Time-coded neurotransmitter release at excitatory and inhibitory synapses

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Communication between neurons at chemical synapses is regulated by hundreds of different proteins that control the release of neurotransmitter that is packaged in vesicles, transported to an active zone, and released when an input spike occurs. Neurotransmitter can also be released asynchronously, that is, after a delay following the spike, or spontaneously in the absence of a stimulus. The mechanisms underlying asynchronous and spontaneous neurotransmitter release remain elusive. Here, we describe a model of the exocytotic cycle of vesicles at excitatory and inhibitory synapses that accounts for all modes of vesicle release as well as short-term synaptic plasticity (STSP). For asynchronous release, the model predicts a delayed inertial exocytotic-endocytotic cycle.

The present study proposes a semiphenomenological multiple-time-scale model to explain the three modes of release as well as STSP in a unified framework. The model is derived via mass action laws and is based on the biological parsimonious view pioneered, in particular, by Thomas Südhof (19) (a summary of the key points of the hypothesized biological model and the detailed derivations of the mathematical equations, which rests upon the assumptions of the biological model, is provided in SI Appendix). The resulting multiple-time-scale mathematical model describes the canonical SNARE and SM protein interaction exocytotic cycle at a mesoscopic scale, and therefore bridges the gap between molecular protein interactions and electrical synaptic activity, as observed in synaptic dual whole-cell recordings.

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Conflict of interest statement

The authors declare no conflict of interest.

Significance

Neurotransmitter exocytosis and short-term synaptic plasticity (STSP) regulate large-scale brain electrical activity. This study is the first, to our knowledge, proposing a multiple-time-scale model that bridges between the microscopic and mesoscopic scales. It is parsimonious, yet with enough descriptive power to express, on the one hand, the interactions between the SNARE and Sec1/Munc18 (SM) protein complexes mediating all forms of neurotransmitter release and STSP, and, on the other hand, the electrical activity required for neuronal communication. A key finding is the discovery of a mathematical structure, termed activity-induced transcritical canard, which quantifies and explains delayed and irregular exocytosis. This structure also provides a previously unidentified way to understand delayed and irregular processes sensitive to initial conditions across various biology processes.

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SNARE-SM Model Assembly
To circumvent the prohibitive complexity of modeling all proteins and detailed (as well as unknown) protein interactions involved in the exocytotic process, we propose to model the interaction of protein complexes semiphenomenologically via first principles of mass action, that is, from a mesoscopic viewpoint. In addition, in an attempt to reduce the time complexity of the physiological processes, the model is based on principles from nonlinear dynamics and multiple-time-scale dynamical systems theory (20–23). This approach results in a deterministic 2D model, with variables \( (p_1, p_2) \) describing the interactions between the canonical SNARE and SM protein complexes; hence, the name SNARE-SM model (SI Appendix). The remaining known exocytotic proteins are considered as regulatory processes, and therefore are treated as parameters that can be tuned to obtain the different modes of release, as idealized in Fig. 2.

There are numerous regulatory proteins; however, only certain proteins are expressed at any given type of synapse (e.g., in Fig. 2, VAMP4 and Syt7 may not be expressed simultaneously). This diversity suggests lumping certain proteins into a single mesoscopic parameter. In contrast, proteins that are shared between different release modes (e.g., Syt1, Syt2, complexin, RIMs, Doc2, TRPV1, voltage-gated Ca\(^{2+}\) channel) remain ungrouped. Altogether, nine parameters are associated with the regulatory proteins (model derivation for further biophysical interpretation of the model’s parameters is provided in SI Appendix).

An important regulatory parameter is the positive small parameter \( 0 < e < 1 \), which induces a separation of time scales between \( p_1 \) and \( p_2 \). Specifically, \( p_1 \) corresponds to a slow-acting protein complex, whereas \( p_2 \) is a fast-acting protein complex. The remaining parameters regulate the interaction strength between \( p_1 \) and \( p_2 \) as well as the conformational changes of the individual protein complexes. The resulting model expresses features of slow, evoked irregular and spontaneous activation. These features emerge from the rules of interaction between the protein complexes \( (p_1, p_2) \) as expressed by the right-hand side of the SNARE-SM model equations (SI Appendix). These interactions are best described (in mathematical terms) by plotting the components of the interaction rules (technically, nullclines) in a 2D space (phase-space) spanned by the actions of \( p_1 \) and \( p_2 \) (Fig. 3A and

![Fig. 1. Parsimonious SNARE-SM molecular exocytotic machinery (modified from ref. 1).](https://www.pnas.org/)

![Fig. 2. Schematic idealization of the SNARE-SM model. The circular center describes the canonical fusion machinery constituted by the SNARE complex and SM proteins, which is ultimately regulated by complexin and synaptogamins (19). This building block is signaled by various proteins and, depending on the proteins involved, the appropriate neurotransmitter release mode is activated (i.e., synchronous, asynchronous, spontaneous). Some of the known proteins associated with each type of release are indicated (reviewed in 17, including a complete description and the latest view on the association between proteins and release modes). The RIM proteins are shared between synchronous and asynchronous release modes, whereas TRPV1, Doc2, and voltage-gated Ca\(^{2+}\) channels (VDCCs) are shared between asynchronous and spontaneous release modes. The remaining proteins are specific to each release mode; however, inhibiting a protein specific to a given release mode will favor the expression of other modes (17).](https://www.pnas.org/)

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Moreover, the model variables can be activated by a presynaptic event to activate the SNARE-SM model, which mediates all of the functions associated with the exocytotic-endocytotic cycle (red curve): priming (P), fusion (F), endocytosis (E), and refilling (R). Note that priming stage I initiates after point U, whereas priming stage II initiates after point TC. Arrows indicate dynamic trajectories in the phase plane. Time course of presynaptic voltage (B1) and p2 activity following a stimulus (B2). Note that, here, t refers to a dimensionless time. (B3) Schematic diagram of an energy landscape where stimulus spikes are required to activate p1 and p2, represented as a particle that initiates movement only if sufficient energy is provided to traverse the energy barrier (U).

**Fig. 3.** SNARE-SM model dynamics and asynchronous mechanism. (A) Interactions between protein complexes p1 and p2 along the vesicle cycle are given by the parabola and the horizontal line (black). These intersections give rise to special points S, U, TC, and SN, which mediate all of the functions associated with the exocytotic-endocytotic cycle (red curve): priming (P), fusion (F), endocytosis (E), and refilling (R). In particular, S can be associated with Munc13-1, forming a homodimer that inhibits priming. Then, U can be related to the action of Munc13 gating the transition from the closed-syntaxin/Munc18 complex to the SNARE complex formation. Subsequently, TC can be linked to the action of complexin, and finally, SN can be connected to the refilling of the vesicle pool. It is noteworthy to observe that the resulting phase-space geometry of the mathematical model shares a great deal of similarity with the schematic diagram of the SNARE-SM biological model by Südhof (19) (compare Fig. 3A and SI Appendix, Fig. S1C), which generate all of the functions associated with each stage of the exocytosis-endocytosis cycle.

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The delay is specifically explained by a previously unexplored mathematical structure that acts as a dynamic (delayed) response to an input via transcritical canards (22, 23), which we denote, “activity-induced transcritical canards” (SI Appendix). This structure quantifies the delay and predicts a delayed inertial protein unbinding associated with the SNARE complex assembly immediately after vesicle priming. Previous modeling attempts introduced stochastic elements or a hardwired delay into the model to account for asynchronous release (24–28). In contrast, the delay in the SNARE-SM model emerges as a result of a dynamic mechanism that resembles a biological process.

In brief, the SNARE-SM model has a mechanistic interpretation because it can be related to processes associated with exocytotic-endocytotic signaling pathways, including intracellular calcium dynamics. Moreover, the delayed irregular activation can be associated, for example, with the action of complexin or Syn I(II) and with the presence of cCB, VAMP4, or even Doc2 in the case of excitatory neurons.

**Extended SNARE-SM Model.** We extend the SNARE-SM model to show how STSP mechanistically integrates within the exocytotic-endocytotic machinery, and also to enable comparison with electrophysiological data. This extension is achieved by feeding the exocytotic-endocytotic signal of the SNARE-SM model into an STSP model, which effectively activates the vesicle pool. In particular, we use the Markram–Tsodyks (MT) STSP model (29–31) (SI Appendix). The MT equations phenomenologically model the time evolution of available resources (vesicles) and how efficiently neurotransmitters are released. In the model there are two quantities, namely, the number of vesicles, \( d \), and the release probability, \( f \), which are updated for every presynaptic spike occurring at time instant \( t_n \). The model predicts the amount of neurotransmitter released, \( T(t_n) = d(t_n)f(t_n) \), which, in reality, is released with a small time delay.

The MT model successfully accounts for the highly heterogeneous STSP dynamics across different brain areas in the context of synchronous release (table S1 of ref. 31). Consequently, the proposed model extends the MT model by incorporating all three modes of neurotransmitter release observed at unitary synapses. However, to complete the model framework and to enable testing against data sampled from whole-cell paired recordings obtained from unitary synapses, an observational variable representing postsynaptic potential is required. This observational variable is modeled with the standard conductance-based (subthreshold) equation, where the action of neurotransmitters on postsynaptic neurotransmitter receptors follows the first-order kinetic equation (SI Appendix). More detailed approaches for modeling receptor dynamics [e.g., detailed kinetics (32)] will be a matter for future consideration.

**Results**

**SNARE-SM Model Dynamics.** The SNARE-SM model has three operating modes. Fig. 3A shows a presynaptic terminal, which encloses the SNARE-SM model’s signaling mechanism. The black arrows labeled \( p_1 \) and \( p_2 \) span the 2D space within which the protein complexes interact. This space is not physical, but...
The stability of the fast nullclines is assessed by looking at the mathematical limit of the model when \( p_1 \) is kept constant \( (\epsilon = 0) \) (details are provided in SI Appendix). In this limit, the only variable left is \( p_2 \), and \( p_1 \) acts as a parameter; the equilibrium states lie on the fast nullclines, and their stability depends on the parameter \( p_1 \) and change at bifurcation points SN and TC. Under normal operating conditions \((\epsilon > 0)\), \( p_1 \) evolves slowly; the points SN and TC are not bifurcation points of the model any longer; however, they still organize dynamic transitions between different levels of quasistationary activity close to \( \Gamma_1 \) and \( \Gamma_2 \). Moreover, the SNARE-SM model possesses two true stationary states, marked S and U (Fig. 3A and SI Appendix, Fig. S1C), which endow it with an excitable structure.

An exocytotic signal (Fig. 3A, red trajectory) is evoked by one or more presynaptic spikes. Input stimuli excite the system away from the functionally inactive state S. However, the protein complexes switch their functional behavior past the switching point (U) only when sufficient energy is available, via action potentials and an increase in calcium influx. In this case, the system passes the TC transition point, which enables the appropriate exocytotic signaling mode to be activated. Fig. 3B illustrates the process in the time domain: Fig. 3B1 shows the presynaptic stimulus; Fig. 3B2 shows the output signal; and Fig. 3B3 is a schematic diagram that depicts a particle (in the abstract sense), initially at a rest point (S), that is driven out of the basin of attraction of S by a sufficient force (blue arrows), enabling it to jump the energy barrier (U). We refer the reader to the article by Kasai et al. (33) for discussion on energy functions associated with the release of neurotransmitters. Thus, a particular amplitude and timing of a perturbation can drive the system away from the equilibrium point and induce it to make a large-amplitude, transient excursion before it settles again to its inactive state (S).

Past the switching point (U), the protein complexes \( p_1 \) and \( p_2 \) begin to interact strongly, activating states associated with vesicle priming I. The passage through the TC point can be associated with the initiation of priming stage II (i.e., SNARE complex assembly and regulation by complexin). Priming can be a fast (synchronous) or slow (asynchronous) process, depending on the time scale parameter \( \epsilon \).

From a mathematical perspective, precise quantitative control of the delay is achieved by the so-called “way-in-way-out function” (SI Appendix). In short, the activity-induced transcritical canard predicts the existence of delayed inertial protein unbinding occurring between priming I and fusion-pore opening stages. This delayed inertial protein unbinding can possibly be related to the clamping action of complexin, or \( \text{Ca}^{2+} \)-activated calcium sensors (e.g., Syt1) competing with complexin for SNARE complex binding (by displacing part of complexin within the SNARE but via a delayed inertial unbinding). Indeed, from the modeling point of view, \( \epsilon \) (which also controls the delayed process), can be associated with complexin or \( \alpha \)-synchronous calcium sensors at a molecular level (SI Appendix). The unbinding between \( p_1 \) and \( p_2 \) (e.g., interpreted mesoscopically as translocation of complexin) initiates fusion (F) and subsequent neurotransmitter release. Following exocytosis, \( p_1 \) and \( p_2 \) begin a second phase of strong interaction that induces endocytosis (E) and subsequent vesicle refilling (R). The final stage is triggered by the SN transition point, which prompts \( p_1 \) and \( p_2 \) to alter their states and evolve toward their inactive state S, where the vesicle pool is replenished.

**SNARE-SM Model Evoked Release Mode.** Evoked synchronous and asynchronous modes of release in the SNARE-SM model are shown in SI Appendix, Figs. S2 and S3, with the parameters specified in SI Appendix, Table S1. For the synchronous mode, SI Appendix, Fig. S3 A–C2 shows that the SNARE-SM model’s output, \( p_2 \), is activated almost instantaneously upon an incoming stimulus, \( V_{in} \). In this case, \( \epsilon \) has a small value. Increasing \( \epsilon \) induces a weaker binding/unbinding that effectively introduces variability (irregular activation via sensitivity to initial conditions) and a strong inertia in the unbinding process, causing a delay. This asynchronous mode is shown in SI Appendix, Fig. S3 B–D2, where the onset of \( p_2 \) is delayed with respect to the stimulus. Note that the output time profile also changes shape and amplitude, with a slower rising phase. These features are crucial, leading to gradual activation of vesicle pools as well as postsynaptic receptors, consistent with the gradual postsynaptic potential response observed in experiments for asynchronous release (1).

**SNARE-SM Model Evoked Synchronous Release Mode.** We now test the full model [extended (E)-SNARE-SM] with paired whole-cell recordings from both inhibitory and excitatory synapses having differential modes of exocytosis. For inhibition, we use recordings from isolated synapses between cholecystokinin (CCK)-positive Shaffer collateral-associating
pressed and delayed IPSP data resulting from spikes occurring at times of CCK-positive SCA interneuron to unitary input spike at time events: Vertical red dashed lines mark spike times, and vertical blue lines release). (Inset) Expansion of the region corresponding to the five release times and amplitudes of the inhibitory postsynaptic potentials (IPSPs) and excitatory postsynaptic potentials; therefore, associated parameter values can vary substantially between release events. The remaining parameters are tuned within a bounded region (inhibitory synapses are shown in SI Appendix, Table S2, and excitatory synapses are shown in SI Appendix, Table S3). Details of the parameter fitting procedures are provided in SI Appendix.

The E-SNARE-SM model successfully reproduces the synaptic dynamics of the SCA inhibitory synapse (Fig. 4). The delayed unitary IPSP in Fig. 4A1 is compared with the output of the inhibitory model (Fig. 4B1). A sequence of IPSPs exhibiting short-term synaptic depression and delay in response to multiple presynaptic stimuli (Fig. 4A2) matches the output of the model in Fig. 4B2. The response to a sequence of IPSPs featuring short-term synaptic facilitation and delay, shown in Fig. 4A3, is compared with the response of the model in Fig. 4B3. The model reproduces the onset of the delays and the temporal profile of the IPSP data. Care was taken with fitting delayed release because the model is sensitive to initial conditions. Completion of an exocytotic-endocytotic cycle brings the system to a different configuration. As a consequence, the parameters of the previous exocytotic-endocytotic cycle will give rise to a different delayed response when a new stimulus occurs. Parameters associated with GABAA-induced currents also undergo changes, albeit minor, because eCBs increase the input resistance of the cell, docking time of neurotransmitters, and affinity.

The parameters of the MT model also depend on the mode of release. Continuity conditions are enforced to ensure that different epochs of data fit with different modes of release (shaded magenta and cyan rectangles in Fig. 4 A2, B2, A3, and B3). Future developments will include the conditions ensured by the way-in-way-out function for an automatic parameter fitting. However, in the limit of complete depletion of neurotransmitters, fitting any continuous mesoscopic model to electrophysiological data becomes increasingly difficult, because noise dominates and expressing microscopic dynamics becomes fundamental (averaging effect is shown in SI Appendix, Fig. S7). In this limit, other theoretical studies reveal that discrete, stochastic, or agent-based models best describe microscopic activity (38).

Comparisons between excitatory postsynaptic currents at the calyx-of-Held synapse and the postsynaptic currents of the E-SNARE-SM model are made in Fig. 5. Specifically, Fig. 5A1 depicts a synchronous activation to a single presynaptic spike, which is matched by the model in Fig. 5B1. Multiple postsynaptic activations elicited by a single input are shown in Fig. 5A2. The first postsynaptic activation is asynchronous, and the two subsequent releases are spontaneous. The model is in good agreement over three epochs shown in different colors (Fig. 5B2). Moreover, the model can also reproduce the WT data from the calyx of Held, mark IPSP response times. The distance between them measures the delay: ~ (2.0, 2.6, 2.5, 9.2, 15.0) ms. (B2) Response of the model to the same input as in A2. (A3) Facilitated and delayed IPSP data. The first epoch (shaded magenta rectangle) is triggered by the first three spikes causing synchronous mode (release within 5 ms); the second epoch (shaded cyan rectangle) is initiated by two subsequent spikes that lead to asynchronous mode (more than 5-ms delayed release). (B3) Response of the model to the same input as in A3.
In particular, the strong synaptic depression seen at this synapse during high-frequency stimulation and the kinetics of recovery from synaptic depression can both be captured. Indeed, our model builds upon the MT framework, which has been shown to account for these phenomena (39).

Discussion

The proposed multiple-time-scale SNARE-SM model extends the MT framework for STSP by incorporating all three forms of exocytosis at the same mesoscopic level of description (37). Moreover, our mathematical model is in good agreement with the biological SNARE-SM model of Südhof (19) (compare again Fig. 3 and SI Appendix, Fig. S1C with SI Appendix, Fig. S1A). Details of the biochemical pathways involved in exocytosis are semiphenomenologically expressed; therefore, predictions of the model can be compared with SNARE-SM physiology, and computational hypotheses can be explored to propose novel experiments. For example, in the model, the three distinct forms of release share the same exocytotic machinery, where the modes of exocytosis are a consequence of parameters in the model. Therefore, in every exocytosis-endocytosis cycle, the release mode may switch due to slowly varying physiological variables that have not yet been identified. However, it is important to be cautious because there may be different vesicle pools or pathways (e.g., different calcium sensors) (4).

The time-scale parameter $\varepsilon$ modulates the activity-induced transcritical canard, which mechanistically explains the ratio between synchronous and asynchronous release. The way-in–way-out function quantifies how the exocytotic-endocytotic signaling pathway fine-tunes the timing of neurotransmitter release, which can be seen as a homeostatic mechanism for efficient neuronal communication. This mechanism is consistent with molecular studies showing that within the canonical fusion machinery, Syt1 and complexin are functionally interdependent and are potentially the key players in regulating all modes of release (19). Specifically, Syt1 mediates calcium-triggered release and controls the rate of spontaneous release (i.e., speed and precision of release by associations with SNARE complexes). Complexin is a cofactor for Syt1 that functions both as a clamp and as an activator of calcium-triggered fusion (19).

Further upstream, other proteins could signal (via yet unknown interactions) this homeostatic system. For example, studies show that Syn I(II), known to coat synaptic vesicles and to have a postdocking role, regulates synchronous and asynchronous release (15). In particular, Syn II interacts directly with P/Q type and indirectly with N-type Ca$^{2+}$ channels to increase asynchronous release. Additionally, Syn I(II) seems to constitute a push/pull mechanism regulating the ratio between synchronous and asynchronous release (15), thus suggesting that they share exocytotic mechanisms. Deeper insight into this mechanism could result from further molecular studies investigating the existence of a signaling pathway between cannabinoid type 1 (CB1) receptor, Syn I(II), RIMs, and RIM-BS proteins, because CB1 also appears to interact with N-type and P/Q-type Ca$^{2+}$ channels (40, 41). Nevertheless, multiple exocytotic mechanisms should not be ruled out, and augmenting the proposed model to allow switching between them is a focus for future research.

The proposed model could also be mapped onto the dual calcium-sensor model (4). Another reported mechanism that should be considered is the VAMP4-enriched vesicle pool, which...
is formed after intense stimulation and enables asynchronous release (11). Surprisingly, the authors show that VAMP4-driven SNARE complexes do not readily interact with synaptotagmin and complexin, which challenges the widely held view that synaptosomal release requires interaction of SNARE complexes (e.g., VAMP4/SNAP-25, syntaxin-1) with Syt1 and complexins. This issue could be resolved by seeking an alternative way to elicit VAMP4-mediated release (identifying a different signaling pathway). In view of the present model, it would be relevant to test for VAMP4 in synapses expressing CCK. Despite these observations, the SNARE-SM model can explain these results without assuming the existence of a second, VAMP4-enriched pool of vesicles (SI Appendix, Fig. S5 B–B2).

Another refinement may emerge from a recent study showing that 2-arachidonoylglycerol (2-AG)/anandamide directly modulates GABA_A postsynaptic receptors, therefore affecting neurotransmitter docking times and possibly contributing to asynchronicity (42). Other forms of synaptic plasticity, such as spike timing-dependent plasticity mediated by differential excitotoxins, could also be explored with the proposed model (SI Appendix, Fig. S6).

Finally, the SNARE-SM model will facilitate large-scale network simulations and consequently explain the functional role of differential excitotoxins and synaptic plasticity on network states underlying memory, cognition, and pathological brain states (e.g., epilepsy) (43). At a microscale, the proposed theoretical approach could provide new insights into the function of other protein–protein interactions. For example, activity-induced transcritical canards can explain recent experiments that identify proteins mediating the asynchronous activation of sodium and potassium channels (44).

**Materials and Methods**

**Inhibitory Synapses.**

**Experimental preparations and observations.** The data are sampled from paired whole-cell recordings obtained from unitary synapses between CCK-positive SCA interneurons in the CA1 region of P18–P21 rat hippocampus (45) (SI Appendix, Fig. S5). These cells possess a modulatory feedback mechanism that allows the postsynaptic cell to control the level of presynaptic GABA_A release via the eCB system, which is composed of cannabinoid receptors, ligands, and the relevant enzymes (45). Specifically, eCB, 2-AG, or anandamide is synthesized and released on demand, involving depolarization of the postsynaptic membrane via the activation of voltage-dependent L-type calcium channels (46). Once synthesized, it diffuses across the synaptic cleft to modulate the activation of CB1 receptors located in the presynaptic cell. Subsequently, CB1 receptors inactivate N-type (and possibly P/Q-type) calcium channels (therefore reducing Ca_2+ concentration) leading to a reduction of GABA_A release (45). Experimentally, the level of CB1 receptor activation and deactivation was controlled by bath application of endogenous cannabinoid agonist anandamide and antagonist AM-251. The endogenous agonist effects could be mimicked by depolarization-induced suppression of inhibition protocols, which involved depolarization of the postsynaptic membrane (45). These modulatory synaptic effects have a direct impact on the timing of synaptic inhibition, specifically asynchronous release and STSP (SI Appendix, Fig. S7). Details of the experimental preparation are explained.

**Slice preparation.** Male Wistar rats (P18–P23; Harlan UK) were anesthetized with sodium pentobarbitone (60 mg/kg Euthatal; Merial) via i.p. injection and perfused transcardially with ice-cold modified artificial cerebral spinal fluid (ACSF) containing 15 mM D-glucose, 248 mM sucrose, 2.5 mM CaCl_2, 3.3 mM KCl, 1.2 mM MgCl_2, 25.5 mM NaHCO_3, and 1.4 mM NaH_2PO_4. Following decapitation, the brain was removed and 300-μm-thick coronal slices of cerebral cortex were cut. These procedures were performed under UK Home Office guidelines by authorized Home Office license holders. The severity of the procedures was classed as moderate. The total number of rats used for this study was 61. Slices were incubated for 1 h before recording, for which they were placed in a submerged chamber perfused with ACSF at a rate of 1–2 mL/min. ACSF contained 20 mM D-glucose, 2 mM CaCl_2, 2.5 mM KCl, 1 mM MgCl_2, 121 mM NaCl, 26 mM NaHCO_3, and 1.25 mM Na_2PO_4 [equilibrated with 95% (vol/vol) O_2 and 5% (vol/vol) CO_2]. All substances used to make ACSF solutions were obtained from VWR International (45). 

**Electrophysiological recordings.** Electodes with resistances of 8 to 10 MΩ were used to make ACSF solutions were obtained from VWR International (45).

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