Synaptotagmin: A Calcium Sensor on the Synaptic Vesicle Surface

Author(s): Nils Brose, Alexander G. Petrenko, Thomas C. Südhof and Reinhard Jahn


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that does not require calculation of derivatives is to look for changes (controlled in appropriate ways) in the parameter values that reduce the error. In the simulations described in this report the model was endowed with a dual, incremental learning mechanism. First, when the model's performance on a new input was markedly inadequate (in comparison with recent history), that input was added as a new component to the model as an additional center (prototype). This happened mainly in the initial trials, with the number of centers eventually reaching an asymptote that depended on the nature of the task and on the parameters that affected the decision to add new centers. The performance of the model during these first trials improved, then stabilized as the number of centers approached the asymptote. Second, further gradual improvement in the performance was obtained by letting the model carry out a local random search in the space of existing HB center coordinates. This search was guided by feedback given to the model (that is, by indicating whether the response at each trial was correct). Details of the learning algorithms, including an extension of the incremental learning algorithm to a situation in which no explicit feedback is available, can be found in Edelman and Kramar (1986).


8. We have also experimented with a different version of the HyperB model, in which orientation-selective receptive fields similar to those of simple cells in V1 played the role of the basis functions. See (7). This version of the model replicated the absolute values and the time course of the improvement of the thresholds found in human psychophysical data in the condition of replicating the data concerning the percentage of correct responses.

9. Hypocretin-level performance was independent of the precise location of the receptors. At the same time, different quasi-random receptor mosaics yielded different thresholds, sometimes by as much as a factor of 2. A similar range of hyperacuity thresholds is observed in human subjects, even at full acuity and with perfectly normal eyes.

10. The model also exhibited learning on a longer time scale (4, 7), similar to the slow long-term learning component found in human subjects (M. Fahlke and S. Edelman, in preparation).


12. The stimulus in the bisection task consists of three dots, arranged in a vertical line, at an approximately even spacing. The subject has to determine whether the middle dot is above or below the midpoint of the segment formed by the other two dots. The hyperacuity module learned this hyperacuity task just as easily as it did in the line vernier case (6). Another simulation made a comparison between the line vernier task and a similar one in which each of the line segments has been replaced by two dots (situated at the endpoints). The network learned this task, as it did previously, in the line vernier and the bisection cases. The better performance of the HyperB module in the dot vernier task for small offsets parallels a recent surprising finding with human subjects (M. Fahlke, unpublished observations).

13. In a recent study, R. Bennett and G. Westheimer (Perception 19, 541 (1990)), we found surprisingly little learning of thresholds in three-dot alignment and grating discrimination. Their experiments used transfer of training across the stimulus range to probe for learning, hiding possible effects of fast learning that may have happened in the baseline session (p. 544). Interestingly, the lack of transfer across the stimulus range in these experiments is consistent with our notion of experience-based learning.


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Synaptotagmin: A Calcium Sensor on the Synaptic Vesicle Surface

Nils Brose,* Alexander G. Petrenko, Thomas C. Südhof, Reinhard Jahn†‡

Neurons release neurotransmitters by calcium-dependent exocytosis of synaptic vesicles. However, the molecular steps transducing the calcium signal into membrane fusion are still an enigma. It is reported here that synaptotagmin, a highly conserved synaptic vesicle protein, binds calcium at physiological concentrations in a complex with negatively charged phospholipids. This binding is specific for calcium and involves the cytosolic domain of synaptotagmin. Calcium binding is dependent on the intact oligomeric structure of synaptotagmin (it is abolished by proteolytic cleavage at a single site). These results suggest that synaptotagmin acts as a cooperative calcium receptor in exocytosis.

Calciun-dependent exocytosis of synaptic vesicles is the central step in the sequence of events from the arrival of an action potential to the release of neurotransmitters. It is generally accepted that Ca2+ enters the nerve terminal via voltage-gated Ca2+ channels in the presynaptic plasma membrane. Intracellular recordings in model synapses such as the squid giant synapse have shown that the latency between Ca2+ entry and the release of transmitter is in the range of 200 μs. This implies that a complex between synaptic vesicles and the plasma membrane must exist in the resting state because the time after Ca2+ entry is too short to allow for vesicle docking before fusion. Furthermore, the dependence of transmitter release on the intraterminal Ca2+ concentration is nonlinear and highly cooperative (1).

The Ca2+ receptor protein for exocytosis has not yet been identified. However, certain predictions about its properties can be made. Because of the short latency between Ca2+ influx and exocytosis, it is likely that the Ca2+ receptor is part of the complex formed between the plasma membrane and the synaptic vesicle and is probably located on one of these membrane compartments. In addition, Ca2+ must induce a change in the properties of the receptor protein, which ultimately causes a rearrangement of the membrane.
phospholipid microdomains at the contact site between the two membranes, leading to membrane fusion. It is unclear how many proteins participate in this process. However, the speed of the event makes it unlikely that major protein rearrangements, such as Ca\(^{2+}\)-induced docking of cytosolic proteins, are involved (1).

In this report, we present evidence that the synaptic vesicle protein synaptotagmin (also referred to as p65) exhibits properties expected of the exocytotic Ca\(^{2+}\) receptor. Synaptotagmin is represented by a family of related integral membrane proteins with widespread distribution (2–4). It is specifically localized in the membrane of synaptic vesicles and that of secretory granules in endocrine cells (2). Structural analysis of this protein family revealed the existence of two copies of an internal repeat that are homologous to corresponding domains in protein kinase C (C\(_2\) domain) (5, 4), in a cystolic form of phospholipase A\(_2\), and, although with less identity, in guanosine triphosphate–activating protein and phospholipase C (5). In protein kinase C and phospholipase A\(_2\), this domain is thought to be responsible for the Ca\(^{2+}\)-dependent binding of these proteins to phospholipid membranes (5). This prompted us to investigate whether native synaptotagmin functions as a Ca\(^{2+}\) binding protein and whether Ca\(^{2+}\) binding by synaptotagmin involves interactions with membrane phospholipids.

To study the properties of synaptotagmin, we isolated the protein from Triton X-100 extracts of rat brain by affinity chromatography, using a newly generated monoclonal antibody (clone Cl 41.1) directed against synaptotagmin I as affinity ligand (6). Synaptotagmin I was purified in a single step, resulting in a protein of more than 90% purity.

We studied the Ca\(^{2+}\) binding to purified synaptotagmin by equilibrium dialysis either in the presence or in the absence of phospholipids (7). As controls, Ca\(^{2+}\) binding to phospholipids alone or to purified synaptophysin, an integral membrane protein of synaptic vesicles (8), was monitored under identical experimental conditions. No Ca\(^{2+}\) binding to synaptotagmin was observed in the absence of phospholipids (Fig. 1). However, when liposomes containing 25% phosphatidylserine and 75% phosphatidylcholine were added, a dramatic increase in Ca\(^{2+}\) binding over that of the phospholipid control was observed (Fig. 1). The Ca\(^{2+}\) binding reached 4 mol per mole of synaptotagmin subunit at a free Ca\(^{2+}\)

![Graph](https://example.com/graph1.png)

**Fig. 1.** Binding of Ca\(^{2+}\) to synaptotagmin in the presence or absence of phospholipid vesicles, determined by equilibrium dialysis (7). As control, Ca\(^{2+}\) binding to protein-free liposomes is shown. (A) Total Ca\(^{2+}\) binding: (●) synaptotagmin plus phospholipid vesicles; (○) phospholipid vesicles alone; (▼) synaptotagmin alone. Note that phospholipid vesicles bind Ca\(^{2+}\) in a linear dependence on the Ca\(^{2+}\) concentration. (B) Net Ca\(^{2+}\) binding to synaptotagmin (●) and, as control, to synaptophysin (▼), in the presence of phospholipids. We obtained the values by subtracting binding to phospholipids alone from total binding. Numbers of moles were calculated on the basis of the molecular weight of the monomers, respectively.

![Graph](https://example.com/graph2.png)

**Fig. 2.** Ca\(^{2+}\)-dependent interaction of purified synaptotagmin with phospholipid vesicles, measured by fluorescence resonance energy transfer between tryptophan residues of phospholipids (excitation at 284 nm) and dansylated phospholipid head groups (emission at 520 nm). Synaptotagmin was added either in micellar form (A and B) or incorporated into liposomes (C). (A) Ca\(^{2+}\) causes an increase of fluorescence resonance energy transfer, which is reversible upon EGTA addition. The phospholipid composition of the liposomes was 50% phosphatidylethanolamine, 40% phosphatidylethanolamine, and 10% dansyl-phosphatidylethanolamine (13). (B) Ca\(^{2+}\)-dependent fluorescence resonance energy transfer is saturable and depends on the phospholipid composition. Values were expressed as a percentage change in emission intensity, I\(_e\) = (I - I\(_0\)) \times 100/I\(_0\), where I is the recorded fluorescence intensity in the experiment and I\(_0\) is the reference fluorescence intensity in the absence of Ca\(^{2+}\). (●) Liposomes containing 50% phosphatidylethanolamine; 40% phosphatidylethanolamine, and 10% dansyl-phosphatidylethanolamine (mean values of three independent experiments); (○) liposomes containing 25% phosphatidylethanolamine, 65% phosphatidylethanolamine, and 10% dansyl-phosphatidylethanolamine; (▼) control, using an assay mixture as in (●) that was digested for 1 hour at 37°C with trypsin (1 μg/ml) before the experiment (14) (data shown are from a representative experiment). (C) Ca\(^{2+}\)-dependent fluorescence resonance energy transfer between synaptotagmin-containing donor liposomes and dansylated acceptor liposomes. The donor liposomes were composed of 45% phosphatidylcholine, 45% phosphatidylethanolamine, and 10% phosphatidylserine; the acceptor liposomes were the same as in (A).

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concentration of $10^{-4}$ M (Fig. 1). In contrast, no significant Ca$^{2+}$ binding was observed up to a concentration of $10^{-4}$ M when purified synaptophysin instead of synaptotagmin was used, regardless of whether phospholipids were present (Fig. 1). This indicates that, contrary to an earlier report (8), synaptophysin does not bind Ca$^{2+}$. Thus, the ability to bind Ca$^{2+}$ is a specific, intrinsic property of synaptotagmin.

To study the interaction between synaptotagmin and phospholipids in more detail, we utilized a fluorescence resonance energy transfer assay. Dansyl-phosphatidylethanolamine was incorporated into liposomes. These liposomes were mixed with purified synaptotagmin. Fluorescence resonance energy transfer between tryptophan residues in synaptotagmin and the dansyl group in the liposomes was measured as a function of Ca$^{2+}$. Energy transfer is highly dependent on the distance between the two fluorophores and is only observed on close contact of the protein and the lipid fluorescent probe. We found that Ca$^{2+}$ triggered fluorescence resonance energy transfer in a dose-dependent manner (Fig. 2A), suggesting that Ca$^{2+}$ causes a close association of synaptotagmin with the dansyl liposomes. The signals were reversed by addition of EGTA (Fig. 2A) and reestablished by subsequent addition of excess Ca$^{2+}$ (not shown), demonstrating that the interaction is reversible. Liposomes alone showed no change in fluorescence on addition of Ca$^{2+}$. Furthermore, no energy transfer was observed up to a Ca$^{2+}$ concentration of 1 mM when synaptotagmin was subjected to mild proteolysis with trypsin before the experiment (Fig. 2B) or when equal amounts of purified synaptophysin or purified immunoglobulin G (IgG) were used instead of synaptotagmin (not shown). These controls demonstrate that this assay measures the specific Ca$^{2+}$-dependent interaction of synaptotagmin with liposomes.

Energy transfer between synaptotagmin and liposomes was specific for Ca$^{2+}$ (Mg$^{2+}$, Ba$^{2+}$, or Sr$^{2+}$ did not evoke a response at concentrations up to 1 mM; these metal ions were also unable to interfere with the Ca$^{2+}$ signal). However, an increase in both the Ca$^{2+}$ sensitivity and the intensity of the signal was observed when the proportion of phosphatidyserine in the acceptor liposomes was increased to 50% (Fig. 2B). This indicates that the acidic head groups may participate directly in the formation of the synaptotagmin-Ca$^{2+}$-phospholipid complex, which is in agreement with the previously determined phospholipid binding specificity of recombinant synaptotagmin (3).

For a given phospholipid composition (25% acidic phospholipids), the dependence of phospholipid binding on the Ca$^{2+}$ concentration was remarkably similar to that of Ca$^{2+}$ binding measured by equilibrium dialysis, with a half-maximal response at $10^{-5}$ M Ca$^{2+}$ (compare Figs. 1B and 2B). Although the precise phospholipid composition of the presynaptic plasma membrane is not known, a proportion of 25% acidic phospholipids is probably a low estimate. Because an increase in acidic phospholipid content drastically shifts the Ca$^{2+}$ sensitivity to lower concentrations, the response lies well within the Ca$^{2+}$ concentration range expected upon excitation at the contact site between synaptic vesicles and the plasma membrane.

To ensure that synaptotagmin can also interact with phospholipid vesicles when the protein itself is incorporated in a phospholipid vesicle, we purified the protein from cholate extracts and reconstituted it into liposomes by a dialysis procedure. When acceptor liposomes containing dansylated head groups were added to these liposomes, Ca$^{2+}$-dependent fluorescence resonance energy transfer was observed, which was similar to that observed with the purified protein alone (Fig. 2C). As an independent confirmation for Ca$^{2+}$-dep-
dent interaction of synaptotagmin with phospholipid vesicles, we measured the binding of radiolabeled liposomes to immuno-
blotted synaptotagmin. Whereas in the pres-
ence of EGTA a small but significant amount of phospholipid binding was ob-
erved, Ca\(^{2+}\) induced a four- to sixfold over the basal binding, which was abolished upon subsequent addition of
EGTA (Fig. 3).

In synaptic vesicles, synaptotagmin is present as a homo-oligomeric complex of probably four subunits containing eight C\(_2\) domains (9). To evaluate whether Ca\(^{2+}\)-
phospholipid binding by synaptotagmin is dependent on an intact oligomeric structure, we subjected the protein to limited proteolysis. Under these conditions, cleavage occurs at a single site adjacent to the membrane spanning domain, creating a large cytoplasmic fragment that contains both C\(_2\) domains (3). This cleavage abol-
ished the ability of synaptotagmin to bind Ca\(^{2+}\) or phospholipids in a Ca\(^{2+}\)-dependent manner as measured with the assays described above (Fig. 2B). To analyze the structure of the proteolytic fragments in more detail, we determined their size by sucrose density gradient centrifugation in CHAPS. For comparison, the migration of the fragments was monitored in SDS, which dissociates all aggregates into monomers. The NH\(_2\)-terminal fragment of syn-
aptotagmin comigrates with uncleaved syn-
aptotagmin in a high molecular weight complex (Fig. 4), clearly separated from the larger COOH-terminal fragment, which migrates at a monomer position. These results suggest that tetramerization of syn-
aptotagmin, mediated by its NH\(_2\)-terminal domain, is necessary for formation of the complex with Ca\(^{2+}\) and phospholipids.

Synaptotagmin is the first Ca\(^{2+}\)-binding protein that has been identified in secretory organelles of the regulated pathway. The widespread distribution of at least one of the synaptotagmin isoforms on every synap-
ic vesicle and probably also every endo-
crine secretory granule (2-4) is in agree-
ment with a general role as putative Ca\(^{2+}\) receptor for exocytosis. The precise nature of the interaction of synaptotagmin with phospholipids and Ca\(^{2+}\) remains to be es-
blished. Synaptotagmin, Ca\(^{2+}\), and
membrane phospholipids probably form a ternary complex or sandwich. This interde-
pendence is similar to that observed for protein kinase C, which binds Ca\(^{2+}\) only when phospholipids are present (10). In the absence of Ca\(^{2+}\), some phospholipid bind-
ing was observed (Fig. 3). This is in agree-
ment with our earlier observation that the recombinant cytoplasmic fragment of syn-
aptotagmin is capable of Ca\(^{2+}\)-independent phospholipid binding after being subjected to denaturing SDS-polyacrylamide gel elec-

trophoresis (SDS-PAGE) and immunoblott-
ing (3).

Because Ca\(^{2+}\)-phospholipid binding was abolished by proteolytic cleavage at a single site, the responsibility of the C\(_2\) domains could not be unambiguously established although it seems likely in analogy to pro-
inase kinase C and phospholipase A\(_2\). The
observation of fluorescence resonance ener-

gy transfer between trypophan and phos-
pholipids strongly supports an involvement of the C\(_2\) domains because these domains contain the only tryptophan residues of the entire structure, the single exception being a trypophan residue located in the mem-
brane-spanning domain, which is unlikely to be included. However, the results indi-
cate that an intact tetrameric structure of the protein is required for Ca\(^{2+}\) binding. This requirement and the presence of mul-
tiple Ca\(^{2+}\)-binding sites may explain the high cooperativity observed for Ca\(^{2+}\) in the transmembrane protein.

How does synaptotagmin function in exocytosis? Although we have demonstrat-
ed Ca\(^{2+}\)-dependent binding of synaptotag-
min-containing membrane vesicles to ac-
ceptor liposomes, we do not believe that this property is solely responsible for vesicle docking to the presynaptic plasma mem-
brane. Instead, we assume that in the rest-
ing nerve terminal where the Ca\(^{2+}\) concen-
tration is low (1), synaptotagmin forms a complex with a specific acceptor protein in the plasma membrane. We have recently reported that synaptotagmin binds specif-
ically to the \(\alpha\)-latrotoxin receptor in vitro (11), suggesting that this protein serves as the vesicle docking protein in the presyn-
aptic membrane. When such a docking complex is exposed to increased Ca\(^{2+}\) con-
centrations, it probably results in an inter-
action of the cytoplasmic arms of synap-
tagmin with the phospholipids of the plasma membrane, causing local rearrange-
ment of phospholipids (12). This may then trigger fusion via interaction with addition-
al proteins that remain to be characterized.

REFERENCES AND NOTES

6. The monoclonal antibody was generated by standard

procedures (G. Köhler and C. Milstein, Na-
tagmin (3) used as antigen. Preparation of the affinity matrix (1-mL final volume, containing ~8 mg of purified IgG) and purification of synaptotag-
min (and synaptophysin) were performed essen-
tially as described for synaptophysin (F. Navone et al., J. Cell Biol. 103, 2511 (1986), by using a Triton X-100 extract of rat brain mem-
brae and starting material was solubilized in 0.05% Triton X-100 and dialyzed for 2 days against six buffer changes before the experi-
ments. Yields were between 0.2 and 0.4 mg of protein per preparation.
7. Ca\(^{2+}\) binding was measured with an equilibrium dialysis assay (10). Dialysis samples contained 0.2 to 0.6 mg of protein (synaptotagmin or synap-
tophysin) per milliliter of phospholipid vesicles (75% phosphatidycholine, 25% phospholipidphosphatidylethylcerine) per milliliter, which were prepared as described in (10). After the intervals needed to follow sample dilution during dialysis, liposomes containing 3H-labeled phosphatidycholine (6 \(\mu\)Ci/mg), which do not bind synaptotagmin or synaptophysin, were added. After incubation for 30 min, the mixtures were exposed to 3H- and \(\alpha\)-sccintillation counting.
9. Evidence that synaptotagmin is tetrameric. Using a variety of detergents, we found that synaptotag-
migrates at a similar position on sucrose gradients. This position is consistent with that of synaptophysin, synaptobrevin, synapsin, synap-
toprotein, and the proton pump, suggesting that synaptotagmin is not part of an antifilarial multiglycoprotein complex due to insufficient sol-
ubilization (3). In the presence of Zwittergent 3-14, synaptotagmin migrates at a dimer position. This is surprising because in the insoluble trisphos-
pholipid complex, Zwittergent 3-14 disrupts interactions between transmembrane regions totally (G. A. Mignery, C. L. Newton, B. T. Archer, T. C. Södhorf, J. Biol. Chem. 270 [1995]). Be-
cause dimers can also be observed in SDS-
PAGE, we think that two strongly bonded dimers form a tetramer.
12. Such lateral rearrangement of phospholipids has been demonstrated by electron microscopy (where association of the protein with membranes in the presence of Ca\(^{2+}\) leads to extensive segregation of acidic phospholipids (M. D. Bazzi and G. L. Nelsenstuen, Biochemistry 30, 7691 (1991)) and for various other proteins and polycations (W. Hartmann and H. Galla, Biochin. Biophys. Acta 709, 474 (1979); D. Carrier and M. Pezzuto, Bio-
chemistry 25, 4167 (1986)); G. B. Birrell and O. H. Griffith, ibid. 15, 2925 (1976); J. M. Boggs et al., ibid., 15, 5420 (1976); T. Ikeda et al., Biochin. Biophys. Acta 1026, 105 (1990)). Furthermore, Ca\(^{2+}\)-binding synaptotagmin was found to calmodulin was reported (J. M. Trifarò, S. Fournier, M. M. Novas, Neuroscience 28, 1 (1989), which, however, could not be reproduced in our labora-
tories.
13. Liposomes were prepared from purified phospholipids from rat brain (phosphatidycholine, chloro-
form/methanol, 9:1). The solvent was evaporated under a stream of nitrogen and then further dried under vacuum for 30 mm. Then 20 mM tris-Cl (pH 7.2) and 100 mM NaCl were added to a final phospholipid concentration of 0.2 mM phospholipid. Phospholipids were resuspended by vigorous vortexing (glass beads). Liposomes were formed by ultrasonication in a Branson bath-sonicator for 5
High-Frequency Network Oscillation in the Hippocampus

György Buzsáki,* Zsolt Horváth, Ronald Urioste, Jamille Hetke, Kensall Wise

Pyramidal cells in the CA1 hippocampal region displayed transient network oscillations (200 hertz) during behavioral immobility, consummatory behaviors, and slow-wave sleep. Simultaneous, multisite recordings revealed temporal and spatial coherence of neuronal activity during population oscillations. Participating pyramidal cells discharged at a rate lower than the frequency of the population oscillation, and their action potentials were phase locked to the negative phase of the simultaneously recorded oscillatory field potentials. In contrast, interneurons discharged at population frequency during the field oscillations. Thus, synchronous output of cooperating CA1 pyramidal cells may serve to induce synaptic enhancement in target structures of the hippocampus.

Much of what is known about the physiological function of the hippocampus is based on in vivo and in vitro studies of sequentially analyzed single neurons (1, 2). Although it has long been believed that the computational power of complex neuronal networks cannot be recognized by the properties of single cells alone (3), experimental access to the emergent properties of cooperating hippocampal neurons has been difficult. Direct investigation of the time-varying organization of neuronal populations requires the simultaneous observation of many individual neurons in the awake animal (4). Using silicon multichannel recording arrays (5), we report here the physiological details of a high-frequency oscillation of the hippocampal CA1 neuronal network that is a specific product of cellular cooperativity.

The data analyzed in this study were recorded from 19 adult male rats. Local field potentials and unit activity were recorded by multichannel microprobes (5, 6) in the rat during spontaneous behaviors and sleep (7). The oscillatory behavior of the recorded cell populations and local field potentials was determined by the periodic modulation of the auto- and cross-correlograms (8).

Local field potentials in the CA1 strata pyramidale and radiatum were related to laminar firing during awake immobility (Fig. 1). Sharp waves in the stratum radiatum of the CA1 network reflect depolarization of the apical dendrites of pyramidal cells by the Schaffer collaterals, which is a result of the synchronous bursting of CA3 pyramidal cells (9, 10). In conjunction with the stratum radiatum sharp waves, fast field oscillations were present in the CA1 pyramidal layer (1, 9, 10). The spindle-shaped oscillatory pattern consisted of 5 to 15 sinusoid waves with 200 Hz intraburst frequency. Neuronal discharges most often occurred during the local field oscillations. Isolated pyramidal cells usually fired a single action potential during the field oscillations but occasionally fired a burst of two to three spikes (II). The probability of spike bursts (complex spikes) was three to eight times higher during the fast field oscillations than during comparable time periods in their absence.

The laminar distribution of the fast field oscillations was determined by advancing linear arrays of electrodes (6) perpendicular to the CA1 pyramidal layer. Amplitude maxima of the fast field oscillations were found in the pyramidal layer (0.2 to 1 mV), and the polarity of the signal reversed in phase about 100 μm below the pyramidal layer, which suggests that the main current source of the extracellularly recorded fast field oscillations is the cell bodies of pyramidal cells.

Typically, less than 15% of the recorded neurons were active during a single oscillatory epoch. When discharges of all neurons

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*G. Buzsáki, Z. Horváth, R. Urioste, Center for Molecular and Behavioral Neuroscience, Rutgers University, 197 University Avenue, Newark, NJ 07102. J. Hetke and K. Wise, Center for Integrated Sensors and Circuits, University of Michigan, Ann Arbor, MI 48109.

To whom correspondence should be addressed.