

# Modeling the resonant release of synaptic transmitter by hair cells as an example of biological oscillators with cooperative steps

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The initial synapses of the auditory system, which connect hair cells to afferent nerve fibers, display two unusual features. First, synaptic transmission occurs in a multiquantal fashion: the contents of multiple synaptic vesicles are discharged simultaneously. Second, synaptic transmission may be tuned to specific frequencies of stimulation. We developed a minimal theoretical model to explore the possibility that hair-cell synapses achieve both multiquantal release and frequency selectivity through a cooperative mechanism for the exocytotic release of neurotransmitter. We first characterized vesicle release as a four-step cycle at each release site, then generalized the result to an arbitrary number of steps. The cyclic process itself induces some degree of resonance, and may display a stable, underdamped fixed point of the release dynamics associated with a pair of complex eigenvalues. Cooperativity greatly enhances the frequency selectivity by moving the eigenvalues toward the imaginary axis; spontaneously oscillatory release can arise beyond a Hopf bifurcation. These phenomena occur both in the macroscopic limit, when the number of release sites involved is very large, and in the more realistic stochastic regime, when only a limited number of release sites participate at each synapse. It is thus possible to connect multiquantal release with frequency selectivity through the mechanism of cooperativity.

afferent fiber | auditory system | multiquantal release | multivesicular release | vestibular system

The principal means of intercellular signaling in the nervous system is chemical neurotransmission, in which an electrically excited presynaptic cell releases transmitter from an intracellular storage site. Upon binding to postsynaptic receptors, the transmitter molecules produce a postsynaptic current that can be recorded with a microelectrode. Observations made in the presence of a reduced extracellular concentration of  $\text{Ca}^{2+}$ , the ion whose entry into a presynaptic terminal initiates exocytosis, established that transmitter release is quantal in nature. Several lines of experimentation further demonstrated that transmitter accumulates in membrane-bounded synaptic vesicles and that each stochastic fusion of a vesicle with the surface membrane yields a quantal postsynaptic event. Exocytosis occurs at a presynaptic active zone that comprises an array of release sites surrounded by hundreds of synaptic vesicles.

Synaptic transmission faces serious challenges in the auditory system, which has evolved to process high-frequency stimuli with great temporal precision. Our ability to localize a sound source by detecting the interval between the arrival times of the signal at the two ears, for example, requires a neural precision of less than 20  $\mu\text{s}$  (1). In a second example, the firing of neurons in some animals displays significant phase-locking to stimuli at frequencies approaching 10 kHz (2). The first synaptic relay of the auditory pathway connects a hair cell, the sensory receptor of the ear, to an afferent nerve fiber of the eighth cranial nerve. As if to underscore the magnitude of the challenge facing this contact, it displays a morphological specialization characteristic of several sensory systems: the ribbon synapse, without which precise

transmission is impaired (3). Although the role of the synaptic ribbon remains obscure, it is plausible that the organelle contributes to the unusual properties of neurotransmission by hair cells.

Two important qualitative features of synaptic transmission by hair cells have yet to be explained. First, the neurotransmitter content of multiple vesicles can be released synchronously, yielding large, multiquantal postsynaptic responses (4–6). Although this behavior might originate from prefusion of several vesicles, it could alternatively stem from the tight coordination of vesicle release at the synaptic ribbon. Second, in keeping with the auditory system's strategies for frequency tuning of its constituent hair cells and neurons, vesicle release appears to be frequency-tuned as well: When a hair-cell is stimulated by sinusoidal oscillation of its membrane potential, the rate of vesicle fusion as a function of stimulus frequency displays a peak (7). To explore a possible basis of multiquantal release and frequency selectivity at synapses, we developed a minimal model of cooperative vesicle release.

## Description of the Model.

Vesicle release is a cyclical stochastic process. Because a presynaptic terminal contains myriad vesicles but only a limited number of release sites, our modeling centered not on the life cycle of a vesicle, but rather on that of a release site (8, 9). For a biologically plausible model, each site must cycle through a minimum of four distinct states (Fig. 1). First, the site is activated by  $\text{Ca}^{2+}$ , which binds to a protein such as synaptotagmin or otoferlin to initiate exocytosis. Next, after the vesicle's membrane has fused with the surface membrane and transmitter has been released by diffusion, the discharged site retains the complex of SNARE proteins that mediate exocytosis. In the third state, the recovered site is prepared by a series of biochemical reactions to accept the next vesicle. Finally, the site is loaded with a new vesicle and primed for exocytosis. Because there are likely to be additional configurations, we defined the state space of the system at a given time by the possibility that any of  $R$  release sites occupies any of  $N$  states.

We examined the system at two levels of detail. The more detailed representation involves a master equation that treats the sites as identical and represents a Markov process (10) defined by the probability of a population of  $n_i$  sites residing in state  $i$  at a given time. We used this approach to conduct stochastic simulations that are described in the second half of this paper. To begin, we considered a more schematic approach that tracks the deterministic evolution of the macroscopic ensemble average of the number of sites in a given state. We defined the dynamical variable

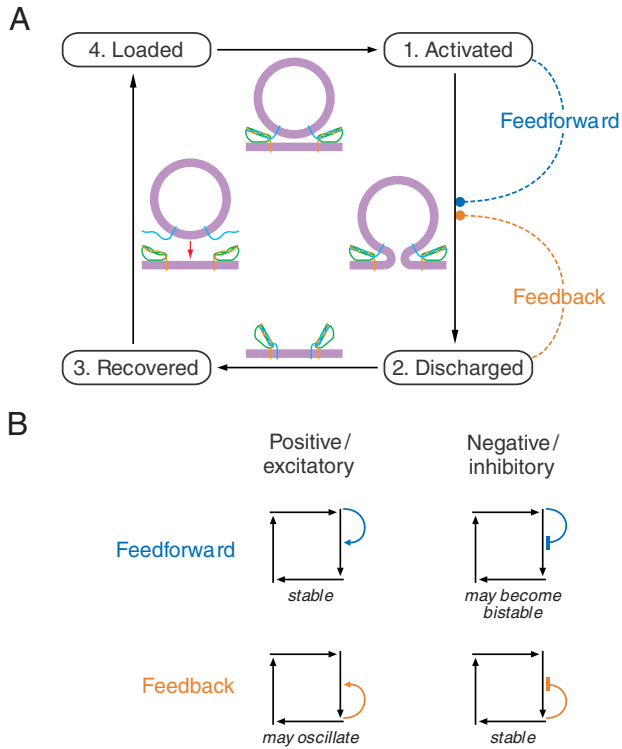
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**Fig. 1.** The Markov ring model expressed as a four-state diagram. **A.** Each release site undergoes a cycle of irreversible, monomolecular steps: *Activated*, the site initiates the fusion of a vesicle to release its contents; *discharged*, the site completes the exocytotic process; *recovered*, the site prepares to accept the next vesicle; and *loaded*, the site docks and primes a new vesicle for release. The central cartoons depict a release site and an associated vesicle during the transitions between successive states. Cooperative effects in the fusion process can take two forms: *Feedforward* in which the population of activated sites influences the rate coefficient for the transition from activated state 1 to discharged state 2, and *feedback* in which this transition is modulated instead by the population of discharged sites. **B.** Positive cooperativity means that a transition's rate coefficient is augmented by an increase in the relevant population, whereas negative cooperativity implies a decrease in the rate coefficient. Flow through the cycle is stabilized by either positive feedforward, in which a larger number of activated sites increases the fusion rate coefficient, or negative feedback, in which a greater number of discharged sites decreases the rate coefficient. Negative feedforward, in which a large number of activated sites decreases the rate coefficient for fusion, exhibits an instability at high cooperativity in which the flow becomes bistable. A large population of activated sites remains large by allowing only a few vesicles to fuse; a small population stays small by permitting more fusions. The case examined in this paper involves positive feedback. For a sufficient amount of cooperative interaction this model becomes oscillatory, and in the vicinity of the oscillatory transition, a Hopf bifurcation, the model displays resonance and frequency selectivity.

$$\dot{\mathbf{x}} = \frac{\langle \mathbf{n} \rangle}{R}, \tag{1}$$

in which the components of the vector  $\mathbf{n}$  are the populations  $n_i$  and therefore a proportion  $x_i$  of sites are in state  $i \in \{1 \dots N\}$ . The dynamics of the system is governed by differential equations of the form

$$\dot{x}_i = k_{i-1,i}x_{i-1} - k_{i,i+1}x_i, \tag{2}$$

with  $N$  transition-rate coefficients  $k_{i,i+1}$ . Arranged in a chain, the states interact only with neighbors and only through irreversible reaction steps; allowing the index to wrap around,  $k_{N,N+1} \equiv k_{N,1}$ , closes the chain into a ring (Fig. 1A). There is a straightforward correspondence between these rate equations

that govern  $\mathbf{x}$  and the master equation for  $\mathbf{n}$  (Supplementary Information). The loss of detail associated with the simpler form is offset by the tractability of the resulting model: The presence of frequency selectivity can be rigorously proven in the deterministic framework and exhibited as a numerical result by stochastic simulation.

The Markov ring model incorporates three different dynamical processes. First, there is a background rate of transitions from each state to the next. This step manifests itself as the linear component of the dynamics of  $\mathbf{x}$ . Second, we introduce an auto-catalytic transition between the states labeled activated and discharged, designated without loss of generality as states 1 and 2, such that the rate of the transition depends on the occupancy of those two states. This term captures the cooperativity between release sites and provides a nonlinear contribution to the dynamics that affects only the two states in question. Finally, we include as the input to our system a periodic parametric forcing of the rate of transition to the *activated* state. The rate equation for  $\mathbf{x}$  can then be rewritten compactly as

$$\dot{\mathbf{x}} = [\mathbf{A} + \varepsilon g(x_1, x_2)\mathbf{B} + F \sin(\omega t)\mathbf{C}]\mathbf{x}, \tag{3}$$

in which the separate contributions of the linear, nonlinear, and parametrically forced components of the dynamics are evident. The function  $g$  captures the nonlinearity,  $\varepsilon$  is its strength,  $F$  is the amplitude of forcing at frequency  $\omega$ , and the matrices  $\mathbf{A}$ ,  $\mathbf{B}$ , and  $\mathbf{C}$  are constant. The rate coefficients of Eq. 2 are connected to the variables of Eq. 3 through  $k_{ij} \equiv A_{ij} + \varepsilon g(x_1, x_2)B_{ij} + F \sin(\omega t)C_{ij}$ . A consequence of Eq. 2 is that, by construction, the total number of release sites is a conserved quantity of the dynamics, such that  $\sum_{i=1}^N x_i = 1$  and the matrices satisfy  $\mathbf{u}^T \mathbf{A} = \mathbf{u}^T \mathbf{B} = \mathbf{u}^T \mathbf{C} = 0$ , with  $\mathbf{u}$  an array of ones.

We set the rate coefficients between states 2, ...,  $N$  as identical and normalized to unity, which is equivalent to rescaling time. The remaining transition, that between states 1 and 2, represents the cooperative vesicle-fusion step for which the rate coefficient  $k_{12}$  is

$$k_{12} = k_0 \left\{ 1 - \frac{\varepsilon}{\nu} + \frac{\varepsilon}{\nu} \left[ c \frac{x_1}{x_1^*} + (1-c) \frac{x_2}{x_2^*} \right]^\nu \right\}. \tag{4}$$

Here  $\nu$  is a Hill coefficient and  $c$  determines the relative contributions of feedforward ( $c = 1$ ) and feedback ( $c = 0$ ). The vector  $\mathbf{x}^*$  is the unique steady state of the linear Markov ring that arises when the strength of the nonlinearity  $\varepsilon$  and forcing  $F$  are set to zero;  $k_0$  is the rate coefficient of the fusion step in that linear case. Because negative cooperativity in this model cannot lead to increased frequency selectivity, we explored only positively cooperative interactions wherein  $k_{12}$  is a monotonically increasing function of  $x_1$  and  $x_2$ . The advantage of writing the nonlinearity in the form of Eq. 4 is that the steady state of the linear problem,  $\mathbf{x}^*$ , remains a known fixed point of the nonlinear system, facilitating the analysis of its stability.

### Response in the Absence of Cooperativity.

Given this simple Markov ring model, we examined the influence of the parameters on the system's ability to resonate. Consider the time dependence of the Markov ring in the linear case,

$$\dot{\mathbf{x}} = \mathbf{A}\mathbf{x}, \tag{5}$$

determined by the sparse  $N$ -by- $N$  transition-rate matrix  $\mathbf{A}$ . For  $N = 4$ ,

$$\mathbf{A} = \begin{bmatrix} -k_0 & 0 & 0 & 1 \\ k_0 & -1 & 0 & 0 \\ 0 & 1 & -1 & 0 \\ 0 & 0 & 1 & -1 \end{bmatrix}. \tag{6}$$



oscillations. In those situations in which a purely real eigenvalue crosses the imaginary axis, a pitchfork bifurcation occurs in the absence of oscillations.

### Phase Diagram of Bifurcations.

To chart the qualitative behavior of the Markov ring, we determined the locations of the bifurcations in  $(k_0, \varepsilon)$  space. The eigenvalues of the central fixed point of an  $N$ -state model are determined by the roots of an  $N$ th-order polynomial in  $\lambda$ :

$$[k_0 + (k_0 - \varepsilon + 1)\lambda + \lambda^2](1 + \lambda)^{N-2} - k_0 = 0. \quad [9]$$

In general, different eigenvalues cross the imaginary axis for distinct sets of parameter values. We are interested in the first such crossing, when all but one of the eigenvalues are stable. One of the roots is always  $\lambda = 0$  owing to the conservation of the number of release sites. To determine the remaining  $N - 1$  roots, we divided the left hand side of Eq. 9 by  $\lambda$ . We sought additional  $\lambda = 0$  roots and thus found that pitchfork bifurcations occur when the parameters satisfy  $\varepsilon_{PB} = 1 + (N - 1)k_0$  (Fig. 3). The Hopf bifurcations, which reflect solutions for which  $\lambda$  is purely imaginary, occur for  $N = 4$  along the line

$$\varepsilon_{HB} = \frac{1}{4}[8 + 5k_0 - \sqrt{k_0(k_0 + 8)}]. \quad [10]$$

### Synchronous Release and Frequency Selectivity.

Upon introducing cooperativity in the fusion step, we expected to see highly correlated and hence synchronous release. We wished to study this phenomenon, along with the anticipated frequency selectivity of the Markov ring's response, both in the macroscopic limit and in the presence of intrinsic noise owing to the finite number of release sites. In order to measure the system's input-output relation, we first specified two details: how an external input couples to the system, and how the frequency selectivity of the output is quantified. We applied a small, periodic, parametric forcing at frequency  $\omega$  and amplitude  $F$ . We depicted the formation of the activated state as linearly dependent on the periodic force, which in terms of vesicle release corresponds to a varying cytoplasmic  $\text{Ca}^{2+}$  concentration. The matrix  $\mathbf{C}$  in Eq. 3 therefore assumes the form

$$\mathbf{C} = \begin{bmatrix} 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & -1 \end{bmatrix}. \quad [11]$$

The output consists of vesicle-fusion events, which are transitions between states 1 and 2 of the Markov ring, that occur at times  $t_j$ . With respect to the phase of the input,  $\omega t$ , the relative phases of the fusion events are  $\theta_j = \omega t_j$  (modulo  $2\pi$ ). We computed the steady-state probability distribution  $P(\theta)$  of event phases in the macroscopic limit by numerically solving differential Eq. 3 and noting that the time-dependent fusion rate is given by  $x_1(t)k_{12}(t)$ . For the stochastic regime in which a finite number  $R$  of release sites participate, we developed a time-dependent Gillespie simulation (14) that allowed us to sample the distribution  $P(\theta)$  (Supplementary Information). Output that is uncorrelated to the input leads to a uniform  $P(\theta)$ . When the output is phase-locked to the input,  $P(\theta)$  exhibits a highly peaked distribution. The extent of phase-locking can be characterized by the vector strength or synchronization index,

$$V = \left| \int_0^{2\pi} e^{i\theta} P(\theta) d\theta \right|, \quad [12]$$

which has the property of being equal to zero for phase-uncorrelated output events and equal to one only when the events always occur at exactly the same phase of the periodic input.

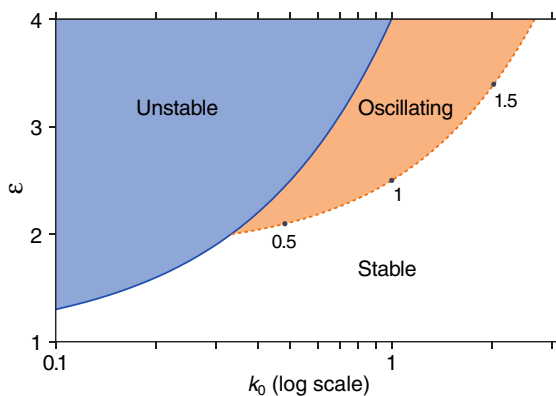
The degree to which events occur in bursts, in a synchronized manner analogous to multivesicular release, can be assessed from the distribution of interevent intervals. The probability that an event  $j + 1$  occurs with a time delay  $\tau$  after an event  $j$  can be written compactly as  $P_{(1)}(\tau)$ . In general, we may consider the delay between events separated by  $m - 1$  other events, with distribution  $P_{(m)}(\tau)$ . For a Poisson process, the waiting-time distribution,  $P_{(1)}(\tau)$ , is an exponential, and the delays between larger separations are determined through the process's Markov property:  $P_{(m)}(\tau) = \int_0^\tau P_{(1)}(\tau - \tau') P_{(m-1)}(\tau') d\tau'$ . Coordinated release becomes apparent in deviations from these distributions.

### Results from Simulations.

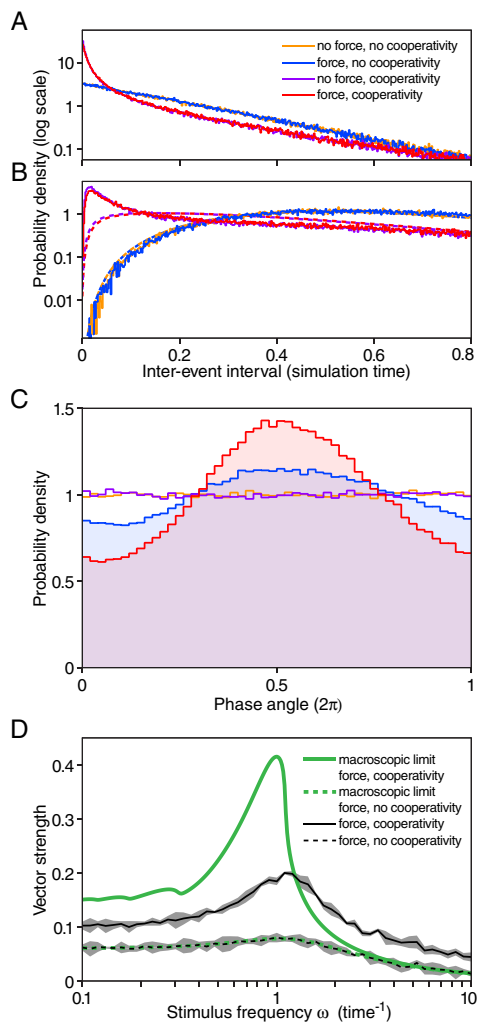
In the absence of cooperativity, release is essentially a Poisson process. By contrast, cooperativity greatly enhances the nearly synchronous release of multiple vesicles (Fig. 4A). By using the freedom to rescale time, we can choose the model system's intrinsic frequency. For a synapse with  $R = 20$  release sites and an intrinsic frequency of 100 Hz, as an example, the probability of releasing four or more vesicles during a 50  $\mu\text{s}$  period increases by a factor of 400 in the presence of cooperativity. As observed experimentally, multiquantal release occurs in the absence of stimulation ( $F = 0$ ) and independently of whether the stimulation is periodic or otherwise (4).

On the timescale set by the mean interevent interval, which is much smaller than the period of stimulation, external forcing has no effect on cooperative or noncooperative release. Stimulation plays a role only on the timescale set by the period  $\omega^{-1}$  of forcing. Even on this much longer timescale, however, cooperative interaction has an effect. The presence of an external force dictates a certain degree of phase synchrony from any system. Cooperativity raises the phase synchrony of the response (Fig. 4B) and does so in a frequency-selective manner (Fig. 4C), even when the system is overwhelmed by intrinsic noise owing to a small number of release sites.

The maximal increase in the vector strength as a function of cooperativity depends on the magnitude of intrinsic fluctuations. Finite-size effects, which blur the macroscopic dynamics and dull the precision of phase-locking, have two sources. Both the number of steps  $N$  in the cycle and the number  $R$  of release sites affect the maximal vector strength and the sharpness of frequency



**Fig. 3.** Stability diagram of the central fixed point in a four-state system. The oscillating region of the diagram corresponds to at least one eigenvalue with a positive real part and a nonzero imaginary part. In the unstable region, at least one of the eigenvalues is positive and real, with zero imaginary part. The boundary separating the stable and oscillating regions represents a line of Hopf bifurcations; the normalized frequencies of the oscillations born there are indicated at three locations. Selecting a specific point along the line of Hopf bifurcations near which to operate tunes the resonance of the system to a specific frequency. A triple point occurs at  $(k_0 = 1/3, \varepsilon = 2)$ .



**Fig. 4.** The effects of cooperativity on release statistics. **A.** The probability of release of two vesicles in quick succession is illustrated by plotting  $P_{(1)}(\tau)$ , which is elevated from its basal state (Blue, Orange:  $\varepsilon = 0$ ) by the addition of cooperativity (Red, Purple:  $\varepsilon = 1.7$ ). The effect is independent of external periodic forcing of amplitude  $F$ , demonstrated by the fact that the purple and orange lines ( $F = 0.5$ ) are identical to their blue and red counterparts ( $F = 0$ ). In the noncooperative case,  $P_{(1)}(\tau)$  follows the exponential distribution expected of a Poisson process, as seen by the straight line on this log-linear plot. Stochastic simulation was performed with  $\omega = 1.1$ ,  $R = 20$ ,  $k_0 = 0.55$ , and  $\nu = 5$ , placing the cooperative system in the stable region near a Hopf bifurcation. **B.** The probability of release of four vesicles in quick succession, illustrated by  $P_{(3)}(\tau)$ , shows even greater elevation owing to cooperativity. The dashed lines show  $P_{(3)}(\tau)$  based on  $P_{(1)}(\tau)$  if the release process were Markovian. The noncooperative case satisfies the Markov condition, whereas the cooperative case does not. **(C)** Phase synchronization in the presence of a periodic stimulus is enhanced by cooperativity. The effect of forcing (Red, Blue) visible in C but not in A and B stems from a separation of timescales: the waiting time between events is dominated by delays smaller than 0.1, much less than the period  $2\pi$  of the forcing. The deviations of the red and blue distributions from the uniform distributions shown in purple and orange, which constitute evidence of phase synchrony, are quantified by the vector strength defined by Eq. 12. The sharper the peak, the greater the vector strength. **D.** The frequency dependence of synchronization is summarized by the vector strength calculated from a series of simulations, such as that in C, in which the stimulus frequency  $\omega$  was varied for a constant force  $F = 0.5$ . The response in the presence of cooperativity is shown in solid black, surrounded by 95% confidence intervals in gray. Traces of subharmonic resonances of the macroscopic limit are visible in the solid green line at frequencies of 0.3 and less. The noncooperative case is portrayed with dashed lines. As indicated by the overlapping of the dashed black and dashed green lines, the macroscopic limit and finite-size simulations agree in the linear noncooperative case. The presence of intrinsic fluctuations limits the maximal synchrony achievable in the cooperative case.

dependence. This effect is apparent as a decrease in vector strength owing to finite values of  $R$  (Fig. 4C).

## Discussion

Inspired by the vesicle-release machinery of auditory synapses, we explored a minimal model of a biological oscillator involving a locally enhanced Markov ring with one nonlinear, cooperative step. By introducing a cooperative step in the form of positive feedback, we were able to explain and connect two previously unrelated observations: multiquantal release, which we associate with temporal synchronization of transitions through the cooperative step; and the frequency selectivity of synchronized release in response to external forcing.

There are numerous models of biological oscillators, many of them based on genetic networks (15, 16). Some involve a time delay and negative feedback (17, 18), whereas others employ a repressive cycle, or rock-paper-scissors mechanism (19). The oscillating quantity is typically the rate of production of a target protein, which in our model is analogous to the rate of fusion events. Further similarities break down, however, because the site-based model that we present here fixes the total number of release sites, whereas the equivalent conservation law usually does not apply in genetic or biochemical networks. The constraint on state space imposed by a fixed population of release sites modifies the dynamics subtly but importantly. For instance, the common negative-feedback mechanism—which resembles negative feedforward in the current formulation—no longer elicits oscillation. It is also worth bearing in mind that the role of oscillation in the Markov ring considered here is not to establish an autonomous rhythm but instead to constitute a resonant filter in a sensory system.

The form taken by the coupling of external stimulation to the Markov ring is highly model-dependent. In the macroscopic limit and for a variety of coupling schemes, the system driven by a periodic stimulus develops chaotic behavior. Parametric forcing of the system can drive it into an unstable region—a likely outcome inasmuch as the system is intentionally poised near an instability—and engender chaos. Of many possibilities, we chose a simple forcing that captures the essentials of  $\text{Ca}^{2+}$  coupling at the synapse and avoids chaos. In any case, noise shifts the critical behavior (20), making it necessary to study the system in the stochastic, finite-size case.

What consequences might our model have for understanding the auditory synapse? Cooperative effects have previously been invoked to explain the dynamics and statistics of synaptic release in other systems. For example, negative cooperativity has been suggested to render release in hippocampal synapses more reliable by decreasing the standard deviation of the number of vesicles released by an action potential (21). The positive cooperativity examined here readily explains the multiquantal release characteristic of hair cells (4–6). More specifically, the large excitatory postsynaptic currents observed electrophysiologically could result from the essentially synchronous fusion of two to perhaps ten synaptic vesicles at adjacent release sites (Fig. 4). Positive cooperativity might arise because the fusion of a first vesicle releases some cytoplasmic factor that triggers the exocytosis of one or more nearby vesicles. Alternatively, as the membrane of a vesicle fuses with the presynaptic membrane, the flow of vesicle proteins or lipids into the presynaptic membrane—or perhaps a consequent change in the tension or fluidity of that membrane—might foster the fusion of additional vesicles. The cyclic nature of vesicle processing at release sites additionally lends itself to frequency tuning, an effect greatly enhanced by cooperativity. The observed sensitivity of the rate of exocytosis to the frequency of stimulation (7) would be expected of a cooperative release system operating near a Hopf bifurcation that would confer frequency selectivity (Fig. 3). The performance of the system depends straightforwardly on a few parameters, offering points for modulation by efferent control. This model

establishes the foundation for future experimental exploration of frequency dependence at the synapse. For a more precise quantitative description of synaptic function, the model could be embellished with additional features such as reversible or deterministic transitions and a two-dimensional geometric arrangement of release sites. The statistics of multiquantal events and synaptic dynamics, including frequency selectivity, could then be fit to the model.

Along with the influence and stability of all the fixed points, the properties of the macroscopic limit might also be investigated in the continuum limit of large  $N$ . It is interesting from the mathematical point of view that this continuum limit corresponds to a modified version of the inviscid Burgers' equation (22); viscosity would arise if reversible components were added to the system.

Whether this limit might shed light on the analysis of synaptic dynamics remains a matter for future investigation. The possible existence of traveling shock waves around the ring and its eventual connection with the frequency selectivity of the dynamics also deserves consideration.

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