**Synaptotagmin-12, a synaptic vesicle phosphoprotein that modulates spontaneous neurotransmitter release**

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Central synapses exhibit spontaneous neurotransmitter release that is selectively regulated by cAMP-dependent protein kinase A (PKA). We now show that synaptic vesicles contain synaptotagmin-12, a synaptotagmin isoform that differs from classical synaptotagmins in that it does not bind Ca\(^{2+}\). In synaptic vesicles, synaptotagmin-12 forms a complex with synaptotagmin-1 that prevents synaptotagmin-1 from interacting with SNARE complexes. We demonstrate that synaptotagmin-12 is phosphorylated by cAMP-dependent PKA on serine\(^{97}\), and show that expression of synaptotagmin-12 in neurons increases spontaneous neurotransmitter release by approximately threefold, but has no effect on evoked release. Replacing serine\(^{97}\) by alanine abolishes synaptotagmin-12 phosphorylation and blocks its effect on spontaneous release. Our data suggest that spontaneous synaptic-vesicle exocytosis is selectively modulated by a Ca\(^{2+}\)-independent synaptotagmin isoform, synaptotagmin-12, which is controlled by cAMP-dependent phosphorylation.

### Introduction

Presynaptic terminals release neurotransmitters in two modes: evoked release, which is induced by Ca\(^{2+}\) flowing into the nerve terminal when stimulated by an action potential, and spontaneous release, which occurs in the absence of massive Ca\(^{2+}\) influx (Katz, 1969). Evoked release is clearly the more important form of release in terms of how the brain processes information, but many observations indicate that spontaneous release may also be physiologically important, and not just an “accident” of turbocharged presynaptic release machinery (for reviews see Otsu and Murphy, 2003; Zucker, 2005). This evidence suggests that “spontaneous” synaptic vesicle exocytosis causing miniature postsynaptic currents (minis) may be mechanistically distinct from evoked exocytosis and independently regulated, and that minis may have a biological function.

Mechanistically, minis appear to derive from a vesicle pool that differs from that which feeds evoked release (Sara et al., 2005). Although evoked and spontaneous release both require SNARE proteins, deletions of the vesicular SNARE protein synaptobrevin/VAMP2 differentially alter evoked and spontaneous release (Deitcker et al., 1998; Schoch et al., 2001), and structure/function studies suggest that the sequences of synaptobrevin required for evoked and spontaneous release differ (Deak et al., 2006). Moreover, spontaneous and evoked release appear to be differentially regulated by Ca\(^{2+}\) (Llano et al., 2000; Angleson and Betz, 2001).

Physiologically, minis may have a substantial function in regulating neural networks. In cultured hippocampal slices, blocking all release by botulinum toxin had a dramatic effect on spine morphology, whereas blocking only evoked release by tetrodotoxin did not (McKinney et al., 1999). Similarly, in cultured hippocampal neurons, spontaneous release was shown to stabilize synaptic function through tonic suppression of dendritic protein synthesis (Sutton et al., 2006). In addition, in small cerebellar interneurons, a single excitatory or inhibitory quantum, which is what is released by a mini event, can trigger or inhibit, respectively, the generation of an action potential (Carter and Regehr, 2002).

In synapses of mouse cortical and hippocampal neurons, and in *Drosophila melanogaster* neuromuscular junctions, evoked synchronous neurotransmitter release is triggered by Ca\(^{2+}\) binding to synaptotagmin-1 (Geppert et al., 1994; Fernández-Chacón et al., 2001; Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004; Maximov and Südhof, 2005), whereas synaptotagmin-2 performs a similar role in brainstem synapses (Pang et al., 2006). In addition to mediating synchronous Ca\(^{2+}\)-triggered release, synaptotagmin-1 and -2 both normally restrict spontaneous release (Pang et al., 2006), suggesting that they are
intrinsic components of the release machinery. We now find that another member of the synaptotagmin family, synaptotagmin-12, is colocalized with synaptotagmin-1 on synaptic vesicles, but is expressed much later in development. Synaptotagmin-12 was originally described as a thyroid hormone–inducible protein (Thompson, 1996) that is homologous to synaptotagmin-1, but lacks its Ca$^{2+}$-binding sequences, suggesting that it does not participate in Ca$^{2+}$ triggering of release. We demonstrate that expression of synaptotagmin-12 in cultured neurons at a time when no endogenous synaptotagmin-12 can be detected causes a dramatic and selective increase in spontaneous release. Moreover, we show that synaptotagmin-12 is phosphorylated by cAMP-dependent protein kinase A (PKA) at a single site, and that mutation of this site blocks the effect of synaptotagmin-12 on spontaneous release, suggesting that this phosphorylation activates its up-regulation of spontaneous release. Finally, we demonstrate that synaptotagmin-12 forms a tight constitutive complex with synaptotagmin-1 on synaptic vesicles, but regulates spontaneous release independently from synaptotagmin-1. Our data suggest a function for a Ca$^{2+}$-independent synaptotagmin isoform in modulating spontaneous release.

Results

Expression of synaptotagmin-12

We generated an antibody against the linker sequence between the transmembrane region and C$_2$ domains of synaptotagmin-12. As expected from the lack of sequence homology between synaptotagmin isoforms in the linker region, the synaptotagmin-12 antibody recognized only this isoform, but no other synaptotagmin (Fig. 1 A). Immunoblotting revealed that synaptotagmin-12 is expressed in adult mouse brain and adrenal medulla. (B) Synaptotagmin-12 could not be detected in cultured cell lines. (C) Expression of synaptotagmin-12 is restricted to postnatal brain and displays progressive increase during development. (E and F) Relative expression levels of synaptotagmin-1 and -12 were determined by quantitative immunoblotting of total brain homogenates isolated from mouse at different stages of postnatal development and cortical neurons isolated from newborn mouse pups and cultured in vitro for 5–15 d. (E) Raw immunoblotting data. (F) Quantification of expression with $^{125}$I-labeled secondary antibodies. The relative expression levels of each protein were plotted as the percentage of expression in adult brains ($n$ ≥ 4). Similar results were obtained with cultured hippocampal neurons (not depicted). Data are shown as the mean ± the SEM.

Localisation of synaptotagmin-12 to synaptic vesicles

Immunoblotting of tissue homogenates isolated from various regions of adult brain (Fig. 2 A) and immunolabelling of brain
sections (Fig. 2 B) revealed that synaptotagmin-12 is abundantly expressed throughout the brain, with the highest expression levels in cerebellum. This labeling was specific because preincubation with the antigen greatly reduced synaptotagmin-12 immunoreactivity in brain sections (Fig. 2 C). To establish the localization of native synaptotagmin-12, we fractionated the brain homogenates and analyzed the distribution of synaptotagmin-12 in different subcellular fractions. Synaptotagmin-12 was highly enriched in the fraction that contains synaptic vesicles (Fig. 2 D).
Because subcellular fractionation is imprecise, we next tested the localization of synaptotagmin-12 by measuring its levels in the brains of synapsin 1/2 double knockout mice. In these brains, synaptic vesicles are selectively decreased in numbers, and thus the levels of all synaptic vesicle proteins are decreased, whereas the levels of other proteins, e.g., active zone proteins, plasma membrane proteins, or cytosolic proteins, are unchanged (Rosahl et al., 1995). Indeed, we found that synaptotagmin-12 was reduced by ~40% in the synapsin-deficient brains, which exactly corresponds to the decrease observed for other synaptic vesicle proteins (Fig. 2, E and F). The levels of synaptic proteins that are not localized on synaptic vesicles, such as the synaptotagmin isoforms 3, 6, and 7 or NMDA-receptors, were not decreased in synapsin double knockout mice (Fig. 2, E and F).

To obtain a higher resolution localization, we cultured cortical neurons from newborn mice or rats. We first attempted to localize endogenous synaptotagmin-12 in the cultured neurons, but surprisingly, could not detect any synaptotagmin-12 in the neurons even after prolonged culture (Fig. 1 E). Quantitations of the levels of synaptotagmin-1 and -12 revealed that whereas the concentration of synaptotagmin-1 continuously increased in the neurons as a function of culture time, synaptotagmin-12 remained at levels below the sensitivity of our assay, even after 15 d in vitro (DIV; Fig. 1 E). Thus, interestingly, synaptotagmin-12 is present in cultured neurons below the levels observed in the brains from which the neurons were obtained, suggesting that additional factors in brain that are absent under culture conditions must contribute to the regulation of synaptotagmin-12 expression.

To be able to localize synaptotagmin-12 in cultured neurons by immunofluorescence labeling, we therefore expressed recombinant synaptotagmin-12 with a lentivirus. We found that recombinant synaptotagmin-12 was targeted to synapses where it precisely colocalized with synaptophysin (Fig. 2, I and J). Synaptic vesicle localization of native synaptotagmin-12 was supported by immunohistochemical analysis of brain sections from adult mice that showed that synaptotagmin-12 is distributed in a typically synaptic pattern (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200607021/DC1), and by immunoelectron microscopy that revealed synaptotagmin-12 immunoreactivity over the vesicle cluster in presynaptic terminals (Fig. 2, G and H). Together, these data show that synaptotagmin-12 is a synaptic vesicle protein.

Biochemical properties of synaptotagmin-12

Vesicular targeting of synaptotagmin-1 is regulated by N-glycosylation (Han et al., 2004). To determine whether synaptotagmin-12 is also glycosylated, we tested the effects of various deglycosylating enzymes on the mobility of native synaptotagmin-12 during SDS-PAGE. In agreement with a previously published report (Han et al., 2004), removal of neuraminic acid and O- and N-linked sugars with sialidase, O-glycanase, PNGase, or different combinations of these enzymes resulted in a dramatic change in the mobility of synaptotagmin-1, but had no effect on the mobility of synaptotagmin-12, suggesting that synaptotagmin-12 is not glycosylated (Fig. 3 A).

To test whether synaptotagmin-12 interacts with phospholipids, we performed phospholipid-binding assays with the recombinant C2AB domain of synaptotagmin-12, using the C2AB domain of synaptotagmin-1 as a positive control. Consistent with the primary sequence analysis that indicated that synaptotagmin-12 lacks Ca2+-binding sequences, no Ca2+-dependent phospholipid binding was observed when the C2AB domain of synaptotagmin-12 was tested alone or in the presence of C2AB domains of synaptotagmin-1 (Fig. 3 B).

PKA-dependent phosphorylation of synaptotagmin-12

A screen of the phosphorylation of different synaptotagmins in synaptosomes that were incubated with [32P]orthophosphate revealed that the strongest labeling with 32P under the conditions used was obtained for synaptotagmin-12 (Fig. 4 B and not depicted). To identify which protein kinase may mediate synaptotagmin-12 phosphorylation, we immunoprecipitated synaptotagmin-12 from nonlabeled brain extracts and incubated the immunoprecipitated synaptotagmin-12 with brain lysate in the presence of 1 mM ATP and 10 μCi γ[32P]ATP alone or with addition of 0.1 mM cAMP, 0.1 mM cGMP, or 1 mM Ca2+. Without additions, no synaptotagmin-12 phosphorylation was detected, but cAMP caused strong phosphorylation (Fig. 4 C). cGMP induced marginal phosphorylation, possibly because cGMP at the high dose used (0.1 mM) can stimulate PKA, whereas Ca2+ did not stimulate any synaptotagmin-12 phosphorylation (Fig. 4 C).

We searched the synaptotagmin-12 sequence for potential PKA phosphorylation sites and identified two such sites in the
synaptotagmin-12 linker region (T87 and S97; Fig. 4 A). To determine whether one or both of these are being used, we first tested whether recombinant fragments of synaptotagmin-12, produced as GST-fusion proteins, were phosphorylated by PKA. Only the linker region, but not the C2 domains, were phosphorylated (Fig. 4 D). We next tested whether mutation of either T87 or S97 alters phosphorylation of the linker by PKA. We found that phosphorylation was completely abolished by substituting serine\(^97\) with alanine, whereas substituting threonine\(^87\) with alanine had no effect (Fig. 4 E), suggesting that synaptotagmin-12 is phosphorylated by PKA on serine\(^97\). To determine whether the PKA phosphorylation site in synaptotagmin-12 is evolutionarily conserved, we compared the linker sequences from different species. Although no significant homology was found between fly and vertebrate synaptotagmin-12 linker sequences (unpublished data), serine\(^97\) with a canonical preceding lysine residue was found to be conserved in all vertebrate synaptotagmin-12 homologues from zebradish to humans (Fig. 4 F).

**Synaptotagmin-12 modulates spontaneous, but not evoked, release**

We next explored whether synaptotagmin-12 regulates synaptic transmission using cultured cortical neurons isolated from newborn mice or rats. Because the cultured neurons do not contain any detectable synaptotagmin-12, even after prolonged culture (Fig. 1, E and F), the cultured neurons resemble a synaptotagmin-12 loss-of-function model. We infected the neurons with lentiviruses encoding full-length wild-type or S97A mutant synaptotagmin-12 and compared the expression levels of recombinant proteins after 15 DIV (and 10 d after lentivirus infection) with the levels of native synaptotagmin-12 in noninfected cultures and in brain (Fig. 5 A). We found that recombinant wild-type and S97A mutant synaptotagmin-12 were expressed in cultured neurons at equal levels, i.e., were properly translated, with an approximately sixfold higher concentration than that of synaptotagmin-12 in P15 brain (Fig. 5 B).

Both wild-type and S97A mutant synaptotagmin-12 were targeted to synapses (Fig. 2 J and not depicted). When compared with noninfected neurons, the neurons infected with synaptotagmin-12 lentiviruses did not display significant changes in general morphology, and the levels of endogenous synaptic proteins (Fig. 5, A and C, and not depicted). To test whether expressed synaptotagmin-12 is phosphorylated under physiological conditions, we analyzed noninfected neurons and neurons infected with wild-type and S97A mutant synaptotagmin-12 lentiviruses by immunoprecipitation. We labeled the neurons with \([^{32P}]\)orthophosphate, lysed them, and measured \(^{32P}\) incorporation into immunoprecipitated synaptotagmin-12. Significant synaptotagmin-12 phosphorylation was only detected in neurons expressing wild-type synaptotagmin-12, whereas noninfected neurons and neurons expressing mutant synaptotagmin-12 exhibited no significant \(^{32P}\) labeling (Fig. 5 A, bottom). These data suggest that under the conditions used, synaptotagmin-12 is phosphorylated only on serine\(^97\) either because another constitutively active protein kinase phosphorylates this amino acid or because, in these cultured neurons, PKA is activated.

![Diagram](https://example.com/diagram.png)
To determine whether synaptotagmin-12 regulates evoked or spontaneous neurotransmitter release, we monitored inhibitory postsynaptic currents (IPSCs) in noninfected neurons or neurons expressing recombinant wild-type or S97A mutant synaptotagmin-12. We found that expression of wild-type synaptotagmin-12 increased the frequency of spontaneous release events threefold, but had no effect on their amplitude (Fig. 5, D–G). In contrast to spontaneous minis, synaptotagmin-12 had no effect on the sizes or kinetics of evoked responses, either when these were elicited by isolated single-action potentials or by high-frequency stimulus trains, indicating that both synchronous and asynchronous components of evoked release were unaffected (Fig. 6).

Several previous studies indicated that spontaneous release is up-regulated by cAMP-dependent pathways (Chavez-Noriega and Stevens, 1994; Capogna et al., 1995; Yoshihara et al., 2000; Doi et al., 2002). To test the possibility that the effect of synaptotagmin-12 on spontaneous release—which depends on the PKA-substrate site at serine97—intersects with the PKA-dependent regulation of spontaneous release, we examined the effect of forskolin (an activator of adenylate cyclase) on spontaneous release. We found that in control cultures, forskolin caused a 4.5-fold increase in the rate of spontaneous release (Fig. 5, D and E), but had no effect on evoked release (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200607021/DC1). In cultures expressing wild-type synaptotagmin-12, the increase in mini frequency induced by forskolin was much more potent than in control cultures, whereas in cultures expressing S97A mutant synaptotagmin-12, the effect of forskolin was identical to that observed in control cultures (Fig. 5, D and E).
Synaptotagmin-12 binding to synaptotagmin-1
To obtain clues to the mechanism of action of synaptotagmin-12 in its selective effect on spontaneous release, we investigated whether synaptotagmin-12 might interact with SNARE proteins because binding to SNARE proteins is a salient property of other synaptotagmins (Bennett et al., 1992; Yoshida et al., 1992; Li et al., 1995; Chapman et al., 1995; Kee and Scheller, 1996; Banerjee et al., 1996; Rickman and Davletov, 2003; Rickman et al., 2004; Bowen et al., 2005; Tang et al., 2005). We performed immunoprecipitations of detergent-solubilized brain proteins with antibodies to synaptotagmin-1 and -12, and analyzed the immunoprecipitates for the presence of SNARE proteins and synaptotagmins. These experiments failed to reveal binding of synaptotagmin-12 to SNARE proteins, but showed that synaptotagmin-1 was coimmunoprecipitated with synaptotagmin-12 (Fig. 7 A), and that synaptotagmin-12 was coimmunoprecipitated with synaptotagmin-1 (Fig. 7 B). SNARE proteins were selectively absent from the synaptotagmin-12 immunoprecipitates, but were present in the synaptotagmin-1 immunoprecipitates. Equal amounts of synaptotagmin-1 and -12 were coimmunoprecipitated in the presence of either 1 mM free Ca\(^{2+}\) or EGTA, suggesting that the interaction between these proteins is Ca\(^{2+}\)-independent (Fig. 7, A and B). Quantitations revealed that the equal amounts of synaptotagmin-1 and -12 were coimmunoprecipitated from nontreated brain homogenates and homogenates preincubated with PKA activator 8-Br-cAMP (1 mM), PKA inhibitor H-89 (5 μM), or PKC activator PDBu (1 μM), suggesting that interaction between synaptotagmin-1 and -12 is not regulated by phosphorylation (Fig. 7, C–F).

Synaptotagmin-12 regulates spontaneous release independent of synaptotagmin-1
To determine whether synaptotagmin-12 regulates spontaneous release through interaction with synaptotagmin-1, we infected cortical neurons isolated from newborn synaptotagmin-1–deficient
mice (Geppert et al., 1994) with lentiviruses expressing wild-type and S97A mutant synaptotagmin-12. We then monitored spontaneous release and release triggered by action potentials (Fig. 8).

Because deletion of synaptotagmin-1 results in an increase in the rate of spontaneous release (Maximov and Südhof, 2005; Pang et al., 2006), we measured spontaneous release in a lower concentration of extracellular Ca\(^{2+}\) that allows us to resolve individual miniature IPSC events. We found that similar to wild-type neurons, expression of synaptotagmin-12 in synaptotagmin-1–deficient neurons enhanced the rate of spontaneous release (Fig. 8, E and F), but did not alter evoked release triggered by action potentials (Fig. 8, A–D). Because evoked release is completely asynchronous in synaptotagmin-1–deficient neurons (Maximov and Südhof, 2005), synaptotagmin-12, thus, does not significantly alter asynchronous release. Although the increase in spontaneous release in synaptotagmin-1–deficient neurons expressing synaptotagmin-12 was not as dramatic as in wild-type cultures, the increase was still dependent on the phosphorylation of synaptotagmin-12 because the S97A mutant synaptotagmin-12 was unable to potentiate the mini IPSC (mIPSC) rate (Fig. 8, E and F). In these experiments, we could not determine the effects of forskolin on mIPSC frequency because the rate of spontaneous release was already too high.

**Discussion**

In this study, we demonstrate that synaptotagmin-12 is a synaptic vesicle protein that is widely expressed in the brain with a developmentally delayed onset (Fig. 1). Although synaptotagmin-12 is highly homologous to synaptotagmin-1 (which functions as the Ca\(^{2+}\) sensor for fast synaptic vesicle exocytosis), and is colocalized with synaptotagmin-1 on synaptic vesicles (Fig. 2 and Fig. S1), synaptotagmin-12 differs from synaptotagmin-1 in that it does not bind Ca\(^{2+}\) and phospholipids (Fig. 3 and not depicted). We also demonstrate that it is phosphorylated in a...
cAMP-dependent manner at a single residue, serine37 (Fig. 4). Because it is expressed in a developmentally delayed pattern, cultured neurons contain very low levels of endogenous synaptotagmin-12. Overexpression of recombinant synaptotagmin-12 in these neurons dramatically increased the rate of spontaneous release (Fig. 5), but had no effect on synchronous and asynchronous components of release triggered by action potentials (Fig. 6).

At least two alternative hypotheses could potentially explain how synaptotagmin-12 controls spontaneous neurotransmitter release: (1) synaptotagmin-12 regulates spontaneous release via its interaction with synaptotagmin-1, and (2) synaptotagmin-12 acts as an independent modulator of spontaneous release.

The first hypothesis is supported by the observation that synaptotagmin-12 forms a constitutive Ca2+-independent heterooligomeric complex with synaptotagmin-1 that is incompatible with the interaction of synaptotagmin-1 with SNARE complexes (Fig. 7). Previous studies indicate that synaptotagmin-1 is associated with SNARE complexes in a Ca2+-independent manner (Kee and Scheller, 1996; Rickman and Davletov, 2003), and that deletion of synaptotagmin-1 increases the rate of excitatory and inhibitory minis (Maximov and Südhof, 2005; Pang et al., 2006), suggesting that freeing SNARE complexes from synaptotagmin-1 may disinhibit spontaneous release. Our finding that synaptotagmin-12 interacts with synaptotagmin-1 suggests that synaptotagmin-12 increases spontaneous neurotransmitter release by pulling synaptotagmin-1 off SNARE complexes, and thereby disinhibiting spontaneous exocytosis. The alternative hypothesis would be that synaptotagmin-12 regulates spontaneous release by a synaptotagmin-1–independent (but phosphorylation-dependent) mechanism, and that the interaction of synaptotagmin-12 with synaptotagmin-1 performs an additional, as yet undetermined, role.

To test the two hypotheses, we determined the effect of overexpression of synaptotagmin-12 in synaptotagmin-1–deficient neurons (Fig. 8). We found that in the absence of synaptotagmin-1, synaptotagmin-12 still produced an increase, albeit a moderate one, in the rate of spontaneous exocytosis. This result favors the second hypothesis, suggesting that the phosphorylation site–dependent modulation of mini release by synaptotagmin-12 does not require synaptotagmin-1. It is also unlikely that the effect of expression of synaptotagmin-12 on spontaneous release is caused by a massive change in synapse density or in the size of the readily releasable pool of synaptic vesicles because we observed no differences between neurons expressing or lacking wild-type or mutant synaptotagmin-12 in evoked synaptic responses; this applies both for responses triggered by single-action potentials or trains of action potentials applied at 10 Hz. A] Typical asynchronous IPSCs triggered by single-action potentials or trains of 100 action potentials applied at 10 Hz. B] Average charge transferred over 1.5 s by asynchronous responses triggered by single action potentials. The total numbers of analyzed neurons are shown on each column. (C) Asynchronous responses triggered by high frequency trains of 100 action potentials applied at 10 Hz. Scale bars apply to both traces. (D) Average total synaptic charge transfer during trains or 100 potentials in control neurons or neurons expressing wild-type synaptotagmin-12. The total numbers of analyzed neurons are shown on each column. (E and F) Synaptotagmin-12 increases the rate or spontaneous release in synaptotagmin-1–deficient neurons. (E) Typical miniPSCs recorded from control neurons (black) or neurons infected with wild-type (blue) or S97A (red) synaptotagmin-12 lentiviruses. MiniPSCs were recorded in 0.2 mM of extracellular Ca2+ by single-action potentials or trains of 100 action potentials applied at 10 Hz. Scale bars apply to all traces. (F) Average frequencies of miniPSCs recorded from different cortical cultures. The total numbers of analyzed neurons are shown on each column. Data are shown as the mean ± the SEM.

Our findings have several implications for the understanding of synaptic transmission. First, the possible biological role of spontaneous release events is widely debated, with opinions ranging from considering such events as mere accidental by-products of turbo-charged fusion machinery to biologically
meaningful processes of synaptic communication (Otsu and Murphy, 2003; Zucker, 2005). Our data, by identifying a synap-

tic vesicle synaptotagmin isoform that selectively regulates spontaneous release in cultured neurons, lend credence to the

notion that spontaneous release is a highly specific and regulated event. Notably, the rate of spontaneous release increases

with age (Hsia et al., 1998), as do the expression levels of synaptotagmin-12. This finding suggests a connection, but sev-
eral other factors such as developmental changes in connectivity and synapse maturation must also play a major role in this

process (Hsia et al., 1998).

Although the effect of synaptotagmin-12 in cultured neu-

rons is specific for spontaneous as opposed to evoked release, this does not exclude the possibility that in an intact brain, synaptotagmin-12 could participate in PKA-dependent forms of plasticity. It is noticeable that synaptotagmin-12 was discovered as a thyroid hormone–inducible gene (Thompson, 1996; Dong et al., 2005) and comes on relatively late in development, sug-

gesting that its role is under further regulation beyond the phos-

phorylation by PKA. Moreover, extensive studies indicate that cAMP-dependent phosphorylation regulates exocytosis in non-

neuronal cells (Koh et al., 2000) and neurons (Chavez-Noriega and Stevens, 1994; Capogna et al., 1995; Yoshihara et al., 2000; Doi et al., 2002). Multiple pathways have been implicated in the cAMP-dependent modulation of synaptic strength (Chavez-

Noriega and Stevens, 1994; Capogna et al., 1995; Carroll et al., 1998; Sakaba and Neher, 2001; Kaneko and Takahashi, 2004).

The requirement of serine for the effect of synaptotagmin-12 on spontaneous release indicates that synaptotagmin-12 may be

generally involved in the regulation of synaptic vesicle exocyto-

sis by PKA-dependent phosphorylation. Finally, expression of synaptotagmin-12 was detected in adrenal glands (Fig. 1 B),

suggesting that synaptotagmin-12 may also be localized on dense core vesicles and play a role in regulating calcium-

independent secretion in nonneuronal cells.

Materials and methods

Plasmid construction

The following vectors were constructed for expression of various regions of rat synaptotagmin-12 as GST-fusion proteins: pGEXKG-synaptotagmin-12-linker (aa 47–150); pGEXKG-synaptotagmin-12-C,A (aa 151–281); and pGEXKG-synaptotagmin-12-C,B (aa 282–421). For construction of synaptotagmin-12 lentivirus, full-length cDNA (aa 1–421) was subcloned into pFUGW shuttle vector. The point mutations were generated by PCR

in independent secretion in nonneuronal cells.

Immunoprecipitations

Rat brains were homogenized in 50 mM Hepes, 100 mM NaCl, 2 mM MgCl2, 4 mM EGTA, pH 6.8, and solubilized for 2 h at 4°C in the same buffer containing 1% Triton X-100. The samples were then centrifuged for 1 h at 50,000 RPM (70 Ti rotor; Beckman Coulter) to remove insoluble ma-

terial and incubated for 2 h at 4°C with protein A–or protein G–Sepharose beads (GE Healthcare) covered with polyclonal antibody to synaptotagmin-12, monoclonal antibody to synaptotagmin-1, corresponding preimmune serum, or control antibody. The protein complexes attached to the beads were washed five times with the extraction buffer, eluted with SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis. The input lanes were loaded with 1% of total protein extract used for immunoprecipitation. To determine the effects of activators or inhibitors of PKA-dependent phos-

phorylation, the brain homogenates were preincubated for 30 min with 1 mM 8-Br-cAMP, 5 mM H89, or 1 μM PD98059 (used as a negative control) before protein extraction, and the immunoprecipitations were carried in the presence of EGTA, as described above.

Cortical and hippocampal primary neuronal cultures

The cortices or hippocampi were dissected from the brains of embryonic day 18 embryos or newborn pups, dissociated by trypsin digestion, and plated on circle glass coverslips coated with Matrigel. The cultures were main-

tained in MEM medium (Invitrogen) supplemented with B-27 (Invitro-

gen), 1-glutamine, 0.5% glucose, 5% fetal bovine serum, and 2 mM Ara-C (Sigma-Aldrich). The cultures were used for experiments at 14–17 DIV.

Immunocytochemistry

Neurons attached to the glass coverslips were rinsed once in PBS, fixed for 15 min in ice in 4% formaldehyde, 4% sucrose in PBS and permeabilized for 5 min at room temperature in 0.2% Triton X-100 (Roche) in PBS. After permeabilization, the neurons were incubated for 30 min in blocking solu-

tion containing 5% BSA (Sigma-Aldrich; fraction V) and 1-h incubation with primary and rhodamine– and FITC-conjugated secondary antibodies diluted in blocking solution. The coverslips were then mounted on glass slides with Aqua-Poly/Mount medium (Polysciences, Inc.) and ana-

alyzed at room temperature using a confocal microscope (DMIRE2, Leica) and 63×/1.32-0.6 oil immersion objective. The images were collected using confocal software (Leica) and processed using Photoshop software (Adobe). The background fluorescence was digitally reduced by 25% us-

ing the “color balance” function in Photoshop. All digital manipulations were equally applied to the entire image. Brain sections from perfusion-

fixed rats were permeabilized for 10 min in 0.5% Triton X-100 in PBS and incubated in blocking buffer containing 2% goat serum and 0.1% Triton X-100 in PBS. The sections were then incubated sequentially for 1 h in pri-

mary and secondary antibodies diluted in blocking buffer and developed in DAB substrate. For deconvolution, the sections were incubated for 30 min in 70% ethanol, followed by 10-min incubation in 90% ethanol and 10-

min incubation in 100% ethanol. The sections were then mounted on glass slides and analyzed by light microscopy.

Immunoelectron microscopy

Adult mouse brains were perfusion fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS, pH 7.4, followed by overnight immersion fix-

ation in 0.12% glutaraldehyde and 2% paraformaldehyde. The brain sec-

tions (120 μm) were permeabilized in 0.2% Triton X-100, blocked in 4% normal goat serum, and incubated overnight with primary antibody alone or primary antibody mixed with the antigen (~1 mg/ml). The sections were then incubated with the secondary antibody conjugated with 1.4-nm gold particles (1:100 dilution; Nanoprobes) for 24 h, and immunogold signal was enhanced with the HQ silver enhancement kit (Nanoprobes). Sections were further fixed with 0.5% osmium tetroxide, dehydrated
through a graded series of ethanol, and embedded in Poly/Bed 812 epoxy resin (Polysciences, Inc.). Ultrathin sections (65 nm) were stained with 5% uranyl acetate solution and examined under a transmission electron microscope (FEI Tecnai; FEI) at 120 kV accelerating voltage.

**Phosphorylation**

Synaptosomes (~1 mg of total protein) were prelabeled for 30 min at 37°C in phosphate-free aerated Krebs-Henseleit-Hepes buffer (118 mM NaCl, 3.5 mM KCl, 1.25 mM CaCl$_2$, 1.2 mM MgSO$_4$, 25 mM Hepes-NaOH, 5 mM Hepes-NO$_2$, pH 7.4, and 115 mM glucose) containing 0.3 mCi of $^{32}$P orthophosphate and incubated for an additional 10 min with 1 μM of okadaic acid. $^{32}$P-Labeled synaptosomes were precipitated by centrifugation at 14,000 rpm on a microcentrifuge (Eppendorf) and solubilized for 1 h at 4°C in Krebs-Henseleit-Hepes buffer containing 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.2% SDS, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM DTT. Solubilized proteins were diluted with equal volume of lysis buffer without detergents and immunoprecipitated with the antibody to synaptotagmin-12 or preimmune serum, as described in Immunoprecipitations. For in vitro phosphorylation of native synaptotagmin-12, brain proteins were extracted in 1% of Triton X-100 and immunoprecipitated with synaptotagmin-12 antibody or preimmune serum. Immunoprecipitates were extensively washed and incubated for 10 min at 37°C with 50 μl of rat brain cytosol (10 mg/ml in Tris buffer without protease inhibitors) mixed with 2.5 mM ATP and 20 μCi γ-$^{32}$P(ATP) alone with addition of 0.1 mM cAMP, 0.1 mM cGMP, or 1 mM Ca$^{2+}$. For in vitro phosphorylation of recombinant proteins, 30 μg of each GST-fusion protein immobilized on glutathione-Sepharose beads were mixed with 500 μl of reaction mixture containing 50 mM Hepes, pH 7.2, 100 mM NaCl, 4 mM EGTA, 2 mM MgCl$_2$, 50 units of catalytic subunit of PKA (Sigma-Aldrich), 1 mM ATP, and 10 μCi γ-$^{32}$P(ATP) and incubated for 30 min at 30°C. The proteins attached to the beads were then washed three times with Hepes buffer (50 mM Hepes, pH 7.2, 100 mM NaCl, 4 mM EGTA, 2 mM MgCl$_2$, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 50 mM NaF) and analyzed by SDS-PAGE. For detection, gels were dried and exposed to x-ray film for 24–48 h at −80°C.

**Electrophysiology**

Cortical neurons were infected with lentiviruses encoding wild-type synaptotagmin-12, brain proteins were extracted in 1% of Triton X-100 and immunoprecipitated with synaptotagmin-12 antibody or preimmune serum. Immunoprecipitates were extensively washed and incubated for 10 min at 37°C with 50 μl of rat brain cytosol (10 mg/ml in Tris buffer without protease inhibitors) mixed with 2.5 mM ATP and 20 μCi γ-$^{32}$P(ATP) alone with addition of 0.1 mM cAMP, 0.1 mM cGMP, or 1 mM Ca$^{2+}$. For in vitro phosphorylation of recombinant proteins, 30 μg of each GST-fusion protein immobilized on glutathione-Sepharose beads were mixed with 500 μl of reaction mixture containing 50 mM Hepes, pH 7.2, 100 mM NaCl, 4 mM EGTA, 2 mM MgCl$_2$, 50 units of catalytic subunit of PKA (Sigma-Aldrich), 1 mM ATP, and 10 μCi γ-$^{32}$P(ATP) and incubated for 30 min at 30°C. The proteins attached to the beads were then washed three times with Hepes buffer (50 mM Hepes, pH 7.2, 100 mM NaCl, 4 mM EGTA, 2 mM MgCl$_2$, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 50 mM NaF) and analyzed by SDS-PAGE. For detection, gels were dried and exposed to x-ray film for 24–48 h at −80°C.

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