications in neurodegenerative and immunological diseases.

2. Methods

2.1 Multiple Sequence Alignment (MSA)

Protein sequences of SNAP-23 and SNAP-25b subfamilies were retreived from the UniProt database (http://www.uniprot.org). Upon removing duplicate entries, the dataset comprised 90 SNAP-23 and 122 SNAP-25b members. The sequences were aligned using the MAFFT software (Katoh and Standley, 2013) with default settings.

2.2 Relative Entropy (RE) and Cumulative Relative Entropy (CRE)

To investigate the functional specificity of the residues in SNAP-23 and SNAP-25, each site's entropies and conservation score in the sequence of linker region present amid SNARE domains were studied. The RE and CRE for the evolutionary conserved amino acid position in SNAP-23/SNAP-25b protein sequences linker region was calculated and verified using in-house information theory (Livingstone and Barton, 1993; Valdar, 2002):

$$H_i = -\sum_{for\ all\ x} p_i(x) \log p_i(x)$$

In the equation $p_i(x)$ is the likelihood of the presence of amino acid residue at position 'x' in column 'i'. The entropy (H_i) of amino acids reaches crest when randomly distributed and trough when fully conserved. Relative Entropy (RE) measures the difference between two residue probability distributions (Cover, 1999),

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assessing the conservation level of amino acid residues (Wang and Samudrala, 2006) in each aligned column compared to the background distribution.

$$RE_{pHq} = \sum_{for\ all\ x} p_i(x) \log \frac{p_i(x)}{q(x)}$$

Here q(x) is the baseline likelihood of the presence of amino acid residue 'x', which denotes the probability (RE_{pHq}) of amino acid x across various protein sequencesavailable in the Swiss-Prot database (Durbin, 1998).

2.3 Relative entropy (RE) and cumulative relative entropy (CRE) scores

Alignments corresponding to the linker domain for subfamilies of SNAP-23 and SNAP-25 were extracted individually from complete MSA. Furthermore, the HMM build program of HMMER (version 3.0) (http://hmmer.wustl.edu) was employed to construct individual alignments. Approach used for RE and CRE calculations has been previously outlined by (Kapoor et al., 2010). We computed RE as a measure to assess the discrepencies in the amino acid distribution between two subfamily within the complete alignment (Hannenhalli and Russell, 2000). The following equation provides the RE scores for y_1 and y_2 (at specified position in the column located in the subfamilies)

$$RE_i y_1 \| y_2 = \sum_{for\ all\ x} p_i(x, y_1) \log \frac{p_i(x, y_1)}{p_i(x, y_2)}$$

here $p_i(x, y_n)$ is the possibility of finding amino acid 'x' in subfamily y_n in column i of the MSA. To further independently inspect the sequence of the linker region of the SNAP-23 and SNAP25 subfamilies for functionally relevant sites, we next employed the information theory measure of CRE. The CRE in column *i* is determinedby the Kullback–Liebler distance between the respective i^{th} columns:

$$CRE_i = \sum_{for\ all\ x} p_i(x, y_1) \log \frac{p_i(x, y_1)}{p_i(x, y_2)} + \sum_{for\ all\ x} p_i(x, y_2) \log \frac{p_i(x, y_2)}{p_i(x, y_1)}$$

The final CRE outcome was obtained after scaling the CRE_i as described previously (Kapoor et al., 2010). All the prior calculations were performed utilizing proprietary Perl scripts developed in house. The method was able to identify functionally relevant sites in sequence alignments that are

differentially conserved between the SNAP-23 and SNAP-25 subfamilies but differ between these two SNARE proteins.

2.4 Recombinant plasmids and site-directed mutagenesis

Human wild type SNAP-23, it's mutants cloned in EGFP-C2 expressing vector have been described before (Agarwal et al., 2019; Naskar et al., 2018). The hGH cDNA was cloned and integrated into the pcDNA3 mammalian expression vector, as reported previously (Puri et al., 2003).

2.5 RBL-2H3 cell culture, transfections and exocytosis assays

RBL-2H3 mast cells (kind gift from Dr. Paul A Roche, NIH, Bethesda, MD, USA) were cultured in RBL medium (Puri et al., 2003) at 37°C in 5% CO_2 incubator. For experimentation, RBL cells, in log phase growth, were harvested and suspended in RBL medium at a density of $20x10^6$cells/ ml for transfection using 20 µg of desired plasmid DNA, following established protocol (Agarwal et al., 2019). The transfected cells were then cultured in RBL medium. Flow cytometry analysis confirmed the transfection efficiencies, which were consistently approximately 50% across all cases (data not shown). 2 µg of pCMV-hGH as a secretion reporter plasmid (Agarwal et al., 2019) and 18 µg of empty vector/ test plasmids (including the EGFP-C2 or EGFP SNAP-23 mutants) was transfected in RBL cells. Exocytosis was induced in transfected cells through cross-linking of FcεRI receptor, following a previously established protocol (Naskar et al., 2018). The cells were sensitized 16-18 hrs with DNP-specific IgE [TIB 142 (ATCC) supernatant]. Sensitized cells were activated by adding 100 ng/ml DNP-BSA (Invitogen, Eugene, Oregon, USA) for 15-45 minutes and non-activated cells (resting). The quantity of hGH secretedin supernatant and lyzates was quantified using ELISA (Roche Diagnostics).Percentage secreted is expressed relative to total hGH in the cells.To analyze the exocytosis from untransfected RBL cells, the cells were cultured and activated as described above or activated with 10□M Ionomycin (Sigma) for indicated time points and secretion was monitored by assaying the release of β-hexosaminidase activity ensued in both the supernatant and lysate fractions, utilizing p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) as the enzymatic substrate, as described before (Naskar and Puri, 2017). The quantification of β-hexosaminidase activity was expressed as a percentage of the net activity, elucidated as the discrepancy between stimulated and resting states, which was then juxtaposed against the total activity, encompassing both released and cell-associated fractions.

2.6 SDS–PAGE and immunoblotting

SDS–PAGE was performed using the Cell lysates obtained from transfected RBL-2H3 cells and transferred to PVDF membranes (Bio-Rad) for immunoblotting analysis (Puri et al., 2003). Anti-SNAP-23 antibody

(Phillips et al., 2001) was utilized to measure the expression of transfected and endogenous SNAP-23. Bands were detected using Immobilon Chemiluminescent HRP Substrate (Millipore), and band intensities were quantified using AlphaEaseFC 4.0 software. Blots for each specific mutant were derived from three independent experiments, with multiple films developed at various exposure times (ranging from 5 seconds to 1 minute). Presented here are representative blots alongside molecular weight markers.

2.7 Neuro-2A cell culture

Murine neuroblastoma (Neuro-2A, ATCC) cells are neural crest cell line. Neuro-2A cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 units/ml) and streptomycin (100 µg/ml; Gibco). The cells were maintained as sub-confluent density at 37°C supplied with 5% CO_2in humidified chamber.

2.8 Neuro-2A exocytosis assay

Neuro-2A cell exocytosis was monitored by studying ATP release by, a luciferin-luciferase assay (Gutiérrez-Martín et al., 2011). This assay quantified the ATP liberated into the extracellular milieu, amid conditions where the process of Ca^{2+}-dependent exocytosis was either augmented or obstructed. Neuro2A cells were cultured within 24-well plates until achieving the desired confluence.The cells were then subjected to a one-hour incubation in a Mg^{2+}-free Locke's buffer, maintained at a temperature of 3°C. Subsequently, the cells were subjected to dual washes with plain Locke's buffer. Stimulation ensued through the introduction of ionomycin, at a final concentration of 10 µM in Locke's buffer or only Locke's buffer, eliciting evoked luminescence. Alternatively, stimulation was executed with 500 µM NEM or DMSO, enabling the measurement of the basal ATP concentration. Supernatants, collected at distinct intervals, were analyzed to ascertain the released ATP concentration. The quantification of ATP concentration was done by the addition of 100 µl of ENLITEN rLuciferase/Luciferin reagent (Promega, Madison, WI) to the samples, followed by luminescence measurement utilizing a GloMax^R 20/20 Luminometer from Promega (Madison, WI). Subsequent to data acquisition, the rate of ATP release ($K_{release}$ (K_{res})) was calculated utilizing SigmaPlot 15.0.

2.9 Statistical analysis

The results are presented as the mean value accompanied by the standard error of the mean (SEM), which was computed based on data from at least three experiments. Statistical evaluation employed a one-tailed distribution alongside a two-sample equal variance Student's t-test. Results were deemed significant if the p-value was less than 0.05.

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3. Results:

3.1 Comparison of the kinetics of secretion from mast cells and neuronal cells

To contrast the kinetics of exocytosis between neurons and mast cells, we induced activation in vitro on the mouse neuroblastoma cell line Neuro-2A and the RBL-2H3 cells, which serve as a model for mast cells.The crosslinking of receptor-bound IgE by multivalent allergens is the physiological trigger used to activate mast cell degranulation. The RBL-2H3 cells were mock sensitized by incubation with complete medium or sensitized with DNP-specific IgE at 1:1000 dilutions for 16-18 hours. The cells were activated by giving 100 ng/ml DNP-BSA for the indicated durations. The supernatants and lysates were collected at various time points (the cells were lysed by 0.2% Triton X-100). The β-hexosaminidase release was calculated. Mast cells showed a net β-hexosaminidase release of 1.7% after 1 minute, 4.6% after 3 minutes, 9% after 5 minutes, 13% after 10 minutes and to 23% at 45 minutes , after which a plateau was reached until 60 minutes post allergen challenge (Figure 1. A).

Mast cells also respond to ionomycin in an IgE-independent manner (Stump et al., 1988). Treatment of RBL-2H3 with ionomycin increases intracellular Ca^{2+} levels in mast cells and induces degranulation. Cells were induced with 10 μM ionomycin for the indicated durations. The supernatants and lysates were collected, and the release of β-hexosaminidase was assessed. The kinetics of degranulation after induction with ionomycin were studied and found that Ionomycin (10μM) caused exponential mast cell degranulation in a time-dependent manner, i.e., 22% net β-hexosaminidase release in 5 minutes and 44% release in 10 minutes, with a maximum release of 80% (Figure 1. B).

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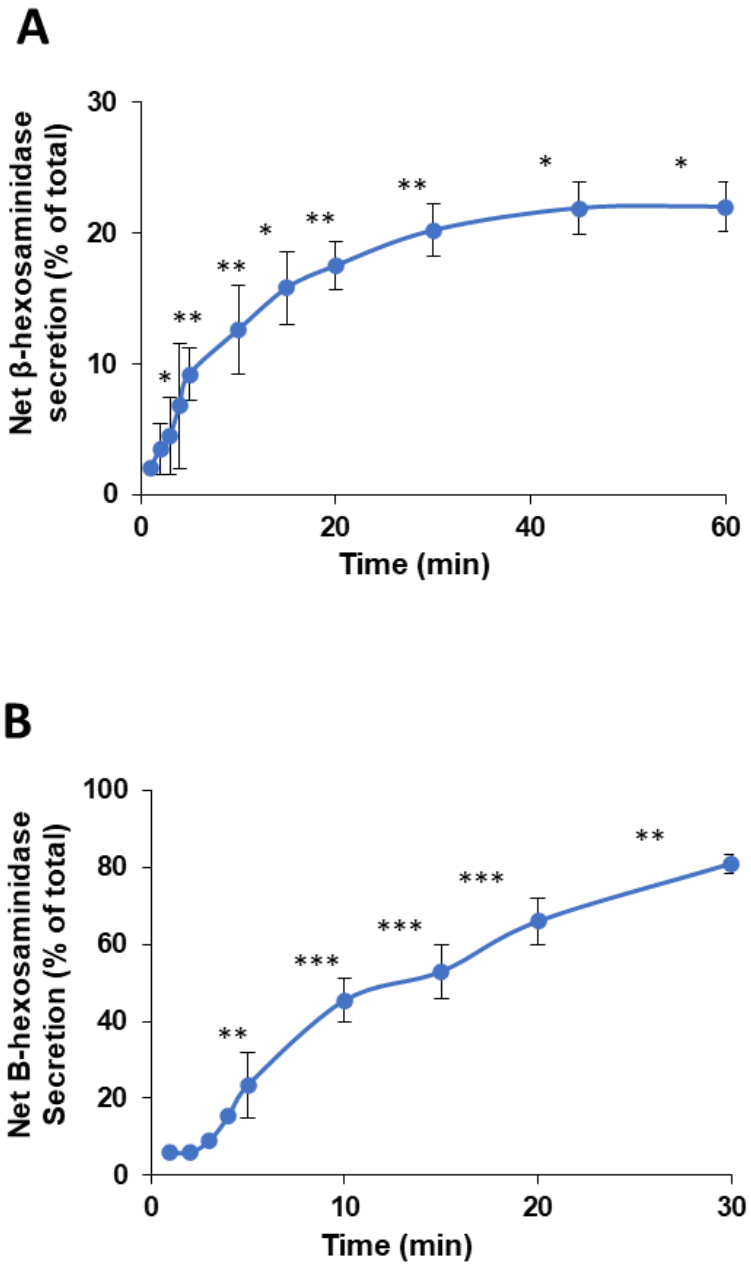

Figure 1. Kinetics of regulated secretion from RBL-2H3 mast cells. (A) Mast cell degranulation was assessed by measuring the net percent secretion of β-hexosaminidase enzyme. DNP-specific IgE sensitized RBL-2H3 mast cells were activated with 100ng/ml DNP-BSA for indicated time points at 37°C. The assay for released and cell-associated β-hexosaminidase was conducted according to the outlined materials and methods.**(B)** RBL-2H3 cells were subjected to treatment with either DMSO or 10µM Ionomycin for indicated time points, and the extent of net secretion was determined using the β-hexosaminidase assay as detailed in the materials and methods.Significance levels (*p<0.05, **p<0.005, ***p<0.0005) are indicated based on t-test results.

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Mouse neuroblastoma cells can release ATP and substance P (as described previously) in the extracellular medium (Gutiérrez-Martín et al., 2011). To studythe kinetics of ATP release (luciferin-luciferase assay kit) in Neuro-2a cells and its effect ineitherpromotionor suppression of Ca^{2+}-dependent exocytosis. Neuro-2a cells were stimulated with 10μM ionomycin, the basal ATP levelswere reasonably low (9.1×10^{-6}M; moles); however, in the presence of the Ca^{2+}-ionophore the ATP release almost doubled (20×10^{-6} M), indicating that ATP release depends on the increase in Ca^{2+} concentration and its involvementin exocytosis mechanism. Furthermore, decrease in ATP release occurred in the presence of a potent inhibitor of SNARE complex formation (NEM). Hence suggesting that SNAREs play a significant role in secretion of various mediators. Furthermore, the rate of ATP release was examined following stimulation with 10 μM ionomycin, resulting in an increase to 17 μmoles at 30 seconds post-induction, followed by a rise to 25 μmoles at 1 minute and 27 μmoles at 2 minutes, with a subsequent decline (Figure 2. A &B). Thus, induction with ionomycin accelerated the kinetics of ATP release in neuroblastoma cells (Figure 3).

3.2 Prediction of structurally and functionally relevant residues in the Q-SNARE protein subfamily using the RE/CRE approach:

RE/CRE scores were utilized to provide insight into the functional significance of residues within the linker region of SNAP-23/25. SNAP-23 and the neuronal SNAP-25, are distinctive in their absence of a transmembrane domain. What sets them apart is a linker region that connects a dyad of SNARE domains, a unique characteristic within the SNAP-25/SNAP-23 subfamily.The SNARE proteins in this family are tethered to the plasma membrane *via* palmitoylated cysteine residues in the linker loop connecting the SNARE domains (Vogel and Roche, 1999). Our earlier studies have already shown the critical role of the highly conserved Cys motif and proline at downstream position in the linker region in the membrane attaching domain of these SNARE proteins (Agarwal et al., 2019). The specific motifs and regulatory mechanisms through which SNAREs govern exocytosis kinetics have not been identified. MSA was performed using the extracted linker subsequences for the SNAP-23/SNAP-25 subfamily, and the RE scores were calculated corresponding to each of the alignment positions in the aligned linker region. The highly conserved cysteine residues in SNAP-23/SNAP-25, i.e., C_{80}, C_{83}, C_{85}, and C_{87}, were selected because of their high RE scores. The other high-RE scoring positions selected in the SNAP-23 linker, i.e., W_{103} (26), P_{86} (9) and G_{81} (4), were also predicted to be structurally conserved and critical for maintaining the folding of the protein (Table 1).

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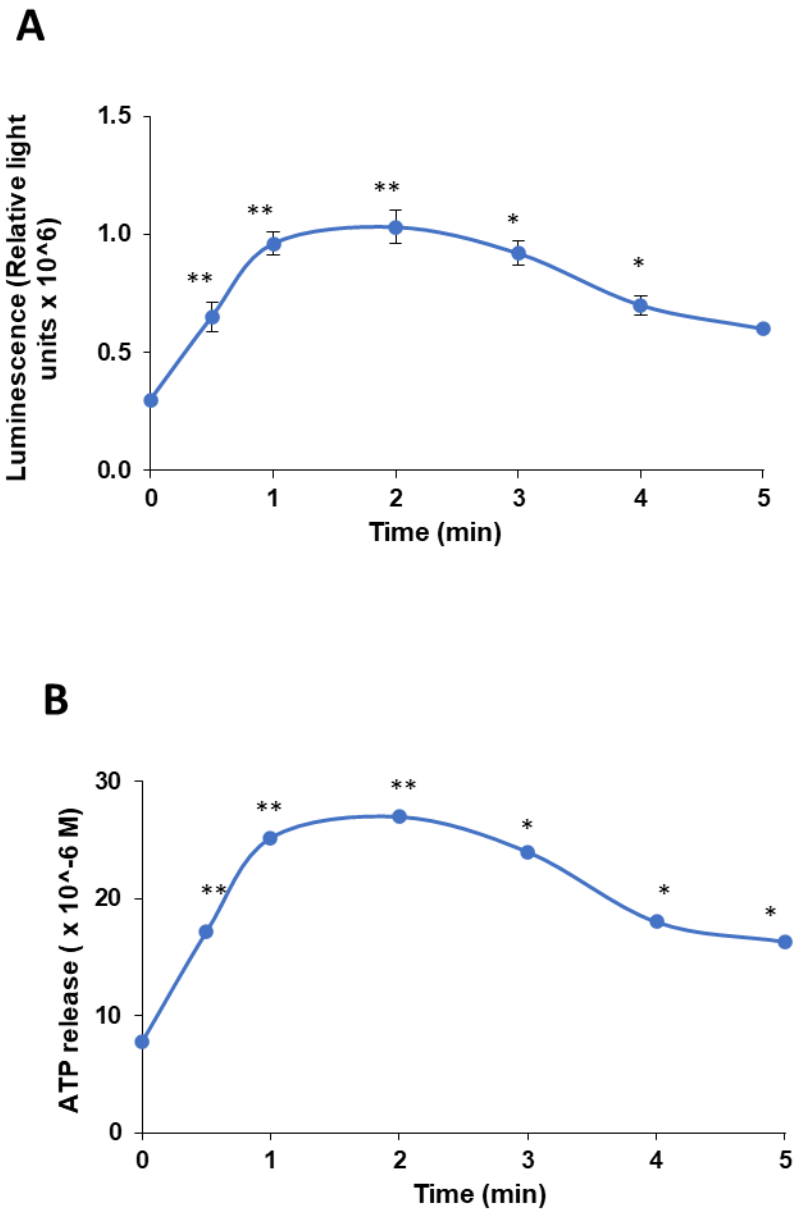

Figure 2. Release of ATP from Neuronal cells (Neura-2a) upon activation. (A) Neuro-2A cells were treated with 10µM Ionomycin for different time points, and the resulting evoked luminescence was measured and calculated for each time point. Subsequently, ATP release was quantified from the standard ATP curve and plotted across different time points. **(B)** The secretion kinetics from Neuro-2A cells during the initial minutes were transformed into net secretion (% of maximum cumulative secretion) for comparative analysis. In panels each time point represents the mean ± SEM of values obtained from at least 3 independent experiments. Significance levels (*p<0.05, **p<0.005, ***p<0.0005) are indicated based on t-test results.

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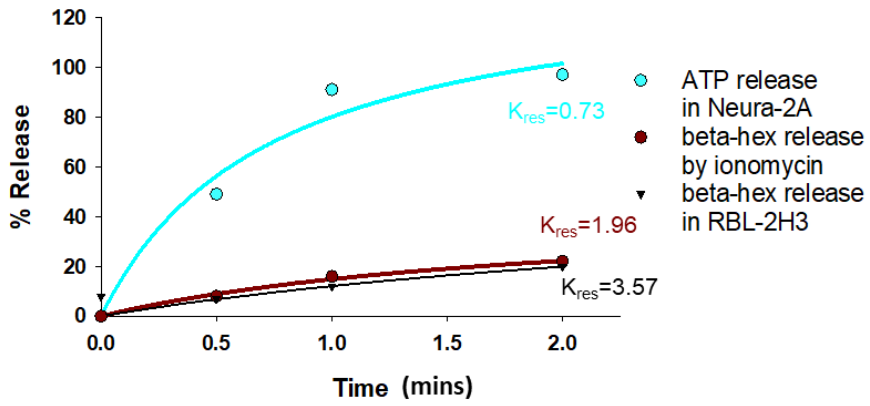

Figure 3. Comparison of kinetics of regulated secretion from Neuro-2A cells versus RBL-2H3 mast cells The kinetics of ATP release from Neura-2A and kinetics of □-hexosamindase release from RBL cells upon activation by ionomycin and ionomycin or allergen, respectively, during the initial minutes were transformed into net secretion (% of maximum cumulative secretion) for comparative analysis.. The kinetics release (K_{res}) was determined drawing the best fit curve using SigmaPlot 15.0 software calculating the release kinetics by both neuronal cells and mast cells upon activation by different means.

The CRE was used, to predict and map differentially conserved residues across subfamilies. Interestingly, the Cys at position 79 was differentially conserved across the SNAP-23/SNAP-25 subfamily. Hence, the other residues with high cumulative relative scores, i.e., R_{89} (12), Q_{126} (49) and Y_{139} (62) etc.), were predicted to be functionally relevant for the SNAP-23/SNAP-25 family (Table 2).

The highly conserved cysteines (C), i.e., C_{79}, C_{80}, C_{83}, C_{85} and C_{87}, were identified by high RE scores, while the differentially conserved cysteine (C) residues at the 79th position in the SNAP-23/SNAP-25 subfamily were identified by high CRE scores. Other residues with high RE/CRE values were also predicted to be functionally relevant for the SNAP-23/SNAP-25 family.

3.3.Overexpression of SNAP-23 mutants modulates the kinetics or overall regulation of exocytosis intransiently transfected RBL cells.

The hydrophobic linker domain of SNAP-23 contains five cysteines crucial for its membrane association through palmitoylation, a process believed to anchor SNAP-23 to the membrane for regulated exocytosis (Agarwal et al., 2019). In our investigation, mutants of SNAP-23 constructed [described before in(Agarwal et al., 2019) ; and in Figure 4. A] were transfected

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Table:1 Relative Entropy Scores of various alignment positions within the linker domain of SNAP-23 and SNAP-25

Amino Acid	Residue No. in Linker	Amino acid No.	RE Score
W	27	103	1.56
C	3	80	1.18
P	10	86	0.68
P	43	119	2.41
G	5	81	1.12
Y	63	139	1.58
Q	42	118	0.57
C	7	83	1.29
C	9	85	0.79
C	11	87	0.80

Table:2. Cumulative Relative Entropy Scores of various alignment positions within the linker domain of SNAP-23 and SNAP-25

Amino Acid	Residue No. in Linker	Amino acid No	CRE Score
R	13	89	0.47
Q	50	126	0.34
C	**3**	**79**	**0.50**
Y	63	139	0.33
C	9	85	2.39
N	48		0.36
C	7	83	0.62
C	11	87	0.68

into RBL cells to study their impact on exocytosis kinetics, revealing intriguing results (Hannenhalli and Russell, 2000).

Our study investigated mutants of SNAP-23 designed to mimic the linker region of SNAP-25, a protein known to modulate exocytosis kinetics in neurons. This C79F mutant did not significantly alter the cumulative net exocytosis from transfected mast cells after 45 minutes of stimulation (Figure 4. B), but they did induce a notable and significant increase in exocytosis after 15 minutes, with approximately 24% net hGH release compared to 18% net hGH release in cells transfected with SNAP-23 wildtype. Even when C was replaced with L (to retain hydrophobicity but loose palmitoylation) the result was increased secretion at 15 mins post activation, with no effect on overall secretion at 45 mins (Figure 4. B). Similar result was not seen when C was mutated to A (which lacks hydrophobicity as well as palmitoylation), This suggests that the kinetics of mast cell degranulation prompted by these mutations are expedited compared to those induced by SNAP-23 wildtype. These observations collectively suggest a pivotal role for specific cysteine residues within the linker region of SNAP-23 in modulating the kinetics of mast cell secretion.

4. Discussion

The nervous and immune systems interact through regulated exocytosis, a process controlled by SNARE fusion proteins. Differences in exocytosis kinetics between neurons and immune cells, like mast cells, are significant. Previous studies have shown that the *t*-SNARE SNAP-25 is involved in rapid exocytosis in neuron, whereas its non-neuronal homolog SNAP-23 is involved in slow exocytosis in mast cells (Nagy et al., 2008). To compare the kinetics of exocytosis in neurons and mast cells, Neuro-2A cells, a mouse neuroblastoma cell line, and RBL-2H3 cells (rat basophilic leukemia), a frequently studied model of mast cells, were activated *in vitro*, after which the kinetics of mediator release were measured. These two cell types exhibited tremendous differences in the early kinetics of regulated exocytosis even when the same trigger (ionomycin) was used. To compare the two *t*-SNAREs involved in secretion in these two cell types and further predict specific motifs involved in regulatory mechanisms of SNAREs that may play a role in governing the kinetics of exocytosis, motif identification was performed using the information theory measures of relative entropy to identify residues that are conserved across families, while cumulative relative entropy was used to identify residues conserved between subfamilies, as done before (Hannenhalli and Russell, 2000). Computational analyses identified key SNARE motifs, with cysteine residues in SNAP-23's linker region (positions 79, 80, 83, 85, 87) along with other novel motifs crucial for its function. The functional relevance of differentially conserved residue in regulating secretion kinetics was then experimentally validated.

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A

Mutants Cloned in EGFP vector	Sequence
SNAP-23 Wildtype	76 LNKCCGLCVCPCN 88
SNAP-23 C79F	76 LNKFCGLCVCPCN 88
SNAP-23 C79L	76 LNKLCGLCVCPCN 88
SNAP-23 C79A	76 LNKACGLCVCPCN 88

B

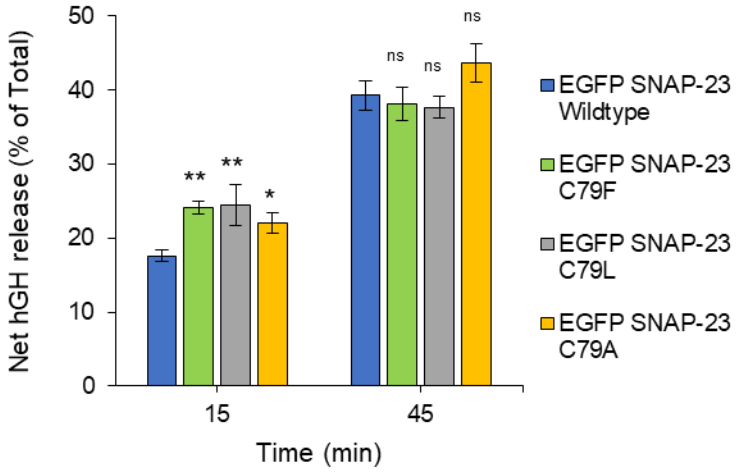

Figure 4. Effect on the secretion of mast cells by point mutations in conserved cys residue at 79th position in linker region of SNAP-23. (A) List of SNAP-23 mutants Cysteine 79 mutant sequences, as cloned in EGFP vector. **(B)** RBL cells were subjected to co-transfection with hGH containing plasmid, alongside either an empty EGFP-C2 vector or an EGFP-tagged wild-type SNAP-23 or its mutants at C_{79} (Phe, Ala and Leu). Following transfection, cells were treated with DNP-specific IgE and subjected to either no stimulation (at rest) or stimulation via DNP-BSA for 15 minutes and 45 minutes. The degranulation was quantified by measuring the release of hGH in the supernatant post-stimulation. Each dataset in the analysis represents the average ± SEM from three experiments. Noteworthy differences in net hGH release from mast cells resulting from the overexpression of SNAP-23 mutants compared to wild-type SNAP-23 were underscored (**$p < 0.005$ and *$p < 0.05$). Notable variations in net hGH release due to the overexpression of SNAP-23 mutants relative to the control/EGFP overexpression were accentuated using hash symbols, with assorted levels of significance ($^{###}p < 0.0005$, $^{##}p < 0.005$ and $^{#}p < 0.05$) (ns = not significant) .

This differential rate of exocytosis reported in mast cells and neurons may be due to qualitative differences in the linker region of the main *t*-SNARE, SNAP-23 in mast cells and SNAP-25 in neurons; the hydrophobicity and hydrophilicity of the amino acids; the number of cysteine residues; and the extent of palmitoylation in the linker region. In a previous study on importance of the linker region in fast exocytosis

in neurons, replacement of SNAP-25 linker with SNAP-23 linker region caused slowing down of exocytosis in a calcium dependent manner. It was also speculated that SNAREs, calcium sensors synaptotagmins and membrane lipids together form a fusion assembly that determines calcium dependence (Nagy et al., 2008). A more recent study divided the linker region of SNAP-25 into N-terminal and C-terminal, and determined that while the C-terminal played important roles in SNARE priming and ternary complex formation; the N-terminal including the conserved cys residues mediated the interactions with membrane lipids affecting membrane curvature for triggering actual fusion by pore formation (Shaaban et al., 2019). Additionally, the differential affinities and associations of SNAP-23 and SNAP-25 with membranes, membrane microdomains and other interacting partners may support slower exocytosis in the case of mast cells and faster exocytosis in neurons (Nagy et al., 2008). The differential rate of exocytosis may also be due to differences in calcium levels at which SNAP-23 and SNAP-25 mediate the docking of secretory granules with the plasma membrane by interacting with different synaptotagmins. Specifically, SNAP-23 has a calcium level of 100 nM, while SNAP-25 has a calcium level of 1μM is higher than that of SNAP-25 (Meldolesi and Chieregatti, 2004). There are already some known mechanistic differences between exocytosis mechanisms in mast cells and neurons. Neurons exhibit pre-docked synaptic vesicles with SNAREs, including SNAP-25, in pre-fusion state already docked for fusion. On the other hand in mast cells, we and others have shown that on receiving an activation signal, *t*-SNARE SNAP-23 gets phosphorylated, moves to granules, and probably mediates granule-granule fusion for compound exocytosis, granules then move with help of motor proteins along the cytoskeleton, then dock and fuse with the plasma membrane releasing the cargo. These events may require a longer time frame and greater regulation than the immediate release of neurotransmitters mediated by SNAP-25 (Baram et al., 2001; Phillips et al., 2001; Südhof, 2013).

While investigating the molecular mechanisms of regulated exocytosis from mast cells we previously demonstrated the importance of the *t*-SNARE SNAP-23 and, specifically, its C-terminal domain in regulating mast cell exocytosis (Agarwal et al., 2019; Hepp et al., 2005; Naskar et al., 2018; Naskar and Puri, 2017; Vaidyanathan et al., 2001). We have also shown that like SNAP-25, SNAP-23 though lacking a transmembrane domain, associates with plasma membrane in mast cells *via* post-translational modifications of (Hepp et al., 2005; Naskar and Puri, 2017) five highly conserved cysteine residues at positions 79, 80, 83, 85 and 87 to varying degrees (Agarwal et al., 2019). Furthermore, to investigate the role of these cysteines of SNAP-23 in the regulation of mast cell exocytosis, we overexpressed SNAP-23 cysteine mutants in RBL mast cells together with a human growth hormone secretion reporter. Overexpression of the SNAP-23 $C_{79}F$ mutant (which mimics the linker region of SNAP-25) (Agarwal et al., 2019) caused a slight increase in the rate of mast cell exocytosis without affecting the extent of degranulation. This result is consistent with the fact that neurons exhibit faster exocytosis (Nagy et al., 2008). However, SNAP-23 and SNAP-25, though similar, exhibit many other differences in sequence and interactions (Salaün et al., 2005). Overexpression of the SNAP-23 $C_{79}L$ mutant also caused increase in the rate of mast cell exocytosis without affecting the extent of degranulation, while overexpression of the SNAP-23 $C_{79}A$ mutant did not affect the rate of mast cell exocytosis. There may be a discrepancy between the membrane association (Agarwal et al., 2019) and exocytosis results due to the presence of endogenous SNAP-23. Nonetheless, our results clearly demonstrate that C79 residue in the cysteine-rich domain of SNAP-23 may regulate the kinetics of mast cell exocytosis.

In our view, the role of SNAP-23 in mast cell exocytosis, dependent on it's dynamic membrane associations, may be regulated by various physiological triggers and intracellular signaling pathways, which ultimately regulate SNAP-23 palmitoylation and phosphorylation dynamics and thereby its function in resting *versus* activated cells. Further study is needed to determine the exact mechanism involved incontrolof the kinetics of secretion. Although *in vitro* studies have shown that SNARE interactions are promiscuous and that SNAP-23 and SNAP25 can substitute for each other (Kádková et al., 2019), these two Q_{bc} SNAREs exhibit subtle but functionally important differences. Both of these *t*-SNAREs mostly

show different locations in terms of the cells in which they are expressed and intracellularly when they may be expressed in the same cell (Yamamori et al., 2011). SNAP-25 forms more stable SNARE complexes than SNAP-23, whereas SNAP-23 shows a much stronger association with lipid raft membrane microdomains than SNAP-25 (Salaün et al., 2005; Yang et al., 1999). A previous study involving SNAP-23 and SNAP-25 expression in PC12 cells has shown that both these proteins show remarkable differences in their association with lipid raft membrane microdomains while being expressed in same cells.

These differences were attributed to differences in cys residues in the linker region (five versus four) (Salaün et al., 2005). Even our earlier study has indicated a very strong association of SNAP-23 with lipid rafts in mast cells. Even ternary SNARE complexes containing phosphor-SNAP-23 were almost completely associated with lipid rafts (Puri and Roche, 2006). Hence from this study, one of the probable reasons for fast versus slow kinetics of secretion could be the stronger association of SNAP-23 to lipid rafts, which could affect its phosphorylation state, ternary SNARE complex formation etc. In lipid rafts on mast cell activation, SNAP-23 may be coming in contact with activated kinases (as many kinase associated signaling mechanisms are known to congregate in lipid raft microdomains). The kinase must be responsible for phosphorylation of SNAP-23 at Ser_{95} and Ser_{120} (Figure 5. A). This transient phosphorylation is very important for inward movement of SNAP-23 to facilitate granule-granule fusion for compound exocytosis, and such phosphorylations have not been reported for SNAP-25 (Naskar et al., 2018). All these dynamic movements of SNAP-23 and sequential membrane fusions during mast cell exocytosis must require additional regulatory mechanisms which are time consuming and slow down the kinetics of mast cell degranulation. The neuronal exocytosis of pre-docked vesicles *via* SNAREs may lack these regulatory and cumbersome steps and hence is very fast (Figure 5. B). This raft association could orchestrate the dynamic regulations and movements of SNAP-23 between various internal organelles in mast cells and may affect interactions with other SNARE regulators like phosphatases, docking proteins like Rabs and calcium sensors like Synaptotagmins. More extensive studies which are ongoing keeping in mind the differences in motifs/residues between these two Q_{bc} SNAREs will reveal the detailed basis of these regulatory and mechanistic differences. Understanding the precise molecular mechanisms of regulated exocytosis from mast cells and neurons and comparing the kinetics of regulated exocytosis will provide novel avenues for exogenously modulating the kinetics of regulated exocytosis, thereby leading to the identification of new drugs for treating various neurodegenerative diseases and various immunological disorders.

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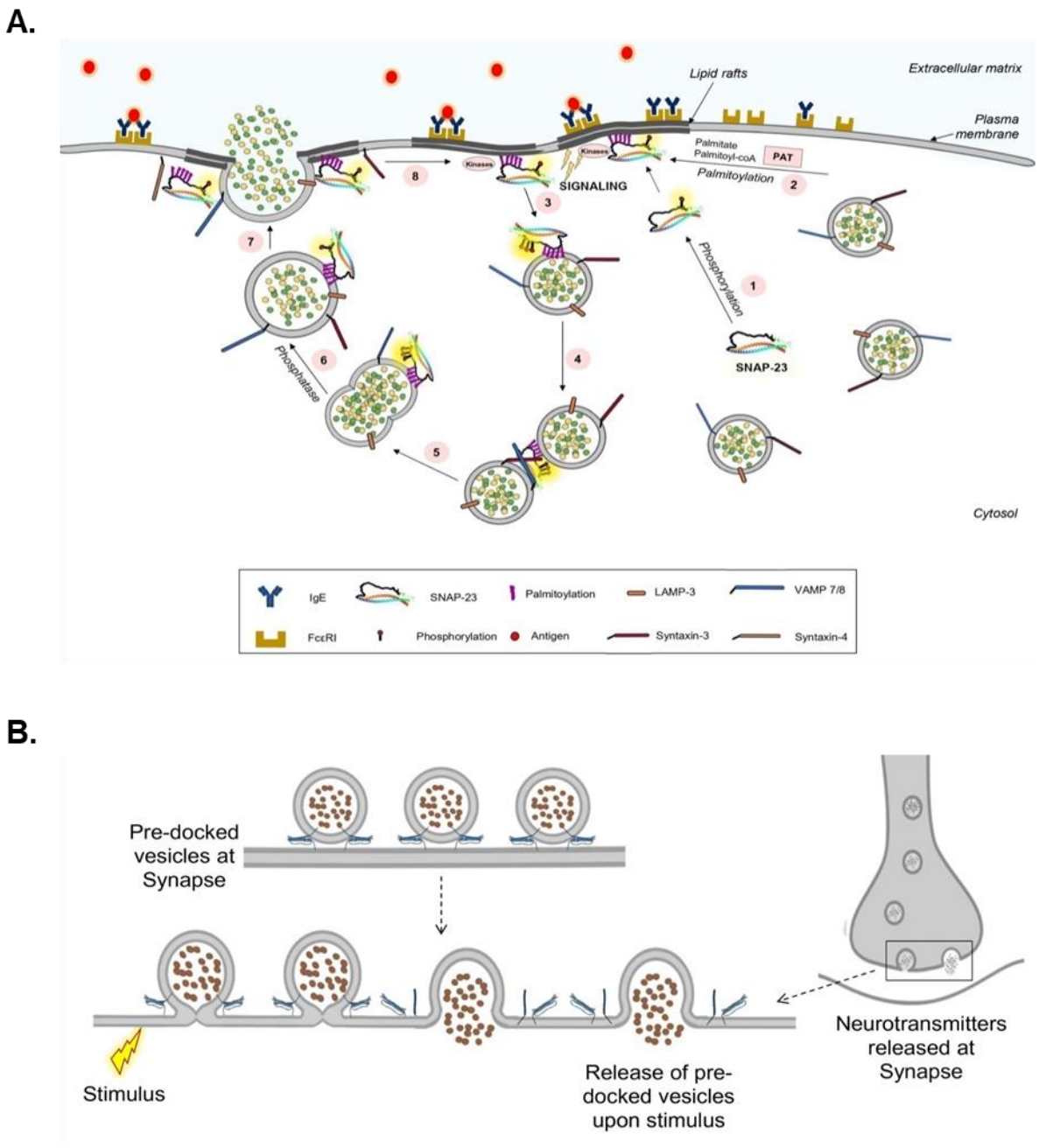

Figure 5. Schematic comparing Neuronal and mast cell exocytosis, highlighting the probable reasons for differences in kinetics of secretion. ***(A) Mast cell exocytosis and its regulation:*** The diagram illustrates the signalling cascade in mast cells triggered by allergen activation *via*IgE, localization of SNAP-23 acrossdifferent cellular compartments, regulation by post-translational modifications. The details are: **1.***Synthesis and Initial Phosphorylation:* SNAP-23 is synthesizedin the cytosol and undergoes phosphorylation at Thr_{102}. **2.***Palmitoylation and Membrane Attachment:* After reaching the plasma membrane, SNAP-23 ispalmitoylated on conserved cysteine residues by palmitoyltransferase (PAT), which isessential for its membrane attachment. **3.***Activation and Further Phosphorylation:* Mastcell activation occurs when an allergen binds to IgE attached to FcεRI receptors. Nearby kinases then phosphorylate

SNAP-23 at Ser_{95} and Ser_{120},enabling its relocation to secretory granules. **4 & 5.***Granule-Granule Fusion:*Phosphorylated SNAP-23 facilitates the fusion of granules, a key step in compoundexocytosis. **6.***Dephosphorylation and Vesicle Movement:* For the granules to movetowards the plasma membrane, SNAP-23 must be dephosphorylated at Ser_{95} and Ser_{120}. Specific phosphatases remove these phosphate groups, signaling granule movement towardsthe plasma membrane. **7.***Granule Fusion and Mediator Release:* The dephosphorylatedform of SNAP-23 is crucial for the final fusion of granules with the plasma membrane,leading to the release of mediators into the extracellular matrix. 8. SNARE ProteinRecycling: The SNARE proteins, including SNAP-23, are then recycled, ready to repeat theprocess. ***(B) Exocytosis of Neurotransmitters by neurons:*** Inneurons, the process of exocytosis is initiated by the depolarization of the cell membrane,which occurs due to a change in the potential difference across the membrane. This depolarization triggers an influx of Ca^{2+} from the extracellular space, as well as the release of Ca^{2+} from the endoplasmic reticulum within the cell. In response to thesecalcium signals, vesicles are already pre-docked at the plasma membrane, where SNAP-25, a key component of the SNARE complex, is in place and ready to facilitate the release ofneurotransmitters or other contents into the extracellular space. The pre-docking of vesiclesand the pre-formed SNARE complex, with SNAP-25 playing a critical role, allow for a rapidand efficient exocytosis process in neurons. This efficiency is enhanced because SNAP-25 interacts more effectively in the SNARE complex compared to SNAP-23, which is associated with other types of cells. As a result, the exocytosis process in neurons is not only faster butalso allows for quicker recycling of vesicles and SNARE proteins, ensuring that neurons can rapidly respond to subsequent stimuli.

5. **Conflict of interest:** The authors declare that they have no conflicts of interest.

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