Function suggests nano-structure: electrophysiology supports that granule membranes play dice

Ilan Hammel1,* and Isaac Meilijson2

1Sackler Faculty of Medicine, Department of Pathology, and 2Raymond and Beverly Sackler Faculty of Exact Sciences, Department of Statistics and Operations Research, School of Mathematical Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Cellular communication depends on membrane fusion mechanisms. SNARE proteins play a fundamental role in all intracellular fusion reactions associated with the life cycle of secretory vesicles, such as vesicle–vesicle and vesicle plasma membrane fusion at the porosome base in the cell plasma membrane. We present growth and elimination (G&E), a birth and death model for the investigation of granule growth, its evoked and spontaneous secretion and their information content. Using a statistical mechanics approach in which SNARE components are viewed as interacting particles, the G&E model provides a simple ‘nano-machine’ of SNARE self-aggregation behind granule growth and secretion. Results from experimental work, mathematical calculations and statistical modelling suggest that for vesicle growth a minimal aggregation of three SNAREs is required, while for the evoked secretion one SNARE is enough. Furthermore, the required number of SNARE aggregates (which varies between cell types and is nearly proportional to the square root of the mean granule diameter) affects and is statistically identifiable from the size distributions of spontaneous and evoked secreted granules. The new statistical mechanics approach to granule fusion is bound to have a significant changing effect on the investigation of the pathophysiology of secretory mechanisms and methodologies for the investigation of secretion.

Keywords: cellular communication; homotypic fusion; porosome; SNARE; unit granule

1. INTRODUCTION

Secretory granules and synaptic vesicles (SVs) are specific intracellular organelles that contain high concentrations of secretory mediators of various molecular weights. Both secretory vesicle types are exocytosed by active fusion of the vesicle with the plasma membrane. Synaptic transmission involves the fusion of neurotransmitter-filled SVs with the presynaptic plasma membrane at the active zone, while the secretory cell sequesters its content to the extracellular neighbourhood. Synaptic vesicles are made locally in the cytoplasmic side of the nerve terminal during recycling of the membrane (i.e. reformation by endocytosis). Synaptic vesicle proteins are sorted and concentrated on the plasma membrane, packaged into a pinocytosed vesicle of an exact and specific quantal size [1–9]. By contrast, secretory granules (SGs) are packaged at the trans-side of the Golgi complex and pass through a few processing steps [10–15] to produce a granule of unitary size [13,16–21]. The SG content ranges from macromolecules (proteins and mucopolysaccharides) to low molecular weight molecules (hormones). The secretion of both types of secretory vesicle is highly regulated and is accomplished through the fusion of the vesicles with the plasma membrane (exocytosis). The secretion of granules and SVs can occur at low levels under resting conditions (basal or spontaneous secretion), and at augmented levels when a stimulus for exocytosis is established (evoked or active secretion, degranulation) [1–15,17,18,20,21].

While granules and SVs have been recognized for almost 60 years as transporters of secretory material, there is still disagreement among researchers on some fundamental questions, such as whether granules exit the cell as originally formed (OF) or undergo homotypic fusion (HF) within the cytoplasm. The mechanisms involved in their HF growth and OF exit from the cell are only beginning to be defined. Morphometric evidence derived from studies of mast cells, pancreatic acinar cells and other cell types (reviewed in [13,17]) supports a model in which the newly formed granule/vesicle has an exact and specific quantal size which can be defined as unit granule (G1). The HF working paradigm on which the current study is based postulates that the pattern of granule–granule fusion occurs solely by the addition of unit granules, either to each other or to larger granules [17,22]. Namely, granule growth is the result of the
fission of SGs of the smallest size with other granules (\(G_1 + G_n \rightarrow G_{n+1}\), termed unit addition mechanism), forming granules whose volumes are multiples of the unit granule volume (\(G_n = n \times G_1\)). Mutations that perturb this process can cause, in a number of cell types, significant pathology resulting in giant SGs, probably the result of random fusion between granules (\(G_n + G_m \rightarrow G_{n+m}\), thus termed the random addition mechanism) [17].

Much of the current knowledge on the secretory apparatus is based on ultrastructural observations in correlation with biochemical and biophysical measurements. Within the last decade, the development of biophysical methods for the investigation of individual biological molecules has reformed our perception of the working principles of few single molecules working in tandem [23]. Complex functions, such as intracellular membrane shuttling, seem to depend on having the right molecules in their proper places at the right time [8,23]. The quantal nature of data provides evidence for the emergence of intracellular molecular machines (e.g. the porosome) which biological membrane-associated processes depend on, such as the SNARE complex and granule fusion [24–35]. Porosomes (or fusion pores), cup-shaped structures measuring only a few nanometres, are the universal secretory machinery in cell membranes of eukaryotic cells. Porosomes contain many different types of protein, especially SNARE proteins, calcium and chloride channels [27,28].

We report here on a Markovian birth and death model for the quantal mechanism of granule growth and elimination (G&E), formulated in terms of the molecular SNARE components which assemble themselves by principles of molecular recognition. Besides intracellular features such as the steady-state distribution of the granule size \((G_n)\), the G&E model determines as well, the distributions of the size of secreted granules under spontaneous and evoked secretion modes. The G&E model is based on the intracellular parameters \(\lambda, \mu, \beta \) and \(\gamma\) that determine for a generic \(G_n\) granule its growth rate (to \(G_{n+1}\)) \(\lambda = \kappa n^\beta\) and its elimination (exocytosis and secretion) basal rate \(\mu_n = \mu n^\gamma\) from the cell [22]. As will be shown, these parameters (that may be estimated from extracellular evoked and spontaneous secretion statistical data) are related to the number \((K_\beta)\) of aggregated SNAREs necessary for HF and the number \((K_\gamma)\) of aggregated SNAREs necessary for basal exocytosis. The simple linear relations are \(\beta = -2(3)/(K_\beta - 1)\) and \(\gamma = -2(3)/(K_\gamma - 1)\). Meta-analysis based on vastly reported measurements conducted by various electrophysiological and fluorescence approaches amply supports the validity of the model. This study has explicit implications on the information content of secretion and on the relationship between the granule–granule SNARE, granule–membrane SNARE and granule size. Its results are in good empirical agreement and provide theoretical confirmation to the observations that porosomes vary in size depending on the cell type [27,28].

The quantal nature of the secreted volume, which has been alternatively explained via OF by the simultaneous exit of unit granules [1], is due under G&E to individual exocytosis of cytoplasmic HF mature granules. Both models admit quantal secreted volume distributions, but differ markedly on the cytoplasmic granule size distribution. Under HF, this distribution should be quantal, while under OF it should be single-peaked and roughly Gaussian, the variability of unit granule formation. The data presented by the earlier studies ([20,21] reviewed in [19]) provide in our view strong evidence in favour of the G&E mechanism, the development of which is the subject matter of this study.

2. THE MODEL

2.1. Quantal basis of vesicle growth and information content

Theoretical modelling and computer simulations can be viewed as experiments conducted on simplified versions of real systems. The complexity of granule growth and elimination can be reduced so that the interactions between the main components can be analysed without having to consider numerous complicating factors present in vivo. By varying the parameters in a simulation, basic structures and mechanisms involved in such a process can be sequestered. The G&E model to be introduced has been developed to emulate parsimoniously a mechanism for the introduction of vesicles of monomer size which grow by a unit addition mechanism and are later eliminated from the system [17–19,22].

A mature granule fuses with a unit granule (to grow into a bigger granule) or with the cell’s membrane (to exit the cell) via the formation of a circular rosette [11] composed of SNARE proteins [24–31]. The self-aggregation capacity of the SNARE protein complex has been reported [26–28,31,36], whereby each unit granule has a limited number of ‘loops’ and the target membranes have a limited number of ‘hooks’. For example, in SVs, the hook is one t-SNARE (SNAP-25 + syntaxin complex), the loop is one v-SNARE (VAMP protein), and a SNARE pair is a complex of one t-SNARE and one v-SNARE [28]. It has been well established that for the formation of a circular arrangement between two fusing membranes, there must be an aggregate of an aggregate of SNARE-pairs (reviewed in [27,28]). The circular arrangement of SNARE–pair complexes is historically denoted as a rosette [11,24,27,28,35] and the pairing of the rosette with the membrane is designated as a porosome [26–28]. Such a membrane fusion structure was investigated by various methods by the Jena group [26–28] leading to the conclusion that vesicle curvature dictates more generally the size of the SNARE ring complex formed. The formation of such a porosome complex of SNARE-pair size \(K\) (figure 1) can be stochastically modelled thus: if a number of ‘loop’ units diffuse randomly on the surface of a granule of size \(n\) (with an area of order \(n^{2/3}\)), then the probability that \(K\) loops will be close to each other and close to \(K\) hooks in the unit granule surface or membrane is of the order

\[
\left(\frac{\text{const.}}{\text{area}}\right)^{K-1} = \text{const.} \times n^{-(2/3)(K-1)}.
\]

In other words, the rate at which a size \(n\) granule grows can be expressed as \(\lambda_n = \lambda n^\beta\) and the rate at which a
size $n$ granule exits the cell can be expressed as $\mu_n = \mu n^\gamma$, where the (negative) fractional dimensions $\beta = -(2/3)(K_{g} - 1)$ and $\gamma = -(2/3)(K_{r} - 1)$ depend linearly on the integer-valued rosette sizes $K_{g}$ (granule HF) and $K_{r}$ (granule–plasma membrane fusion).

Hua & Scheller [25] calculate the fraction of fusion-competent SNARE complexes in the VAMP2 domain, express the probability of fusion as this fraction raised to the number of SNARE complexes whose cooperative activity is required for fusion, and view global fusion events as a Poisson process with this probability as the rate. We resort to a very similar argument and essentially to the same type of cooperative activity probability, but for a different modelling purpose, the evaluation of the size-dependent fusion rate. The fraction raised to the number of SNARE complexes expresses the relative area of the cap, where SNARE coils have to form SNARE pair complexes, to the total granule area. Since the granule size is quantified as the volume, the effective power becomes the number of SNAREs times $-2/3$.

The volume of secreted mature granules has been repeatedly measured and the empirical distribution of this volume (based on samples of hundreds of secretion events) has been recorded under each of the two conditions ‘spontaneous’ secretion and ‘evoked’ secretion.
The secreted granule volume is 'quantal' [16–21] in the sense that these two empirical distributions are multimodal, with equally spaced modes that are identical in the two conditions. These quantal volumes are to be interpreted as representing integer multiples of the unit granule volume.

2.2. The growth and elimination model

The three-parameter G&E model \((\mu, \lambda, \gamma, \beta)\) tracks the granule size as a Markov process [22]. The stationary and exit granule size distributions in this model are derived with the intention of relating the exit distribution to spontaneous secretion and the stationary distribution to active or evoked secretion. From level \(n\), the granule \((G_n)\) can either make the transition to level \(n+1\) (i.e. to \(G_{n+1}\)) or be eliminated. As motivated above, the corresponding transition rates are \(\lambda_n = \lambda n^\beta\) and \(\mu_n = \mu n^\gamma\). This model effectively describes the distribution of the granule population size at steady-state and upon elimination (see the electronic supplementary material, figures S1–S3) as functions of three parameters: the effective kinetics factor \((\mu/\lambda)\), the effective surface factor \((\gamma - \beta)\) and the surface tension \((\gamma)\).

2.2.1. Stationary distribution

The steady-state condition is that the flow into state \(n+1\) (from state \(n\)) must equal the flow out of \(n+1\) (into \(n+2\) or as exit). Formally (as in [22]),

\[
\lambda \times n^\beta \times \text{STAT}(n) = [\mu \times (n+1)^\gamma + \lambda \times (n+1)^\beta] \times \text{STAT}(n+1).
\]

The value \(\text{STAT}(1)\) is set arbitrarily as any positive number, all other \(\text{STAT}(n)\) are then identified inductively, their sum is evaluated, and, finally, the sequence is normalized, dividing each entry by this sum, provided it is finite, as is the case if and only if \(\gamma - \beta > 1\). Thus, a stationary distribution exists only if \(\gamma - \beta > 1\).

2.2.2. Exit distribution

Following Nitzany et al. [22], the probability that a granule of size \(m\) will grow to size \(m+1\) is \(P_{\text{GROW}}(m) = \lambda \times m^\beta/([\mu \times m^\gamma + \lambda \times m^\beta] = 1/(1 + (\mu/\lambda) \times m^\gamma)^\beta)\). Letting \(P_{\text{GROW}}(0) = 1\), the probability \(\text{EXIT}(n+)\) that a granule will exit at size at least \(n\) is the product of \(P_{\text{GROW}}(m)\) for \(m\) from \(0\) to \(n-1\). The EXIT distribution is then obtained as \(\text{EXIT}(n) = \text{EXIT}(n+) - \text{EXIT}((n + 1)+)\).

As evident from the expression for \(P_{\text{GROW}}(m)\), the exit distribution depends on the two parameters \(\mu/\lambda\) and \(\gamma - \beta\) only. The stationary distribution, while, in principle, depending on all three, is nearly two-dimensional parametrically [22]. The conjunction of the two distributions, stationary and exit, determines the three parameters \(\gamma, \mu/\lambda\) and \(\gamma - \beta\). Maximum-likelihood estimation applied to empirical spontaneous and evoked data gives satisfactory results (see the electronic supplementary material, figures S1–S3).

If \(\gamma = 0\), the exit and stationary distributions are identical. If \(\gamma < 0\), stationary granules are stochastically bigger than exit granules [22]. Thus, the parameter \(\gamma\) determines granule propensity for elimination, while the parameter \(\beta\), a fractional dimension of the granule as an attractor of unit granules, affects the ability of granules to fuse.

Spontaneous and evoked granule size distributions have been repeatedly measured and analysed, although analysis has been mostly restricted to implications and the range of mean granule size (MGS) and the question of whether the distribution is 'geometric-like' or 'Poisson-like', i.e. memoryless or the result of rare-event count accumulation. Section 2.3 validates the need to deepen secretion analysis.

2.3. Detection of evoked exocytosis

The cell’s 'state' is its dichotomous mode, spontaneous or evoked (e.g. the neural cell should be quiescent or actively firing). Under the paradigm that given the state, the secretion size and time-spacing are random, the only information carried by secretion size and time-spacing is the state. Extracellular receptors must swiftly and with high probability detect the advent of an evoked mode, while keeping a low rate of false detections. The statistical theorem sub-discipline 'detecting a change-point in distribution' deals precisely with such a problem and solves it optimally by the cumulative SUM control chart (CUSUM) statistic method, explained in §5. To understand and quantify information content, it is imperative to model, accurately enough, the distributions of granule size under each mode.

Consider a 'random walk' that is incremented at each secretion by the log-likelihood ratio \(\ln(\text{STAT}(n)/\text{EXIT}(n)) = \ln(\text{STAT}(1)/\text{EXIT}(1)) - \gamma \times \ln(n)\), where \(n\) is the size of the secreted granule. Since \(\gamma < 0\), this increment is negative for monomers and increases with the granule size. The CUSUM statistic is the (non-negative) current gap of this random walk over its minimal value so far. Every time the CUSUM statistic exceeds a pre-assigned threshold \(TH\), a detection of the evoked mode is declared. The random walk has a negative drift \(-KLD(\text{EXIT}, \text{STAT})\) in the spontaneous mode and a positive drift \(KLD(\text{STAT}, \text{EXIT})\) in the evoked mode. Thus, the mean number of granules to detect an evoked mode is linear in the threshold (approx. \(TH/KLD(\text{STAT}, \text{EXIT})\)), while the mean number of granules between false detections is exponential in the threshold (approx. \(\exp(TH)/KLD(\text{EXIT}, \text{STAT})\)). Hence, a relatively minor delay in detection can drastically reduce false alarms. The two drifts are known as the Kullback–Leibler divergence \((KLD(f, g) = \int [\ln(f(t)/g(t)]f(t)g(t)dt)\) in the information theory and statistics literature. The increment \(\ln(\text{STAT}(n)/\text{EXIT}(n))\) that monitors secreted granules based on the granule size only, should be added a second summation, the contribution to detection based on a change in the secretion rate. This summation is the sum of two terms, one linearly decreasing in time, and the other linearly increasing in the number of secreted granules (see §6 for further details). In neural cells, the CUSUM statistic can be viewed as implemented by the action potential under integrate-and-fire neural management, and the additional temporal linear term is similar to the membrane time constant. In relation to endocrine
cells, the spontaneous mode is termed ‘baseline process’ and the evoked mode ‘pulsatile hormone secretion’ [44].

3. METHODS

3.1. Quantitative microscopy

In the absence of published data on granule size distribution, we used published calibrated transmission electron microscopy micrographs and measured the outermost membrane of the vesicles.

3.2. Quantification of the multi-modal distributions

Figures were magnified to the full page and each bin was quantified. Data consist of ‘single vesicle event’ counts in equal-length micro-bins, to be clustered into equal-length macro-bins corresponding to unit granules. For each possible macro-bin size, two mixtures of Gaussian distributions were fitted by means of the expectation-maximization (EM) algorithm [45,46], one for the evoked secretion data and the other for the spontaneous secretion data. The means of the component Gaussian distributions are constrained to be a homogeneous arithmetic sequence (i.e. multiples of the unit granule mean volume \(v\)) and their variances are constrained to be an arithmetic sequence (i.e. a measurement error variance \(\tau^2\) plus multiples of the unit granule variance \(\sigma^2\)). The three parameters \(v\), \(\tau\) and \(\sigma\) are common to both distributions. Each of the two standard deviations \(\tau\) and \(\sigma\) is of the order of magnitude of 10–15% of the unit granule mean volume \(v\). The two (pseudo) ‘empirical’ quantal granule size distributions are defined as the mixture probabilities delivered by the EM algorithm. These two distributions are the input for a maximum-likelihood estimation program that estimates the three parameters \(\mu/A, \beta\) and \(\gamma\), assuming that the pseudo-empirical secretion data are random samples from the stationary and exit distributions. The program has the option of letting \(\beta\) and \(\gamma\) be real or integer multiples of 2/3. With SNARE in sight, we report only the latter.

4. RESULTS

4.1. Rosette size

The number of SNARE complexes that cooperate to mediate vesicle fusion is under dynamic investigation [28–35]. Using various model systems including liposome fusion assays and titration of SNARE inhibitors, 1–15 SNARE complexes are estimated to be needed for membrane fusion (reviewed in [31,34]). The G&E model provides a simple explanation for this range. A scattergram display (figure 1c) of \(K_\gamma\) and \(K_\beta\) versus vesicle diameter \(D_v\) supports the simple granule size relation \(K_\beta \approx (0.9 + 0.2)\sqrt{D_v}\) to be theoretically justified in §4.2. Since \(\gamma - \beta > -1\) is necessary for the existence of a steady state [22], it follows that \(K_\gamma \leq K_\beta + 1\) (graphical explanation in figure 1a). The smallest \(G_1\) that has been observed in neurosecretory cells is within the diameter range 25–30 nm. Thus, in secretory cells, the minimal value for \(K_\beta\) should be three or four SNARE pairs \((D_v=25\text{ nm}; \text{ integer } K_\beta = K_\gamma \approx 0.9\sqrt{D_v} = 3 - 4)\). In secretory cells with large unit granules (diameter > 200 nm), data is available only on the evoked (but not on the spontaneous) granule size distribution. As a result, reliable estimates for large \(K_\beta\) and \(K_\gamma\) are unavailable at this stage. However, freeze-fracture data for mast cell granules reveal rare rosettes of at least 20 SNARE pairs (some ‘line’ along the fracture plane is devoid of particles) [11]. The peritoneal mast cell unit granule volume is in the range of 0.050–0.065 \(\mu m^3\) (equivalent to a diameter range of 4.6 \(\times\) \(10^2\)–5.0 \(\times\) \(10^2\) nm; [18,19]), which would result in a rosette estimated as composed of 18–20 SNARE pairs depending on the membrane width.

An interesting case has been reported with experimental evidence for only one SNARE necessary for granule docking and fusion [34] in liposome models. Using liposomes in which the number of SNAREs was progressively reduced to an average of below one molecule per liposome, it was found that liposomes bearing only a single SNARE molecule were still capable of fusion with other liposomes, or with purified SVs [34]. The G&E model supports this observation. In fact, since \(\gamma = 0\) \((K_\gamma = 1)\) makes the exit distribution equal to the stationary distribution [22], the G&E model suggests the postulate that the evoked distribution is a manifestation of the stationary distribution, via a process that temporarily switches the exit intensity \(\mu_m = \mu n^2\) by another, independent of granule size \(n\), such as membrane fusion with a single SNARE pair.

Support for the identification of the evoked and stationary distributions has been provided by comparing the amperometric estimation of secreted granule content with the granule diameter obtained via high-resolution fluorescence microscopy [47]. Individual exocytosis events of a single granule in intact adrenal medulla [48], pancreatic β-cells [49] and exocrine cells [50] were visualized by two-photon extracellular polar-tracer imaging. Exocytosis of SGs often occurred in a sequential manner, involving first the granules located at the cell periphery and then those present deeper within the cytoplasm. The full-width at half-maximal diameters of the primary \((0.50 \pm 0.08\text{ µm})\) and secondary exocytotic granules were similar \((0.50 \pm 0.09\text{ µm})\) and were consistent with those of the stationary chromaffin granules stained with acridine orange \((0.51 \pm 0.08\text{ µm})\) [48]. Granule–granule fusion did not precede primary exocytosis, indicating that secondary fusion events may require a plasma-membrane factor [48–50]. This sequential replenishment mechanism of exocytosis allows the cell to take advantage of a large supply of fusion-ready granules without needing to transport them to the plasma membrane. Interestingly, although the SNARE protein SNAP25 was found to be necessary for sequential exocytosis, it was absent from the primary granules prior to cell stimulation, being supplied via lateral diffusion from the plasma membrane to the membrane of primary vesicles after their fusion [48–50].

4.2. Ultrastructural ‘choreography’ of SNARE proteins

Membrane fusion, obligatory in granule life, requires the remodelling of two phospholipid bilayers. Our model
may generate some insight on the SNARE aggregate size \( (K) \) as a function of the granule size \( (D_V = 2r > 25 \text{ nm}, \text{figure 1c}) \). Since the SNARE rosette is approximately circular and \( K \) should be roughly proportional to the perimeter \( 2\pi r \) of the rosette, it is possible to arrive at a relationship between \( K \) and vesicle diameter \( D_V \) via the representation \( a = [h(D_V - ph)]^{0.5} \) of the radius of the rosette (figure 1c). The lipid width of most membranes is within \( 3.4 - 4.5 \text{ nm} \) (figure 1d), depending on the lipid composition [41–43]. Thus, the distance \( h \) from the surface of the granule to the centre of the membrane bilayer is nearly independent of the granule size, and may be estimated as \( h \approx 2 \text{ nm} \), small enough with respect to \( D_V \) so that \( a \approx (hD_V)^{0.5} \), yielding a relation \( K \approx \text{const} \times \sqrt{D_V} \). Figure 1e shows the linear correlation between the maximal rosette size and the granule size (at \( \sqrt{D_V} \) scale) for various \( h \) values. Meta-analysis generated 12 rosette size estimates for granule homotypic growth \( (K_g) \) and for granule elimination \( (K_e) \) (figure 1e inset). All 12 data groups conform to the steady-state condition \( K_g \leq K_h + 1 \). Correlation of rosette size data with the granule diameter (\( \sqrt{D_V} \)) is in good agreement with the assumption that hemifusion events occur within the membrane hydrophobic centre, since all data points are within the narrow zone of \( 0.5 \text{ nm} < h < 3 \text{ nm} \) (i.e. near the bilayer centre).

These derivations may provide some insight on the structure of the porosome complex at the nanometre scale. Cho et al. [26] established that the size of the \( t/v \)-SNARE porosome complex \( (D_p) \) is directly proportional to the vesicle diameter \( D_V \) (i.e. \( D_p/D_V \approx 300/800 \)). The SNARE complex that mediates exocytosis at the plasma membrane in the yeast *Sacccharomyces cerevisiae* is a long rod \( (4 \times 5 \times 11.2 \text{ nm}) \) [40]. Assuming that all SNARE complexes have similar dimension \( (D_k = 4 - 5 \text{ nm}) \), a simple geometric calculation (figure 2a) of the ratio \( D_p/D_V \) coupled with \( K_g \approx (0.9 \pm 0.2) \sqrt{D_V} \) suggests that the effective arc length \( (L) \) between the centres of adjacent SNARE complexes is about \( L \approx (1.46 \pm 0.32)K \) (in nanometre scale).

Using atomic force microscopy, Cho et al. [26] detected porosome formations at the plasma membrane of growth hormone (GH)-secreting cells of the pituitary and implicated their involvement in hormone secretion. Pits containing fusion pores or depressions 100–200 nm in diameter in resting GH-secreting cells were observed to enlarge following the stimulation of secretion. Depressions that measured in resting GH cells 154 ± 4.5 nm (mean diameter ± s.e.), increased to 215 ± 4.6 nm \((p < 0.01)\) after the stimulation of secretion. After the completion of secretion, the dilated depression returned to the resting size. Pancreatic acinar cells degranulation (induced by mastoparan) results in a time-dependent increase of 20–35% in the depression diameter, returning to the resting size on completion of secretion. Thus, \( L_{\text{open state}} \approx (1.46 \pm 0.32)K \times 1.3 = (1.9 \pm 0.4)K \), which may partially explain the linear correlation between the granule size and the complete pore size [40].

### 4.3. The growth and elimination benchmark

The optimal mean number of granules needed for correct detection (MGCD) was numerically evaluated, keeping the mean number of granules between false alarms (MGFA) under control. The evaluation was performed on a wide benchmark of parameters: \( K_h \) from 3 to 32, Stationary MGS (in unit granules) from 1.25 to 5.75, MGFA = 10^4 for \( d \) from 2 to 7, and the secretion rate magnified by a factor 2^f for \( f \) from 0 to 6. The MGCD needed for the detection of the evoked mode fixing the MGFA as 10^5 is displayed in figure 3 as a function of the MGS. It is evident that, for small \( K_h \) (4–5), at least 8–10 granules (on average) are needed for detection, while, for \( K_h > 8 \), many fewer granules are needed. However, since the total volume of 10 granules with \( K_h = 4 \) is about 6% per cent of the volume of one granule with \( K_h = 16 \), the drive for miniaturization of granules is apparent. Differentiation of cells by secretory activity (e.g. neuro-secretion, hormones and immune activity) is coupled with differentiation by the granule diameter size (i.e. by rosette size \( K \)), where small \( K \) corresponds to a high granule turnover [22].

#### 4.3.1. Dependence on the number of rosette petals

For \( K_g = K_h \), the MGS decreases if the common value of \( K_g \) and \( K_h \) is increased. However, with minor exceptions (MGS < 2 and \( K_g > 4 \)), the MGS decreases further if \( K_h \) is reduced by one rosette petal with respect to \( K_g \). In the exceptional cases, the improvement by not reducing \( K_h \) is below 0.3 per cent. Thus, according to the G&R model, the granule–membrane porosome size \( K_g \) should optimally exceed the granule–granule porosome size \( K_h \) by one rosette petal, although equality between the two is close to optimal. It may be biologically safer to keep them equal, since \( K_g = K_h + 1 \) is borderline stable in the sense that \( K_g > K_h + 1 \) would not admit a steady state. In most examples analysed, the two were estimated to be indeed equal (figure 1e, inset).

#### 4.3.2. Effect of magnification of the secretion rate

Figure 3 demonstrates that secretion magnification factors above eight achieve MGCD quite insensitive to the granule size distribution or further magnification factor. Indeed, the literature reports that evoked secretion rates are commonly 10 times or higher above basal secretion rates.
In principle, $S_n$ or above (TDTH) be the first time. Let $S_n \geq 5.1$. Mean time to correct detection
more data, hitherto unavailable. This is a subject for further study, based on the 12 examples in the electronic supplementary material. This is a subject for further study, based on the 12 examples in the electronic supplementary material.

4.3.3. Small is beautiful
The benchmark discloses that the product MGCD $\times K_B$ is roughly constant. This finding supports miniaturization, since, although fewer bigger granules are needed for detection, their total volume (MGCD $\times$ volume, proportional to MGCD $\times K_B^3 = MGCD \times K_B \times K_B^2 \approx$ const. $\times K_B^3$) grows like the square of the granule diameter. Thus, neuronal SVs have an advantage over larger granules, as their smaller volume enables miniaturization of the synapse contact.

4.4. Is $K$ a fixed integer?
In principle, $K_B$ and $K_\gamma$ could be random integers, varying slightly from cell to cell or even from fusion to fusion. In this case, fractional values of $K$ would have to be considered too. Randomness could add support to the argument above, rendering the optimal choice $K_\gamma = K_B + 1$ unstable, and dictate the equality between the two or even smaller values of $K_\gamma$ in agreement with the 12 examples in the electronic supplementary material. This is a subject for further study, based on more data, hitherto unavailable.

5. PAGE'S CUSUM APPROACH FOR THE DETECTION OF CHANGE

5.1. Mean time to correct detection
Let $S_n$ be a random walk with increments $X_n$ starting at $S_0 = 0$. For a threshold $TH > 0$, let time to reach a value $TH$ or above (TVTH) be the first time $n$ with $S_n \geq TH$. Similarly, let time to experience a draw-up $TH$ or above (TDTH) be the first time $n$ with $S_n = \min_{m \leq n} S_m \geq TH$ (a value at least $TH$ above the minimal value so far, as the CUSUM method dictates). Wald’s well-known identity [51], a direct generalization of the deterministic-time identity $E[S_n] = nE[X]$, states that, for a stopping time $\tau$, $E[S_\tau] = E[\tau]E[X]$.

Thus, $E[TVTH] = E[S_{TVTH}]E[X]\geq TH/E[X] = TH/KLD$. Clearly, TDTH $\leq TVTH$. Hence, the mean number of granules MGCD for the CUSUM statistic to signal a correct detection is a little smaller than something a little bigger than TH/KLD (STAT/EXIT).

5.2. Mean time between false alarms
For Brownian motion $B_t$ with negative drift $\nu$ and standard deviation $\sigma$ per unit time, the expected TDTH is $[\exp(\rho TH) - 1 - \rho TH]/\rho$ [52], and expression 7 in [53]), where $\rho = -2\nu/\sigma^2$ is such that $E[\exp(\rho B_1)] = 1$. For random walks, this result holds only approximately. Meilijson [54] has proved that the expected time for a random walk to experience a draw-up $TH$ is at least $[\exp(\rho TH) - 1 - \rho TH]/\rho$ and at most $[\exp(\rho (TH + d)) - 1 - \rho TH]/\rho$ for some constant $d$ that depends on the increment $X$ distribution, where $\rho$ (known in risk theory as the coefficient of adjustment) [55] is defined by $E[\exp(\rho X)] = 1$. It is a remarkable fact that $\rho = 1$ whenever $X$ is the negative of a log-likelihood ratio:

1. $\int \exp \left[ -\ln \frac{g(t)}{f(t)} \right] g(t)dt = \int \left[ \frac{f(t)}{g(t)} \right] g(t)dt = \int f(t)dt = 1.$
Assuming that TH is relatively big (so that $1 + TH$ may be neglected in \(\exp(TH) - 1 \sim TH\) with respect to \(\exp(TH)\)), this establishes the validity of the proposed approximation \(MGFA \approx \exp(TH)/KLD(EXIT\,STAT)\) for the mean number of granules between false alarms of the CUSUM method.

### 5.3. The effect of increased secretion rate

Suppose that spontaneous (respectively, evoked) secretion occurs at rate $\eta_2$ (respectively, $\eta_3$), with $\eta_3 > \eta_2$, and both secretion processes are Poisson. Assuming that in each mode, the granule size and time-spacing are independent, the log-likelihood function is the sum of the granule size and time-spacing log likelihoods. Hence, an equivalent relation also holds for the KLD. The log likelihood for a period of length $T$ during which $n$ granules (of volumes $V_i$) have been secreted is \[22\]

\[-(\eta_2 - \eta_1)T + n\ln\left(\frac{\eta_2}{\eta_1}\right) + \sum \ln \left(\frac{\text{STAT}(V_i)}{\text{EXIT}(V_i)}\right),\]

expressible more explicitly as $-(\eta_2 - \eta_1)T + n[\ln(\eta_2/\eta_1) + \ln(\text{STAT}(1)/\text{EXIT}(1))] - \gamma_S\ln(V_i)$, of the form $-C_1T + (2/3)(K_s - 1)\Sigma\ln(C_2V_i)$, reminiscent of an action-potential, additive inputs per secretion dampened by a membrane time constant.

Its mean per granule in the evoked mode, the effective KLD($S,E$), obtained replacing $T$ by $1/\eta_2$ and substituting $n = 1$, is $KLD(S,E) + \eta_2/\eta_1 - 1 - \ln(\eta_2/\eta_1)$, while its mean per granule in the spontaneous mode, the effective $KLD(E,S)$, is $-KLD(E,S) + \eta_2/\eta_1 - 1 - \ln(\eta_2/\eta_1)$. Since the convex function $x - 1 - \ln(x)$, defined on $(0,\infty)$, has a minimum and vanishes at $x = 1$, each effective KLD progressively exceeds the corresponding granule size KLD as $\eta_2/\eta_1$ is increased.

Summarizing, \(MGCD \times KLD_{EFF}(STAT,EXIT) \approx TH \approx \ln(MGFA \times KLD_{EFF}(EXIT,STAT))\) leads to the following approximate formula for the MGCD to correctly detect the evoked mode, in terms of the required MGFA between false alarms in the spontaneous mode.

\[\ln[MGFA] + \ln[KLD(EXIT,STAT)] \]
\[MGCD \approx \frac{\eta_2/\eta_1 - 1 - \ln(\eta_2/\eta_1)}{\ln[STAT] - \ln(EXIT,STAT)} + \frac{\eta_1}{\eta_2} - 1 - \ln(\eta_1/\eta_2)\infty, since the numerator and the denominator are asymptotic to $\ln(\eta_2/\eta_1)$.

### 6. DISCUSSION

#### 6.1. Granule size dictates the rosette size and fusion rates

This report has introduced a discrete Markovian stochastic model for granule growth and elimination. This model identifies the stationary and exit granule size distributions and relates these to the rosette size. Various alternative approaches have been applied to define the granule stationary size distribution (reviewed in [17]), based on classical biophysical continuous models, in which the fusion rates could only decay as a small power of the radius (up to 6). By contrast, data consistently suggest much faster decay. This has led us to apply a statistical mechanics approach in which fusion rates correspond to the probability of self-aggregation of SNARE units. Thus, fusion rates are exponential in the rosette size. Our model enables for the first time, to our knowledge, the estimation of the SNARE aggregate size needed for granule HF. In conclusion, the rosette size is statistically identifiable from secreted granule size distributions, and dictates cell morphology, as displayed in figure 2.

Electrophysiological measurements on live secretory cells suggest the existence of fusion pores at the cell plasma membrane [27,28]. Membrane-bound secretory vesicles are assumed to dock and fuse at the fusion sites, to release their contents [7–14]. High-resolution studies using TEM on fixed tissues and atomic force microscopy (AFM) on live secretory cells confirmed the presence of such plasma-membrane pores, revealing their ultrastructure (TEM and AFM) and dynamics (AFM) at near nanometre-resolution and in real time. The G&E model and the meta-analysis results support such porosome structure flexibility. The vesicle-vesicle SNARE aggregate $K_p$ seems to be the key parameter for the estimation of the porosome size. Based on the meta-analysis data, it is estimated that for a granule size within the range of 25–100 nm, 3–9 SNAREs can form the porosome at the cell resting state. Such an estimate is in excellent correlation with published data [31,34].

Our calculations show that the distance ($L$) between rosette petals is roughly $L \approx (1.46 \pm 0.32)K$ nm. This finding is consistent with the prevailing empirical evidence on the range of the number of granule-plasma membrane SNARE aggregates needed for fusion (reviewed in [28,31]). For $K = 3$, the distance is approximately $3 \times 1.46 \approx 4.5$ nm, equivalent to the width of a single SNARE unit [40], which is in excellent agreement with the estimate of Cho et al. [56] for the minimal requirements for porosome formation. A 3-SNARE ring complex at the neuronal porosome base allows room for a central channel measuring just 1–1.5 nm in diameter, enough for only low weight molecules to pass through (e.g. neuroamines).

#### 6.2. Granule size is correlated with granule lifespan

Previous studies [57] using pulse-labelling techniques have suggested that secretory proteins may be stored and secreted heterogeneously. Although most of these studies found that newly synthesized proteins are secreted selectively, little is known about how non-random non-parallel secretion results from the integration of synthesis with the sequential cellular processing, storage and mobilization of this protein. Granules are a heterogeneous vesicular population even within a single cell type. For instance, they can differ in size [17], content [12–14] and in the kinetics of content release [57–60]. Several of these differences are correlated with the granule age [10,14,61]. Ageing
can also affect the probability of SGs undergoing exocytosis, with newly generated SGs being preferentially released [10,22]. For example, Duncan et al. [61] investigated the age-dependent distribution of secretory vesicles within chromaffin cells by tagging SGs with the fluorescent protein dsRed-E5, which changes its emission from green to red over time. These authors point out that the granule age is a critical factor that segregates granules with respect to their localization and mobility, and affects the probability of undergoing exocytosis in response to different stimuli. Our theoretical findings offer a simple explanation for the phenomenon of size disparity under which smaller granules remain within the cell for a shorter time when compared with granules of larger size, inducing an almost ‘last-in, first out’ queue management: transition rates decrease as a function of the granule volume, and so transitions (whether growth or exit) occur more frequently to smaller granules. Such a preferential release of smaller granules means that newly synthesized secretory material will have a better opportunity to be secreted.

6.3. Membrane curvature and the hemifusion state

The G&E model supports the concept that macromolecular scaffolding of proteins is responsible for bringing the plasma membrane close to the SG membranes, generating the architecture that enables the hydrophobic force to cause fusion. Kunding et al. [39] correlated the influence of membrane curvature on the efficiency of intermembrane docking reactions. These authors monitored the docking of single vesicle-vesicle pairs of various diameters (30–200 nm) as mediated by SNAREs. The intermembrane docking efficiency exhibited 30- to 60-fold augmentation as a function of curvature. This well-documented phenomenon [27,28] shows that membrane curvature can regulate intermembrane tethering reactions and other downstream processes, including the fusion of vesicles. Under G&E, higher curvature means smaller granules, for which the rate of growth is bigger. A n-mer has a rate of growth \( n^{-2/(2/3)(K-1)} \) relative to a monomer. For \( K = 5 \) and \( n = 4 \) this is 1/40, for \( K = 6 \) and \( n = 3 \) this is 1/39, while for \( K = 7 \) and \( n = 4 \) this is 1/64. Thus, the G&E model seems to be in good qualitative correlation with measurements by Kunding et al. [39].

As the vesicle size distribution is not presented in their work, it does not provide a reliable estimate for \( n \).

Experiments with lipid vesicles and planar bilayers have shown that bilayer fusion progresses through hemifusion [37,38]. Since most experimental points are located very near the centre bilayer (\( h \approx 2 \) nm), it is tempting to suggest that the hemifusion non-bilayer intermediates are located at the centre of the newly formed ‘bilayer’ (figure 1a, pathway II, D intermediate).

6.4. Synapse evolution dictates granule evoked burst

For bigger granules (mast cells and pancreas), the G&E stationary distribution is spread out and quite different from the exit distribution [22], affording reliable yes–no information. Information theory quantifies the argument that miniaturization (e.g. nervous system) brought about the need for magnified evoked secretion rates, to increase the KLD between the two secretion modes to the level that permits efficient detection at the synaptic cleft (figure 3). Since the granule size is highly correlated with the logarithm of the granule lifetime [22], it means that miniaturization has a price tag of high granule turn-over.

Presynaptic nerve terminals contain between several hundreds to tens of thousands of vesicles. Typical synapses may have approximately 10 vesicles (4–32, depending on the cell type) immediately available for fusion [62]. The maintenance of an inventory of large amounts of reserve vesicles suggests that these are relevant for synaptic communication: as such, the granule size should behave according to a distinctive, temporally stable steady-state distribution that will manifest itself in each short evoked burst, well differentiated from an equally stable spontaneous mode. This ergodicity requirement can only be met if the granule buffer is large enough. The number of vesicles and the fusion speed with the plasma membrane impose limits on the time periods and frequencies with which information can be transmitted by short bursts during repetitive stimulation. The data in figure 3 are in good agreement with our theoretical calculations that a burst of at least eight vesicles will generate enough data for reliable yes–no communication.

6.5. ‘Nash equilibrium’ and comparative rosette size

As explained in §6.4, the KLD between basal and evoked granule size distributions measures the efficiency of transmission of a yes/no message. The evaluation of KLD in a large benchmark of scenarios disclosed that, for fixed \( K_B \); \( K_Y \) should be as large as possible (but \( K_Y < K_B + 1 \) would generate unstable huge granules), while for fixed \( K_B \), \( K_B \) should equal \( K_Y \). It is as if in the evolution game, the equality of the two SNARE rosette sizes is a Nash equilibrium. These theoretical findings were matched by the 12-example meta-analysis: the scattergram of \( K_Y \) and \( K_B \) reveals that \( K_B = K_B + 1 \) (borderline stable equilibrium) in two out of the 12 examples, while \( K_Y = K_B \) in the other 10 examples. In other words, for evolutionary safety of granule fusion kinetics, the SNARE aggregate size generally resulted in one step ‘backwards’ from the borderline stable optimum, to guarantee stability.

This work was supported by Tel Aviv University Intramural Research Programme Funds.

REFERENCES


Granule membranes play dice. I. Hammel and I. Meilijson 2525


54 Meilijson, I. 2003 The time to a given drawdown in Brownian Motion. In Seminaire de Probabilites XXXVII, Springer Lecture Notes in Mathematics, no. 1832, pp. 94–108. Berlin, Germany: Springer.


