

the preassociation locus to be  $<10 \text{ \AA}$  ( $r$ ) from the linker origin (Fig. 3E and fig. S3). This indication of strongly enriched local CaM seems robust, as fits based on measurements of free intracellular [CaM] in these cells ( $\sim 50 \text{ nM}$ ) (25–27) diverge from our data by orders of magnitude (Fig. 3E). Also, the suggestion of increased local [CaM] is insensitive to the particular theory used for data analysis. Even if linked CaM was uniformly distributed in a sphere, estimated  $[\text{CaM}]_{\text{local/endo}}$  would still be  $\sim 1.3 \text{ mM}$  (fig. S4). Thus, the concentration of local endogenous CaM near channels appears greater by several orders of magnitude than that for free cytoplasmic CaM.

Our experiments establish two critical parameters for channel regulation and signaling. That one CaM is both necessary and sufficient for CDI constitutes a simplifying advance for establishing a molecular mechanism of CaM-Ca<sup>2+</sup> channel regulation. In general, the approach of linking regulatory modules to reveal functional stoichiometry is a valuable adjunct to structural biology. Even if structures resolved multiple CaMs per channel (28), the number of functionally important CaMs would still be indeterminate without a complementary strategy as illustrated here. Second, our results suggest that numerous CaMs are positioned near each channel, within earshot of the local Ca<sup>2+</sup> signal. Assuming a hemispheric CaM domain with a 400 Å diameter (7, 29), a 2.5 mM concentration entails a local cadre of  $\sim 25$  free CaM molecules. Alternatively, the enrichment of effective local CaM concentration could reflect direct transfer of CaM from multiple nearby CaM buffer sites to the preassociation locus (30). In either case, the local Ca<sup>2+</sup> plume of one channel need not signal through a solitary local CaM (fig. S5A). Instead, it is likely that the local Ca<sup>2+</sup> influx of one channel can activate multiple CaMs, either to diversify the consequences of channel activity by triggering different signaling pathways (fig. S5B) (9, 10, 15) or to afford parallel amplification of a single type of pathway (fig. S5C). In the CaM translocation hypothesis, wherein local Ca<sup>2+</sup> drives CaM to the nucleus and thereby activates CREB (15), the amplification scenario suggests that CaMs hailing from L-type channels could supply a substantial fraction of the nucleus-bound contingent.

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- We thank B. Alseikhan for the gift of rCaM<sub>MUT</sub>. G. Yellen for *Shaker* cDNA, R. Blaustein for advice on polymer-chain statistical theory, and Henry Colecraft with members of the Ca<sup>2+</sup> Signals Laboratory for comments and discussion. This work was supported by grants from the NIH (D.T.Y.).

#### Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S5

References

10 November 2003; accepted 12 February 2004

## Reconstitution of Ca<sup>2+</sup>-Regulated Membrane Fusion by Synaptotagmin and SNAREs

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We investigated the effect of synaptotagmin I on membrane fusion mediated by neuronal SNARE proteins, SNAP-25, syntaxin, and synaptobrevin, which were reconstituted into vesicles. In the presence of Ca<sup>2+</sup>, the cytoplasmic domain of synaptotagmin I (syt) strongly stimulated membrane fusion when synaptobrevin densities were similar to those found in native synaptic vesicles. The Ca<sup>2+</sup> dependence of syt-stimulated fusion was modulated by changes in lipid composition of the vesicles and by a truncation that mimics cleavage of SNAP-25 by botulinum neurotoxin A. Stimulation of fusion was abolished by disrupting the Ca<sup>2+</sup>-binding activity, or by severing the tandem C2 domains, of syt. Thus, syt and SNAREs are likely to represent the minimal protein complement for Ca<sup>2+</sup>-triggered exocytosis.

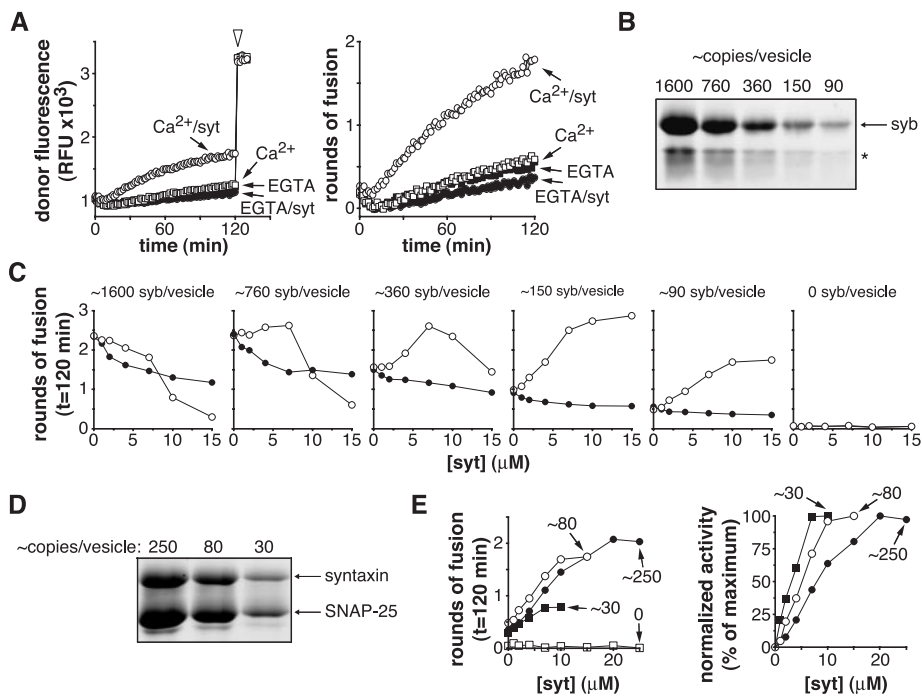
Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are thought to form the minimal machinery needed to mediate intracellular membrane fusion (1). In neurons, the SNARE complex is composed of the target membrane SNAREs (t-SNAREs), syntaxin and SNAP-25, and the vesicle membrane SNARE (v-SNARE), synaptobrevin (syb) (2). Reconstituted v-SNARE vesicles fuse with t-SNARE vesicles during the assembly of cognate *trans*-SNARE complexes (1, 3).

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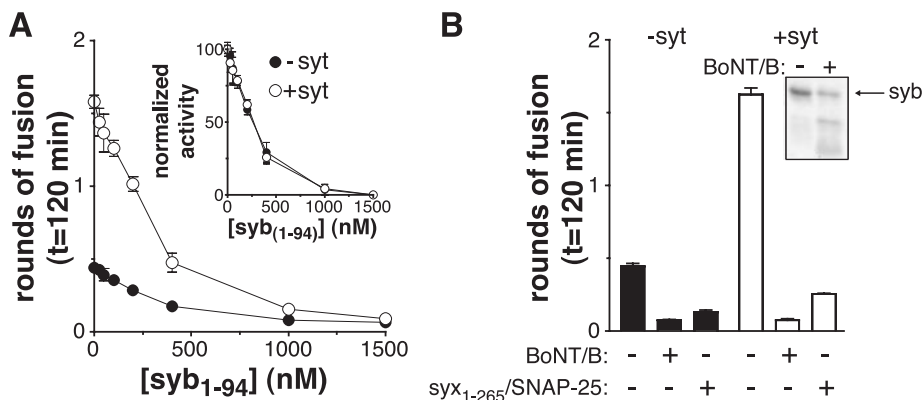
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Fusion occurs with a half-time on the order of minutes (4) and is not regulated by Ca<sup>2+</sup>.

At synapses, exocytosis of neurotransmitters is strictly controlled by Ca<sup>2+</sup> (5). The integral membrane protein, synaptotagmin I, binds Ca<sup>2+</sup> and has been proposed to function as the Ca<sup>2+</sup> sensor that triggers rapid release (6, 7). Ca<sup>2+</sup> promoted interactions of synaptotagmin with anionic phospholipids and t-SNAREs have emerged as putative coupling steps in evoked secretion (6–8). Here, we directly addressed this issue by examining the ability of synaptotagmin to regulate SNARE-catalyzed membrane fusion, using a defined reconstituted model system (1). In this assay, lipidic fluorescence resonance energy transfer (FRET) donor and acceptor pairs are incorporated into v-SNARE vesicles. Fusion of the labeled v-SNARE vesicles with unlabeled



**Fig. 1.** Reconstitution of  $\text{Ca}^{2+}$ -dependent membrane fusion. (A) Syt ( $10 \mu\text{M}$ ) stimulates SNARE-mediated fusion between v-SNARE vesicles ( $\sim 90$  syb/vesicle) and t-SNARE vesicles ( $\sim 80$  syntaxin/SNAP-25 complexes per vesicle) in the presence of  $1\text{mM}$   $\text{Ca}^{2+}$  but not in the presence of  $0.2\text{mM}$  EGTA. Raw donor fluorescence (left) was normalized by using the maximum fluorescence obtained after addition of the detergent *n*-dodecylmaltoside ( $0.5\%$ ; open arrowhead) and converted to rounds of fusion (right), as described (4, 10). (B) v-SNARE vesicles were reconstituted with varying amounts of syb. Purified vesicles ( $7.5 \mu\text{l}$ ) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie blue. The asterisk denotes proteolyzed syb. (C) t-SNARE vesicles ( $\sim 80$  syntaxin/SNAP-25 complexes per vesicle) were incubated with v-SNARE vesicles containing the indicated amounts of syb in the presence of increasing concentrations of syt in either  $1\text{mM}$   $\text{Ca}^{2+}$  (open circles) or  $0.2\text{mM}$  EGTA (filled circles). Plots depict the total amount of fusion obtained after 2 hours at  $37^\circ\text{C}$  as a function of [syt]. (D) t-SNARE vesicles reconstituted with varying amounts of syntaxin and SNAP-25. Purified vesicles ( $5 \mu\text{l}$ ) were subjected to SDS-PAGE and visualized by staining with Coomassie blue. (E) v-SNARE vesicles ( $\sim 90$  syb/vesicle) were incubated with t-SNARE vesicles containing 0,  $\sim 30$ ,  $\sim 80$ , or  $\sim 250$  copies of syntaxin/SNAP-25 complexes per vesicle in the presence of increasing [syt] plus  $1\text{mM}$   $\text{Ca}^{2+}$ . The amount of fusion ( $t = 120$  min) was plotted as a function of [syt] (left) or normalized to the maximum amount of fusion obtained for each t-SNARE vesicle condition (right).



**Fig. 2.**  $\text{Ca}^{2+}$ •syt stimulation of membrane fusion proceeds through the formation of fusion-competent SNARE complexes. (A) v-SNARE and t-SNARE vesicles were incubated with increasing amounts of the cytoplasmic domain of syb ( $\text{syb}_{1-94}$ ) in the absence (filled circles) or presence (open circles) of syt ( $10 \mu\text{M}$ );  $1\text{mM}$   $\text{Ca}^{2+}$  was present throughout all reactions. The total amount of fusion ( $t = 120$  min) was plotted as a function of  $[\text{syb}_{1-94}]$ ; data were also normalized to the maximum amount of fusion obtained in the absence of  $\text{syb}_{1-94}$  (inset). (B) BoNT/B treatment of v-SNARE vesicles, or incubation with soluble t-SNAREs ( $\text{syx}_{1-265}$ /SNAP-25;  $5 \mu\text{M}$ ), leads to loss of membrane fusion both in the absence (black bars) and presence of syt (white bars);  $1\text{mM}$   $\text{Ca}^{2+}$  was present in all the samples. (Inset) Untreated or BoNT/B-treated v-SNARE vesicles ( $7.5 \mu\text{l}$ ) were subjected to SDS-PAGE, and protein was visualized by staining with Coomassie blue; syb was efficiently cleaved by the toxin (13). Some syb was protected from digestion because of the luminal orientation of its cytoplasmic domain (7). Error bars indicate standard deviations for three independent determinations.

t-SNARE vesicles results in dilution of the donor and acceptor. Thus, fusion can be monitored by following the increase in donor fluorescence (1, 9, 10).

The cytoplasmic domain of synaptotagmin I (syt) was mixed with t-SNARE and v-SNARE vesicles at  $37^\circ\text{C}$ . In the presence of  $\text{Ca}^{2+}$ , syt markedly enhanced both the rate and extent of fusion (Fig. 1A). In the absence of  $\text{Ca}^{2+}$ , syt suppressed fusion to a small but reproducible extent. Thus,  $\text{Ca}^{2+}$  triggered a transition in the activity of syt. In the absence of syt, fusion was largely unaffected by  $\text{Ca}^{2+}$ .

The ability of syt to stimulate membrane fusion was dependent on the density of syb on the v-SNARE vesicles (Fig. 1, B and C).  $\text{Ca}^{2+}$ •syt stimulation of membrane fusion was most evident at or below  $\sim 150$  syb/vesicle, similar to densities reported for native synaptic vesicles (11, 12). At much higher densities of syb, syt inhibited fusion. However, at  $\sim 760$  syb/vesicle, the mass ratio of syb to lipids would be  $\sim 1:2$ ; thus, the surface of the liposome would be strongly influenced by molecular crowding, and the observed inhibition might be secondary to complex effects of non-physiological levels of syb.

$\text{Ca}^{2+}$ •syt stimulated SNARE-mediated fusion at all t-SNARE densities tested (Fig. 1, D and E). Increasing the number of t-SNARE complexes per vesicle did, however, require higher concentrations of syt to saturate the reaction (Fig. 1E), which suggested that syt acted, at least in part, via direct interactions with the t-SNAREs, syntaxin and SNAP-25.

Substitution of either v-SNARE or t-SNARE vesicles with protein-free vesicles resulted in complete loss of fusion; under these conditions, syt and  $\text{Ca}^{2+}$  were without effect (Fig. 1, C and E). Furthermore, fusion in the presence and absence of  $\text{Ca}^{2+}$ •syt was efficiently blocked by the cytoplasmic domain of syb ( $\text{syb}_{1-94}$ ) (Fig. 2A), presumably because it prevents *trans* SNARE pairing (1). The  $\text{syb}_{1-94}$  dose responses in the presence and absence of syt were identical. Thus  $\text{Ca}^{2+}$ •syt acts by facilitating the SNARE-mediated fusion pathway, rather than by causing lipid mixing via an alternative mechanism.  $\text{Ca}^{2+}$ •syt stimulation of membrane fusion was also inhibited by soluble t-SNARE complexes and by cleavage of reconstituted syb by botulinum neurotoxin B (BoNT/B) (Fig. 2B) (13).

We incubated a mutant version of syt, in which the  $\text{Ca}^{2+}$ -binding sites in both C2 domains had been disrupted (14–16), with v- and t-SNARE vesicles. The  $\text{Ca}^{2+}$  ligand mutant ( $\text{syt}_{\text{CLM}}$ ) was completely unable to stimulate fusion in the presence of  $\text{Ca}^{2+}$  (Fig. 3A). Thus,  $\text{Ca}^{2+}$  binding to syt is critical for regulation of SNARE-mediated fusion.

The tandem C2 domains of syt must be tethered together in order for syt to bind t-SNAREs—severing the linker that connects C2A and C2B disrupts t-SNARE-binding activity (17, 18). Isolated C2A and C2B failed to stimulate fusion, even when added simultaneously (Fig. 3A). Because isolated C2A retains robust lipid-binding activity (18), stimulation of fusion by  $Ca^{2+}$ •syt is not due to perturbation of the

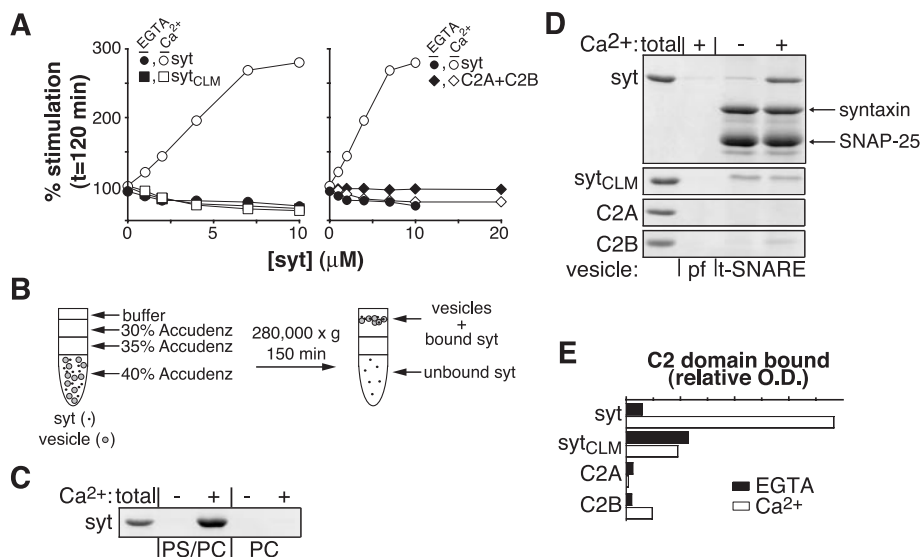
vesicle through, for example, coating of membranes with C2 domains. These data are consistent with the idea that  $Ca^{2+}$ •syt stimulates fusion, at least in part, via interactions with t-SNAREs.

The interaction of syt with membrane-embedded t-SNAREs has not been measured directly. To assay for these interactions we used a coflotation assay (Fig. 3B). When the vesicles were composed of 15%

phosphatidylserine (PS) and 85% phosphatidylcholine (PC), syt efficiently cofloated with vesicles in a density gradient, which reflected its ability to bind anionic phospholipids in the presence of  $Ca^{2+}$  (Fig. 3C) (7). Syt did not bind nor cofloat with vesicles composed of 100% PC. Next, t-SNARE vesicles that had been reconstituted in 100% PC were incubated with syt. Syt efficiently bound the reconstituted t-SNARE vesicles in the presence of  $Ca^{2+}$ ; only weak binding was observed in the absence of  $Ca^{2+}$  (Fig. 3D). Disruption of the  $Ca^{2+}$ -binding sites of syt abolished  $Ca^{2+}$ -stimulated coflotation with t-SNARE vesicles. C2A exhibited no detectable binding, whereas C2B exhibited a faint amount of  $Ca^{2+}$ -dependent binding, similar to levels previously shown in pull-down experiments (17, 18). Thus, syt efficiently and stoichiometrically binds to t-SNAREs that are embedded in lipid bilayers, and loss of  $Ca^{2+}$ -dependent t-SNARE-binding activity is correlated with a loss of  $Ca^{2+}$ -dependent stimulation of membrane fusion (Fig. 3E).

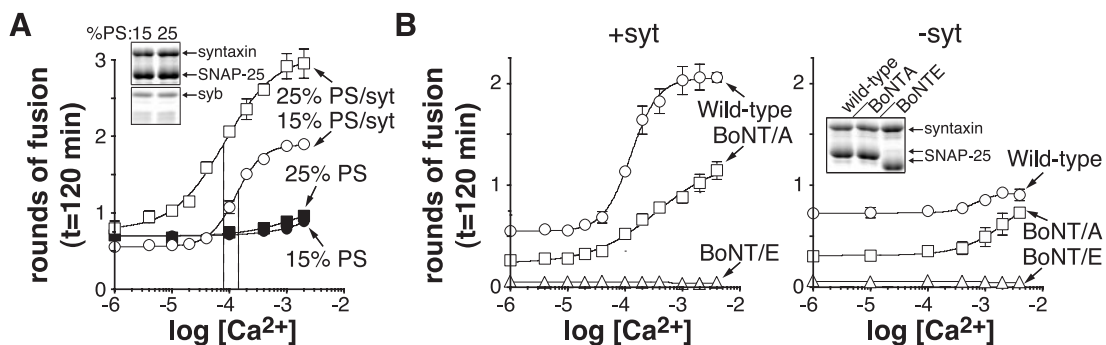
We explored the  $Ca^{2+}$  requirements for fusion in the minimal regulated fusion assay. When we used vesicles that contained 15% PS,  $Ca^{2+}$ •syt stimulated fusion with a  $[Ca^{2+}]_{1/2}$  of 129  $\mu$ M (Fig. 4A); increasing the PS to 25% shifted the  $[Ca^{2+}]_{1/2}$  to 82  $\mu$ M. These findings agree with previous studies showing that the apparent affinity of syt for  $Ca^{2+}$  is strongly dependent on the mole fraction of PS (7). Thus, the interaction of syt with membranes, and the ability of syt to simulate membrane fusion in vitro, occurs at physiologically relevant  $[Ca^{2+}]$  (6).

Finally, we asked whether alterations in t-SNAREs could impact the  $Ca^{2+}$  sensitivity of the fusion reaction. Cleavage of SNAP-25 by BoNT/A causes a reduction in secretion that can be overcome, at least in part, by elevating  $[Ca^{2+}]$  (13, 19, 20). t-SNARE complexes containing truncated



**Fig. 3.** Stimulation of fusion involves the  $Ca^{2+}$ - and t-SNARE-binding activities of syt. (A) v-SNARE and t-SNARE vesicles were incubated with increasing amounts of wild-type syt (circles), syt that harbors  $Ca^{2+}$ -ligand mutations ( $syt_{CLM}$ ) in both C2 domains (C2A: D232,230N; C2B: D363,365N) (squares, left), and a mixture of isolated C2A and C2B domains (diamonds, right; [syt] refers to the concentration of each C2 domain). The amount of stimulation obtained after 2 hours as compared with control ( $-syt$ ,  $+Ca^{2+}$ ) was plotted as a function of the [syt]. (B) Syt was incubated with vesicles before mixing with Accudenz density medium. The mixture was overlaid with decreasing concentrations of Accudenz. After centrifugation, the vesicles floated to the 0/30% Accudenz interface along with any bound syt. Samples collected from the 0/30% Accudenz interface were analyzed by SDS-PAGE and stained with Coomassie blue. Unbound syt remained in the bottom portion of the tube. (C) Syt (10  $\mu$ M) cofloated with vesicles composed of 15% PS, 85% PC (PS/PC) in the presence of  $Ca^{2+}$  (1 mM) but did not cofloat with vesicles composed 100% PC (PC). (D) The indicated syt constructs were incubated with t-SNARE vesicles (100% PC) or protein-free (pf) vesicles (100% PC) in the presence or absence of 1 mM  $Ca^{2+}$ ; samples were applied to density gradients as described in (B). (E) The t-SNARE-binding activity shown in (D) was quantified by densitometry.

**Fig. 4.** The  $Ca^{2+}$ -dependence of syt-mediated stimulation of fusion can be modulated by changes in membrane composition or by C-terminal truncation of SNAP-25. (A) v-SNARE and t-SNARE vesicles, reconstituted with either 15% (circles) or 25% (squares) PS, were incubated in the presence (open symbols) or absence (filled symbols) of 10  $\mu$ M syt at the indicated  $[Ca^{2+}]$ . The total amount of fusion ( $t = 120$  min) was plotted as a function of  $[Ca^{2+}]$ . At 15% PS, the  $[Ca^{2+}]_{1/2}$  (as indicated by the dashed line) was 129  $\mu$ M, and the Hill slope was 1.9; at 25% PS the  $[Ca^{2+}]_{1/2}$  was 82  $\mu$ M, and the Hill slope was 1.0. (Inset) v-SNARE (7.5  $\mu$ l) and t-SNARE (5  $\mu$ l) vesicles were subjected to SDS-PAGE, and protein was visualized by staining with Coomassie blue. (B) We generated t-SNARE complexes using either wild-type SNAP-25 or truncated SNAP-25 constructs that correspond to the



BoNT/A and BoNT/E cleavage products (residues 1 to 197 and 1 to 180, respectively) (13). The t-SNARE complexes were reconstituted into vesicles, and fusion assays were carried out in the presence (left) or absence (right) of 10  $\mu$ M syt at the indicated  $[Ca^{2+}]$ . (Inset) t-SNARE vesicles (5  $\mu$ l) were subjected to SDS-PAGE and stained with Coomassie blue. Error bars indicate standard deviations from three independent determinations.

versions of SNAP-25 that mimic cleavage by BoNT/A (residues 1 to 197) were reconstituted into vesicles (with 15% PS). As a control, a truncated version of SNAP-25 that mimics cleavage by BoNT/E (corresponding to residues 1 to 180), was tested in parallel [this cleavage event results in a more profound block of exocytosis (13)]. Fusion was abolished by the “BoNT/E” truncation (Fig. 4B). In contrast, the “BoNT/A” truncation supported a low level of fusion that could be enhanced by increasing  $[Ca^{2+}]_i$ ; the  $Ca^{2+}$  response was too impaired to determine the precise  $[Ca^{2+}]_{1/2}$ , but this value is  $>360 \mu M$  (Fig. 4B). Thus, the reconstituted system recapitulates the functional effect of BoNT/A and E treatment on neurons (13, 19, 20).

Membrane-embedded syt has been reported to stimulate membrane fusion in a  $Ca^{2+}$ -independent manner (21). We have repeated these experiments and observed the same phenomena. The lack of an effect of  $Ca^{2+}$  is surprising, because the ability of syt to interact with its targets in the reduced fusion assay is promoted by  $Ca^{2+}$ . The easiest explanation, however, is that the bacterially expressed full-length syt is not fully functional. Variants of a number of isoforms of syt, including syt I, that lack a transmembrane domain are expressed in cells where they may also regulate membrane traffic in vivo, supporting the idea that studies with the cytoplasmic domain of syt are physiologically relevant (22–24).

The data reported here indicate that a complex of syt, membranes, and SNARE proteins forms the core of the  $Ca^{2+}$ -triggered fusion apparatus. With this reconstitution approach, it should be possible to test additional factors to construct a  $Ca^{2+}$ -triggered membrane fusion complex that operates on the rapid (millisecond) time scale observed during synaptic transmission.

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25. We thank M. Dong for technical assistance with the BoNT/B experiments and A. Riccio for assistance with

preparation of t-SNARE heterodimers. We also thank M. Jackson, M. Edwardson, J. Weisshaar, and all members of the Chapman lab for comments and discussions. This study was supported by grants from the NIH (NIGMS GM 56827 and NIMH MH 61876 to E.R.C.; NIGMS GM 66313 to T.W.), AHA0440168N (E.R.C.), and the Milwaukee Foundation (E.R.C.). E.R.C. is a Pew Scholar in the Biomedical Sciences. W.C.T. is supported by a Postdoctoral Individual NSRA from the NIH.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1097196/DC1  
Materials and Methods  
References and Notes

25 February 2004; accepted 17 March 2004  
Published online 25 March 2004;  
10.1126/science.1097196  
Include this information when citing this paper.

# Integration of Word Meaning and World Knowledge in Language Comprehension

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Although the sentences that we hear or read have meaning, this does not necessarily mean that they are also true. Relatively little is known about the critical brain structures for, and the relative time course of, establishing the meaning and truth of linguistic expressions. We present electroencephalogram data that show the rapid parallel integration of both semantic and world knowledge during the interpretation of a sentence. Data from functional magnetic resonance imaging revealed that the left inferior prefrontal cortex is involved in the integration of both meaning and world knowledge. Finally, oscillatory brain responses indicate that the brain keeps a record of what makes a sentence hard to interpret.

Language is used, among other things, to exchange information about the world. This entails that, during online comprehension, the meaning of a phrase or sentence is derived and, in many cases, its truth is verified. For this to be possible, usually information about the words of a language and about the facts of the world need to be retrieved from memory.

At least since Frege (1, 2), theories of meaning have made a distinction between the semantics of an expression and its truth value in relation to our mental representation of the state of affairs in the world (3, 4). For instance, the sentence “the present queen of England is divorced” has a coherent semantic interpretation, but it contains a proposition that is false in the light of our knowledge in memory that she is married to Prince Phillip. The situation is different for

the sentence “the favorite palace of the present queen of England is divorced.” Under default interpretation conditions, this sentence has no coherent semantic interpretation, because the predicate *is-divorced* requires an animate argument. This sentence mismatches with our representation of the world in memory, because the descriptive features of the purported state of affairs are inherently in conflict. The difference between these two sentences suggests the distinction that can be made between facts of the world and facts of the words of our language, including their meaning. Although theories of semantic memory usually do not make this distinction (5), accounts of online language processing often do, and they distinguish between the retrieval and usage of world knowledge and of knowledge of word meaning.

Relative to the distinction between facts of the world and facts of the words of one’s language, some aspects of word meaning might be characterized as linguistic in nature, whereas other aspects relate to world knowledge. In linguistic theory, the latter is referred to as the domain of pragmatics, and the former as the domain of semantics. Based on

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