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Overexpression of Complexin in PC12 Cells Inhibits Exocytosis by Preventing SNARE Complex Recycling

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Abstract—Complexin is an important protein that functions during Ca^{2+}-dependent neurotransmitter release. Substantial evidence supports that complexin performs its role through rapid interaction with SNARE complex with high affinity. However, α-SNAP/NSF, which can disassemble the *cis*-SNARE complex in the presence of MgATP, competes with complexin to bind to SNARE complex. In addition, injection of α-SNAP into chromaffin cells enhances the size of the readily releasable pool, and mutation disrupting the ATPase activity of NSF results in the accumulation of SNARE complex. Thus, whether high concentrations of complexin could result in a reverse result is unclear. In this paper, we demonstrate that when stably overexpressed in PC12 cells, high levels of complexin result in the accumulation of SNARE complex. This in turn leads to a reduction in the size of the readily releasable pool of large dense core vesicles. These results suggest that high levels of complexin seem to prevent SNARE complex recycling, presumably by displacing NSF and α-SNAP from SNARE complex.

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Key words: complexin, SNARE complex cycle, readily releasable pool, neurotransmitter release

Neurotransmitter release is triggered by an influx of calcium following the arrival of an action potential, a rapid process that is tightly regulated by a variety of proteins [1]. Complexin, one of these factors, is a small and hydrophilic protein that is enriched in the brain [2]. Previous research suggested that complexin plays an important role during exocytosis [3-6]. In addition, a peptide that prevents complexin from binding to SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) complex also inhibits evoked transmitter release [7]. Biochemical and biophysical studies revealed that complexin binds rapidly to the SNARE complex along the groove between VAMP (vesicle associated membrane protein, or synaptobrevin) and syntaxin in an anti-parallel conformation with high affinity [8, 9]. This interaction might stabilize the *trans*-SNARE complex, enabling the extraordinarily high speed of Ca^{2+}-evoked neurotransmitter release [10]. Recent reports have shown that complexin inhibits the fusion of liposomes reconstituted with SNAREs or cells expressing "flipped" SNAREs; and this inhibition can be relieved by synaptotagmin in the presence of calcium [11, 12]. Moreover, synaptotagmin/calcium could trigger the fast exocytosis by displacing complexin from SNARE complex in a calcium-dependent manner [13]. All these studies supported the idea that complexin plays its role by interaction with SNARE complex.

As the essential machinery that mediates exocytosis, SNARE complex contains three membrane proteins, which bind to each other to form an SDS-resistant, highly twisted and parallel four-helix bundle [14-17]. During the cycle of a synaptic vesicle, SNARE complex also undergoes the cycle of assembly and disassembly. It is now clear that after fusion, the *cis*-SNARE complex is disassembled by α-SNAP (α-soluble N-ethylmaleimide-sensitive factor attachment protein) and NSF (N-ethylmaleimide-sensitive fusion protein) in the presence of

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Abbreviations: LDCV) large dense core vesicle; NSF) N-ethylmaleimide-sensitive fusion protein; RRP) readily releasable pool; α-SNAP) α-soluble N-ethylmaleimide-sensitive factor attachment protein; SNAP-25) synaptosomal-associated protein of 25 kD; SNAREs) soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors; VAMP) vesicle associated membrane protein, or synaptobrevin.

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MgATP [18, 19]. In addition, injection of α-SNAP into a neuron enhances the size of the readily releasable pool (RRP), while injection of the inhibitory α-SNAP or NSF peptide reduces the size of the RRP [20-24]. Moreover, mutation disrupting the ATPase of NSF results in the accumulation of SNARE complex [25, 26]. These results suggested a post-docking and pre-fusion role of α-SNAP and NSF. A previous report showed that complexin binds SNARE complex competitively with α-SNAP [2], which suggests that high concentrations of complexin may lead to an adverse result.

In present study, we established a PC12 cell line that stably expresses complexin II to investigate the role of complexin during exocytosis. We demonstrated that overexpression of complexin II in PC12 cells reduces the size of the RRP of LDCV (large dense core vesicle). This is not because the overexpression of complexin II changes the numbers or the distributions of LDCV, but may be due to complexin-dependent SNARE complex accumulation, which suggests that high levels of complexin II may prevent the SNARE complex from recycling, besides arresting hemifusion.

MATERIALS AND METHODS

Materials. Recombinant fusion proteins, α-SNAP, NSF, and syntaxin, were prepared as His_6-tagged fusion proteins and purified using Ni^{2+}-Sepharose affinity chromatography and ion-exchange chromatography. The purified proteins were then used as an immunogen for the production of rabbit antiserum. Antibodies for synaptotagmin, VAMP, and actin were purchased from Santa Cruz Biotech (USA). Mouse anti-SNAP-25 (synaptosomal-associated protein of 25 kD) monoclonal antibody (MAB331) was from Chemical (Germany). Affinity purified rabbit antiserum of complexin was from Synaptic Systems (Germany).

cDNA of complexin II was kindly provided by D. Fasshauer. $[7,8,^3H]$Dopamine was purchased from Amersham (England). Fluo-3/AM and PluronicF-127 were from Biotium (USA). G418 sulfate was from Amresco (USA). BCA protein assay kit was from Pierce (USA).

PC12 cell culture and transfection. PC12 cells were plated onto poly-L-lysine coated dishes and were maintained in RPMI-1640 medium supplemented with 10% horse serum and 5% fetal calf serum at 37°C in 5% CO_2 incubator.

To establish the cell line stably expressing complexin II, 15 µg of linearized pcDNA3.1(+)-CPXII plasmid was transfected into PC12 cells by electroporation using an ECM830 electroporator. G418 (0.5 mg/ml) in the growth medium was used to screen the positive clones, and a single clone was isolated and determined by immunoblotting with complexin antiserum.

Calcium imaging. The calcium imaging experiment was performed according to the procedure as described previously with modification [27]. After loading with 6 µM Fluo-3/AM in Krebs–Ringer buffer (145 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes, pH 7.4) at 37°C for 30 min, the cells were washed three times and incubated for 15 min to further de-esterify Fluo-3/AM before imaging. Cells were then directly imaged using a Nikon (Japan) inverted microscope (TE300). $[Ca^{2+}]_i$ transients were expressed as fractional amplitude increase ($\Delta F/F_0$, where F_0 is the baseline fluorescence level and ΔF is the rise over baseline).

Measurement of $[7,8,^3H]$dopamine release. Cells were plated with a density of $2.5 \cdot 10^5$ per ml on poly-L-lysine coated 24-well dishes and cultured in the medium for 2 days. The cells were then incubated in 500 µl/well RPMI-1640 medium with 0.5 µCi of $[7,8,^3H]$dopamine and 0.5 mM ascorbic acid at 37°C for 3 h. After washing twice with Krebs–Ringer buffer without calcium, the cells were incubated with culture medium for approximately 30 min in order to reduce the amount of spontaneous release, followed by another two washes. Release of dopamine was measured in 500 µl of high KCl content buffer (95 mM NaCl, 56 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 15 mM Hepes, pH 7.4, 5.6 mM glucose, and 0.5 mM ascorbic acid) or in 500 µl of Krebs–Ringer buffer with 0.75 M sucrose for 10 or 5 min, respectively. Release reactions were initiated by warming at 30°C and terminated by chilling on ice. The $[7,8,^3H]$dopamine released into the medium was determined. $[7,8,^3H]$Dopamine remaining in the cells was measured by lysing the cells with 500 µl 0.5% Triton X-100. The $[7,8,^3H]$dopamine content in both the cell and supernatant fractions was counted in a scintillation counter, and dopamine release was expressed as a percentage of total cell content for each well.

Transmission electron microscopy and morphometry. After rinsing twice with PBS, PC12 cells and clone 1 cells were fixed with 2.5% glutaraldehyde in PBS at 4°C overnight. The cells were then washed extensively with PBS, post-fixed with 1% OsO_4 in PBS for 1.5 h, dehydrated through a series of increasing ethanol concentration, and embedded in Epon. Ultrathin sections (≈60 nm) were collected on Formvar (polyvinyl formaldehyde)-coated copper grids, stained with lead citrate and uranyl acetate, and observed with a Philips CM 120 transmission electron microscope. Numbers and the distribution of LDCV were counted by two investigators in a double-blind fashion.

Preparation of cell extracts and immunoblotting. To compare the level of SNARE complex in clone 1 cells and PC12 cells, cellular membrane fraction was separated according to a procedure described previously with modification [28]. After washing with PBS, the cells were resuspended with hypotonic buffer (10 mM Tris-HCl,

pH 7.4, 10 mM $MgCl_2$, 2.5 mM EGTA) supplemented with protein inhibitor, followed by freeze-thaw twice with liquid nitrogen and sonication. The cell membrane fractions were collected by centrifugation at 500*g* at 4°C for 10 min and subsequent centrifugation at 80,000*g* at 4°C for 30 min, and the membrane pellet was resuspended with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS). For the total cell extract, the cells were lysed with SDS sample buffer followed by sonication and centrifugation at 20,000*g* at 4°C for 10 min, and the supernatant was collected. The total protein concentration in the total cell extract or the membrane proteins extract was determined using the BCA protein assay kit. Then 20 mM dithiothreitol (DTT) and 0.001% Bromophenol Blue (final concentration) were added. All samples were then subjected to SDS-PAGE and visualized by immunoblotting.

RESULTS

Establishment of a PC12 cell line stably overexpressing complexin II. To explore the function of complexin II during the process of exocytosis, we established a PC12 cell line that stably overexpresses complexin II. Clones stably expressing complexin II were screened with G418 based on the expression level of complexin compared with control PC12 cells. At least two individual clones overexpressing complexin II were selected for further experiments with similar results, and representative data from one of the complexin II-overexpressing clones (clone 1) is presented in this study.

As shown in Fig. 1, expression level of complexin in clone 1 cells was much higher than that of control PC12 cells. In addition, the expression levels of other proteins that play important roles in neurotransmitter release were also compared between the clone 1 cells and control PC12 cells. SNARE proteins, which include syntaxin, SNAP-25, and VAMP, are the minimal machinery for membrane fusion. Synaptotagmin is thought to be the calcium sensor, which couples the calcium signal with the membrane fusion. α-SNAP and NSF are the proteins that disassemble the *cis*-SNARE complex into three individual proteins, which will enter the next fusion cycle. Western blotting showed that overexpression of complexin II in clone 1 cells did not affect the expression level of these proteins.

Overexpression of complexin II in PC12 cells inhibits dopamine release by reducing the size of the RRP. Next, we compared the [7,8,^{3}H]dopamine release between clone 1 cells and PC12 cells induced by 56 mM KCl. From Fig. 2a, we can see that the release of [7,8,^{3}H]dopamine from clone 1 cells was remarkably lower than that from PC12 cells. This was not due to the different uptake of [7,8,^{3}H]dopamine, because there was no significant difference of [7,8,^{3}H]dopamine uptake between clone 1 cells and PC12 cells (data not shown). However, as the release was induced by the high KCl, which resulted in an increase in intracellular calcium level, it is possible that overexpression of complexin II in clone 1 cells impedes the increase in intracellular calcium level, which results in the reduced [7,8,^{3}H]dopamine release. Thus, we determined the increase in intracellular calcium level after treatment with 56 mM KCl using the Ca^{2+}-sensitive dye Fluo-3. Figure 2b shows that overexpression of complexin II in clone 1 cells did not markedly affect the increase in intracellular calcium level. These data indicate that the reduced [7,8,^{3}H]dopamine release directly results from the overexpression of complexin in clone 1 cells.

Previous studies showed that injection of α-SNAP into chromaffin cells increases the size of the RRP [20, 21]. Moreover, complexin competes with α-SNAP to bind to SNARE complex [2]. These phenomena led us to

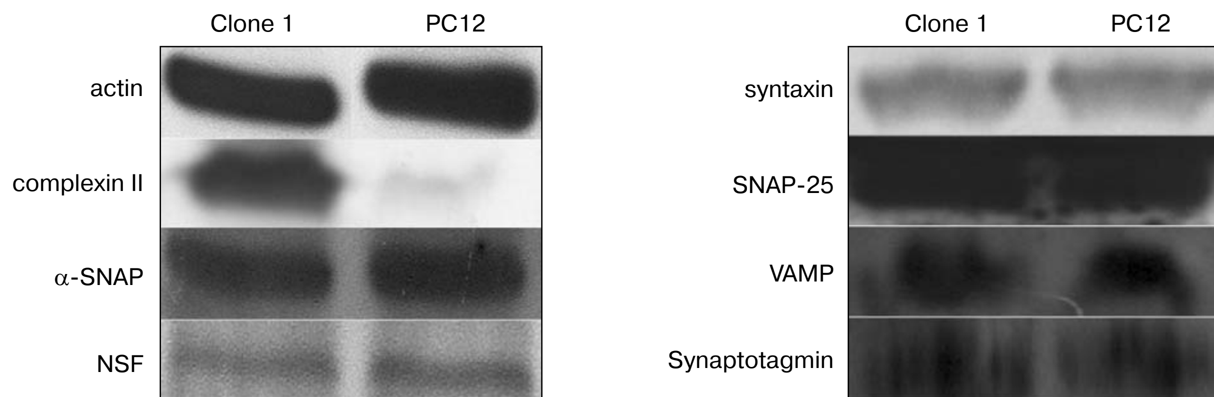

Fig. 1. Determination of proteins expression levels in clone 1 cells and control PC12 cells. After washing with PBS, the cells were lysed with loading buffer (50 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS), followed by sonication. The lysate was then centrifuged at 20,000*g* at 4°C for 10 min, the supernatant was collected, and the quantities of total proteins were determined using the BCA protein assay kit. Before loading, each sample was supplemented with 20 mM DTT and 0.001% Bromophenol Blue (final concentration). For each targeted protein assay, the same amounts of total proteins from clone 1 cells and PC12 cells extracts were subjected to SDS-PAGE and visualized by immunoblotting.

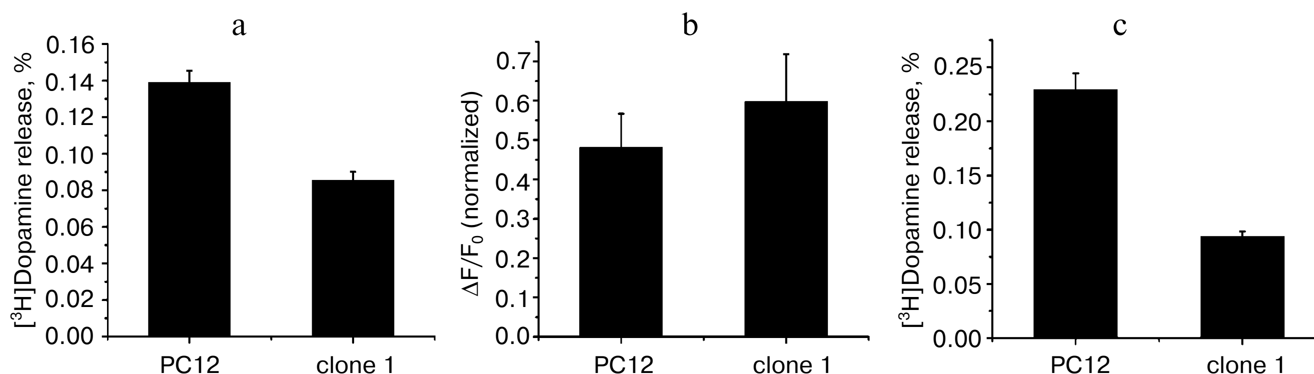

Fig. 2. Overexpression of complexin II in PC12 cells reduces the size of RRP. a) Overexpression of complexin II in PC12 cells inhibits the [7,8,^{3}H]dopamine release induced by 56 mM KCl. Quantifications of [7,8,^{3}H]dopamine release from the clone 1 cells and control PC12 cells were determined after induction by 56 mM KCl at 30°C for 10 min. Two independent experiments were performed, and each experiment was repeated three times ($n = 6$, $p < 0.01$). b) Overexpression of complexin does not remarkably affect the increase in intracellular calcium level in clone 1 cells induced by 56 mM KCl at 30°C compared with that in PC12 cells ($n = 8$ for PC12 cells, $n = 12$ for clone 1 cells, $p > 0.05$). c) Overexpression of complexin II in PC12 cells reduces the size of RRP. The level of [7,8,^{3}H]dopamine release induced by 0.75 M sucrose at 30°C for 5 min from the clone 1 cells and PC12 cells was determined. Two independent experiments were performed, and each experiment was repeated three times ($n = 6$, $p < 0.01$).

speculate that overexpression of complexin II might affect the size of the RRP. In the neuron, high concentrations of sucrose induce the fusion of docked vesicles with presynaptic membrane, which has been employed to determine the size of the RRP [29]. Therefore, we used 0.75 M sucrose to induce exocytosis in clone 1 cells and PC12 cells. As shown in Fig. 2c, complexin overexpression in clone 1 cells greatly reduced the size of the RRP. In addition, the release of [7,8,^{3}H]dopamine induced by 0.75 M sucrose from clone 1 was similar to that induced by 56 mM KCl, while release from control PC12 cells did not, which indicates that the vesicles releasing [7,8,^{3}H]dopamine induced by KCl in clone 1 cells were the same vesicles in the readily releasable pool. However, in the previous study, the release induced by 0.75 M sucrose from PC12 cells was about 36%, while it was only about 23% in our work [30]. This apparent discrepancy is possibly because the passages of PC12 cells in our work were as many as that of clone 1 cells when the clone 1 cells were screened and determined, which may cause the loss of some properties, such as a small number of LDCV [31].

Overexpression of complexin II in PC12 cells does not affect the number or the distribution of LDCV. The above results show that overexpression of complexin II in PC12 cells reduces the size of the RRP. But what is the mechanism underlying this inhibition by high concentrations of complexin? As previous results indicate that there is a direct relationship between the size of the RRP and the numbers and distributions of the LDCV [32], we then compared the numbers and distributions of the LDCV between clone 1 cells and PC12 cells (Fig. 3). However, as

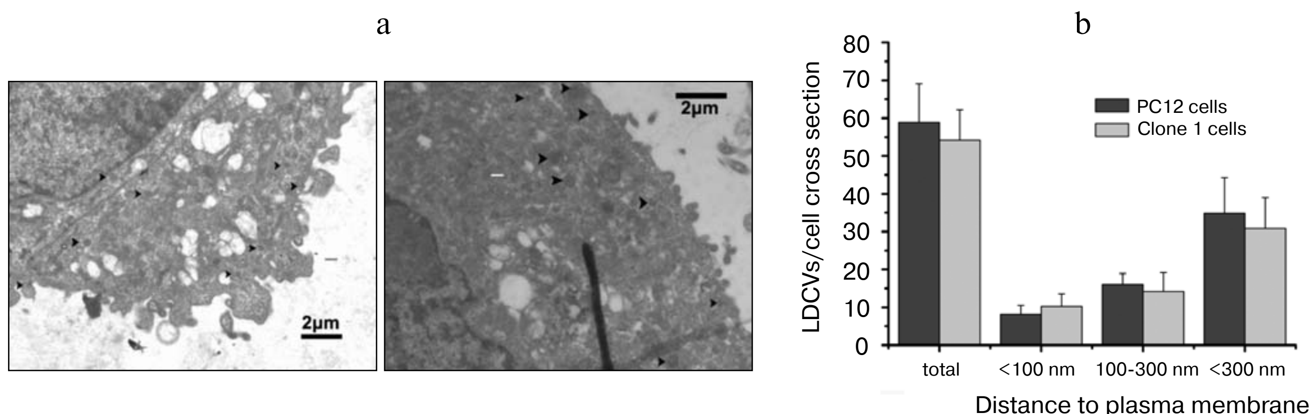

Fig. 3. Comparison of the number and the spatial distribution of LDCV between clone 1 cells and control PC12 cells. a) Representative section micrographs of control PC12 cells (left) and clone 1 cells (right) (the arrowheads indicate the LDCV). b) Morphological comparison between clone 1 cells and PC12 cells ($n = 32$ for PC12 cells; $n = 36$ for clone 1 cells, $p > 0.1$).

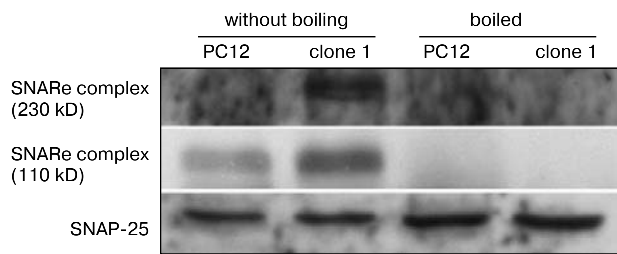

Fig. 4. Overexpression of complexin II in PC12 cells results in the accumulation of SDS-resistant SNARE complex. After the membrane fractions from clone 1 cells and PC12 cells were extracted, the total proteins were quantified by BCA protein assay kit. Before loading, each sample was supplemented with 20 mM DTT and 0.001% Bromophenol Blue (final concentration). For each lane, the loading of total proteins from clone 1 cells and control PC12 cells were identical. The samples were subjected to SDS-PAGE and immunoblotted with Mouse anti-SNAP-25 monoclonal antibody (MAB331).

shown in Fig. 3b, there was no significant difference in the total numbers of LDCV in the two cell lines. Moreover, although overexpression of complexin II reduces the size of the RRP, the distributions of LDCV in the two cell lines are also similar. Thus, the difference in the size of the RRP between the PC12 cells and clone 1 cells cannot be explained by changes in the number or distribution of LDCV.

Complexin II overexpression in PC12 cells results in the accumulation of SNARE complex. α-SNAP and NSF are the factors that disassemble the *cis*-SNARE complex after the exocytosis, which releases the SNARE proteins to enter the next cycle [18, 19]. Temperature-sensitive paralytic mutations in NSF lead to the accumulation of 7S SNARE complex [25, 26]. In addition, complexin competes with α-SNAP to bind to SNARE complex [2]. Thus, it is postulated that complexin may prevent the interaction between α-SNAP and SNARE complex, resulting in the accumulation of SNARE complex in clone 1 cells. Although previous studies reported that complexin does not affect the disassembly of SNARE complex by α-SNAP and NSF *in vitro* [8], it is still unclear whether this case also appears *in vivo*. Thus, in the next experiment, we compared the level of SNARE complex in clone 1 cells and PC12 cells. Consistent with previous report, we detected the presence of SDS-resistant SNARE complex in both cell lines [28]. However, the amount of SNARE complex in clone 1 cells was higher than that in PC12 cells (Fig. 4). This result indicates that overexpression of complexin II in clone 1 cell may inhibit the recycling of SNARE complex.

DISCUSSION

Complexin is a small cytoplasmic protein that functions in neurotransmitter release by its interaction with SNARE complex [2, 7-10]. Previous studies suggested that complexin stabilizes the SNARE complex and functions in a late step during the calcium-dependent release [4, 10-13]. However, we could not eliminate the possibility that complexin functions at other steps with different mechanism. As α-SNAP competes with complexin to bind to SNARE complex, overexpression of α-SNAP enhances the size of the RRP and release [2, 20, 21]. Thus, overexpression of complexin might lead to an adverse result. In this work, we show that overexpression of complexin II in PC12 cells results in the accumulation of SNARE complex, which may further reduce the size of the readily releasable pool. Moreover, although overexpression of complexin reduces the size of the readily releasable pool, the number or distribution of LDCV is not affected in our assay. All these data suggest that, in addition to arrest the hemifusion, complexin may also take part in an early step in the calcium-dependent release, presumably by inhibiting SNARE complex recycling, which leads to reduced release from RRP.

Although studies done *in vitro* showed that complexin did not affect the disassembly of SNARE complex [8], we showed here that overexpression of complexin II resulted in the accumulation of SNARE complex. How can we explain this apparent contradiction? Previous reports have confirmed that exocytosis can be divided into a MgATP-dependent step and a MgATP-independent step, which correspond to the priming step and triggering step, respectively [33, 34]. Although complexin overexpression in PC12 cells does not change the expression level of α-SNAP and NSF (see Fig. 1), ATP and Mg^{2+} may be depleted by complexin overexpression, which may inhibit the disassembly of SNARE complex and reduce the size of the RRP [35].

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