Regulation of Quantal Size by Presynaptic Mechanisms

David Sulzer and Emmanuel N. Pothos

Departments of Neurology and Psychiatry, Columbia University, Department of Neuroscience, New York State Psychiatric Institute, New York, USA

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Reprint address:
Dr. David Sulzer
Black Building, Room 305
650 West 168th St.
New York, NY 10032, USA
e-mail: ds43@columbia.edu
Dr. E. Pothos: e-mail: ep155@columbia.edu

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SYNOPSIS

Quantal size is often modeled as invariant, although it is now well established that the number of transmitter molecules released per synaptic vesicle during exocytosis can be modulated in central and peripheral synapses. In this review, we suggest why presynaptically altered quantal size would be important at social synapses that provide extrasynaptic neurotransmitter. Current techniques used to measure quantal size are reviewed with particular attention to amperometry, the first approach to provide direct measurement of the number of molecules and kinetics of presynaptic quantal release, and to CNS dopamine neuronal terminals. The known interventions that alter quantal size at the presynaptic locus are reviewed and categorized as (1) alteration of transvesicular free energy gradients, (2) modulation of vesicle transmitter transporter activity, (3) modulation of fusion pore kinetics, (4) altered transmitter degranulation, and (5) changes in synaptic vesicle volume. Modulation of the number of molecules released per quantum underlies mechanisms of drug action of L-DOPA and the amphetamines, and seems likely to be involved in both normal synaptic modification and disease states. Statistical analysis for examining quantal size and data presentation is discussed. We include detailed information on performing nonparametric resampling statistical analysis, the Kolmogorov-Smirnov test for two populations, and random walk simulations using spreadsheet programs.

KEY WORDS

box-and-whisker, cumulative distributions, cyclic voltammetry, non-parametric, normal probability plots, VMAT2

PART 1: INTRODUCTION AND BACKGROUND

The quantal release event is the basic unit of synaptic transmission. Early studies, most prominently by Katz and colleagues /111,112/, demonstrated that histograms of endplate potential amplitudes produced bins that could be simulated using a Poisson distribution reflecting integer multiples. The size of the apparent fundamental unit was identical to the size of spontaneously occurring miniature endplate potentials. An important insight was that the miniature potentials could be emulated by iontophoresis of acetylcholine (ACh), suggesting that they were due to the nearly simultaneous release of many ACh molecules. This unit was labeled a quantum in analogy to a concept in particle physics that describes the discrete energy levels by which molecules absorb or emit energy.

Concurrently with these physiological studies, electron microscopy demonstrated the presence of vesicles in synapses and neurosecretory cells, and it was soon suggested that quantal release was due to the exocytosis of a single vesicle. This hypothesis was confirmed by numerous findings: a) compounds that cause vesicle fusion also induce transmitter release; b) the presence of transmitter in isolated synaptic vesicle fractions; c) the presence of the osmophilic transmitter analogue 5-hydroxydopamine in synaptic vesicles observed with the electron microscope; d) the finding that inhibition of exocytosis abolishes transmitter release; e) inhibition of vesicular uptake transporters inhibits release; f) stoichiometric release of neurotransmitter with other intravesicular components such as chromogranins; g) a delayed inhibition of release by inhibition of vesicle recycling; h) ultrastructural images of secretory vesicles observed in the act of fusion (omega profiles); i) uptake of endocytic tracers into synaptic vesicles followed by destaining during subsequent transmitter release. These findings are detailed in numerous reviews /38,95, 98,102,113,178,219,238,273/.

ABBREVIATIONS

ACh acetylcholine; AChE acetylcholinesterase; ATP adenosine 5'-triphosphate; BDNF brain-derived neurotrophic factor; CNS central nervous system; DA dopamine; DAT dopamine transporter (plasma membrane); EAAT excitatory amino acid transporter; GDNF glial-derived neurotrophic factor; H7 1-[5-isouquinoliny1-sulfony1]-2-methyl-piperazine; H8 N-[2-(methylamino)ethyl]-5-isouquinolinesulfonamide; L-DOPA L-dihydroxyphenylalanine; NT-3 neurotrophic factor-3; NT-4 neurotrophic factor-4; PKA protein kinase A; PKC protein kinase C; PMA phorbol 12-myristate 13-acetate; VMAT1 peripheral vesicular monoamine transporter; VMAT2 central vesicular monoamine transporter.
A long-held assumption in neuroscience is that the amount of transmitter represented by a quantum at a given synapse is invariant. This assumption has had a huge influence on the interpretation of data in synaptic physiology, focusing many studies on interpreting data on altered magnitude of postsynaptic response per quantum (this is the classical definition of quantal size) and modulation of the number of quanta released (quantal content). Indeed, for reasons discussed in the following section, the assumption of invariance of quantal size if measured by postsynaptic charge is often accurate, particularly for fast-acting ionotropic transmitter systems. However, the assumption of invariance of presynaptic quantal size has always been an attempt to simplify a truly complex situation by modeling one parameter as constant.

In models in which presynaptic quantal size is treated as invariant, numerous possible mechanisms of presynaptic plasticity are overlooked. There has been a long-standing effort on the part of Van der Kloot and colleagues to detail possible presynaptic mechanisms that lead to altered quantal size in the neuromuscular junction. Their reviews, which precede the advent of amperometric quantal detection in the CNS, are highly recommended as companions to this article /242,248/ and we intend to extend rather than duplicate the information in those reviews.

A note on nomenclature is necessary. Katz coined the term quantal size to refer to the amplitude of the miniature postsynaptic potential. Following the introduction of presynaptic quantal recording by Wightman and colleagues in the early 1990s /127,259/, Ewing with our group adapted this technique to examine alterations in the amount of transmitter released per vesicle exocytosis /118, 220/. We elected to use the term quantal size to report the number of transmitter molecules released per quantal amperometric event. Regardless of the wisdom of our decision, the term is now incorporated into the field; for brevity, we use the term quantal size in this article to refer to presynaptic quantal size, i.e., the number of molecules released per quantum, rather than alterations in postsynaptic responses.

An additional focus of this review is to examine quantal size regulation in catecholamine systems that are adapted for providing neurotransmitter that flows beyond its synapse. Until the advent of amperometric recordings of quantal release, observation of quantal release had not been observable in catecholamine neurons, due to lack of rapid postsynaptic currents. Now, with direct presynaptic observation of quantal release, there is striking evidence that quantal size of monoamine transmitters is extremely plastic and regulated by multiple mechanisms. We categorize currently identified mechanisms of plasticity as:

1. Alterations of the transvesicular free energy (concentration gradient and protonotive force) that regulate transmitter accumulation (Section V);
2. Regulation of vesicle transmitter uptake systems (Section VI);
3. Regulation of fusion pore expression (Section VII);
4. Modulation of degranulation from the intravesicular matrix (Section VIII);
5. Regulation of vesicle recycling and/or vesicle volume (Section IX).

II. ARE THERE FUNCTIONAL CONSEQUENCES OF PRESYNAPTIC QUANTAL SIZE REGULATION?

A. Does receptor saturation occur after release of a quantum?

To model effects that could be mediated by altered quantal size, we adopt parameters from the literature on dopamine (DA) release in the nucleus accumbens. This site is selected since the strongest evidence for plasticity of quantal size in the CNS is for the terminals of DA neurons that make up the mesolimbic projection. The following models describe how altered quantal size could mediate profound changes in synaptic input.

Collectively, the models show that for synapses in which transmitter release does not flow beyond its synapse (private synapses), altered quantal size has an effect if receptors are not normally saturated, and that there is also an effect on the duration that transmitter is present at the receptors. In contrast, for synapses that donate transmitter beyond their synapses (social synapses), changes in quantal size always affect receptor activation. These concepts
extend back to Ramon y Cajal, who suggested point-to-point neurotransmission in the brain, and Golgi, who proposed diffuse transmission /274/. Increases in quantal size in both models are functionally similar to releasing a greater number of quanta per action potential. However, in a social synapse, increased quantal size has an effect similar in part to actions of plasma membrane uptake blockers, such as cocaine, as both interventions would enhance the distance neurotransmitter diffuses and the duration extracellular neurotransmitter is present following synaptic release. Of course an individual synapse may exhibit aspects of both characteristics.

The relevance of extrasynaptic overflow at some synapses remains controversial. Kullman and colleagues have been active in addressing this question for glutamate transmission, studying the Schaeffer collateral-CA1 pyramidal cell synapse; to date, it appears that extrasynaptic spillover occurs at room temperature /124/ but is negligible at physiological temperature /11/. If this is true, a version of the private synapse model is pertinent to glutamatergic CNS synapses under physiological conditions. However, it should be noted that there is a large amount of literature, particularly using microdialysis, that supports extrasynaptic diffusion of transmitter for CNS cholinergic, GABAergic and glutamatergic systems. It is generally claimed that the overflow is of neuronal origin as it is abolished by tetrodotoxin and it is calcium-dependent; however, there is calcium-dependent glutamate release from astrocytes /25/.

There is evidence in support of saturation of postsynaptic sites from the contents of a single vesicle in glutamatergic systems /66,228/ as well as indications that saturation may not occur in synapses of hippocampal cultures and slices /77,132/. As mentioned, if there is normally no receptor saturation (clearly often true at the neuromuscular junction), increased quantal size would have an effect in such private synapses.

In contrast, evidence in support of diffusion of transmitter beyond DA and serotonin presynaptic terminals is very strong. This phenomenon has been examined and reviewed in studies by Fuxe, Agnati, and colleagues who suggest that neurotransmission can be either “wired”, i.e., activating a constrained neuronal chain, or “open” with diffusion beyond the synaptic cleft /274/. There have been several additional reviews of extrasynaptic neurotransmission /14,31,252/.

Diffusion of DA and serotonin beyond their synapses must occur, as these transmitters would not otherwise be detected by cyclic voltammetry in the extracellular space following electrical stimulation. This fundamental observation has been detailed in elegant studies by Wightman and colleagues /30,81/ and the following models are based on these reports. It is also instructive to examine the review by Stjarne et al. on quantal release in sympathetic tissue /216/. They conclude that released monoamines in sympathetic synapses diffuse beyond the synapse, whereas the fast acting transmitter ATP does not overflow, due to rapid metabolism by extrasynaptic ectoATPase.

Another indication that extrasynaptic transmitter release occurs is that quantal DA release from midbrain DA axonal varicosities in culture can be detected by amperometric recording /187/. Since the electrode is far too bulky (5 μm diameter) to be embedded within the cleft of a conventional synapse (~20 nm), and these rapid events must be very close to the electrode to be detected (see Fig. 10), vesicle exocytosis from DA neurons can occur at sites distal from a classical synapse.

Extrasynaptic transmitter would seem to have a physiological role since the DA uptake transporter (DAT) and most DA receptors including the D2 DA receptor are generally not found close to the morphologically identified presynaptic sites /62,94, 164,209/. DAT in particular may always be found outside of the synaptic cleft, although this point is controversial. Therefore, extrasynaptic transmitter must occur for these receptors to be activated. However, it should be noted that monoamine axonal varicosities at the ultrastructural levels often show relatively sparse and less obvious synaptic specializations in comparison to fast-acting transmitter terminals /163,164/. In contrast, receptors for amino acid transmitters tend to be highly localized in the synaptic cleft.

In the remainder of this section, we estimate consequences of altered quantal size using two models that predict D2 receptor exposure following quantal DA release in the nucleus accumbens. D2

REVIWES OF THE NEUROSCIENCES
receptors, which are found both pre- and postsynaptically in the striatum, mediate the reward pathway and voluntary motor control. These receptors are the target of the family of antipsychotic drugs used to treat schizophrenic psychosis, and as related below, it is possible that alteration of quantal size may be involved in antipsychotic action. We model D₂ receptors in the high affinity state, since it is now thought that the high affinity state is actively coupled to G proteins, and so plays a more important physiological role than D₂ receptors in the low affinity state.

The following estimates are adapted from the literature. Note that background DA levels are in dispute /181/.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>background extracellular [DA]</td>
<td>5 nM</td>
<td>/177/</td>
</tr>
<tr>
<td>extracellular [DA] following 5 mg/kg amphetamine</td>
<td>5 μM</td>
<td>/50/</td>
</tr>
<tr>
<td>Kₘ for DA at high affinity D₂ receptor</td>
<td>10 nM</td>
<td>/166/</td>
</tr>
<tr>
<td>Km of DAT in nAcc</td>
<td>0.16 μM</td>
<td>/141/</td>
</tr>
<tr>
<td>Vₘₐₓ of DAT in nAcc</td>
<td>3 μM/sec</td>
<td>/81/</td>
</tr>
<tr>
<td>DA synapse density in striatum</td>
<td>1 x 10⁹/mm³</td>
<td>/182/</td>
</tr>
<tr>
<td>diffusion coefficient for DA in aqueous solution</td>
<td>6.9 x 10⁻⁶ cm²/sec</td>
<td>/160/</td>
</tr>
<tr>
<td>apparent diffusion coefficient for DA in brain</td>
<td>2.7 x 10⁻⁶ cm²/sec</td>
<td>/160/</td>
</tr>
<tr>
<td>extracellular volume fraction</td>
<td>0.2</td>
<td>/161/</td>
</tr>
<tr>
<td>DA molecules released per vesicle</td>
<td>2,400</td>
<td>/187/</td>
</tr>
</tbody>
</table>

### B. The private synapse

In this model there is no transmitter flow between postsynaptic areas, and transmission is a form of “wired” communication between cells /274/ (Fig. 1). The perisynaptic volume is selected as the spherical volume per DA synapse in the nucleus accumbens. From the reported density of DA synapses in the nucleus accumbens, the perisynaptic volume would be

\[ 1 \times 10^8 \text{ synapses/mm}^3 = 1 \text{ synapse/10 μm}^3 \]

If the perisynaptic synapse is a sphere,

\[ \frac{4}{3} \pi (r \text{ μm})^3 = 10 \text{ μm}^3 \]

and the perisynaptic radius \( r = 1.34 \text{ μm} \).

The perisynaptic volume through which transmitter can diffuse is restricted by the extracellular space. This extracellular volume fraction for compounds <500 mw is about 0.2 /161/, so that the extracellular perisynaptic volume is \( 2 \text{ μm}^3 = 2 \times 10^{-18} \text{ m}^3 \).

Adopting estimates of receptor and transporter densities derived from binding studies from Garris et al. /81/, each 10 μm³ volume would contain 1750 DAT uptake sites, 1655 D₁-like receptor sites, and 433 D₂-like receptor sites, a total of 3838 sites. Normally, one would suppose that a significant fraction of high affinity D₂ receptors would be bound at background levels (5 nM), which are close to the dissociation constant for the receptor (10 nM). However, in the private synapse there would be negligible receptor binding because the volume is so small. The number of DA molecules within the perisynaptic volume at background levels would be

\[ \frac{5 \times 10^{-9} \text{ mole}}{(0.1 \text{ m})^3} \times (2 \times 10^{-18} \text{ m}^3) \times \frac{6.02 \times 10^{23} \text{ molecules}}{\text{mole}} \]

\[ = 6 \text{ molecules} \]

Therefore, receptor binding at background levels in this model is \( 6 ÷ 3838 = 0.15% \).

If the number of molecules in a DA quantum in the nucleus accumbens is similar to that in culture medium, the modal value is \( \sim2,400 \) molecules (mean value \( \sim3,000 \) molecules) released in < 200 μsec. This would bind 62.5% of the receptors. As discussed below, if supplemented with glial-derived neurotrophic factor (GDNF), the modal value is \( \sim4,400 \) molecules (mean value \( \sim7,000 \) molecules), which would temporarily saturate all postsynaptic binding sites. (Garris et al. estimated 1,000 molecules released per quantum using MFB stimulation and cyclic voltammetry in vivo. Estimates of quantal size associated with synaptic
vesicle exocytosis at the neuromuscular junction are 7,000-10,000 (242).

The kinetics of DA flux within the perisynaptic space can be determined by a random walk simulation (Fig. 2). When a nearly heterogeneous concentration is reached (< 5 msec for the DA diffusion coefficient in brain) the local DA concentration would be

\[
\frac{2,400 \text{ molecules}}{2 \times 10^{-18} \text{ m}^3} \times \frac{1 \text{ M}}{(6.02 \times 10^{23} \text{ molecules} / (0.1 \text{ m})^3)} = 2 \text{ } \mu\text{M}
\]

far above the \( K_D \) of D₂ (10 nM); still, only 2,400/3838 = 62.5% of the perisynaptic receptor sites would be bound. The concentration predicted in this model is strikingly close to that measured experimentally in the ventral striatum using cyclic voltammetry following single pulse stimulation of the DAergic terminals with an extracellular bipolar electrode /81/.

In addition to altering the initial binding of dopamine receptors, altered quantal size would affect the duration the transmitter was present in the perisynaptic space. Since diffusion is restricted to the perisynaptic space, DAT would be expected to limit the duration. To calculate this duration, DAT is assumed to follow Michaelis-Menten kinetics. The Michaelis-Menten equation can be integrated over time.

\[
v = \frac{\partial[DA]}{\partial t} = \frac{V_{\text{max}} [DA]}{K_M + [DA]}
\]

\[
V_{\text{max}} \frac{\partial t}{\partial t} = \frac{K_M x \partial[DA]}{[DA]} - \partial[DA]
\]

\[
V_{\text{max}} \int_{t_i}^{t_f} \partial t = -K_M \int_{t_i}^{t_f} \frac{\partial[DA]}{[DA]} - \int_{t_i}^{t_f} \partial[DA]
\]

\[
V_{\text{max}} t = -K_M [DA]_l [DA]_h - ( [DA]_h - [DA]_l )
\]

\[
K_M \ln \frac{[DA]_h}{[DA]_l} + [DA]_h - [DA]_l
\]

\[
t = \frac{[DA]_h - [DA]_l}{V_{\text{max}}}
\]

[Eq. 1]

As can be seen from Equation 1, the relationship between the initial neurotransmitter concentration and the duration required for reuptake is logarithmic at low concentrations and becomes linear as higher levels begin to saturate the transporter.

From this relationship, for a quantal size of 2,400 molecules (2 \( \mu\text{M} \) in the perisynaptic volume), the time at which DAT action would reduce the concentration to 10 nM (12 molecules; the dissociation constant of a D₂ receptor) would be 940 msec.

**Effects of decreased quantal size**

A decrease in quantal size would have a consequence if the number of molecules released is lower than the number of binding sites. With a decrease in quantal size to ~20% (480 molecules; 0.4 \( \mu\text{M} \)) as occurred following reserpine exposure /122/, only 12.5% (480/3838) of perisynaptic binding sites would be occupied. DAT activity would bring DA levels to 10 nM in 325 msec.

**Effects of increased quantal size**

A quantal event that was increased to 500% of control levels (12,000 molecules; 10 \( \mu\text{M} \)), as observed following L-DOPA /187/, would saturate all receptor sites and would require almost 4 sec before DAT action would decrease it to 10 nM.

Although there may not be transmitter diffusion beyond fast-acting amino acid transmitter synapses, there are conditions where these synapses appear not to be saturated by a quantum /20,77,78,132, 133,233/. In these cases, regulation of presynaptic quantal size would play an important role. Most convincingly, non-saturation must be common at the neuromuscular junction since altered quantal size can be measured, as shown by Van der Koot’s studies.

Central glutamate synapses at the ultrastructural level are surrounded by astrocytes that express excitatory amino acid uptake transporters (EAATs) that should limit diffusion beyond the synapse. Therefore, a private synapse model readjusted for the smaller perisynaptic volume for a fast-acting transmitter system would be better adapted for this synapse than for a central monoamine synapse. GABA synapses expressed by cultured striatal neurons show highly variable quantal sizes, and this
Fig 1: In the *private synapse*, we assume a perisynaptic sphere with a boundary as shown, beyond which dopamine (DA; red) transmitter diffusion beyond the sphere does not occur (white area beyond the sphere). Quantal release would activate both postsynaptic and presynaptic receptors (black objects), including those on DA or non-DA presynaptic elements within the sphere. Since DAT (grayed object) activity decreases extracellular DA much more avidly than extracellular metabolism, DA remains within this volume until taken up by the DAT.

Fig 2: Random walk simulation to model diffusion of 2400 DA molecules from a quantal event in a 2.68 μm diameter perisynaptic space. This uses the diffusion coefficient for DA in the brain which takes into account the extracellular fraction. The molecules are released at the midpoint (1.34 μm: the distribution at time 0 would be represented by a single point at 2,400 molecules). Near-homogeneous concentration is rapidly reached. An abbreviated version of this calculation is in Appendix A.

Fig 3: In the *social synapse* model there is no obstacle to the diffusion of DA beyond the 10 μm³ perisynaptic space. DA released from the axonal terminals would bind to receptors on multiple neighboring pre- and postsynaptic structures.
could be controlled by maintenance of different presynaptic quantal sizes at different sites /18,78/.

C. The social synapse:

Extrasynaptic diffusion model

In this model (Fig. 3) there is no restriction on the flow of extrasynaptic DA beyond the perisynaptic volume, and so is equivalent to “volume transmission” where transmitters diffuse in three dimensions beyond the synaptic cleft. For clarity, we first discuss consequences of extrasynaptic diffusion for a single quantum and later for release from multiple neighboring sites. The second condition seems more relevant from the finding that appetitive stimuli elicit action potentials in ∼70% of midbrain DA neurons /149/.

For a single quantum with no volume restriction on diffusion, diffusion over a short distance is extremely rapid and would reduce the concentration more quickly than reuptake. To characterize diffusion for a population of molecules, Fick’s first law can be solved for a set of particular conditions. These derivations can be quite complex /24,55/. For three-dimensional diffusion of a population of particles released instantaneously from a point source, such as diffusion of a drop of dye from a micropipette, Fick’s first law has been solved as

$$C = \frac{N}{(4\pi D t)^{3/2}} \times e^{-r^2/4Dt}$$

where $r$ is the radial distance from the point source and $N$ is the number of molecules /24/.

The random walk has some advantages over solving Fick’s law. These simulations can be easily performed using a spreadsheet program (Appendix A). Additionally, changing the position or nature of the boundary conditions (specifying a reflective or absorbing boundary) requires solving Fick’s first law for each new situation, whereas a random walk requires only substituting a calculation in the appropriate bin.

If a quantum is released at a reflective boundary on the X axis and diffuses in an unrestricted manner in the Y and Z axes, the local density of molecules can be predicted by running a random walk for each dimension and determining the product of the fractional proportion of molecules in each three dimensional bin (Appendix A). For 2,400 DA molecules in a 2 μm³ water droplet placed in water, free diffusion would reduce the maximum local concentration to 10 nM in 65 msec. Even using the smaller diffusion coefficient for DA in the brain to take into account the extracellular volume fraction and tortuosity /161/ and a 10 μm³ volume, it would require only 140 msec for 2,400 molecules to reach a maximal local concentration of 10 nM.

However, cyclic voltammetry studies of stimulated DA release in the slice indicate that it takes substantially longer than 140 msec for extracellular DA to reach baseline following stimulation. Apparently, diffusion does not primarily regulate the duration that DA is present in the extracellular milieu. One reason for this is probably because quanta are released from multiple nearby synapses so that diffusion from the perisynaptic volume is compensated by diffusion into the perisynaptic volume. Empirically, DAT activity has the most prominent effect on extracellular DA levels following electrically stimulated release /84,109/.

The relationship between diffusional distance and time for molecules was shown by Einstein to be

$$t = \frac{x^2}{2D}$$

[Eq. 2]

where $x$ is the distance the average molecule has traversed along the x axis, $D$ is the diffusion coefficient, and $t$ is the time after release /73/. The $x$ represents the midpoint of a Gaussian distribution.

From Equation 1, a quantum of 2,400 DA molecules would reach 10 nM at 942 msec by DAT action. During this period, from Equation 2, a typical DA molecule in the brain would travel along the x dimension

$$x = \sqrt{2Dt}$$

$$= \sqrt{2 \times (2.7 \times 10^{-4} \text{cm}^2/\text{sec})(0.942 \text{sec})}$$

$$= 0.00225 \text{ cm} = 0.0225 \text{ mm}$$

Along the x dimension, this molecule would encounter

$$0.0225 \text{ mm} \times \frac{1 \times 10^7 \text{ synapses}}{\text{mm}} = 10.46 \text{ synapses}$$
If motions in each plane are independent, the number of synapses available to the molecules would increase with $n^3$. Using the midpoint of the distribution of distance traversed from Equation 1, DA molecules released from a single synapse over the course of 942 msec would encounter $(10.46)^3 = 1147$ perisynaptic volumes (Fig. 4).

**Effects of increased quantal size**

A quantum of 12,000 molecules would reach 10 nM at 3,678 sec. During this period, DA molecules would encounter 8,852 synaptic volumes. This would be similar to the increased temporal and spatial exposure of DA caused by a DAT blocker.

**Effects of psychostimulants**

As detailed in Section V, amphetamine decreases quantal size and evokes reverse transport, as well as acting as a competitive inhibitor for DA uptake. The very high extracellular DA levels measured after amphetamine administration should result in occupation of all binding sites. In this case, there would be virtually no normal stimulation-dependent response to quantal release. However, the effects on quantal size may last longer than the effects on extracellular levels, since the free energy harnessed for vesicular monoamine uptake will be dissipated (see Section V). The predicted loss of discrete, focused input could have important long-lasting effects. For instance, amphetamine disrupts working memory, a process in which regulated spatial and temporal DA input are required /262/.

In the case of cocaine, there is a slow decrease in quantal size in PC12 cells, probably due to limiting the amount of transmitter available for vesicular uptake /190/ (Section V). This may also occur in the brain, as suggested by the decreased stimulation-dependent DA efflux following stimulation in the DAT knockout animal /109/. In the case of both psychostimulants, the net effect would be to increase the noise for stimulation-dependent DA input by decreasing quantal transmitter release and simultaneously increasing stimulation-independent activation of synapses in addition to activating distant synapses that would otherwise not be exposed to a given input.

In summary, in a social synapse, a change in quantal size would always alter temporal and spatial characteristics of neurotransmitter flux. This condition seems to be met for modulatory transmitters such as DA and serotonin. These effects would be quite similar to those that arise by altering the number of quanta released (i.e., quantal content) or regulating uptake transporter activity. These effects would likely modulate actions of other

![Graph showing relationship between quantal size, duration extracellular DA, and number of synapses encountered.](image-url)
transmitter systems at private synapses. Potentiation of quantal size or frequency at a private synapse to the point that uptake transporters are saturated might convert the system to a social synapse.

Equivalent conclusions were reached by Stjarne et al. in studies of norepinephrine transmission in smooth muscle. He suggests that repeated quantal release into the junctional space saturates uptake transporters, further increasing the distance the transmitter diffuses, which allows the artery to contract briskly during high frequency bursts /216/.

III. TECHNIQUES

Until quite recently, all quantal recordings were conducted by recording rapid ionic currents or potentials from postsynaptic cells. This entails several disadvantages. First, the recorded duration of the event is inevitably much longer than the release event itself since the kinetics of the ion channels are measured rather than the release event itself. Second, postsynaptically recorded quanta are affected by factors including desensitization /86/, rundown during the recording, and alterations of second messenger systems. As a consequence, the number of molecules released and the duration of the quantal events in the CNS have been subjects of conjecture /3,215,233,242/. A third drawback is that many transmitters do not activate fast ionic currents so that their release cannot be observed at the quantal level.

Lately, amperometric techniques have allowed the direct presynaptic recording of quanta with vastly improved kinetics and without the concern that postsynaptic receptors may be altered. Amperometric techniques have other disadvantages, as they can be used only for a few neurotransmitters and only record release close to the electrode. Nevertheless, this approach has provided much information on quantal size regulation and kinetics. Other recently developed optical and physiological approaches, while unlikely to directly record changes in quantal size in the very near future, provide corroboratory information on presynaptic function. There are several recent reviews on presynaptic detection of exocytosis or quantal release /8,93,234/.

A. Postsynaptic recordings: neuromuscular junction

Quantal recordings from muscle fibers at neuromuscular junctions have provided a wealth of material on altering quantal release /111,242,248/. Here, two more recently developed approaches are mentioned.

Quantal studies at sympathetic nerve terminals in smooth muscle were introduced by Stjarne, Bennet, and their colleagues. The rat tail artery preparation is a sympathetically innervated system that releases at least three transmitters; norepinephrine, adenosine 5'-triphosphate (ATP) and neuropeptide Y. Amperometry is used to measure norepinephrine release due to nearly synchronous release of quanta from multiple sites while extracellular recording of the smooth muscle electrical response is used to measure quantal ATP release /216/. While little has been done so far to address modulation of quantal size, this preparation is quite promising for such studies.

A preparation for quantal recordings in Drosophila melanogaster is clearly desirable for examining genetic modulation of quantal release. A useful approach has been to record glutamate-mediated spontaneous miniature currents at the body-wall muscle of the third instar larval stage /103,104/. The abdominal hemisegments of the larva contain roughly forty motoneurons that synapse with thirty identified muscle fibers /60/. This preparation has recently been used to study modulation of quantal size in a clathrin adaptor mutant (LAP, homologous to mammalian AP180) that regulates endocytosis and the volume of synaptic vesicles by altering vesicular endocytosis /271/. There are also changes in quantal size regulated by protein kinase A (PKA) in this system. However, these changes may be due to postsynaptic alterations /59/ and a recent paper from Davis and Goodman on regulation of quantal size by innervation was unable to conclude whether the regulation was pre- or postsynaptic /60/.

An intriguing use of postsynaptic recording is to record human neuromuscular junction obtained from muscle biopsies. In anococus muscles from ten patients with amyotrophic lateral sclerosis, quantal size was smaller than in muscle from control patients, which could explain the impaired
neuromuscular function /144/. In intercostal muscle from a patient with congenital myasthenic syndrome, ACh receptors and morphology were normal but the miniature endplate potential was very small, suggesting a presynaptically decreased quantal size /236/. Muscle from familial infantile myasthenia displayed decreased quantal size following tetanic stimulation, which may be presynaptic /72/. Together, these studies indicate that alterations in quantal size may play a role in neuromuscular disorders.

B. Postsynaptic recordings: CNS preparations

It has proven far more difficult to characterize quantal size by postsynaptic recording in the CNS than at the neuromuscular junction. Since CNS synapses receive numerous synaptic inputs from many neurons, the origin of a given quantal event is generally unknown. The currents measured are variable due to electrotonic attenuation as the current traverses the cell, and because the events arise from multiple incoming synaptic contacts. Due to these difficulties, statistical methods of quantal analysis are used to estimate quantal release. The parameters are borrowed from neuromuscular junction studies; the probability $p$ of release of a single quantum, the total pool of available vesicles $n$, and the mean number of quanta released $m$, also known as quantal content. Different models for these statistical treatments in experiments in brain slice preparations have led to a variety of controversial and conflicting interpretations /21,120,197,213,255/.

To more clearly estimate these parameters, researchers have recorded from neurons with large endbulb synapses /99/ and hippocampal microcultures in which small numbers of neurons form autaptic synaptic connections /22,208/. A very promising direction is the use of loose patch recording of individual boutons in hippocampal cultures /77,139/ which greatly reduces problems from electrotonic attenuation and overlapping events. Another system is the organotypic culture preparation, in which a brain slice is cultured. This destroys neurites extending from neurons outside of the slice and thins the slice, providing improved recording conditions. This approach has been explored by Thompson and Gahwiler to characterize glutamate and GABA quantal release in hippocampal preparations /35,61,80,185,231/.

At the time of writing, nearly all alterations in synaptic strength by postsynaptic recording in mammalian CNS have been identified as being due to altered release frequency /39/ or altered postsynaptic response /229/. In long-term potentiation and long-term depression, there can be corresponding changes in both quantal size and frequency /172/ apparently due to alterations of NMDA receptors /162,173/, so that alterations in quantal size appear due to postsynaptic mechanisms.

C. Amperometric recordings

Amperometric techniques for presynaptic recording of quantal release events were introduced by Wightman and collaborators in studies of catecholamine release by large dense core granule exocytosis in adrenal bovine chromaffin cells /127,259/. These studies reported the first direct recording of quantal release events from the release site and introduced an approach by which the number of released molecules could be counted.

Detection of monoamines

Amperometry is most often performed by applying a potential sufficient to oxidize neurotransmitter at a carbon fiber electrode. The technique has been mostly used with the monoamines DA, serotonin, epinephrine, norepinephrine, and histamine. Following the initial studies in chromaffin cells, amperometric recordings of quantal monoamine release have used the leech Retzius cell /28/, the giant DA neuron of the pond snail Planorbis corneus /41/, peripheral neurons of the sympathetic ganglia /118,272/, glomus cells of the carotid artery /237/, and the pheochromocytoma PC12 cell line /42/. CNS recordings have been conducted at midbrain DA neurons at presynaptic terminals in culture /187/, apparently from substantia nigra somata in midbrain slices /101/, and acutely isolated dopaminergic retinal amacrine cells /96/. There are reviews on the use of amperometry in various systems /40,46,49/.

Detection using false transmitters. While electrodes have been designed that provide enzymatic
reactions to oxidize byproducts from other classical transmitters, particularly glutamate /119/, these approaches have so far provided responses too slow to distinguish quanta. However, at least two approaches using "false transmitters" appear promising for adapting amperometry in non-monoamine neurons. Hille and collaborators found that exposing a variety of secretory cell types, including hippocampal neurons, to very high exposures of DA (70 mM for 30 min) or other catecholamines could produce quantal release events measurable with amperometry /114/. Therefore, a system exists by which transmitter can be packaged even in the absence of the appropriate vesicular transmitter transporter /56,189/. At the time of writing, this intervention appears to be loading secretory vesicles that undergo constitutive rather than stimulation-dependent release, and are therefore likely not small synaptic vesicles.

Another approach is to convert secretory cells to a monoamine-secreting phenotype by inducing expression of the CNS vesicular monoamine transporter (VMAT2). Then the vesicles can be loaded using the DA precursor L-dihydroxyphenylalanine (L-DOPA) if there is endogenous aromatic acid decarboxylase as in AtT-20 cells (Fig. 5) /189/ or otherwise with DA itself (Sulzer and Pothos, unpublished results). This intervention may provide the means for quantal release from many other cell types to be measurable by amperometry.

Fig. 5: Effects of VMAT2 cotransfection on amperometric recordings of quantal release from the non-monoaminergic ACTH-secreting AtT-20 cell line /189/, transfected to express tyrosine hydroxylase /91a/. Traces of amperometric recordings from a representative tyrosine hydroxylase-only cell (a) and a VMAT2/tyrosine hydroxylase-cotransfected cell with nine release events (b) are shown. The arrows indicate the starting point of an application of 40 mM K+ (3 sec). These events fell into two categories, i.e., > 1 msec duration and at ~200 μsec, and may be due to the presence of two types of secretory vesicles /28/ or to altered expression of the fusion pore. Examples are shown of a wide duration peak from a tyrosine hydroxylase-only culture (c), a representative event from a VMAT2/tyrosine hydroxylase-cotransfected culture (d), and an event from a cotransfected culture exposed to L-DOPA (100 mM for 30 min) (e). f indicates a sample rapid event from a cotransfected culture.
Detection of neuropeptides

Quantal release of some neuropeptides has also been recorded by amperometry. Electrochemical oxidation of tryptophan and tyrosine residues in small proopiomelanocortin-derived peptides was measured amperometrically from cultured melanotrophs of the intermediate lobe of the rat pituitary /175/. Insulin has been detected from pancreatic beta-cells by preparing electrodes with a coat of ruthenium oxide and cyanoruthenate /97/.

Relationship of current to quantal size

The relationship between the Faradic current arising from oxidation of catecholamines or serotonin and the number of molecules released depends on two oxidation reactions that can take place per molecule (Fig. 6) /48,69/. The first oxidation reaction, conversion of the monoamine to a monoamine-o-quinone, occurs rapidly and donates 2 e-.

Following this, the amine group can either undergo deprotonation and cyclization to a leuco form or undergo another oxidation reaction to form a dopaminochrome.

\[
\begin{align*}
\text{DA} & \quad \rightarrow \quad \text{DA-o-quinone} \\
\text{DA-o-quinone} & \quad \rightarrow \quad \text{leucodopaminochrome} \\
\text{leucodopaminochrome} & \quad \rightarrow \quad \text{dopaminochrome}
\end{align*}
\]

Fig. 6: Oxidative reactions for dopamine (DA). The first reaction is rapid and donates 2 e- to the carbon fiber. The second reaction is a pH-sensitive cyclization, favored at low pH /69/. The forward step in the second reaction is rate-determining because leucodopaminochrome is efficiently converted to dopaminochrome in the third reaction, donating an additional 2 e- to the carbon. The leucodopaminochrome together with a quinone also reacts to regenerate DA (a disproportionation reaction). Therefore, depending on the time and pH, DA at the carbon fiber electrode can donate from 2 to 4 e-.

However, at physiological pH the second reaction is too slow to allow the disproportionation reaction to contribute current during the quantal amperometric event /48/.

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dopaminochrome or be re-reduced to DA. A second fast oxidation reaction involving oxidation of
leucodopaminochrome to dopaminochrome results in the donation of 2 additional e⁻.

The cyclization is strongly favored at acidic pH and is quite slow at physiological pH. This is
because for cyclization to proceed, both (1) the amino group must be deprotonated (pK = 9.9), and
(2) the quinone function must be protonated. Since quinones absorb UV but leucodopaminochrome
is UV transparent, the cyclization rate can be estimated by examining appearance and disappearance
of the quinone, which absorbs at 380 nm /69/. The observed rate of cyclization of the quinones to
leucoaminochrome is modeled as

$$\frac{-\delta[\text{quinone}]}{\delta t} = k_{\text{observed}}[Q]_{\text{total}}$$

at physiological pH with periodate used as an
oxidant. $k_{\text{observed}}$ for leucodopaminochrome formation $\equiv 0.1 \text{ sec}$, or a time constant $\tau$ of 1 / 0.1
sec $\equiv 10 \text{ sec}$. Similar results have been shown to occur with noradrenaline oxidation /70/.

In a study by Wightman’s group /48/, the rate constant for formation of the second set of elec-
trons from DA at physiological pH was $k = 0.07 \pm 0.03 \text{ s}^{-1}$, i.e., $\tau = 14.3 \text{ sec}$. The fraction of molecules
expected to undergo this reaction during the duration of a CNS amperometric event, $\sim 200 \mu\text{sec}$, would be

$$1 - e^{-kt} \equiv 0.001\%$$

The Faradic current that results from the second pair of oxidation reactions would occur long after
the amperometric spike resulting from the initial pair of oxidation reactions, and so would be lost in the
background noise. For longer duration events, such as those seen in adrenal chromaffin cells
($\sim 100 \mu\text{sec}$), the second reaction would still only account for $< 1\%$ of the DA or norepinephrine
during the amperometric event. However, there
would be significant effects on epinephrine current
spikes (the rate constants $k$ for norepinephrine and
epinephrine are 0.49 and 44 s⁻¹ respectively).

In summary, $2 e^-$ donated per molecule of
monoamine is the best estimate to relate charge and
number of molecules in the experimental con-
ditions used for these studies. Nevertheless, the
second reaction plays an important role under conditions of highly restricted diffusion. This was
pointed out by Bruns and Jahn /28/ who showed that if the catecholamines were trapped in a water
droplet surrounded by oil, the Faradic current was
well fit by 4 $e^-$ donated per molecule.

To determine quantal size from Faradic current,
the number of moles N is related to the total charge
of the event (the current integral) by

$$N = Q / zF$$

where $Q$ is the charge (Coul; amp x sec), $z$ is the
number of electrons donated per molecule, and F is
Faraday’s constant (96,485 Coul per mole equi-
valent). It is convenient to express this as
molecules/pCoul.

$$\left( \frac{6.023 \times 10^{23} e^-}{10^{12} \text{Coul}} \right) \times \frac{2e^-}{\text{mole}} = \frac{96,485 \text{Coul}}{\text{mole}}$$

$$= 3.121 \times 10^6 \text{ molecule/pCoul}$$

**Kinetics of large dense core granule signals**

The carbon fiber electrode response produces
little kinetic filtering. To characterize the electrode
response, we applied a 5 mV square pulse to the 5
μm diameter carbon fiber electrodes. The pulse rise
time was 5 μsec, the maximum sampling rate of the
boards we use (ITC-18, Instrutech, Great Neck,
NY). The resulting current showed exponential
growth and decay, as expected from the relationship

$$I = C \frac{\delta V}{\delta t}$$

where $C$ is the capacitance. The time constant of
the response was $\tau = 7 \mu\text{sec}$ ($143 \pm 9 \text{kHz}$, $n = 4$
electrodes). The rapid response of the carbon fiber
electrode indicates that there was essentially no
time distortion for events of duration in excess of
20 μsec. While this level of accuracy is not
achieved in practice unless there is suitable filtering
and rapid sampling, the estimated kinetics of the
release event are several orders of magnitude more
accurately measured than from postsynaptic
currents.

The utility of amperometric recording to
indicate kinetics of the release event was demon-
strated by Chow et al. /47/ who showed that in large
dense core granule exocytosis from chromaffin
cells, the expression of a low charge feature often
preceded the full amplitude spike. This feature, which they labeled a foot, is suggested to reflect a lower conductance state of the fusion pore preceding the full fusion event. This prediction has recently been borne out by patch amperometry techniques /1/ (Fig. 7).

![Image of Fig. 7: Appearance of a foot in a PC12 quantum /186/. The half height is indicated by the middle broken line. The foot, filled by an array of points, is estimated by integrating the portion of the event preceding the slope (broken line) between the 60% and 90% rise-times, similar to the approach by Chow /46/.

We have observed features in vesicular monoamine transporter-expressing neurosecretory (AtT-20) cells that may show extreme examples of the foot, including rear feet (Fig. 8). This seems unlikely if the rapid portion of the event were due to full fusion with the plasma membrane, suggesting that for some vesicles, the fusion pore may be expressed in multiple conformations.

**Kinetics of small synaptic vesicle signals**

Quantal release can be measured from axonal varicosities in midbrain DA neurons in culture (Fig. 9) /187,189,190/. The material measured by the amperometric recordings appears to be DA based on 1) reserpine blockade, 2) colocalization with tyrosine hydroxylase immunolabel, 3) dependence on sufficient oxidation potential, 4) absence of events recorded in hippocampal cultures that lack DA neurons, 5) events are recorded in non-monoamine neurons following heterologous expression of the vesicular monoamine transporter in the presence of DA, and 6) elevation of quantal size following L-DOPA or increased VMAT2 expression.

![Image of Fig. 8: In the AtT-20 adrenocorticotroph derived cell line, following induced VMAT2 expression (see Fig. 5), some amperometric spikes show extreme examples of feet (the slower, low amplitude component), suggesting that fusion pores can exist in multiple conformations or exhibit multiple conductances. a indicates one such event. The high amplitude portion is shown with increased time resolution in b.

Amperometric recording provided the first presynaptic observation of quanta from CNS neurons, and made it possible to directly calculate the number of molecules and the duration of release during exocytosis (3,000 DA molecules over 200 microseconds under control conditions when glial-derived neurotrophic factor is not included in the medium; Fig. 9A). The total duration of the events is generally < 0.5 msec, in contrast to those from large dense core vesicles, which are usually in the tens to hundreds of msec.

**What fraction of the released molecules is measured?**

How accurate are the kinetic readings of very fast events resulting from small synaptic vesicle release? Random walk simulations allow us to predict the role diffusion would play in these measurements. For quantal events the size measured in CNS preparations, the electrode needs to be very close to the release site or the event is buried in the background noise (Fig. 10).

With large quantal events in chromaffin cells recovery is better than 99% even at 5 μm distance from the cell. However, at such distances changes
Fig. 9: Representative examples of individual quanta measured at midbrain DA neuron axonal varicosities. A indicates examples of control quanta, B quanta from cultures treated with GDNF, and C from cultures treated with L-DOPA /187/.

Fig. 10: Random walk simulation modeling the relationship between the distance of the electrode from the release site and spike shape. 7000 DA molecules are released during a time bin of 1 μsec. The resulting amperometric current is simulated at electrodes 333 nm and 1 μm away from the release site. The membrane at the release site is modeled as a reflecting surface and the amperometric electrodes as absorbing surfaces. At 333 nm, the maximal amplitude is 28 pA and the t½ is 62 μsec. The amplitude and shape of the spike at this distance closely resembles that actually measured at synaptic varicosities in midbrain DA neuronal culture. At 1 μm distance from the electrode, it can be seen that this event would be lost in the noise, ~7 pA peak-to-peak in our real world system (using a 10 kHz 4-pole Bessel filter followed by a three point low-pass digital smoothing filter at 8 kHz). These calculations indicate that for any such event exhibiting a t½ of 62 or less, the electrode must be closer than 333 nm from the release site. The random walk simulation is explained in Appendix A.
in spike shape due to diffusional filtering are profound /203/.

The flux rate of transmitter through the fusion pore can also be estimated. (It should be noted that the expression of a fusion pore mediating exocytosis of small synaptic vesicles is controversial, although it is now accepted for large dense core vesicles.) While CNS events are too fast to estimate steady state flux, as in the foot preceding the full spike in chromaffin cells /1/, maximal flux can be estimated if the filtering is at a high frequency, diffusion is over short distances, and sampling rates are used that achieve a reasonably good estimate of amplitude. (This would be convincing if sampling is sufficiently rapid to measure several data points close to the maximal amplitude.) For the amperometric event from a VTA neuron shown in Fig. 11, the maximum flux recorded during the amperometric spike is 52 pA during a single 20 μsec bin, equivalent to

\[
\frac{3.12 \times 10^6 \text{ molecules}}{\text{pA} \times \text{sec}} \times 52 \text{ pA} = 1.62 \times 10^8 \text{ molecules/sec}
\]

We model the fusion pore as having a cross-sectional area \(a\) and length \(b\). The flux \(j\) of molecules per second through a circular aperture of diameter \(a\) in a non-absorbing barrier separating two media is

\[j = aD\Delta C\]

where \(D\) is the diffusion coefficient and \(\Delta C\) is the concentration gradient /24/. If the extracellular concentration is much lower than the vesicular concentration, \(\Delta C\) is close to the initial free catecholamine intravesicular concentration \(C_0\). Since transmitter flux through the plasma membrane should be inversely proportional to pore length /24/, in our model

\[j = \frac{aDC_0}{b}\]

We estimate a membrane spanning pore of \(b\) as 15 nm /1/. The synaptic vesicle volume for a 50 nm diameter sphere is

\[\frac{4}{3} \pi (2.5 \times 10^{-6} \text{ cm})^3 = 6.5 \times 10^{-17} \text{ cm}^3\]

If all of the transmitter were released during this event, the \(C_0\) in the synaptic vesicle (50 nm diameter) would be

\[
\frac{15,300 \text{ molecules}}{6.5 \times 10^{-17} \text{ cm}^3} \times \frac{1 \text{ M}}{6.02 \times 10^{23} \text{ molecules/(1000 cm}^3)} = 390 \text{ mM}
\]

---

**Fig. 11:** a. An amperometric spike recorded from a midbrain DA neuron in culture. This spike has a \(t_{90}\) of 70 μsec and represents 15,300 molecules. This recording was performed with a 4-pole Bessel filter at 10 kHz. b. The spike is transformed so that the bars indicate the difference of the time integral of the current in 20 μsec bins from the averaged baseline. The highest amplitude bin is 52 pA over the baseline.
This can be used to estimate the cross sectional area of the fusion pore.

\[
a = \frac{jb}{C_0D} = \frac{(1.6 \times 10^8 \text{molecules/sec})(15 \times 10^{-7} \text{cm})}{(2.35 \times 10^{20} \text{molecules/cm}^3)(6.9 \times 10^{-9} \text{cm}^2 / \text{sec})} = 1.48 \times 10^{-13} \text{cm}^2
\]

The fusion pore diameter is then

\[
2r = 2\sqrt{2a + 3\pi} = 3.5 \times 10^{-7} \text{cm} = 3.8 \text{nm}
\]

This is very close to the fusion pore diameter calculated from the steady state conductance and amperometric current during the fusion event during chromaffin granule exocytosis, \(~2.4 \pm 0.7 \text{nm} /1/\). It is striking that while large dense core vesicles in adrenal chromaffin cells have radii of \(~0.150 \mu \text{m} \) (volume of \(4/3 \pi r^3 = 1.4 \times 10^{-3} \mu \text{m}^3\)), whereas small synaptic vesicles have radii of \(~0.025 \mu \text{m} \) (volume of \(6.5 \times 10^{-5} \mu \text{m}^3\)), a difference of over three orders of magnitude, fusion pores of both types of secretory granules may have roughly the same dimensions. This would add further support to the suggestion by Neher that small synaptic vesicles may nearly always release their contents via a transient fusion pore rather than full fusion into the membrane /159/.

Lindau suggested that a similar approach can be used to estimate the intravesicular concentration /1/.

A related issue is to determine the velocity at which the fusion pore expands. Recordings of miniature endplate currents at motor endplates were used to estimate the rate of expansion of the fusion pore of \(>25 \mu \text{m/sec} /215/\). The very fast rise times observed in CNS amperometric recordings confirm this as a low estimate (see Fig. 10, in which release is modeled as instantaneous). This issue has been analyzed most thoroughly for dense core granules, for which capacitance recordings can resolve the opening of single pores during exocytosis /136, 202/.

**How can small events be recognized against the background?**

In CNS preparations, in which there is extensive vesicle recycling and refilling, there are expected to be vesicles recently emptied of transmitter that provide very small quanta when detected. It is therefore important to set a careful selection criterion for spikes that should exclude stochastic jiggling in the background. As a minimum amplitude threshold for spike detection in our laboratory, we generally use \(4.5 \times \text{the root mean square noise of the background} \). We use background subtraction so that the baseline is set at 0 pA, and the root mean square is the standard deviation of the noise. As the noise has a Gaussian distribution around 0 pA, the fraction of data points that would be \(>4.5 \times \text{the root mean square noise}\) can be determined from the normal probability density function for \(z \) standard deviations from the mean

\[
f(z;0,1) = \frac{1}{\sqrt{2\pi}}e^{-z^2/2}
\]

From this distribution, only \(3.4 \times 10^{-6} \) of background data points should have higher amplitudes than \(4.5 \times \text{root mean square noise} \). Empirically, we almost never observe noise of this amplitude in the medium or in cells that do not release monoamines.

An alternate approach, the “sliding template”, which has been used to identify miniature post-synaptic currents, could be useful for amperometric detection /51/. A short wave with the time course of an idealized event is slid along the trace and scaled to fit the data at each position. A reasonable duration of this wave was suggested to be about 4 time constants of the idealized spike. An event is identified if it crosses the threshold of the detection criterion (DC) defined as

\[
DC = \frac{\text{scale}}{\sum (\text{data point} - \text{ideal point})^2}
\]

where \(\text{scale} \) is the peak amplitude of the event. A threshold of \(DC = 4\) was suggested to produce reliable results for miniature currents. While the dependence on an ideal spike shape is clearly a concern, a good reason to consider this approach is that less filtering is required. Another advantage is that it “compensates” for background noise and can thus be used for every recording in an experiment even if the background is different from site to site. In contrast, detection using a threshold of a product of the root mean square noise will result in a variety of detection thresholds, depending on variation between electrodes and other factors.
D. Cyclic voltammetry

Cyclic voltammetry differs from amperometry in that a changing rather than constant potential is applied to the electrode. Most often the potential applied is a triangle wave (Fig. 12). For detection of monoamine transmitters, the applied potentials are generally capable of alternately oxidizing and reducing the molecule in question. In some protocols, the wave shape can provide relative selectivity to different oxidizable species, e.g., better distinction between DA and serotonin /30/ or DA and ascorbic acid /33/.

There are several advantages of cyclic voltammetry over amperometry. With cyclic voltammetry, compounds can be discriminated by their characteristic oxidation and reduction peaks. Recently, Wightman introduced a graph scheme that displays time on the x axis, applied voltage on the y axis, and resulting currents as a color scale /82/. This has the advantage that multiple molecular species and pH shifts are observed.

Due to alternating oxidation and reduction cycles, there is essentially no concentration gradient at the electrode face in cyclic voltammetry /260/. This non-destructive nature allows the concentration of the anolyte at the electrode face to be measured by comparison with known concentrations. This can yield an accurate kinetic estimate of transmitter in the extrasynaptic space. However, the temporal detail of the recording is limited by the rate that the oxidation potential is applied, so that quantal events may be lost in the background and kinetic resolution severely attenuated in comparison to amperometry. To utilize advantages of both techniques, our laboratory conducts both amperometry and cyclic voltammetry with the same electrode in the brain slice preparation.

Cyclic voltammetry with high frequency voltage waves has been used to demonstrate the identity of

![Graphs and figures]

Fig. 12: a. A triangle wave applied to the carbon fiber electrode. b. The current response to the triangle wave in a. The solid trace indicates the background response in saline, and the broken trace the response in DA. c. Background subtracted cyclic voltammogram derived from b represents the oxidation and reduction of DA. 5 μM DA was measured in this calibration; the shape confirms the identity of the transmitter, and the amplitude the concentration. Figure supplied courtesy of Dr. Yvonne Schmitz.
the monoamine transmitters released during the quantal release events from adrenal chromaffin cells /260/ and PC12 cells /122/.

E. Patch amperometry, a welding of quantal exocytosis and quantal release

Since biological membranes have a specific capacitance, Neher and colleagues developed capacitance recording in the patch clamp mode to detect vesicle exocytosis for large secretory vesicles /83/. A sensitive form of phase analysis to monitor capacitance known as the “Lindau-Neher” technique uses a sine wave voltage applied to the patch electrode. The resulting current is analyzed by a phase-sensitive detector to produce the real Re and imaginary Im current components (i.e., magnitude and phase components of the signal). The membrane capacitance $C_v$ and pore conductance $G_p$ are determined from the relationships

$$C_v = \frac{\left[ (Re^2 + Im^2) / Im \right]}{\omega}$$

$$G_p = \frac{(Re^2 + Im^2)}{Re}$$

where $\omega$ is the applied sinusoid frequency in radians.

Until recently, only very large vesicles such as mast cell granules had sufficiently large membrane surface areas to be observed at the quantal level following exocytosis. Mast cell granules have diameters of 0.7 $\mu$m, and a membrane surface area of $3 / 2 \pi (0.35 \mu m)^2 = 0.51 \mu m^2$. As membranes have a specific capacitance of $\sim 9$ fF/$\mu m^2$, the expected capacitance increase would be $\sim 5.2$ fF, which is detectable using conventional techniques.

However, exocytosis of smaller insulin-containing granules, which have diameters of 0.2 $\mu$m (0.042 $\mu m^2$, $\sim 0.38$ fF), is below the resolution of these techniques. Even more challenging, small synaptic vesicles in the CNS have diameters of 50 nm (0.003 $\mu m^2$, $\sim 0.024$ fF), or only $\sim 0.5\%$ of the area of mast cell granules. For these reasons, whole cell recording has not provided sufficient sensitivity to resolve individual small synaptic vesicle exocytosis in neurons, as seen studies of exocytosis in saccular hair cells /179/, photoreceptor cone synapses /199/, and retinal bipolar neurons /146/.

There have been statistical manipulations of data designed to estimate quanta embedded in multiple events in whole cell capacitance recordings /154, 90, 154/. However, these approaches cannot indicate whether there is a variety of quantal release events within the population.

Two recent developments by Lindau and colleagues provide recording of quantal exocytic events by vesicles the size of small synaptic vesicles and fundamental insights into quantal transmitter release. In cell attached capacitance recording /1, 136/ the membrane is not ruptured by the patch pipette, so that only the plasma membrane under the electrode is recorded. In this case, there is a lower likelihood of temporally overlapping exocytic events and the noise of the system is lower than in whole cell recording, $< 0.025$ pF, allowing individual events as small as 0.1 fF to be discerned.

Second, patch amperometry combines capacitance measurements and amperometric detection of catecholamine in the cell-attached configuration by introducing a carbon-fiber electrode (CFE) into the patch pipette /1/ so that vesicles fusing under the patch secrete their contents into the pipette. This provides excellent temporal coordination between exocytosis and transmitter release. Using this approach, the conductance of the fusion pore during the expression of the foot preceding the full amperometric spike has been calculated as 5.15 pS. Often the conductance during the full spike became infinite, suggesting full fusion of the vesicle with the plasma membrane. This study also suggested that a proportion of the amperometric events were from exocytic events that did not completely fuse with the plasma membrane and so were ‘kiss-and-run’ events. Confirmatory results demonstrating coordination of capacitance and amperometric traces have been reported by several groups /5, 44, 90, 170, 171, 225/.

F. Optical techniques

Optical techniques hold great promise for the study of synaptic preparations. Under conditions of limited stimulation, and using statistical approaches, quantal staining of the endocytic marker FM1-43 was demonstrated in hippocampal cultures /200/ and rates of small synaptic vesicle endocytosis estimated /117/.
Native and mutant forms of green fluorescent protein (GFP) have a highly pH-dependent emission. As synaptic vesicles maintain an acidic intravesicular milieu, exocytosed vesicles can be identified following exposure of the intralumenal GFP to the more alkaline extracellular environment /147,148/. While these techniques indicate vesicle exocytosis, they do not measure the amount of transmitter released.

An alternate approach is to use fluorescent false transmitters. Evanscent-wave fluorescence microscopy provides observation of large dense-core granule secretion using compounds such as the weak base vital dye acridine orange /214/ and may eventually allow optical observation of fluorescent transmitter analogs. A particularly promising technique is to use three photon microscopy to provide observation of endogenous serotonin in secretory granules in living preparations in culture /138,210/. This could indicate the amount of transmitter within a vesicle, and destaining due to exocytosis might estimate the extent of release.

Another avenue of great promise is to conduct postsynaptic quantal recordings by calcium indicator imaging in dendrites. This relatively non-invasive approach has been used to observe postsynaptic sites that undergo changes in activity by glutamate or protein kinase C (PKC)-mediated mechanisms /156/ and quantal calcium currents in hippocampal dendritic spines /269/.

IV. DATA ANALYSIS

A. Comparison of two populations of quantal sizes

No matter the experimental approach used for quantal recording, one cannot help but be impressed at the remarkable variability in synaptic responses. In experimental protocols in which there are large effects, such as exposure of DA neurons or PC12 cells to L-DOPA, all of the tests discussed easily reach statistical significance. However, in protocols where mean value differences are small (say, < 2-fold), the choice of an appropriate statistical analysis is important, and different tests can yield different conclusions /244/. Nonetheless, these smaller effects may be important physiologically; e.g., most studies of long-term potentiation explore < 2-fold differences in synaptic strength. In these cases, one should carefully consider the appropriate statistical analysis. With the exception of Van der Kloot’s reviews, there is little written on appropriate statistical methodology for this problem, and standard statistical references are incomplete in addressing some of the relevant issues. For these reasons, we discuss these approaches in detail. We also give advice on how to conduct these tests, and go into extensive detail for useful tests that are either not commercially available (resampling) or are incorrectly implemented in commercially available packages (Kolmogorov-Smirnov test for two populations).

A fundamental consideration is experimental design. In principle, it would be best to show an effect of a given intervention at a site and its recovery, and to replicate the finding several times while showing that the absence of the intervention has no effect on control sites. Essentially, this is a mixed design that both compares group differences and repeated measures within each group. In practice, this approach cannot always be performed, often because the experiments test genetic manipulations or other interventions that cannot be altered. Moreover, in another troublesome analogy to particle physics, the very act of experimental observation of stimulated release can alter the response to a later trial, since there are often profound stimulation-dependent effects. In such cases, it is necessary to compare results from multiple sites.

A second consideration is based on assumptions about the data distribution, since parametric tests such as ANOVA assume a normal distribution and homogeneous variance to estimate probability. Unless these conditions are met, a non-parametric test is called for.

A third consideration is that since there can be highly variable results at different sites, it may be desirable to use a robust statistical method, meaning a test that is relatively resistant to influences from outliers. A classic example of a robust nonparametric test is the Mann-Whitney rank sum or U test.
Non-parametric tests

The commonly used non-parametric tests for comparing two populations are the Mann-Whitney rank sum test and the Kolmogorov-Smirnov two-sample test. The Mann-Whitney is a distribution-free test, with its calculations performed on the ranks of the data values rather than the actual values. This feature makes the test robust. A commonly used test for comparing cumulative quantal populations is the Kolmogorov-Smirnov test, which is used by the laboratories of Thompson, Gahwiler, and Van der Kloo as well as our laboratory. This test makes only the assumption that the sample is random and continuous /57/, considers each value in the population, and in theory will identify differences at any point of the population. Another non-parametric approach that has yet to be widely applied for quantal analysis, randomization or resampling, has the advantages that it considers each value in many combinations and it can arrive at exact probabilities rather than probability estimates. However, these tests all assume that the samples are selected randomly and obtained independently /155/. These assumptions often do not hold for studies on quanta because the distribution of these events is often cell-dependent /52/.

Kolmogorov-Smirnov test for two populations

This test estimates the probability that two populations are different by examining the greatest difference between two cumulative distributions. Other tests could be designed following this approach, such as measuring the total area between two distributions. While the Kolmogorov-Smirnov is a popular test, standard statistics references do not explain how to perform the calculations, although there is a volume that supplies code and additional information for performing these calculations using FORTRAN or C /194/. We examined two commercially available programs, both of which performed the calculations incorrectly. For this reason, we compile the required information here. The following provides sufficient guidance to perform the test in a spreadsheet program.

First, the data from each of group A are ranked in ascending order and each data point assigned a rank \( n \). Each data point from group A is then assigned a fractional value \( n/N \) where \( N \) is the total number of data points in group A, \( i.e. \ N = N_A \). The procedure is repeated for each data point in group B. These fractional values derived from groups A and B are plotted in a cumulative plot where the y axis indicates \( n/N \) for each data point and the x axis indicates that point's quantal size (Fig. 13). This method is robust because the distributions are always identical at \( y = 0 \) and \( y = 1 \), close to outlier values.

The greatest absolute value of the difference \( D \) between the cumulative distributions along the y axis for a given \( x \) is then determined (in some texts this is labeled the supremum /57/). One way to determine \( D \) is simply measure the distance on the y axis between the two distributions. Another way is to use the \( n/N \) values already assigned. The values from the two groups \( (n/N_A \) and \( n/N_B \) with their original quantal sizes) are combined and resorted in order of increasing quantal size. A simple way to estimate \( D \) is then to subtract each neighboring \( n/N \) value and then sort the results to indicate the largest absolute value of the difference of these values. In our example, this estimates \( D = 0.298 \).

However, the simple approach to estimating \( D \) may slightly underestimate actual significant differences if one group has multiple data points clustered near the true statistic, which in the example shown is \( D = 0.304 \). Nevertheless, the estimated value is extremely close and more accurate than the values returned in the commercially available programs we tried. If an accurate \( D \) is desired in a spreadsheet program, one can use a nested logic function that reiteratively subtracts prior data points until the absolute value of the difference no longer returns greater values. In Microsoft Excel 5.0, which allows 7 nested iterations, a function for testing cumulative fractions in column C can be written at cell 8 as

\[ =\text{ABS(IF(C8-C7>C8-C6,C8-C7,IF(C8-C6>C8-C5,C8-C5,IF(C8-C5>C8-C4,C8-C4,IF(C8-C4>C8-C3,C8-C3,C8-C3>0,C8-C3))))} \]
C2,C8-C3,IF(C8-C2>C8-C1,C8-C2,IF(C8-C1>C8-C0,C8-C1))))

For C or FORTRAN compilers, code for determining $D$ is available /194/.

The effective N value used to calculate the probability for a two-sided test, in which differences that result in either increase or reduction of quantal size will be tested, is given by

$$N_{\text{eff}} = (N_A \times N_B) / (N_A + N_B)$$

where $N_A$ and $N_B$ are the number of data points in each set. For the example shown below $N_{\text{eff}} = 80.7$. This approximation is good for groups with $n > 4$ /194/, far below the limit of what one would examine for quantal size studies.

For a two-sided test, the null hypothesis is that any differences between the two populations are due to chance. A low $p$ value suggests that it is likely that the populations are different and the null hypothesis is rejected. The probability $p$ that $D$ is due to chance is determined from the test statistic $\lambda$ (sometimes called KS-Z) /194/.

$$\lambda = (\sqrt{N_{\text{eff}}} + 0.12 + (0.11 / \sqrt{N_{\text{eff}}})D$$

For the example, $\lambda = 2.73$.

Finally, the probability $p$ that the two groups are drawn from the same population is determined from the Poisson limit function determined by the relationship

$$p = Q_{KS}(\lambda) = 2\sum_{i=1}^{\infty} (-1)^{i-1} \times e^{(-2\lambda)}i!$$

Since this calculation is tedious to perform, we have prepared a table that relates the probabilities for different levels of $\lambda$ (Appendix B). For the data in Figure 13, the probability that the two sets of measurements are from the same population is $\sim 6.7 \times 10^{-7}$.

The Kolmogorov-Smirnov approach can also be used to produce confidence intervals to assist in determining differences in populations /244/.

Resampling/randomization tests

Randomization/resampling approaches are conceptually straightforward and well established, but until recently required too many calculations to enter wide use for comparisons between large populations /155/. Attractive features of the exact randomization test, and the similar Fisher's exact test, are that they make no assumption about data distribution and that they return the exact probability that two populations are equal. However, these tests are impractical to run in the contexts discussed in this review, as shown below. Resampling or approximate randomization, a non-parametric approach, was proposed by Van der Kloot as a similar practical approach for testing differences between quantal size populations /244/.

To perform an exact randomization, one first determines the mean values of two populations. For the data displayed in Figure 13, the mean quanta for controls ($A$) is 7,400 molecules and the mean
VMAT2 overexpressor (B) quanta is 19,300 molecules (apparent overlapping events, as below, were not included, so that for quantal size \(N_A = 74\) and \(N_B = 790\)). One combines all of the events, which in this case would yield \(N_A + N_B = 74 + 790 = 864\) quanta. Now comes the tricky part: one makes every possible combination of data points for groups of the size of the original \(N_s\). How many ways are there to select 74 quanta from 864? For the first selection, there are 864 possibilities, for the second 863, for the third 862, ... until one reaches 791. This number is about \(8 \times 10^{215}\), explaining why the technique is not widely used! Actually, this number is order dependent; to correct the number of combinations that could occur in any order, one divides the sum by 74! = \(3 \times 10^{107}\), the number of different orders in which each group of 74 measurements could be arranged. Therefore, to make an exact randomization test of these data, one would order each combination of \((8 \times 10^{215}) + (3 \times 10^{107}) = 3 \times 10^{108}\) groups and take the mean of each. Then one would find the fraction of these sets that would produce a mean the size of the original mean or smaller. The percentage of such combinations would yield an exact test of the null hypothesis, and at the rate of one trillion calculations per second would require only \(\sim 10^{60}\) years to perform.

Resampling is an analogous approach of great benefit to mortal investigators with finite durations between grant renewals. Van der Kloot’s approach is attractive due to its conceptual simplicity for quantal size comparisons /244/. Rather than determine the mean of every combination, one combines the values from groups A and B and then randomly selects a set of \(N_A\) values and then a set of \(N_B\) values. If the difference between the means of the two randomly selected sets is equal to or larger than the difference in means of A and B, a counter is incremented by one unit. This protocol is then repeated, say, 1000 times. Empirically, we found that by re-running this protocol on genuine data several times, 1000 means resulted in fairly reproducible distributions; the larger the \(N\), the more narrow the distribution, which in all cases we have examined is very close to normal (not shown).

The probability of the null hypothesis that the means of the two original populations are identical can be estimated by the fractional value of the counter. For instance, for the data in Figure 13, the difference between the mean values of groups A and B is 4233 molecules. In 1000 paired randomly shuffled values, no difference was greater than this value, so \(p < 0.001\). If 5 values > 4233 were observed, \(p\) would be \(5/1000 = 0.005\). If greater accuracy is desired, the test could be repeated, say, 10,000 rather than 1000 times. We demonstrate how to perform resampling with a spreadsheet program in Appendix C.

Van der Kloot provides a strong endorsement for resampling from an experiment in which he recorded four sets of measurements from neuromuscular junctions, two in the presence and two in the absence of nicotine /244/. He then compared each set with the other sets (six comparisons) by ANOVA of the mean values, Kolmogorov-Smirnov, and resampling. Resampling was the only test to correctly identify the different sets in every case (\(p<0.5\)).

**Comparing mean values: Box and whiskers plot**

There are several ways to compare mean values of quantal sizes at a descriptive statistics level. We prefer to show the distribution of the means of the cells or sites using a “bar and whiskers” plot (Fig. 14), which clearly indicates the median values and the distribution around the mean. We find this somewhat easier to interpret than simply displaying each data point, which requires significant effort from the reader. Outlying values can be shown as points.

**Mann-Whitney rank sum test**

The Mann-Whitney rank sum test (a.k.a. Wilcoxon rank sum test, Mann-Whitney U test) is advantageous because it is relatively robust. However, an underlying assumption is that the two distributions have the same shape which is not necessarily Gaussian /155/, in contrast to the Kolmogorov-Smirnov and resampling tests, which make no assumption about the distribution. Therefore it may not be appropriate for interventions that induce a subpopulation of new events.

The first step for the Mann-Whitney rank sum test is to combine and then rank the values from both groups from 1 ... \(N_{A+B}\). If there are ties, each
Fig. 14: Box diagrams are used to display the range of all mean values for each site recorded. Median values are indicated as a point within each box. In a variant of this approach, all mean values can be overlaid as points, which makes outliers obvious. In this example, 25% and 75% values of each distribution are reported as the box edges, and 10% and 90% values by the ends of the upper and lower lines; cells that released < 5 events within the 20 sec post stimulation were excluded from analysis. The distributions of mean values of quantal sizes per cell from control PC12 cells (n = 49 cells) and L-DOPA-treated (50 μM, 40 min) cells (n = 26 cells) are shown. Two conceptually straightforward ways to characterize the differences between the displayed values are by (1) the non-parametric Mann-Whitney rank sum test, which yields a z value = 3.697 and p = 0.00012 or (2) the parametric one-way ANOVA of the log(mean quantal size per cell), which for two groups is equivalent to a t-test of the log(mean quantal size per cell), and yields F = 6.75 and p = 0.0003. In the following sections, we explain how to conduct these tests.

of the tied values receives the average of the ranks for which they tie. Next, the ranks for each of the two groups are summed.

For example, to explore effects of L-DOPA on mean quantal size per cell, we recorded from 49 cells (2316 quantal events) in control and from 26 cells (1330 quantal events) in L-DOPA-treated PC12 cultures (Fig. 14). Therefore, there are 49 + 26 = 75 ranks assigned. The total sum of ranks would be 1 + 2 + 3 + 4 ... + 75 = 2850.

In this example, the sum of the ranks for controls = T1 = 1530 with a mean rank of 1530/49 = 31.22, and for L-DOPA the sum of ranks is T2 = 1320 with a mean rank of 1320/26 = 50.77. The sums of ranks can be compared with tables in statistics books for "Mann-Whitney", "rank sum", or "Wilcoxon rank sum" tests. Do not use tables for the "Wilcoxon signed rank test", as that test is for paired data!

In some tables, U values are used instead of T values. U is calculated as the smaller of the two terms

\[ U = T1 - Na (Na + 1) \]
\[ 2 \]

\[ U = T2 - Nb (Nb + 1) \]
\[ 2 \]

where Na and Nb are the number of data points in the two groups. For our example, U =305.

Often the published tables do not indicate values for the large Ns recorded in these experiments. If more than 10 data points are recorded per group, p can be closely approximated from the z value /155/, since the z values are assumed to follow a near-Gaussian distribution, in contrast to the data points.

\[ z = \frac{|U - (n1/2)|}{\sqrt{n1 n2 (n1 + n2 + 1)/12}} \]

In our example, \( z = 3.697 \).

The z value is used to estimate a p value from a Gaussian distribution table from the fraction of the
population that is within $z$ standard deviations from the mean. Or one can use a spreadsheet program to determine the standard normal cumulative distribution function. This formula is the same as the one describing the Gaussian distribution of the noise in the preceding section.

$$f(z;0,1) = \frac{1}{\sqrt{2\pi}} e^{-z^2/2}$$

In Excel 5.0, this formula is the NORMDIST function, and one can determine a two sided $p$ value using the command

$$=2*NORMSDIST(z)-1$$

and for a one sided $p$

$$=NORMSDIST(z)-1$$

Using this formula, 0.99978 of the population is within 3.697 standard deviations from the mean. Then the probability of the null hypothesis that the means of the two populations are drawn from the same population is $p = 1 - 0.99978 = 0.00012$.

**ANOVA/$t$-test**

If the data or transformed data are normally distributed, independently obtained, and the variance is homogeneous across groups, differences between mean or individual values per group can be determined by a one-way completely randomized analysis of variance (ANOVA). This design examines two or more different levels of a single independent variable (e.g., drug). The ANOVA test allows one to determine the extent that differences are due to variation within the groups and variation within the groups.

If more than one measurement is taken from the same cell (e.g., before and after a treatment on the same cell), an ANOVA for repeated measures is necessary. If more than one independent variable is tested, a two-way or factorial design ANOVA should be used. For example, if a study is on effects of a drug on cultures treated with three different growth factors, the design is a $2 \times 3$ factorial (total of 6 groups of data), where 2 refers to the levels of the drug factor (control vs. drug) and 3 to the levels of the growth factor. In some circumstances, levels of one factor are measurements between-groups, while levels of the other factor are repeated measures. For example, if a study is on the effects of a drug over time and measurements are taken from the same cell at $n$ time points, the design is a $2 \times n$ factorial with $n$ repeated measures on one factor (time), and the appropriate test is a two-way ANOVA with repeated measures. The ANOVA summary tables in each of these designs provide different information, as explained below.

Since in most experiments under discussion the samples are not independent of the treatments, i.e., a given cell is tested in only one experimental condition, an appropriate test is a nested ANOVA. In this case, each cell is considered to be nested within each treatment. The cells chosen would be a random effect, whereas the drug exposure would be a fixed effect. The nested ANOVA test is very cumbersome to run with many cells and events; a reference on how to conduct these tests is available /211/. The main drawbacks of this test are that it is relatively impractical to run and it can be difficult to make assumptions about nested variables. There are commercially available programs for nested ANOVA tests, including MINITAB (State College, PA, USA) and STATISTICA (Statsoft, Tulsa, OK).

Most often, pooling the data from different cells would be desirable. There are two problems with this: (1) different $n$s are represented from each cell, (2) violation of independent sampling, i.e., the population of quanta is dependent on the cell /52/. The first issue can be addressed by trimming each cell to the same number of events /261/. In initial trials, we found that one-way ANOVA on trimmed pooled data gave similar results to nested ANOVA. However, the second problem cannot be addressed if data are pooled.

The one-way ANOVA test of differences in mean values of the cells has the advantage that is easier to perform, and does not require data to be pooled. We use the log transforms of the quantal sizes, because the data are then normally distributed (Section IV.C), which is rarely the case in untransformed distributions of quantal sizes.

For the example shown in Figure 14, for means of the log quantal sizes from each cell, the one-way ANOVA is conducted as follows:
where SS = sum of squares (the sum of (each value - mean)$^2$), MS = mean squared, and df = degrees of freedom. In this experiment, SS drug = 1.767 and SS cells = 9.041. Together, the data from this experiment yield:

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>drug</td>
<td>2 - 1</td>
<td>SS$<em>{drug}$/df$</em>{drug}$</td>
<td>14.28 (MS$<em>{drug}$/MS$</em>{cell}$)</td>
</tr>
<tr>
<td>cells</td>
<td>(49 - 1)+(26 - 1)</td>
<td>SS$<em>{cells}$/df$</em>{cells}$</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The null hypothesis for this test is that there is no difference between treatments. Using a table of F values or a commercial statistics program, for F = 6.75 and 1 and 73 degrees of freedom, P = 0.0003, meaning there is a 0.03% probability that the means of the control and L-DOPA groups are the same.

In a one-way ANOVA for repeated measures, for an example where quanta (Q) are measured at cells at multiple time points (TP), the summary table looks as follows:

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells</td>
<td>nCells-1</td>
<td>SSCells/nCells=1</td>
<td></td>
</tr>
<tr>
<td>quanta/point</td>
<td>nQ-nCells</td>
<td>SSQ/TP-1</td>
<td>MSTP/MSError</td>
</tr>
<tr>
<td>time points</td>
<td>TP-1</td>
<td>SSQ/TP-1</td>
<td>MSTP/MSError</td>
</tr>
<tr>
<td>error (Q-TP)</td>
<td>dQ-dTP</td>
<td>SSQ=SSTP/dQ-dTP</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>dfCells + dfQuanta</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The F-ratio reports whether there is a significant difference in the means before and after a treatment on the same cells.

Summary of statistical approaches:
Which should be used?

For experiments where effects are robust and the $n$ of both cells and individual quanta is high, most tests will reliably demonstrate statistical significance. However, many interesting phenomena are unlikely to produce such large changes. For instance, long-term potentiation in the hippocampus is often examined with increases in postsynaptic current of ~150% of controls.

The first important prerequisite when designing experiments to test changes in quantal size is a sufficiently large $n$. For PC12 cells, adrenal chromaffin cells, or midbrain dopamine neurons, our laboratory's rule is to record from at least 9 cells from each experimental group from sister cultures, i.e., cultures derived from the same parent colony or litter. Then we choose a minimum number of quanta per cell that we will accept as a representative population. For secretory cells, we usually include cells that provide at least 15 quanta per stimulation. For central dopamine neurons we include all quanta in the analysis since most sites yield few events. The above guidelines cannot always be followed, however. For instance, we have found it very difficult to record quantal release from PC12 cells following reserpine.

Most often, the question to be tested is whether means of groups are different. It is tempting to pool the quanta from all cells in each group since this provides a large $n$. Indeed, most studies use this approach. However, as mentioned in the section on ANOVA, the quanta are often not independent since some cells in the same group can produce different size quanta from others /52/, i.e., there is no homogeneity of variance. Second, different cells have different $n$s of quanta and are thus represented at different levels in the pooled data. If an outlier cell has a very high $n$, it can lead to a false conclusion being drawn. The nested ANOVA is a powerful method as it examines all data points and does not pool together data from different cells (i.e., it does not violate the requirement for independent sampling). However, there are drawbacks to its use, as mentioned above.

One answer to this problem is to analyze mean values for each cell in a group. Each mean value would be independent of the others in the same group and each would be equally represented. To display this graphically, we prefer the box-and-whiskers plot. The Mann-Whitney rank sum test is appropriate for statistical analysis. If the $n$ of mean values is sufficiently large and can be log transformed to an apparently normal distribution, one-way completely randomized ANOVA (identical to a t-test if two groups are compared) can be used. The disadvantage of using the mean analysis for either a non-parametric or a parametric test is the reduction in statistical power because each cell is represented by only one point. If, however, the $n$ of
cells is adequate, the analysis of the means can reliably show the presence or the lack of an effect.

We also recommend that for groups that show differences in mean values, the pooled individual quantal events within the groups be demonstrated graphically to indicate the data distribution. The best approach is usually a normal probability distribution of the log transforms, detailed below. In most cases, this leads to a startlingly linear population distribution with the attractive feature that the entire population can be described by a linear equation of the form \( y = mx + b \). By displaying the two experimental groups in the same figure, it becomes clear whether the entire population was altered. An alternative approach is to use cumulative distribution plots, particularly for data with low \( n_s \) of events recorded per site. Histograms remain in wide use, but can be misleading /242/.

The optimal use of the Kolmogorov-Smirnov or resampling tests would be to analyze data from a given site tested before, after, and following recovery from a given treatment. Resampling and the Komolgorov-Smirnov can both be conducted on any data distribution, although resampling only tests differences between means. There are variations of the Kolmogorov-Smirnov test that can be used to better examine differences far from the mean /194/ as well as graphical representations of cumulative data that can be used to indicate new subpopulations /242/.

Finally, it is striking that when the effect is robust and the \( n \) sufficiently high, these different statistical approaches tend to yield similar probability values. This is seen in the examples in which we ran the Komolgorov-Smirnov and resampling on the pooled values in Figure 13 and the Mann-Whitney and one-way ANOVA on the mean values in Figure 14. When possible, the best approach is to increase the number of observations and/or design the interventions to produce a larger effect.

B. Interpreting overlapping events

In postsynaptic recordings of systems such as the hippocampal slice or mouse vas deferens in which input from many terminals can occur, there is a high likelihood of temporally overlapping events. The classic approach to analyzing the frequency of overlapping events is to examine whether the data fit a Poisson distribution. An approach to determine the basic quantal unit within overlapping events is to use Monte Carlo simulations /23,26,63,71,126/. Such approaches have been addressed in numerous recent reviews /19,89, 100,121,131,197,232,253/.

Overlap of quanta are far less likely in amperometric recordings from axonal varicosities or cell bodies, where one or a small number of sites are recorded rather than many synaptic inputs. Moreover, in CNS axons, the duration of amperometric events is much shorter than postsynaptically recorded events.

An approach to determine whether the temporal distribution of secretory events is stochastic was suggested by Fernandez and colleagues /4/. The expected frequency of overlapping release events recorded is estimated from a fusion probability distribution function determined by the latency times between consecutive events (Fig. 15). A close fit to an exponential decay is consistent with independent events. The decay is characterized by its time constant \( \tau \) and the probability \( p \) of finding two events that would occur within an interval \( t \) is given by

\[
1 - p(t) = 1 - e^{-t/\tau}
\]

To date in amperometric recordings, these analyses have shown a low likelihood of overlapping events, far too low to explain estimated changes observed in quantal size.

Additional experiments can be conducted to determine whether one is measuring true quantal events or overlapping quanta. The size of true quantal events should be independent of release frequency. One way we have examined this is to record at different levels of \( \text{Ca}^{2+} \), since the number of events tends to increase with \( \text{Ca}^{2+} \) concentration to the fourth power; therefore, this should greatly increase the possibility of multiple simultaneous events. In CNS preparations, we have not yet noted any change in quantal size using 1.2 or 6 mM \( \text{Ca}^{2+} \) /187/. Similarly, spontaneously released quanta can be examined to determine whether the size is identical to those resulting from stimulation.

Despite these arguments, we do not take sides on the issue of whether all "quantal" release events are genuinely stochastic. Indeed, it seems quite
possible that conditions exist in which multiple vesicles may be released in close temporal coordination /12,233/. Moreover, it may be that phenomena such as flickering or repetitive transient formations of the fusion pore are smeared together to constitute a quantum.

![Graph showing the relationship between interspike intervals and the probability of two successive events occurring within an interval of 1 msec.](image)

Fig. 15: An approach to estimate frequency of stochastically overlapping quantal events. The data are from VTA neurons induced to overexpress VMAT2, which exhibit a greater frequency of release than control VTA neurons /189/. Intervals are measured between each pair of successive events in the data. The fraction of interspike intervals within each 20 msec bin is indicated on the y axis. The best-fit exponential decay, determined by the least squares method, is the solid line. From the single exponential decay of the interspike intervals \( y = (0.84/72) e^{-0.005t} \), \( r^2 = 0.950 \) for intervals from 975 events, the probability \( p \) of two successive events occurring within an interval \( t \) of 1 msec = \( -0.005 \).

C. Modality of the quantal populations: Relationship between vesicle volume and quantal size

In nearly all studies, quantal sizes do not follow a normal distribution, but rather a right-hand skew reflecting a broader distribution of larger granule sizes. The distribution approaches normal if either the cubed root or the log transformation of the quantal sizes is plotted /74,242/. The suggested rationales are that: (1) Vesicle diameters are distributed normally; therefore, if transmitter filling is proportional to volume, the population distribution should produce a cubed root normal distribution /20/. (2) If multiplicative deviations from the mean occur, this would produce a lognormal distribution /242/; an example would be if differences in the number of uptake transporters per vesicle resulted in multiplicative transmitter accumulation rates. (3) The skew could be accounted for by a "second-stage" loading of transmitter into vesicles positioned at the active zones, coupled with random release without regard to transmitter content /249/.

Investigators continue to design studies to distinguish between these possibilities.

A recent study suggests that the cube roots of quantal sizes recorded at chromaffin cells are less skewed than the diameters of chromaffin granules. This suggests that the intravesicular transmitter concentration is not uniform, but lower for granules of larger size /87/.

In PC12 cells, we find that a lognormal distribution results in a substantially closer fit to a normal distribution than the cubed root transformation, with all of the log transformed populations studied well described by a single regression line with \( r^2 > 0.98 /186/ \).

Normal probability plots

A concern for determining whether raw or transformed quantal sizes follow a normal distribution is that there are often too few events in quantal recordings to produce a good histogram. In such cases, the choice of bin size can dramatically change the appearance of a histogram. Van der Kloot recommends that a normal probability function is better suited for displaying the population of quantal sizes /242/. In this approach, every data point is displayed, and deviation from a normal distribution is easily seen by eye. No arbitrary bin size is selected. Values that depart from linearity represent events that are not part of the overall population. Finally, a shift in the linear distribution illustrates altered quantal size.

To produce a normal probability plot, the standard deviation of the data is determined. Then each point is plotted on the y axis in units ± standard deviations from the mean on the x axis, i.e., for a value \( x \), the position is \([x - (\text{mean value})] \) ± standard deviation. When displayed in this form, a normal distribution is represented by a straight line. If the normal probability plot of the data fits with more than one line, multiple populations are present. Generally, we perform this with the log transformation of data (Fig. 16a).
Following the characterization of the normal probability distribution and determination of the fit by linear regression, one can determine whether a given intervention altered the quantal size of the entire population or a subpopulation. To do this, the populations compared are scaled for the slope and y intercepts indicated by the linear regression. Then, either the quantal sizes or log quantal sizes can be displayed as cumulative distributions, since the untransformed or transformed data show the same shape. If the entire population has shifted, the distributions shown in this manner should be identical.

**Fig. 16:** Normal probability distributions of log transforms of quantal sizes. Every point in the data is indicated. On the x axis, "0" represents the mean value, and other values represent standard deviations from the mean, i.e., for a value x, the position is [x - (mean value)] ± standard deviation. The y axis reports the log transforms of the quantal sizes. **a.** Examples of amperometric data contrasting release from ventral midbrain DA neurons in culture. Data from VMAT2 over-expressing ventral midbrain dopamine cultures are in the upper trace (smaller points), and controls in the lower trace (hollow points). The upper distribution represents the overexpressors (975 events) and the lower the controls (n = 88 events). It appears that nearly the entire population of quantal events is shifted to a greater size. The log transforms of the data are consistent with a different unimodal lognormal distribution of quanta in each group (y = 3.7669 + 0.289x, r² = 0.977, ANOVA F = 3150 for controls; y = 3.9644 + 0.2938 x, r² = 0.995, ANOVA F = 81,902 for overexpressors). **b.** An example of multimodal populations observed in a normal probability distribution. These quanta are recorded from AtT-20 cells induced to express tyrosine hydroxylase or tyrosine hydroxylase and VMAT2 (Fig. 5) (n = 175 events).

**Fig. 17:** The log transforms of the quantal sizes in Figure 16a were normalized according to the best fit regressions of the normal probability distributions. When replotted by their normalized sizes, their cumulative distributions are nearly identical, suggesting that nearly the entire population of control quantal sizes was shifted to larger values.

**PART 2:**
**MECHANISMS OF QUANTAL SIZE REGULATION**

**V. REGULATION BY THE TRANSESICULAR FREE ENERGY AND CONCENTRATION GRADIENTS**

In the 1980s, work by several groups characterized how chromaffin granules maintain high levels of monoamines against a large concentration gradient /107,165/. Empirical studies of isolated granules showed that the relationship was due mostly to a proton gradient (ΔpH) driven by an
ATP-driven proton pump and a vesicular Cl⁻ channel. In short, the uptake of protons increases both the concentration gradient ΔH⁺ and the electrical gradient ΔΨ of charges across the vesicle membrane.

From work on the protonmotive force maintained by mitochondria /150/, the electrochemical potential difference experienced by the protons across the vesicle membrane (ΔµH⁺) can be expressed as

\[ ΔµH⁺ = FΔΨ - 2.3RTΔpH \]

where \( F \) = Faraday's constant, \( ΔΨ \) = the electrical gradient, \( R \) = the gas constant, and \( T \) = the absolute temperature. When a vesicular Cl⁻ channel is opened, the ΔΨ is reduced by Cl⁻ moving to its equilibrium, so that ΔµH⁺ is reduced and additional H⁺ accumulation is favored. This Cl⁻ channel mechanism appears to control the extent of acidification throughout many compartments in the cell, including the Golgi apparatus and lysosomes /13, 88/.

In isolated chromaffin granules, monoamines distribute according to the electrochemical gradient as

\[ \log([A]_\text{in}/[A]_\text{out}) = ΔΨF/RT + 2ΔpH \]  
[Eq. 3]

In chromaffin granules, granule pH is often estimated to be ~5.6, the cytosolic pH ~7.2, and ΔΨ to be ~+80 mV. Assuming RT/F = 59 mV, this indicates an equilibrium transvesicular gradient of ~36,000:1. The relationship between the electrochemical gradient and transmitter accumulation is somewhat different for glutamate, which is more dependent on the ΔΨ, and for GABA, which is dependent on both ΔpH and ΔΨ /145/.

This relationship hints at many interventions that might alter quantal size. First, we discuss effects on the right-hand side of Equation 3, the H⁺ and electrical gradients. Then, we discuss effects of the left side of the equation, the concentration gradient across the membrane.

A. Regulation by the intravesicular pH gradient

Several experimental methods are used to reduce or collapse transvesicle electrochemical gradients. These include (1) proton pump inhibitors and protonophors, which collapse the pH gradient, (2) weak bases that are membrane permeable in their uncharged state, which reduce the pH gradient, (3) membrane-permeable compounds such as isothiocyanate, which collapse electrical gradients.

The effect of modulation of the chloride channel conductance, a physiological mechanism by which electrical gradients would be collapsed, has not been studied in relation to quantal size. However, the investigations of Tamir et al. on the role of chloride channel on serotonin uptake by secretory granules /226,227/ imply that effects on vesicular chloride flux could regulate the packaging of transmitter in vesicles.

**Protonophore and proton pump inhibitors**

While exposure of isolated chromaffin granules to protonophores rapidly redistributes transmitter from inside to outside the vesicle /107,224/, effects by protonophores and proton pump inhibitors on quantal size have been little explored. We find that the proton pump inhibitor baflomycin reduces quantal size in chromaffin cells (Pothos and Sulzer, unpublished data). However, protonophores can be problematic as they collapse all cellular pH gradients. For example, the electrochemical gradient across the mitochondrial membrane, which is due to the action of a different proton pump than that in secretory vesicles, is collapsed by protonophores, leading to a loss of mitochondrial ATP synthesis and neurodegeneration. Nevertheless, protonophores such as monensin that do not induce complete loss of proton gradients /221/ may be useful for studies of this sort under controlled conditions.

**Weak bases**

For a weak base with a single proton-accepting group with a pK >> physiological pH, membrane-permeable in its neutral form and membrane-impermeable in its cationic form, at equilibrium

\[ \frac{[\text{base}]_\text{out}}{[\text{base}]_\text{in}} = \frac{[\text{H}^+]_\text{out}}{[\text{H}^+]_\text{in}} \]

Therefore, weak bases are distributed across the membranes according to the pH gradient /140/. As their concentration becomes sufficiently high, they exceed the buffering capacity of the vesicle interior.
and collapse the pH gradient. This would be expected to decrease quantal size by redistributing transmitter from the vesicles to the cytosol. For example, weak bases such as tributylamine or ammonia (added as ammonium chloride) and protonophores such as monensin, cause release from DA neurons by reverse transport /221/.

In the neuromuscular junction, the weak base ammonium reduces quantal size /239/.

Amphetamine, a weak base psychostimulant

Interesting examples of the weak base mechanism are provided by the amphetamines, including D-amphetamine, methamphetamine, fenfluramine, and methylenedioxyamphetamine (MDMA; "Ecstasy"). The amphetamines are the only widely used class of drugs that elicit transmitter release by a non-exocytic mechanism /204,220-224/. Accumulation of amphetamines into synaptic vesicles would reduce vesicular DA uptake and result in competition for protons between amphetamine and vesicular DA. The resulting unchanged DA would diffuse out of the vesicular compartment following its concentration gradient. In contrast to the exchange-diffusion hypothesis of amphetamine /75/, redistribution of vesicular DA would result in elevated cytosolic DA, which in turn would promote reverse transport by increasing the concentration gradient across the DAT /204,220,221/. It seems likely that as both vesicular monoamine transporter and DAT substrates /6,183,180/ amphetamines would be quite effectively sequestered in vesicles.

Amphetamine provided the first instance of pharmacological manipulation of DA quantal size. Amperometric recording demonstrated that a pharmacologically appropriate level of amphetamine exposure (10 μM for 10 min) decreased quantal size by 50% in PC12 cells /220/. Cyclic voltammetry recordings in the acute striatal slice strongly suggest that similar actions occur in vivo /108/. Ewing’s group found that two classes of DA vesicles were differentially depleted by amphetamine in the giant DA neuron of Planorbis corneus /7/.

B. Regulation by the cytoplasmic/intravesicular concentration gradient

From Equation 3, if the electrochemical gradient is unchanged, a two-fold increase in cytosolic levels of transmitter should result in a two-fold increase in quantal size. Early studies that contrasted with this simple prediction showed that loading presynaptic neurons with ACh by intracellular injection in Aplysia synapses increased quantal frequency but not quantal size /192,193/. However, exposure of neuromuscular junctions to extracellular ACh increased quantal size. This would be expected if ACh is converted to choline and taken up into the cell and then into vesicles /242/. Further evidence that elevated cytosolic transmitter can increase loading into vesicles is seen in PC12 cells, in which exposure to extracellular DA increased quantal size /186/, presumably following uptake by DAT.

Modulation by elevated cytosolic transmitter levels: L-DOPA. The DA precursor L-DOPA is the most widely used clinical intervention for Parkinson’s disease. It had long been presumed that synaptic vesicles are not loaded by L-DOPA /32/ and that increased DA release was due to other mechanisms. However, amperometric recordings demonstrated that L-DOPA rapidly elevates quantal size in midbrain neurons (Fig. 18A,B) /187/ and PC12 cells /188/. The rapid increase in quantal size appears consistent with in vivo cyclic voltammetry results, where L-DOPA rapidly increased action potential-mediated DA release /81/.

Autoreceptor ligands, second messengers, and altered transmitter synthesis

Typically, the synthesis of L-DOPA from tyrosine via tyrosine hydroxylase provides the rate-limiting step in catecholamine synthesis. Long-lasting D2 DA autoreceptor-mediated mechanisms that play an important role in synaptic release are inhibition of transcription and phosphorylation of tyrosine hydroxylase. PKA activity increases tyrosine hydroxylase activity by phosphorylation of its ser40 residue /125/. D2 receptor activation inhibits cAMP activation of PKA, and so inhibits tyrosine hydroxylase activity. Moreover, there is decreased
activation of a cAMP response element (CRE) 45-38 bp upstream of the tyrosine hydroxylase coding region, which is required for both basal and cAMP-regulated transcription /116/.

Studies using in vivo microdialysis and cyclic voltammetry indicate that the D₂ agonist quinpirole and cAMP antagonists attenuate or abolish stimulation-dependent DA release /174/, whereas the D₂ antagonist sulpiride elevates DA release /266/ by blocking tonic activation of D₂ autoreceptors by extracellular DA. D₂ antagonists, as well as forskolin or dibutyryl cyclic AMP, increase tyrosine hydroxylase phosphorylation in striatal slices /201/ and forskolin increases L-DOPA formation in striatal slices /264/. However, there are conflicting findings regarding whether D₂ receptors are decoupled from this action, particularly in meso-cortical DA terminals /43,263/. In striatal slices and synaptosomes derived from them, D₂ agonists decreased tyrosine hydroxylase activity, forskolin increased tyrosine hydroxylase activity, and sulpiride blocked the effects of endogenous DA stimulation /68,201/.

D₂ receptor-mediated inhibition of release is also due to effects on calcium and potassium currents, which would lower quantal frequency /36, 115/. To examine the specific effects of D₂ blockade on quantal size, we used amperometric recordings on PC12 cells, a line that expresses tyrosine hydroxylase, D₂ and D₄ autoreceptors. Under conditions of D₂ activation that decrease tyrosine hydroxylase activity by 50%, quantal size was also decreased by 50% /186/ (Fig. 18C, D). This effect was occluded by L-DOPA, the product of tyrosine hydroxylase, or exposure to high extracellular DA, which would be supposed to load vesicles in the absence of new DA synthesis.

D₂ antagonists are used as antipsychotic drugs, and the antipsychotic sulpiride increases both stimulation-dependent and amphetamine-induced release in striatal slices /67/. In preliminary studies, we observed similar effects of sulpiride on quantal size in cultured midbrain DA neurons /191/. It may be that altered DA synthesis by D₂ blockade is a mechanism by which antipsychotic drugs alter DA neurotransmission.

A possible similar example of autoreceptor action was observed at the frog neuromuscular junction, where application of nicotinic receptor agonists blocks quantal size increases due to insulin or hypertonic solution /243/. This would appear to be due to an action at autoreceptors at the cholinergic terminal.
Uptake blockers

Cyclic voltammetry in striatal brain slices indicates that DAT is chiefly responsible for clearance of neurotransmitter released following electrical stimulation /109,258/. However, in quantal recordings in culture, the shape of the amperometric event is not regulated by uptake transporters (Fig. 19). Presumably, a salient difference between the systems is that numerous events contribute to the extrasynaptic transmitter measured in the brain slice.

![Graph showing I (pA) vs t (msec) with control and 30µM cocaine traces.]

Fig. 19: Averaged spike shapes representing quantal DA release from PC12 cells before and following exposure to 30 µM cocaine for 30 min (n = 10 spikes each from controls and cells; spikes of the same amplitude were selected). The downward slope, representing the decrement of DA level at the electrode face, is not altered by cocaine and therefore not by reuptake. (Pothen and Sulzer, unpublished).

Longer-term exposure to uptake blockers eventually alters quantal size in PC12 cells, probably by decreasing cytosolic transmitter available for vesicular accumulation. The DAT blockers cocaine, nomifensine, and amfonelic acid each decrease quantal size by 50% (10 µM, 40 min for each compound) /190/. Since amfonelic acid is not a weak base and does not collapse intracellular pH gradients, the effect is likely due to reuptake blockade. The effect of cocaine is not blocked by sulpiride or the D1 antagonist SCH23390, indicating it is not due to D1 or D2 binding. A similar effect may occur in the CNS since DAT knockout mice have very low levels of stimulation-dependent DA release as measured by cyclic voltammetry /109/.

In analogous findings at the neuromuscular junction, the decrease of quantal size observed following increased Na+ gradients may be due to reduced uptake of choline /242/ that would otherwise be used to synthesize ACh. The choline uptake blocker troxopyrrolic reduced quantal size at the neuromuscular junction without altering the coefficient of variance /206/.

Second messenger systems

Second messenger systems and growth factors mediate a wide range of physiological mechanisms and it can be a daunting task to assign a response to a simple mechanism. We discuss this literature in this section as it seems likely that these pathways often alter quantal size by actions on the transvesicular concentration gradient or protonmotive force.

In the frog neuromuscular junction, a possible increase in quantal size was initially observed with epinephrine /106/. This was confirmed in later studies, and the elevation was shown to be blocked by PKA inhibitors /245,246/. The prevalent form of PKA is an inactive tetramer of two catalytic and two regulatory subunits. cAMP binds the regulatory subunit, which disrupts the tetramer and releases the catalytic units. The permeable cAMP analogue Sp-cAMPS increased quantal size in frog neuromuscular junction /246/. Moreover, the protein kinase inhibitor H8 (N-[2-(methylamino)ethyl]-5-isoquinolonesulfonamide) blocked the effects of epinephrine, norepinephrine, and Sp-cAMPS. In contrast, the PKA inhibitor Rp-cAMPS by itself had no effect on quantal size, but antagonized the increase in quantal size due to longer term exposure to hypertonic solution (see below).

Interestingly, anandamide, an endogenous ligand of the cannabinoid receptor, decreased quantal size at the neuromuscular junction, apparently by inhibiting adenylate cyclase /248/.

Transient exposures to hypertonic solution (>15 min) increased quantal size at both rat and frog...
neuromuscular junctions /240,246,268/. Another study reported an initial decrease in quantal size, followed by a gradual recovery, followed by an increased size /65/. These results are apparently due to an effect on PKA, since the increase is prevented by PKA inhibition /246/.

In the Xenopus cultured neuromuscular junction, a potentiation by ATP of spontaneous release was blocked by a P2 purinoceptor antagonist. The action of the antagonist alone reduced the postsynaptic currents, while exposure to H7 (1-[5-isquinolinyl-sulfonyl]-2-methyl-piperazine) and H8 both decreased quantal size, suggesting that endogenously released ATP, via action with protein kinases, can regulate quantal size /79/.

In hippocampal slices, adenosine (5-100 μM) decreased transmitter release, although this appeared to be due mostly to changes in release frequency rather than quantal size /195/. PKA activation has been found to increase the frequency but not the size of quantal release, or to promote the response at previously silent synapses in hippocampal preparations /26,34,39/.

Since D2 autoreceptor activation decreases intracellular cAMP levels, we examined effects of cAMP analogs at neuronal terminals in culture. In pilot findings using amperometric recording of quantal DA release in midbrain DA culture, the PKA activator Sp-cAMPS and the D2 antagonist sulpiride appear to elevate quantal size. Although preliminary, this would be consistent with the possibility that PKA activation at these synapses increases quantal size through tyrosine hydroxylase activation.

PKA may also regulate degranulation of other dense core components in addition to classical neurotransmitters. Betz reported that stimulated rat pituitary lactotrophs in the presence of FM1-43 resulted in intercalation of the probe in dense cores as well as vesicular membrane /9/. Normally, the dense core, which contains numerous components including prolactin and a variety of glycosaminoglycans, was not released during vesicle fusion but remained attached to the cell and eventually endocytosed. However, exposure to dopamine, the D2 analog bromocriptine, or cAMP antagonists induced release of the dense core. This effect was partially reversed by cAMP analogs, suggesting that the response could be due to altered cytosolic cAMP levels. The mechanism of this action is unknown, but it has been suggested that modulation of the intravesicular proton gradient could play a role.

Other second messenger systems have not been as extensively explored as PKA. PKC activation can modulate quantal release in a number of systems, e.g. /123,151/ including quantal size as well as frequency in chromaffin cells /1/. Increased release has been reported after exposure to phorbol esters in frog neuromuscular junction /196/, although this may not be due to PKC but to an unidentified protein that also binds phorbol esters /207/.

However, PKC did not change quantal size in the hippocampus /34/ or chick ciliary ganglion /267/. It may be that PKC regulates the kinetics of fusion pore expression /92,202/. PKC also appears to regulate some vesicular Cl- channels /226/ which as mentioned above regulate the vesicular electrochemical gradient. Phorbol esters that activate PKC reduce quantal size at the neuromuscular junction, an effect blocked by the protein kinase inhibitor H7 /242/. Due to multiple and complex attendant pathways (NOS, PKG, PI, AA), it is possible that there are numerous PKC-mediated pathways that could control quantal release.

A variety of interventions at frog neuromuscular junction that alter quantal size would seem likely to be mediated by second messengers, including treatment with insulin or 2 hours of high K+ levels /241, 243/. These changes were not seen at mouse neuromuscular junction /268/.

In summary, there are likely to be many pathways of second messenger modulation of quantal size to be uncovered. Those illustrated to date appear cell type specific. Clearly, these pathways could provide an enormous variety of plastic responses in the CNS.

Growth factors and hormones

Using postsynaptic recordings, brain-derived neurotrophic factor (BDNF) glial-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and ciliary neurotrophic factor (CNTF) have been shown to modulate quantal release at cultured Xenopus neuromuscular junction /129,218,256/. In
most studies there has been an increased quantal frequency, rather than size, and this is usually interpreted to mean that the change is at a presynaptic locus /135,217/.

However, alterations in quantal size have been observed in two studies /129,256/. Liou and Fu suggested that an effect of growth factors on the number of molecules released per quantum might occur in situ since neurons that formed contacts with myocytes (natural synapses) produced larger quanta than neurons that did not spontaneously form synapses with myocytes. To form such "non-synaptic" contacts, they performed postsynaptic recordings by manipulating myocyte tissue (myoballs) so they would encounter the axons. The effect on quantal size was unlikely to be due to a postsynaptic change, since the myoballs maintained a uniform distribution of ACh receptors. The effect could be imitated by NT-3 while antibodies to NT-3 blocked the increase in quantal size observed in natural synapses. Similar increases in quantal size in this developing system occurred after exposure to BDNF, neurotrophin-4 (NT-4), CNTF or GDNF. Insulin-like growth factor-1 (IGF-I) and basic fibroblast growth factor (bFGF) had no effect /130/.

Postsynaptic responses to growth factors or hormones in hippocampal preparations have so far been found to increase quantal frequency rather than quantal size /37,110,128/.

A clear effect of a growth factor on quantal size in a CNS preparation was identified using amperometric recordings of cultured midbrain DA presynaptic terminals, where exposure to culture medium containing GDNF increased quantal size and the release frequency of midbrain DA neurons by fourfold. This effect lasts at least one month after a single GDNF exposure /187/. A possible explanation of this is increased DA synthesis, since GDNF is known to increase tyrosine hydroxylase levels /17/.

Corticosteroids and adrenocorticotropic hormone increase quantal size in rat neuromuscular junction, possibly by a presynaptic mechanism /242,250,251/. These effects were attributed to an increase in synaptic vesicle volume /250/ and elevated choline uptake /251/.

VI. REGULATION BY VESICULAR TRANSPORTERS

Although vesicular accumulation of transmitter is generally assumed to reach a steady state based on the electrochemical gradient, expression of vesicle transporters provides additional regulation /198/. This could occur if there is an endogenous leak of transmitter following its concentration gradient, as appears to occur with isolated small synaptic vesicles /76/. In contrast, large dense core vesicles appear to retain transmitter over periods of an hour or longer /140/. If there is indeed a leak, the equilibrium should reach a higher level if there are more active transporters. Picture refilling a leaky bucket of sand on the beach; a higher level of sand can be maintained in the bucket using two shovels rather than one.

A pharmacological approach to explore this issue is to use vesicle transporter uptake blockers. In the neuromuscular junction, the vesicular ACh transport blocker vesamicol begins to reduce quantal size /158/ following 15-30 min of tetanic stimulation /137,178,248/. Comparable results were observed in the garter snake, in which some classes of quanta showed decreased amplitudes with vesamicol /205/. In the rat, vesamicol was suggested to selectively decrease the quantal size of the population of recycling vesicles /206/.

A similar response was seen in amperometric recordings from PC12 cells, in which the VMAT inhibitor reserpine induced a reduction in quantal size in 5-15 min. By 50 min exposure to 1 µM reserpine, total K+-stimulated release was only 10% of initial levels /222/. The apparent frequency as well as quantal size was decreased, perhaps due to exocytosis of "empty" vesicles.

That vesicles that contain little or no transmitter still fuse with the plasma membrane was shown by stimulation-dependent loss of the endocytic marker FM2-10 in the presence of the weak base methylamine, or the ATPase inhibitor bafilomycin /54/. While methylamine and bafilomycin should deplete vesicular transmitter, they did not affect the field-evoked uptake, recycling, or destaining of the vesicle-specific dye FM2-10. Therefore, "empty" vesicles apparently recycle in the axon terminal.

In the Xenopus neuromuscular junction culture model, overexpression of the vesicular ACh transporter was induced by injection of transporter...
cDNA or mRNA into embryos. This led to increased quantal size and frequency. As discussed above, the increased frequency could have been due to loading of vesicles that would otherwise release undetectable levels of ACh /212/.

To examine how overexpression of the monoamine transporter VMAT2 affects quantal size, an adenoviral vector harboring a VMAT2 expressing sequence was used to introduce VMAT2 into AtT-20 cells, PC12 cells and neurons /189/. Amperometric recordings in AtT-20 cells, which normally do not release DA, demonstrated that cotransfection of VMAT2 and tyrosine hydroxylase was sufficient to convert vesicles to a DA-releasing phenotype. Amperometric recordings in PC12 cells, which normally express tyrosine hydroxylase and a vesicular monoamine transporter subtype, VMAT1, showed a 400% increase in quantal size (Fig. 20), but no change in release frequency, presumably because all of the vesicles already had detectable levels of monoamine. In cultured ventral midbrain neurons, VMAT2 overexpression increased both quantal size and frequency, consistent with recruitment of synaptic vesicles that do not normally release DA. Therefore, vesicle transporters limit the rate of transmitter accumulation and can alter synaptic strength through at least two distinct mechanisms.

VII. REGULATION BY FUSION PORE EXPRESSION

There are recent reviews on the action of vesicular fusion pores in mediating release /152,153/. While the fusion pore can be modeled as an ion channel /136/, modulation of fusion pore open time has been little explored. One mutation, ruby-eye in mice, may alter the duration of vesicular fusion pore expression as these animals exhibit a three-fold increase in the duration of transient fusion events of mast cell granules /167/.

As discussed in Section III, amperometric recordings from chromaffin cells also suggest that the fusion pore can exist in at least two states. Recordings of dense core exocytosis often show a longer-lasting, lower conductance portion, known as a foot, followed by a full amperometric spike /47/. Recently, we identified an extreme example of two types of conductance in single quanta in VMAT2/tyrosine hydroxylase-coexpressing AtT-20 cells (Fig. 5) /189/. Moreover, as discussed above, Fernandez and coworkers showed that transmitter release from large dense core vesicles can occur without full fusion /4/. These findings were extended by Lindau’s group using patch amperometry /1/ (Section III). Together, these findings suggest that fusion pore modulation should be capable of affecting the amount and kinetics of transmitter release.

As discussed in Section V, modulation of PKC may alter fusion pore kinetics. In a study at frog neuromuscular junction /92/, staurosporine inhibited or abolished activity-dependent destaining of terminals preloaded with the endocytic tracer FM1-43, but did not block synaptic transmission. Nevertheless, the dye was taken up into vesicles in terminals pre-exposed to staurosporine. These results suggest that the exocytotic fusion pore can behave like a valve, in some cases letting FM1-43 in, but not out. However, an alternate possibility is that staurosporine somehow recruits unlabelled vesicles to a releasable pool.
Using cell attached capacitance recording in horse eosinophils, Lindau showed that while the PKC activator phorbol-12-myristate-13-acetate (PMA) had no effect on the amplitude and dynamics of degranulation, fusion pore expansion was accelerated ~two-fold. The PMA effect was abolished by staurosporine. Elevated \( \text{Ca}^{2+} \) also accelerated pore expansion by two-fold; however, this effect was not prevented by staurosporine, indicating that intracellular \( \text{Ca}^{2+} \) may activate fusion pore expansion by distinct mechanisms. The phosphatase inhibitor \( \alpha \)-naphthylphosphate slowed fusion pore expansion /202/. A recent study in chromaffin cells finds that elevated \( \text{Ca}^{2+} \) potenti ated kiss-and-run events /2/.

Thus, it may be that intracellular \( \text{Ca}^{2+} \), in addition to its well known role in initiating exocytosis, may regulate the kinetics of the fusion pore. We have noted that under conditions in which the secretagogue \( \alpha \)-latrotoxin would be expected to greatly increase \( \text{Ca}^{2+} \) flux /134/, the quantal size in PC12 cells is doubled (Pothos and Sulzer, in preparation) (Fig. 21). This appears to be due mostly to an increased event duration rather than amplitude, consistent with an effect on pore open time.

The fungal metabolite brefeldin A inhibits guanine nucleotide exchange on ADP-ribosylating factors involved in vesicle membrane budding for secretory vesicles. In chromaffin cells, amperometric measurements showed that brefeldin exposures that disrupted Golgi membranes increased the mean quantal size and the occurrence of the foot and stand-alone signals. The authors conclude that brefeldin A may slow the fusion kinetics of some vesicles /265/.

VIII. EFFECTS ON THE GRANULAR MATRIX

The interior of dense core vesicles, and possibly small synaptic vesicles, is composed of a gelatin/water-like expandable matrix of transmitter proteins, and other small molecules. In some cases, the isolated matrix from giant mast cell granules preserves the characteristic appearance of the dense core /176/. The intravesicular matrix contains sulfated anions as can be observed by cation binding at pH 1, at which only sulfate groups would tend to be charged /222/. Likely this is due to the presence of glycosaminoglycan molecules, including heparan sulfate and chromogranin A, which are negatively charged due to carboxyls and/or sulfate groups /257/. Intravesicular ATP may also contribute to the matrix /107/.

* Note added in proof: The secretory protein CAPS may regulate fusion pore kinetics in chromaffin cells (Elhamdani A, Martin TJF, Kowalchyk JA, Artalejo CR. \( \text{Ca}^{2+} \)-dependent activator protein for secretion is critical for the fusion of dense-core vesicles with the membrane in calf adrenal chromaffin cells. J Neurosci 1999; 17: 7375-7383).
In mast cell granules, ion exchange mediates the dissociation of the transmitter from the gel matrix /143/. The isolated mast cell granules can fuse even in a cell-free model system /167/ and the release of transmitter from such isolated granules has been characterized amperometrically /142/. It remains an open question whether a similar ion exchange occurs with small synaptic vesicles, although the high concentrations of transmitter seem to imply that this occurs.

Dense core components aggregate at mildly acidic pH (< pH 6.5) in the presence of Ca^{2+} /53/. Therefore, one form of degranulation may involve alkalization of the vesicle interior shortly prior to exocytosis /9,91/. As discussed above, it has been suggested that PKA may mediate this response /9/.

Given these results, it would seem that altering the extracellular environment could modulate the degranulation of transmitter from its matrix. While broad changes in the extracellular milieu may not occur under physiological conditions, the effects lend insight into the nature of exocytosis. The parameters that have been studied to date are altered pH, temperature, osmolarity, and species of cation. The voltage across the vesicle membrane would also seem to play a role, as the matrix responds to applied negative voltages by swelling and to positive voltages by condensing /143,157/.

**Extracellular pH**

Since the catecholamines are positively charged, the rate of dissociation of the dense core matrix would be predicted to increase with higher pH. In chromaffin cells, quantal events elicited by Ba^{2+} in Ca^{2+}-free medium were of larger quantal size at pH 8.2 than at pH 7.4 /105/. Quanta obtained after Ba^{2+} exposure at an extracellular pH of 5.5 had a different shape than those obtained in more basic solutions /105/.

Interestingly, high pH accelerated the rate of release of the peptide insulin as well, although in this case the total amount of transmitter released was unchanged /10/.

**Temperature**

The opening and closing of fusion pores in patch-clamped mast cells is temperature-dependent as measured by whole cell capacitance recording /169/. The rate of closure is discontinuous with the break at approximately 13°C. Above this point, closure is weakly temperature dependent, while below this point, the closure is temperature independent. This was suggested to be due to a fluid-solid phase transition at the break temperature, so that pore closure is regulated by lipids that become phase separated at 13°C.

In mast cells, there was a decrease in the time required for complete transmitter release at 37°C compared to room temperature /184/. In chromaffin cells, amperometric recordings determined that the quantal size was greater at physiological temperature than at room temperature /184/. In two subsequent studies /52,254/ the amplitude and quantal size of the amperometric spike were not different, but rise and decay times were substantially shortened as the temperature was raised from 15°C to 35°C. Nevertheless, all of these studies conclude that increased temperature may potentiate dissociation of catecholamine from the matrix.

At the neuromuscular junction, quantal size is mostly independent of temperature /247/.

**Osmolarity and ion species**

**Osmolarity**

Hypertonic solutions would be predicted to reduce the rate of dissociation of the granule matrix. In a study using chromaffin cells, smaller quanta were released under hypertonic conditions (630 mosM) than with isotonic physiological solution (315 mosM). These events were broadened with hypertonic solution. At 970 mosM, even smaller amplitude secretory events were evoked that resembled the amperometric foot (see above) /27/.

**Cation species**

It may be that cations could substitute for the amines at their cationic residues. In chromaffin cells, induced spikes in Ca^{2+}-free medium were broader and shorter but had the same area as spikes in Ba^{2+} medium /105/. Interestingly, release induced by K⁺ after Ba^{2+} exposure elicited events of larger quantal size than those prior to Ba^{2+} exposure.
Fig. 22: A. In the third instar larval NMJ synapses of *Drosophila*, synaptic vesicles cluster around a dense body at active zones. In wildtypes, the synaptic vesicles are uniform in size. B. In *lap* mutants, the vesicles are variable in size and tend to be enlarged. Scale bar = 0.2 μm. C. Cumulative distribution of spontaneous miniature currents from body wall muscles of the third instar larval stage. The *lap* mutants show larger quantal size, and events with amplitudes $>$ -2 nA are found only in the mutants. Courtesy of Dr. Bing Zhang [27].
In a study of insulin granule exocytosis, increasing extracellular Zn\(^{2+}\) concentration from 0 to 25 μM increased the width and decreased the quantal size. This was suggested to be due to a common ion effect on Zn\(^{2+}\)-insulin dissociation /10/. In chromaffin cells, quantal size was decreased with Zn\(^{2+}\), but increased with the monovalent cation Cs\(^+\) /184/.

IX. VESICLE RECYCLING AND VESICLE SIZE

The total amount of transmitter should be proportional to the vesicle volume. This was noted in a study of Drosophila larval neuromuscular junction, using a mutation of the Drosophila homologue of the clathrin adaptor protein AP180 (lap), a protein thought to mediate synaptic vesicle endocytosis. The mutant shows severely impaired endocytosis and altered clathrin localization. The synaptic vesicle volume was increased by nearly two-fold in the mutants, and the quantal size was increased by 1.6-fold /271/ (Fig. 22). Presumably, other proteins that mediate the initial budding or recycling of secretory and synaptic vesicles, including those associated with clathrin or dynamin, will alter vesicle volume and quantal size /270/.

Other interventions reviewed that alter quantal size have not yet been found to regulate vesicle volume /242,250/. However, it is possible that the effects of brefeldin A /265/ discussed in the section on fusion pore kinetics may also involve membrane trafficking.

X. ADDITIONAL INTERVENTIONS

Stimulation

Activity-dependent synaptic modifications are crucial for synaptic function, particularly for the development or acquisition of new behavior and memory. Extensive tetanic stimulation initially decreases quantal size /64,85,205/, presumably because there has not been sufficient opportunity for vesicle recycling, refilling the vesicular stores, or replacing the readily releasable vesicle pool. In chromaffin cells, repetitive stimulation by high K\(^+\) can elevate quantal size /74/. Increased quantal size has also been reported in some cases with long-term potentiation /162,172,173/ although stimulation-dependent increase in quantal amplitude in the mammalian CNS is most often reported to be due mostly to postsynaptic alterations /235/.

In the Drosophila larval neuromuscular junction, decreased innervation due to the mutations of the fasclin-II gene, which controls nerve-muscle innervation, result in an increased quantal size, although it could not be concluded whether the change was due to a pre- or postsynaptic mechanism /60/. In a cricket CNS preparation, it has been suggested that there is modulation of quantal size in a sensory to interneuron synapse by a stimulation-induced retrograde signal from the postsynaptic cell /58/.

Oxygen deprivation

Amperometric recordings in PC12 cells showed that chronic hypoxia for 21-26 hours increased acute hypoxia-evoked quantal frequency and size /230/. The altered frequency seemed to be due to the action of an oxygen-sensitive K\(^+\) channel, while the basis of the increased quantal size is unknown.

Anion species

It has long been observed at the neuromuscular junction that quantal size is strikingly increased by the anion gluconate. The mechanism of this effect remains unclear /242/.

XI. SUMMARY

As with many past generalizations on nervous system function, the assumption of invariability of quantal size breaks down following closer examination. There are a broad variety of interventions that modulate quantal size at the presynaptic locus. We think it highly unlikely that the brain would reject these mechanisms as a means to provide plasticity.

For L-DOPA and amphetamine in particular, altered quantal size appears to provide their fundamental mechanism of action. For social synapses that provide extrasynaptic neurotransmitter or private synapses unsaturated by release of a single quantum, alterations in quantal size are likely to have extensive physiological implications.
APPENDIX A

Using a spreadsheet program to simulate a random walk

Diffusion of molecules is surprisingly non-intuitive. John Crank's classic volume on diffusion /55/ is very useful for showing examples of how to derive formulae to describe molecular diffusion under different conditions. This approach is quite useful, and one such formula has been used to estimate the relationship between distance and amperometric spike shape /46/. Nevertheless, the complexity of deriving the appropriate formulae as conditions are altered is a drawback to its use.

In contrast, the so-called random walk or numerical approach, discussed by Berg /24/, provides the same answers but in a manner in which it is simpler to manipulate conditions. This was used in a study by Schroeder et al. in a study of amperometric spike shape and fractional recovery /203/. An advantage to a random walk approach is that it is easily performed on spreadsheets. As the above references do not show precisely how this is performed, we show an example here.

A spreadsheet modeling diffusion in one dimension over time can be made with columns representing distance and rows representing time intervals. Since the vectors of the diffusion are random, for a population of molecules diffusing in one dimension (X), one half will move to the right and one half will move to the left. This can be simulated by making the bins in a row t (x+1) = the average of the two neighboring bins in row t (x).

An absorbing surface at t (x+1) will retain its previous population of molecules and inherit half of the molecules from its (only) neighboring bin at t (x). A reflecting surface will inherit half of the molecules from its neighboring bin and donate half of its molecules to its neighboring bin. This is simulated by making a bin at t (x+1) = the average of itself and its neighbor at t (x).

For a release event at x = 0 μm from a reflecting surface and an absorbing surface at 3 μm, a short random walk of 4 reiterations can be modeled as:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μm</td>
<td>0.5 μm</td>
<td>1.0 μm</td>
<td>1.5 μm</td>
<td>2.0 μm</td>
<td>2.5 μm</td>
<td>3.0 μm</td>
</tr>
<tr>
<td>time 0</td>
<td>initial # molecules</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>time 1</td>
<td>av(a0,b0)</td>
<td>av(a0,c0)</td>
<td>av(b0,d0)</td>
<td>av(c0,e0)</td>
<td>av(d0,f0)</td>
<td>e0/2</td>
<td>f0/2+g0</td>
</tr>
<tr>
<td>time 2</td>
<td>av(a1,b1)</td>
<td>av(a1,c1)</td>
<td>av(b1,d1)</td>
<td>av(c1,e1)</td>
<td>av(d1,f1)</td>
<td>e1/2</td>
<td>f1/2+g1</td>
</tr>
<tr>
<td>time 3</td>
<td>av(a2,b2)</td>
<td>av(a2,c2)</td>
<td>av(b2,d2)</td>
<td>av(c2,e2)</td>
<td>av(d2,f2)</td>
<td>e2/2</td>
<td>f2/2+g2</td>
</tr>
<tr>
<td>time 4</td>
<td>av(a3,b3)</td>
<td>av(a3,c3)</td>
<td>av(b3,d3)</td>
<td>av(c3,e3)</td>
<td>av(d3,f3)</td>
<td>e3/2</td>
<td>f3/2+g3</td>
</tr>
</tbody>
</table>

... where "av" is an averaging function, the columns represent distances, and the rows are reiterations. The reflecting surface is column A and the absorbing surface is column G.
In the following example, 7000 molecules are released from the reflecting column A at \( t = 0 \). The above calculations are used to predict the diffusion of the number of molecules per 0.5 \( \mu \)m bin to 10 reiterations:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( x = 0 ) ( \mu )m</td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>( t = 0 )</td>
<td>7000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>3500</td>
<td>3500</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3500</td>
<td>1750</td>
<td>1750</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2625</td>
<td>2625</td>
<td>875</td>
<td>875</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2625</td>
<td>1750</td>
<td>1750</td>
<td>438</td>
<td>438</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2188</td>
<td>2188</td>
<td>1094</td>
<td>1094</td>
<td>219</td>
<td>219</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2188</td>
<td>1641</td>
<td>1641</td>
<td>656</td>
<td>656</td>
<td>109</td>
<td>109</td>
</tr>
<tr>
<td>7</td>
<td>1914</td>
<td>1914</td>
<td>1148</td>
<td>1148</td>
<td>383</td>
<td>328</td>
<td>164</td>
</tr>
<tr>
<td>8</td>
<td>1914</td>
<td>1531</td>
<td>1531</td>
<td>766</td>
<td>738</td>
<td>191</td>
<td>328</td>
</tr>
<tr>
<td>9</td>
<td>1723</td>
<td>1723</td>
<td>1148</td>
<td>1135</td>
<td>479</td>
<td>369</td>
<td>424</td>
</tr>
<tr>
<td>10</td>
<td>1723</td>
<td>1436</td>
<td>1429</td>
<td>813</td>
<td>752</td>
<td>239</td>
<td>608</td>
</tr>
</tbody>
</table>

While the dimensions of the distance axis can be set arbitrarily, more columns between the surfaces will yield smoother shapes.

The relationship between the time intervals and iterations on the spreadsheet can be determined from the diffusion coefficients for the molecule in the appropriate media. The diffusion coefficients for classical transmitters in water are available from standard references. For single molecules, kinetic motion is

\[
t = \frac{x^2}{2D}
\]

For bins of 0.5 \( \mu \)m and the apparent diffusion coefficient \( D \) for DA in the brain of 2.7 \( \times 10^{-6} \) \( \text{cm}^2/\text{sec} \), the time \( t \) represented by each row is

\[
t = (0.5 \times 10^{-4} \text{ cm})^2 / 5.4 \times 10^{-6} \text{ cm}^2/\text{sec}
\]

\[= 463 \times 10^{-6} \text{ sec}
\]

In the example above, an absorbing surface 3 \( \mu \)m distant from the release site absorbs 608/7000 = 9% of the molecules at 4.63 msec.

To estimate the relationship between the number of molecules and the resulting current, one uses Faraday's law as in Section III, which indicates that 1 pCoul = 3.121 x 10^6 molecules of DA. For 463 \( \mu \)sec time bin intervals as in our example, this would correspond to

\[
3.121 \times 10^6 \text{ molecules} \times 463 \times 10^{-6} \text{ sec}
\]

\[= 1445 \text{ molecules} / \text{pA}
\]

Using this approach, the iterations can be expressed as time intervals and the number of molecules as current. Then the simulated events can be compared to genuine amperometric recordings.
In some cases, the random walk will lead to interpolated zero values. In these cases, we “smooth” the data by averaging each of three successive points (the bin in question and its two neighbors) at the \( t \) (x-1) iteration. This smoothing function has little effect on the time course of a random walk.

To adapt a one-dimensional random walk to a two- or three-dimensional model, one decides on the type and shape of each surface in that bin and performs another random walk. Since diffusion in each direction is independent, the fraction of molecules in each bin\(^2\) or bin\(^3\) at a given time is the product of the fractions in the bins in each of the two or three dimensions.

One can adapt the random walk procedure to extremely complex situations. For example, one could scan electron micrographs of the CNS, produce a 3-dimensional reconstruction, guess the sites of absorption (e.g., receptor or uptake transporter) and reflecting surfaces, choose release sites, and then model temporal snapshots of the distribution of neurotransmitter /16/.

\[\begin{array}{|c|c|}
\hline
\lambda & P \\
\hline
0.5 & 0.96394524 \\
0.75 & 0.62716704 \\
0.9 & 0.39273071 \\
1 & 0.2699997 \\
1.1 & 0.1771182 \\
1.2 & 0.1122497 \\
1.25 & 0.0878664 \\
1.3 & 0.0680922 \\
1.35 & 0.0522419 \\
1.375 & 0.0455878 \\
1.4 & 0.0396819 \\
1.425 & 0.0344548 \\
1.45 & 0.0298415 \\
1.475 & 0.0257813 \\
1.5 & 0.0222180 \\
1.525 & 0.0190993 \\
1.55 & 0.0163774 \\
1.575 & 0.0140083 \\
1.6 & 0.0119520 \\
1.625 & 0.0101721 \\
1.65 & 0.0086357 \\
1.675 & 0.0073120 \\
1.7 & 0.0061774 \\
1.725 & 0.0052052 \\
1.75 & 0.0043750 \\
1.775 & 0.0036680 \\
1.8 & 0.0030676 \\
1.9 & 0.0014636 \\
2 & 0.0006709 \\
2.1 & 0.0002955 \\
2.2 & 0.0001250 \\
2.3 & 5.0839E-05 \\
2.4 & 1.9859E-05 \\
2.5 & 7.4533E-06 \\
3 & 3.0460E-08 \\
3.25 & 1.3383E-09 \\
3.5 & 4.5795E-11 \\
4 & 2.5328E-14 \\
5 & 3.8575E-22 \\
\hline
\end{array}\]
APPENDIX C

Using a spreadsheet program for resampling

Resampling to test differences in quantal size between groups was introduced by Van der Kloot /249/. We are not aware of commercial programs that run resampling protocols or available code, although the protocol below could easily be adapted. In our system (Excel 5.0 for Macintosh running on a Power Macintosh G3, 300 MHz) these calculations usually require ~5-10 min for ns of ~100.

To perform the resampling test:

1. Determine the average values of the populations (A and B) to be compared and the difference between the averages. For this example, n_A = 200, and n_B = 300, and the difference in means = 1000 units.

2. Copy the original values from both A and B into the same column and sort by size.

3. Set up a table with the following structure

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>q size</td>
<td>random #</td>
<td>lookup</td>
</tr>
<tr>
<td>1</td>
<td>1677</td>
<td>112</td>
<td>5217</td>
</tr>
<tr>
<td>2</td>
<td>1770</td>
<td>32</td>
<td>2885</td>
</tr>
<tr>
<td>3</td>
<td>1848</td>
<td>128</td>
<td>5718</td>
</tr>
<tr>
<td>4</td>
<td>1860</td>
<td>96</td>
<td>4684</td>
</tr>
<tr>
<td>5</td>
<td>1930</td>
<td>112</td>
<td>5199</td>
</tr>
<tr>
<td>6</td>
<td>2207</td>
<td>103</td>
<td>4975</td>
</tr>
<tr>
<td>7</td>
<td>2226</td>
<td>132</td>
<td>5790</td>
</tr>
<tr>
<td>8</td>
<td>2271</td>
<td>79</td>
<td>4280</td>
</tr>
</tbody>
</table>

where the sorted quantal size values are copied into column B, and numbered 1...n_{A+B} in column A. (The data in column B and D are a portion of those used in Fig. 13.) We create random numbers from 1 to 500 in column C, here using a random number generator in Excel, but other formulae for selecting random numbers can be used /194/. As the random number generator used in this case assigns fractional values from 0 to 1, we multiply the random number by the n_{A+B} of the values and then add 1, since fractional values are adjusted to the next lower value. The random number generator column in Excel in this case is written as

\[=((\text{RANDOM})*500)+1\]

The lookup column D uses the randomly generated number in column C to select a random data value from column B. The form for a lookup function is dependent on the protocol of the program. In Excel 5.0, the format is

\[=\text{LOOKUP}($C1,$A$1:$C$500,2,\text{TRUE})\]

This will take the random number generated in the first row of column C, and from the table (in this case 500 values in each of column A-C), search for the same number in column A, and then report the corresponding value from column B. The lookup instruction is copied into the number of bins of n_A; i.e., if n_A = 200, the lookup command is repeated 200 times to produce 200 randomly selected quantal sizes from the combined population in column D.

4. A new column E is set up to record the average of the 200 values in the lookup column.

5. At this point, a macro should be written that will copy the value from step 4 into a new column and repeat this action 1000 times. In the spreadsheet program we use, the macro must use the “paste special: values” command, or the numbers will change with each iteration. This will result in the new column being filled with 1000 values of 200 randomly selected subsets of the total data.

6. Steps 3-5 are repeated but with the lookup values copied into = n_B bins. Therefore, the lookup command is repeated 300 times to produce 300 randomly selected quantal sizes, and column D will contain 300 values.

7. There should now be two sets of 1000 values. These are placed in neighboring columns and a third column uses a logical function to determine whether the difference is > than the difference between the means of A and B. In Excel, this function is written

\[=\text{IF}(\text{G1-H1}>1000,\text{"1")}\]

That is, if the difference between the data pairs in the first row of columns G and H is greater than 1000, the bin will be labeled “1”.

8. The column with the logical function is summed. The p value = the sum ÷ 1000.
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