Synaptogyrins Regulate Ca\(^{2+}\)-dependent Exocytosis in PC12 Cells*  

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Synaptogyrins constitute a family of synaptic vesicle proteins of unknown function. With the full-length structure of a new brain synaptogyrin isoform, we now show that the synaptogyrin family in vertebrates includes two neuronal and one ubiquitous isoform. All of these synaptogyrins are composed of a short conserved N-terminal cytoplasmic sequence, four homologous transmembrane regions, and a variable cytoplasmic C-terminal tail that is tyrosine-phosphorylated. The localization, abundance, and conservation of synaptogyrins suggest a function in exocytosis. To test this, we employed a secretion assay in PC12 cells expressing transfected human growth hormone (hGH) as a reporter protein. When Ca\(^{2+}\)-dependent hGH secretion from PC12 cells was triggered by high K\(^+\) or α-latrotoxin, co-transfection of all synaptogyrins with hGH inhibited hGH exocytosis as strongly as co-transfection of tetanus toxin light chain. Synaptophysin I, which is distantly related to synaptogyrins, was also inhibitory but less active. Inhibition was independent of the amount of hGH expressed but correlated with the amount of synaptogyrin transfected. Inhibition of exocytosis was not observed with several other synaptic proteins, suggesting specificity. Analysis of the regions of synaptogyrin required for inhibition revealed that the conserved N-terminal domain of synaptogyrin is essential for inhibition, whereas the long C-terminal cytoplasmic tail is largely dispensable. Our results suggest that synaptogyrins are conserved components of the exocytic apparatus, which function as regulators of Ca\(^{2+}\)-dependent exocytosis.

In presynaptic nerve terminals, synaptic vesicles accumulate transmitters and release them by exocytosis (reviewed in Refs. 1–3). Studies of synaptic vesicles over the last 10 years have elucidated the structures of their major protein components, and the functions of many vesicle proteins are now being investigated. Two families of distantly related synaptic vesicle proteins, synaptogyrins and synaptophysins, are among the most abundant vesicle components (4–9); together they account for more than 10% of the total vesicle protein. Synaptogyrins and synaptophysins contain four transmembrane regions and a cytoplasmic C-terminal tail that is tyrosine-phosphorylated by pp60c-src and fyn kinases (6–12). The exact size of the synaptogyrin and synaptophysin families is unclear. The two families comprise members that are enriched in synaptic vesicles and endocrine granules (synaptogyrin I, synaptophysin I, and synaptoporin/synaptophysin II) and members that are ubiquitously expressed in all cells (cellugyrin and pantophysin (4–9, 12, 13)). In addition, a partial human sequence for a possible third synaptogyrin isoform (synaptogyrin III) was recently reported (14). The recently completed genome sequence of Caenorhabditis elegans contains a single homolog of synaptogyrins but no direct synaptophysin homologs, suggesting that synaptogyrins but not synaptophysins are evolutionarily conserved.

The presence of neuronal and ubiquitous isoforms for synaptogyrins and synaptophysins suggests that, similar to other synaptic vesicle proteins (e.g. synaptobrevin/cellubrevin, SCAMPs, rab3), synaptogyrins and synaptophysins may have general functions in exocytosis in all cells. The fact that the synaptic vesicle-specific isoforms are components of all synaptic vesicles, and abundant components at that, indicates a role in neurotransmitter release for these isoforms. However, despite a large number of genetic and biochemical studies, the precise functions of synaptogyrins and synaptophysins are still unknown. Analysis of knockout mice lacking synaptophysin I and synaptogyrin I revealed that these proteins are not essential for exocytosis but may regulate neurotransmitter release (15, 16). Several forms of synaptic plasticity, including posttetanic potentiation and long term potentiation, were defective in mice that lack both synaptophysin I and synaptogyrin I. Although this result agrees well with previous studies indicating a role for tyrosine phosphorylation in long term potentiation (17–19), they do not provide a definitive description of the functions of synaptogyrins and synaptophysins. The effects of the synaptogyrin/synaptophysin deletion are reminiscent of the results obtained with knockouts of rab3A, another highly abundant vesicle protein (20, 21). Mice lacking rab3A also exhibit selective changes in the regulation of neurotransmitter release, although the types of changes were different from those observed in synaptogyrin/synaptophysin knockouts.

Holz and co-workers (22) developed an assay for protein function in exocytosis in which plasmids encoding human growth hormone (hGH)2 and a second protein of interest are transiently co-transfected into PC12 cells or chromaffin cells. In the transfected cells, hGH is secreted as a function of stimulation and serves as a reporter for exocytosis. A high probability of co-transfection of two distinct plasmids into the same cell makes it possible to investigate the effect of the protein of...
interest on hGH secretion. In previous studies in PC12 cells, we demonstrated that secretion of transfected hGH is Ca\textsuperscript{2+}-de-
pendent, triggered by membrane depolarization or \alpha-latro-
toxin, inhibited by tetanus toxin, and dependent on phosphati-
dylinositol kinase activity (23). Using this and similar assays, the
effects of several proteins have been examined, including
rab3A and rabphilin in bovine chromaffin cells (24, 25) and
DOC2 and RIM in PC12 cells (26, 27). If a co-transfected protein
changes Ca\textsuperscript{2+}-regulated hGH secretion, this is taken as
evidence for a direct involvement of the transfected protein in
exocytosis. Thus, transiently transfected secretory cells such as
PC12 cells may be useful models to study the function of syn-
aptic proteins in exocytosis.

In the current study, we first analyzed the size of the syn-
aptogyrin family. We then examined the functions of various
synaptogyrins in Ca\textsuperscript{2+}-dependent secretions using
transfected PC12 cells and applying a number of controls to
validate the effects observed. Our results demonstrate that all
synaptogyrins potently inhibit Ca\textsuperscript{2+}-dependent exocytosis
when overexpressed. Inhibition was as strong as that produced
by tetanus toxin. Synaptophysin I exerted a lesser inhibitory
effect, and other unrelated proteins caused no inhibition. Our
observations reveal a direct participation of synaptogyrins in
exocytosis that correlates with their roles in synaptic plasticity
observed in the knockout mice.

EXPERIMENTAL PROCEDURES

Miscellaneous Procedures—SDS-polyacrylamide gel electrophoresis
and immunoblotting experiments were performed as described (28–30).
RNA blots with rat multitissue blots (CLONTECH) were hybridized
with specific probes as reported (6, 31).

Eludication of the Primary Structure of Synaptogyrin III—Mouse
synaptogyrin III was identified in EST data banks (accession numbers
AU035767 and AU035405). The corresponding cDNA clones MNCB-
0851 and MNCb-0414 were obtained from the Division of Genetic Re-
sources, National Institute of Infectious Diseases in Tokyo 162-8460,
Japan, and fully sequenced using standard procedures (31) to elucidate
the amino acid sequence of synaptogyrin III (submitted to GenBank—
accession number AF117207). Sequence analyses were performed using
the BLAST program suite at NCBI.

Plasmid Construction—A 2.6-kilobase BamHI-EcoRI fragment en-
coding hGH was cloned into the blunted XhoI site of the pCMV5 (32)
or pCMV-myc vector to create phGHCMV5 or phGHCMV-myc in which
hGH expression is driven by the SV40 promoter. The coding regions of
various proteins were then subcloned into the polymer of phGHCMV5
or phGHCMV-myc. In this way, the same plasmid encodes hGH
driven by the SV40 promoter and a second protein driven by the CMV
promoter. In addition, synaptogyrin cDNA was cloned into a separate
pCMV vector to systematically vary the relative amounts of hGH and syn-
aptogyrin expressed. Furthermore, synapsin cDNAs were subcloned
directly into pCMV5 and co-transfected with phGHCMV5 for analysis.
Proteins whose effects on the secretion of hGH were examined along
with the names of the constructs were as follows: cellubrevin, phGH-
CMV8–5; cellugyrin, phGHCMVცgyr(1717/1522); rab3A, pC-
MVh25–1; tetanus toxin, phGHCMV게Tx; synapsins Ia and Iib, pC-
MVsynIa and pCMVSynIb; synaptobrevin II, phGHCMV18–1;
synaptogyrin I, phGHCMVп29–21 and pCMVSynп29–21; synaptogyrin
III, pCMVSynyгIII; synaptophysin, phGHCMVп38–1; synaptotagmins
I and III, phGHCMVпyлI and phGHCMVпyлIII.

PC12 cell transfection and secretion experiments were performed
essentially as described (23, 27). Briefly, PC12 cells were maintained
in 75-cm\textsuperscript{2} flasks (uncoated) in RPMI 1640 with 10% horse serum (heat-
inactivated), 5% fetal bovine serum (heat-inactivated), penicillin (50
units/ml), and streptomycin (50 units/ml) at 37 °C in 5% CO\textsubscript{2}. Confluent
cells were detached by gentle trituration through a 25-gauge needle
pipette, plated onto collagen-coated 35-mm dishes (Costar) at 40–50% conflu-
ency, and used in 40–48 h (70–80% confluency). In a standard experi-
ment, 2.4 \mu g of plasmid DNA was transfected by LipofectAMINE (Life
Technologies, Inc.) according to the manufacturer’s instruction. Dulbe-
cco’s modified Eagle’s medium devoid of serum and antibiotics was used
instead of RPMI 1640 for transfection to increase the transfection
efficiency. In experiments in which the effects of increasing amounts
of hGH or synaptogyrin I were tested, the total amount of DNA trans-
fected varied as described in the figure legends. After 6 h, 4 ml of
complete RPMI 1640 medium was added to the dishes. Two days after
transfection, PC12 cells were harvested and re-plated, with the cells
resting in serum-free medium for 22–24 h (26). One day after re-plating, secretion experiments were conducted. One dish served
as control and was treated with physiological saline solution containing
145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 5.6 mM
sucrose, 15 mM HEPES, pH 7.4. The other dish served as test sample;
secretion was induced by a 15-min incubation with high K\textsuperscript{+}
 saline solution (physiological saline solution containing 95 mM NaCl and
56 mM KCl) with a 10-min incubation at 3 mM \alpha-latrotoxin in physio-
logical saline solution. At the end of the experiment, dishes were trans-
fected to ice, and the supernatant was removed and centrifuged in an
Eppendorf centrifuge. hGH in the supernatant from this centrifugation
was taken as secreted hGH. The cells from the dishes were taken up
in 1.2 ml of phosphate-buffered saline containing 1 mM EDTA and 0.1
\mu g of phenoxyethylsulfonyl fluoride and added to the pellet of the
Eppendorf centrifugation of the medium. Cells were then lysed by three
freeze-thaw cycles (in a dry ice/ethanol bath and a 37 °C bath), and
insoluble material was pelleted in an Eppendorf centrifuge. The mate-
rial from the supernatant of this step was taken as the cellular hGH
that was not secreted. hGH levels in the various samples were mea-
sured by a radioimmunossay kit (Nichols Institute, CA). Briefly, 0.1–
0.3 ml of the medium and cell extracts were mixed with \textsuperscript{125}I-
labelled hGH and with avidin-coated beads containing a second monocular
hGH antibody. Samples were incubated overnight at room temperature,
beads were washed twice with the wash solution provided in the kit,
and radioactivity associated with the beads was determined using an
LKB1272 gamma counter. Standard curves were made with known
amounts of purified recombinant hGH. All experiments were carried
out in duplicates or triplicates, and the average percent of total hGH
released was calculated. Statistical analyses were performed with the
two tailed Student’s t test.

Protein Expression in COS-7 Cells—COS-7 cells were maintained
in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum
under 5% CO\textsubscript{2} at 37 °C and transfected using DEAE-dextran with
choroquin and a 2-min glycerol shock as described (30) with 6.6 \mu g of
DNA for 900,000 cells in a 10-cm dish. Cells were washed with phos-
phate-buffered saline 72 h after transfections and harvested in 0.4 ml of
HEPES buffer (20 mM HEPES, 1 mM EGTA) per dish. Proteins were ex-
tracted after the addition of 0.4 ml sample buffer and passage through
a 25-gauge needle (10 times), and aliquots (10 \mu l) were analyzed
using standard SDS-polyacrylamide gel electrophoresis and immunoblotting
using ECL detection and antibodies described previously (9, 12, 30).

RESULTS

Molecular Analysis of Synaptogyrin III Reveals Conserved
Structure of Synaptogyrins—In the original studies a single
form of synaptogyrin localized to synaptic vesicles and a sec-
ond, ubiquitous form expressed in all cells tested were identi-
ﬁed (9, 12). Both isoforms are composed of four transmembrane
regions ﬂanked by cytoplasmic N- and C-terminal sequences,
with tyrosine phosphorylation sites in the C terminus (12).
Searches of sequence data banks for synaptogyrin-related se-
quences uncovered a third member of the synaptogyrin family,
referred to as synaptogyrin III. Partial sequences from the
human ortholog of synaptogyrin III were recently published
together with sequences of the human ortholog of cellugyrin,
which in that study was named synaptogyrin II (14). To avoid
confusion, we propose to retain the name cellugyrin for the
ubiquitous isoform of synaptogyrin and the name synaptogyrin
III for the new isoform even though, as a consequence, there is
no synaptogyrin II in this terminology.

To ensure that synaptogyrin III indeed belongs to the syn-
aptogyrin family and to compare it with other synaptogyrins,
we determined the sequences of two overlapping murine syn-
aptogyrin clones. Alignment of the deduced amino acid
sequence of synaptogyrin III with the sequences of synap-
 togyrin I, cellugyrin, and an invertebrate synaptogyrin homo-
log from C. elegans (accession number AF078373) unequivocally
identified synaptogyrin III as a member of the

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synaptogyrin family (Fig. 1). Both cDNA clones sequenced appeared to be full-length based on the similarity of the N termini of their predicted sequences with those of other synaptogyrins (Fig. 1). Synaptogyrin III exhibits 52% identity with synaptogyrin I and 44% identity with cellugyrin, whereas synaptogyrin I shares 47% sequence identity with cellugyrin. This suggests that all three vertebrate synaptogyrins are similarly related.

The alignment confirms that synaptogyrins form a family of homologous membrane proteins with four transmembrane regions. Interestingly, if the C. elegans sequence is included in the analysis, the short cytoplasmic N-terminal region of synaptogyrins and the linker sequence between the second and third transmembrane regions are the most conserved sequences of synaptogyrins. The transmembrane regions are also conserved, especially among the vertebrate isoforms in which they exhibit an unusual abundance of phenylalanine residues (Fig. 1). In contrast, the two intravesicular loops are highly variable except for two conserved cysteine residues in the first intravesicular loop, which may form an intramolecular disulfide bond (Fig. 1). The C-terminal cytoplasmic tail differs among various synaptogyrins and may be alternatively spliced in human (14). Previous studies showed that the C-terminal cytoplasmic domains of synaptogyrin I and cellugyrin are phosphorylated in transfected cells by c-src and fyn tyrosine kinases (12). Although there are tyrosine residues that are conserved in all four synaptogyrin sequences, the overall preservation of the cytoplasmic C-terminal tails is rather low.

We also analyzed the tissue distribution of synaptogyrin III expression. As reported for human synaptogyrin III (14), mouse synaptogyrin III mRNA was only detected in brain among the five tissues tested (Table I). Mouse synaptogyrin III mRNA was only detected in brain among the five tissues tested (Table I). Thus, similar to synaptogyrin I, synaptogyrin III is predominantly expressed in the brain.

Synaptogyrin I Inhibits Ca²⁺-triggered Exocytosis from PC12 Cells—To test if synaptogyrin I function in exocytosis, we studied the effect of synaptogyrin I overexpression on Ca²⁺-regulated exocytosis. For this purpose, we used a transfection assay in which hGH was co-expressed in PC12 cells with a protein of interest. Similar transfection assays have been used previously in a large number of studies of proteins implicated in exocytosis (e.g., see Refs. 22–27). Most previous studies used co-transfection of two separate vectors, one for hGH and one for the test protein or a control. We modified this procedure by employing a single test vector for both hGH (driven by the SV40 promoter) and the test protein (driven by the CMV promoter) and an identical control vector containing hGH but lacking the coding region for the test protein. A single vector was used to reduce possible variability in experiments in the co-expression of the two proteins in the same cell. Only in some experiments the single vector approach was replaced by the two-vector procedure, for example when we examined the secretory consequences of variable amounts of hGH and the protein of interest.

We first analyzed the effect of synaptogyrin I on the time course of Ca²⁺-dependent hGH secretion from transfected PC12 cells. Secretion was stimulated by two secretagogues with distinct mechanisms of action: KCl at high concentrations, which induces Ca²⁺ influx by membrane depolarization (Fig. 2A), or α-latrotoxin, which triggers exocytosis by an unknown mechanism (Fig. 2B). Previous studies showed that both secretagogues act by a Ca²⁺-dependent mechanism that is sensitive to inhibition by tetanus toxin or blockers of phosphatidylinositol kinases (23). hGH secretion as a percentage of total cellular hGH was measured as a function of time by radioimmunoadassay.

KCl depolarization or α-latrotoxin triggered robust, time-dependent hGH secretion from PC12 cells transfected with the control vector. Secretion exhibited a slow time course that required ∼10–15 min for completion and resulted in a total release of ∼40–50% hGH. The time course was similar for the two stimulation paradigms; its slow speed suggests that secretory vesicles are being continuously recruited and exocytosed during the reaction until the vesicle supplies are exhausted. In the absence of secretagogues, the amount of hGH in the PC12 cell medium (∼5–10%) did not increase markedly with time (Fig. 2). The lack of a time-dependent increase in hGH in the medium when cells are not stimulated by KCl depolarization or α-latrotoxin indicates that there is not a significant amount of constitutive secretion of hGH. Instead, most of the hGH in the medium appears to be derived from dead or detached cells.

PC12 cells transfected with synaptogyrin I exhibited a markedly different response from control cells. hGH secretion trig-
cells triggered by KCl depolarization or α-latrotoxin. PC12 cells were transfected with a plasmid encoding only hGH (Control) or both hGH and synaptogyrin (Sgyr I). Transfected cells were incubated at 37 °C for various times either in control medium and a high K+ solution (56 mM) (panel A) or 0.3 mM α-latrotoxin (panel B). At the end of the incubations, cells were transferred to ice. The amounts of hGH secreted into the medium and retained in the cells was measured by radioimmunoassay, and hGH release was calculated as a percentage of total hGH synthesized. Experiments were performed in duplicates.

We co-transfected increasing amounts of an hGH expression vector with a constant amount of a synaptogyrin I or control vector. As a result, we observed a dose-dependent increase in total hGH synthesis with or without synaptogyrin I (Fig. 4A). At the same time, we detected a significant depression (>50%) of hGH synthesis by co-expressed synaptogyrin I. Despite these disparities in expression levels, the degree of inhibition of secretion by co-expressed synaptogyrin I was independent of the amount of hGH synthesized (Fig. 4B). Even when we compared transfections in which more hGH was produced in the synaptogyrin I-transfected samples than in the control samples, inhibition was still profound.

We also reversed this experiment and tested the effects of variable levels of synaptogyrin I on the synthesis and exocytosis of hGH produced by a constant amount of hGH plasmid (Fig. 5). When we transfected more than 1 μg of synaptogyrin plasmid DNA (amounts usually used for all transfections in PC12 cells in our experiments), there was only a marginal effect of changing the amount of transfected DNA on exocytosis. However, when we transfected considerably less synaptogyrin plasmid DNA (<0.5 μg) than usual, the inhibitory effect of synaptogyrin decreased (Fig. 5). We observed a direct correlation at low amounts of transfected plasmid DNA between the magnitude of inhibition and the amount transfected. Together these data suggest that there is a direct relation between the amount of synaptogyrin expressed and the inhibition of exocytosis by synaptogyrin and no relation between the total synthesis of hGH and inhibition of exocytosis.

Domains of Synaptogyrin Required for Inhibition—Synaptogyrins are composed of four transmembrane regions associated with conserved cytoplasmic sequences and variable intravesicular loops and followed by a nonconserved tyrosine-phosphorylated cytoplasmic tail. To determine which of these regions are involved in the inhibition of exocytosis, we constructed deletion mutants of synaptogyrin I that lack defined sequences from its N or C terminus and tested the ability of these mutants to inhibit hGH secretion (shown schematically in Fig. 6A). Some of the constructs used were designed with an N-terminal myc tag to allow detection of the transfected protein when the epitope of our synaptogyrin antibodies was deleted. We confirmed that the various constructs expressed well and had the appropriate size by transfection into COS cells. These experiments also showed that the C-terminally truncated forms were not tyrosine-phosphorylated (data not shown).

When we examined the effects of the various synaptogyrin mutants on hGH secretion triggered by α-latrotoxin, we found that deletion of most of the cytoplasmic C-terminal tail did not decrease the inhibitory activity of synaptogyrin (constructs...
III-V, Fig. 6). This indicates that most of the cytoplasmic C-terminal tail is not important for the inhibitory effect, a finding that agrees well with lack of conservation between synaptogyrins in this region (Fig. 1). However, deletion of all of the cytoplasmic C-terminal tail (constructs VI and VII) or of the short N-terminal sequence (construct VIII) abolished the inhibition of exocytosis by synaptogyrin I. Thus most of the C-terminal cytoplasmic tail of synaptogyrin I is not involved in its regulatory effect in the PC12 cells, but the conserved N-terminal and C-terminal sequences adjacent to the transmembrane region are indispensable for inhibition.

**DISCUSSION**

The Function of Synaptogyrins—Synaptogyrins and synaptophysins constitute families of abundant synaptic vesicle proteins that are distantly related to each other (4–9). Members of both families contain four transmembrane regions and a tyrosine-phosphorylated cytoplasmic tail. Furthermore, as shown here, both families are composed of two neuronal isoforms (synaptogyrins I and III versus synaptophysin I and synaptoporin/synaptophysin II) and one ubiquitous isoform (cellugyrin versus pantophysin). The functions of synaptogyrins and synaptophysins have remained obscure despite considerable efforts. In recent studies of knockout mice, we observed that mice lacking both synaptogyrin I and synaptophysin I are viable and fertile, suggesting that they are not essential for synaptic vesicle exocytosis. 3 When we analyzed these mice electrophysiologically, however, we detected major changes in synaptic plasticity in the double knockout mice that lack both synaptogyrin I and synaptophysin I. These results suggested that synaptogyrins and synaptophysins, although not required for exocytosis as such, are essential for regulating neurotransmitter release.

3 R. Janz and T. C. Südhof, unpublished observations.
In the current study, we have taken a completely different approach to analyzing the functions of synaptogyrins. We tested the effects of overexpressing synaptogyrins and synaptophysin I on Ca\textsuperscript{2+}-regulated exocytosis in PC12 cells. To evaluate how general our findings are for various members of the synaptogyrin family, we first sought to define the characteristics of this protein family. For this purpose, we determined the full-length structure of a new member of the synaptogyrin family, synaptogyrin III (Fig. 1). Sequence comparisons and RNA blots showed that with this new synaptogyrin, the synaptogyrin family comprises at least two neuronal and one ubiquitous isoform with a selective pattern of sequence conservation. Most strikingly, sequences associated with the membrane are conserved, whereas the intravesicular loops and the cytoplasmic C-terminal region of synaptogyrins are variable, indicating that the functions of synaptogyrins are executed by their transmembrane regions and adjacent cytoplasmic sequences.

Next we analyzed the effect of overexpressing synaptogyrins and synaptophysin I on Ca\textsuperscript{2+}-regulated exocytosis in transfected PC12 cells. Our data show that all synaptogyrins and the distantly related synaptophysin I severely inhibit exocytosis (Figs. 2 and 3). The extent of inhibition was similar to that produced by tetanus toxin, a powerful inhibitor of secretion. Mutational analysis demonstrated that inhibition does not require most of the C-terminal domain of synaptogyrin, which is tyrosine-phosphorylated but depends on the sequences immediately surrounding the transmembrane regions and may also involve the transmembrane regions, thereby mirroring the conservation of the synaptogyrin sequences (Fig. 5). Several other synaptic trafficking proteins such as synaptotagmins, synapsins, and synaptobrevins that we tested did not inhibit exocytosis.

Is it possible that the inhibitory effect of synaptogyrins is the result of an artifact relating to the transfection technique and PC12 cell secretion? To address this concern, we strove to validate the transfection assay. The tetanus toxin sensitivity and Ca\textsuperscript{2+} dependence of regulated secretion showed that hGH secretion results from exocytosis. In addition, to avoid stimulation-specific artifacts, we applied two different stimulation conditions (KCl depolarization and \(\alpha\)-latrotoxin), which trigger exocytosis by distinct mechanisms. These two conditions gave identical results. Finally, we addressed the possibility that synaptogyrin expression may interfere with the synthesis of...
hGH and thereby influence the percent of hGH that is available for stimulated secretion. Indeed, the amount of hGH synthesized is reduced after synaptogyrin expression (Fig. 4). Nevertheless, this appears to have no effect on the assay. The same inhibition of hGH synthesis was observed after transfections of a number of proteins that do not inhibit exocytosis, suggesting that there is no correlation of hGH synthesis with inhibition of secretion. Furthermore, titration of the transfections with different amounts of hGH or synaptogyrin plasmids demonstrated that inhibition was independent of the ratio of hGH to synaptogyrin. Thus the inhibition observed is not a transfection artifact, suggesting that all synaptogyrins specifically inhibit secretion of hGH and are thus directly involved in exocytosis.3

Comparison of Knockout and Transfection Assays in Analyzing Secretion—It is striking that the transfection studies reported here give diametrically opposite results to those obtained in knockout mice. In the synaptogyrin and synaptophysin knockout mice, only changes in synaptic plasticity were observed without major impairments in Ca$^{2+}$-regulated exocytosis.3 In transfected PC12 cells, in contrast, synaptogyrin I or synaptophysin I severely inhibited Ca$^{2+}$-regulated exocytosis, on par with the effect of tetanus toxin light chain. Interestingly, this apparent discrepancy is not limited to the proteins examined in the current study but also emerges from previous investigations of other proteins, which are summarized in Table I. Together with the results from the current paper and unpublished studies, there is a total of 10 proteins that were analyzed in transfected cells and in knockout mice. Four of these proteins are associated with mild phenotypes in knockouts but severe effects in transfections (synaptogyrin I, synaptophysin I, rab3A, and rabphilin). Conversely, four other proteins are associated with lethal phenotypes in knockouts but mild effects in transfections (synaptotagmin I, munc18–1, synaptobrevin II, and munc13–1). Finally, two proteins display a significant knockout phenotype which, however, is not lethal and also causes no changes in transfected PC12 cells (synapsins I and II); these proteins are therefore intermediate between the two other groups. Thus there appears to be a negative correlation between knockout and transfection phenotypes. Proteins that cause major effects in transfections are usually nonessential in knockouts, and conversely, proteins that are associated with essential func-

![Figure 6](image_url)

**FIG. 6.** Mutational analysis of the inhibitory role of synaptogyrin on α-latrotoxin induced secretion of hGH in transfected PC12 cells. A, schematic diagram of the transmembrane structure of synaptogyrin I and location of mutants. The constructs are identified by letters and plasmid names on the left, with the top construct (I) depicting wild type protein. The shaded areas in the bar diagrams denote transmembrane regions that are identified by roman numerals. The amino acid residue numbers are shown at the boundaries of the transmembrane regions and at the beginning and end of each construct. Some constructs contain an N-terminal myc-tag (myc) for immunological identification of the protein product. B, the constructs shown in A were analyzed as described in Fig. 3. Letters above each data set identify the protein encoded by the plasmids with the same letters in A.
tions in exocytosis elicit no change in secretion upon transfection.

The apparent discrepancy between the results of knockout and transfection studies raises the question of which analysis is more physiologically relevant. There are several possible explanations for the discrepancies between the assays, and both approaches have inherent limitations that need to be considered. The case where a protein causes lethality in knockouts but no effect in transfections is most easily explained. In the transfection assays, the overexpressed protein is already present endogenously. It is probable that most endogenous proteins that function as effectors in exocytosis are not rate-limiting and that expressing more of such a protein would at best enhance the normal function of this protein. In this view, the lack of a phenotype in transfection assays for a protein that has a central function in executing exocytosis is not surprising.

The lack of a knockout phenotype for proteins that have major effects in the transfection assay is more difficult to explain. One hypothesis is that a protein may be functionally redundant in mice, which would not be a problem in the transfection assay. However, two arguments speak against this hypothesis as a general explanation. First, it seems too much of a coincidence that several proteins that have major effects in the transfection assay is more difficult to explain. One hypothesis is that a protein may be functionally redundant in mice, which would not be a problem in the transfection assay. However, two arguments speak against this hypothesis as a general explanation. First, it seems too much of a coincidence that several proteins that have major effects in the transfection assay is more difficult to explain. One hypothesis is that a protein may be functionally redundant in mice, which would not be a problem in the transfection assay. However, two arguments speak against this hypothesis as a general explanation. First, it seems too much of a coincidence that several proteins that have major effects in the transfection assay.

**TABLE I**

Comparison of loss-of-function and gain-of-function analyses of vertebrate synaptic trafficking proteins

Table I shows a summary of the current and previous studies on trafficking proteins using knockout experiments in mice or overexpression experiments in PC12 or chromaffin cells. The table includes only proteins for which data from both mouse knockouts and overexpression experiments were available. Data on synaptotagmin, synaptobrevin, munc18–1, and rab3A homologs were also obtained genetically in Drosophila and/or C. elegans but are not included in the table because corresponding overexpression analyses with the same proteins in transfected cells are not available. Abbreviations in table: PPF, paired pulse facilitation; PTP, post-tetanic potentiation; mLLTP, mossy fiber LTP.

| PROTEIN         | Loss of function (mouse knockout) | PHENOTYPE | Ref. | Gain of function (overexpression) | Ref. |
|-----------------|----------------------------------|-----------|------|----------------------------------|------|
| Synaptogyrin I  | No morbidity or mortality; selective decrease in PTP | Footnote 1 (in text) | | Strong inhibition of exocytosis | This study |
| Synaptophysin I | No morbidity or mortality | 15 | Strong inhibition of exocytosis | This study |
| Rab3A           | No morbidity or mortality; increased neurotransmitter release; loss of mLLTP | | | Moderate inhibition of exocytosis | 24 |
| Rabphilin       | No major phenotypic effects | Unpublished | | Moderate increase in exocytosis | 25 |
| Synaptotagmin I | Lethal | 38 | | No effect | This study |
| Synaptobrevin II| Lethal | Unpublished | No effect | This study |
| Munc18–1        | Lethal | Unpublished | No effect | This study |
| Munc13–1        | Decreased synaptic vesicle stability; increased PPF | 39 | No effect | Unpublished |
| Synapsin I      | Decreased synaptic vesicle stability; changes in PTP and PPF | 39 | No effect | This study |
| Synapsin II     | Epilepsy; decreased synaptic vesicle stability; changes in PTP and PPF | | | | |

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