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Synaptotagmin I and IX function redundantly in controlling fusion pore of large dense core vesicles

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Abstract

Synaptotagmins (Syts) constitute a large family of at least 16 members and individual Syt isoforms exhibit distinct Ca^{2+}-binding properties and subcellular localization. It remains to be demonstrated whether multiple Syt isoforms can function independently or cooperatively on certain type of vesicle. In the current study, we have developed NPY-pHluorin to specifically assess exocytosis of large dense core vesicles (LDCVs) and studied the requirement of Syt I and Syt IX for LDCV exocytosis in PC12 cells. We found that down-regulation of both Syt I and Syt IX resulted in a significant loss of Ca^{2+}-dependent LDCV exocytosis. Moreover, our results suggest Syt I and Syt IX play redundant role in controlling the choice of fusion modes. Down-regulation of both Syt I and Syt IX renders more fusion in the kiss-and-run mode. We conclude that Syt I and Syt IX function redundantly in Ca^{2+}-sensing and fusion pore dilation on LDCVs in PC12 cells.

Keywords: Synaptotagmins; Large dense core vesicles; Exocytosis; Kiss-and-run; Full fusion

Secretory exocytosis is the major mechanism of transmitters and hormones release. The classic view of neurotransmitter release holds that initially formed fusion pore expands rapidly and leads to a full merging of synaptic vesicle and plasma membrane [1]. Alternatively, fusion pore formation can be transient leaving an intact synaptic vesicle, which represents of the "kiss-and-run" mode of release [2]. The regulatory release of peptide hormones has been originally thought to require the full fusion of the large dense core vesicle (LDCV) with the plasma membrane [3]. However, accumulating evidences suggest that LDCV can also proceed in the "kiss-and-run" mode of fusion [4,5]. Despite its critical role in exocytosis, very little is known about the structure and regulation of fusion pore.

Synaptotagmins (Syts) are proposed to function as Ca^{2+} sensors of the molecular fusion machinery. Syts harbor two tandem cytoplasmic C2 domains which interact with Ca^{2+} and phospholipids [6]. At least sixteen isoforms of Syts have been identified in mammalians [7]. Structurally similar Syts localizing differentially, have distinct apparent Ca^{2+}-affinities and membrane-binding activities, and may form fusion machineries for different secretory granules [8]. Among them, Syt I is best characterized as an abundant synaptic vesicle-associated protein essential for rapid synchronous synaptic vesicle exocytosis [9,10]. Syt I also exists on LDCVs in neuroendocrine and endocrine cells [11], and has been shown to be involved in Ca^{2+}-dependent endocrine exocytosis. The exact role of other members of Syts, however,

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remains less clear. Syt IX, a close homologue to Syt I on sequence alignments, has been postulated to function as a major Ca^{2+} sensor for LDCV exocytosis in neuroendocrine cells [12]. Nevertheless, the unique binding properties of Syt IX with Ca^{2+}, phospholipid and SNARE complex suggest a biological role different from that of Syt I [13]. Multiple Syt isoforms (I, IV, V, VII, and IX) have been detected in PC12 cells and previous studies have yielded conflicting results as to which isoform functions in Ca^{2+}-dependent LDCV exocytosis in PC12 cells [8,12,14].

Syts not only serve as the Ca^{2+} sensors for exocytosis, but are also postulated to function in controling fusion pore [15–17]. It has been suggested that the interaction between Syt and *t*-SNARE regulates fusion pore dynamics [15]. Transfection of Syt I in PC12 cells has been shown to prolong the open state of the fusion pore, whereas transfecting with Syt IV has the opposite effect [16]. Moreover, the same group showed that overexpression of different domains of Syt I and IV control the choice between kiss-and-run and full fusion [17]. It should be noted that fusion pore is very sensitive to overexpression as overexpression of exogenous proteins tends to change membrane composition or tension, hence altering fusion pore non-specifically [18,19]. Thus, it is necessary to verify the role of Syts in fusion pore control by intervening with endogenous Syts *in vivo*. To this end, we have reduced the protein level of Syt I and IX in PC12 cells by RNAi mediated gene silence. By assessing exocytosis and fusion pore dynamics, we show that Syt I and IX co-localize in LDCV and function redundantly in LDCV exocytosis. Down-regulation of both Syt I and IX shifts more fusion events to the kiss-and-run mode.

Materials and methods

Constructs. A 19-nucleotide target sequence of Syt I and Syt IX genes: GCT GAA GCA GAA GTT TAT G [12], and GCG CTG TCG GAG ACG GAT G were chosen to silence the expression of Syt I and Syt IX, respectively. Annealed double stranded oligonucleotides encoding the target-sequences were subcloned into the vector pRNAT-H1.1/RFP to generate the plasmids expressing shRNAs against Syt I and Syt IX. NPY and pHluorin were cloned in frame into pcDNA3.1 (Invitrogen) vector. All constructs were verified by sequencing.

Total internal reflection fluorescence microscopy (TIRFM) and image analysis. The TIRF microscope setup was constructed based on the prismless and through-the-lens configuration as previously described [20]. Images were recorded at 10 Hz with a 50-ms exposure time using a cooled CCD (PCO Computer Optics). Imaging was controlled using TILLvisION 4.01 (TILL Photonics) and analyzed in Matlab (Math Works) or Igor Pro (WaveMetrics).

Exocytotic events were manually selected based on the abrupt increase in fluorescence intensity of NPY-pHluorin upon fusion. We placed two concentric circles (18 and 27 pixels, corresponding to approximately 1.2 and 1.8 μm in diameter), to center the selected vesicle and its surrounding. The mean brightness of the inner circle was measured as fluorescent intensity of individual vesicle [20]. The time course of fluorescence changes in outer concentric annulus was defined as kinetics of diffusion of fluorescent peptides through fusion pore.

Amperometry. Carbon fiber electrodes used for amperometry were produced as described by Zhou et al. [21]. A holding potential of 700 mV was applied in the voltage-clamp mode. The current was sampled at 16.7 kHz and filtered at 2.9 kHz, and then further digitally filtered at cut-off frequency of 600 Hz and analyzed in Ampz v 4.6.

Result

Knockdown of both Syt I and Syt IX, but not each single isoform alone, reduced LDCV exocytosis

We found a partial co-localization of Syt I or Syt IX with NPY-DsRed, a specific LDCV marker [20], in PC12 cells under TIRFM. Moreover, we observed about 85.0% of Syt IX co-localized with Syt I, demonstrating the co-residence of both Syt isoforms on same vesicles (See Supplementary Figure 1).

Real-time TIRFM imaging has been successfully monitored of single LDCV fusion by a diffusion pattern of luminal LDCV marker, NPY-EGFP [4]. However, the fusion events detected is considerably sparse [22], due to the low detection rate of EGFP fluorescence. To overcome this problem, we have substituted pHluorin [23] for EGFP. NPY-pHluorin is expressed and sorted to the lumen of LDCVs. Thus, the NPY-pHluorin fluorescence is expected to increase dramatically upon fusion when the acidic vesicle lumen encounters neutral extracellular fluid.

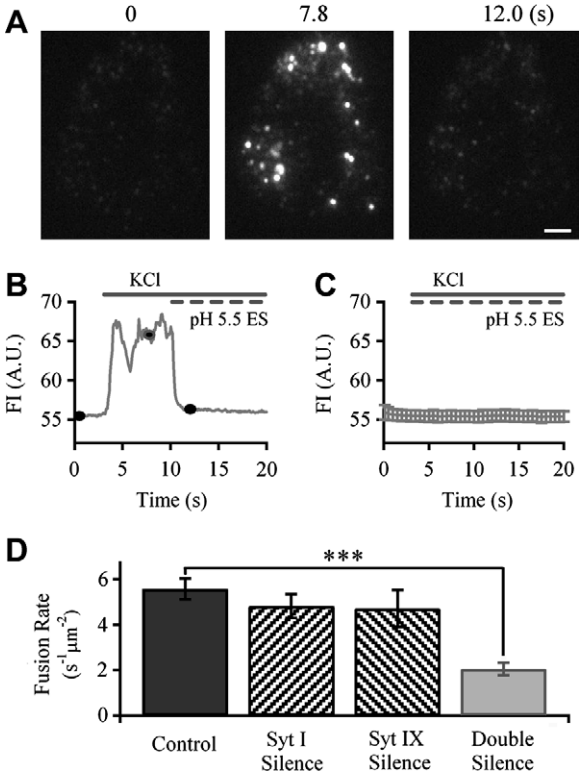

Fig. 1. Double silence of Syt I and Syt IX reduces Ca^{2+}-stimulated LDCV fusion. (A) The footprint of a live PC12 cell under evanescent-field before (left) and after the application of high KCl (middle) and then quenched by pH 5.5 external solution (ES) (right). The scale bar indicates 2 μm. (B) Time courses of the mean fluorescence intensity of the cell showed in (A). The solid and dashed lines mark the applications of high KCl and pH 5.5 ES, respectively. Filled circles indicate the time at which images were recorded. (C) Average time courses of the mean fluorescence intensity for six cells, each of which was perfused with high KCl buffered at pH 5.5. (D) Comparison of the fusion rate per second per μm^2 among the control, Syt I silence, Syt IX silence and double silence groups. ***, $p < 0.001$ compared to the control.

Fig. 1A shows example of fusion events from a PC12 cell expressing NPY-pHluorin observed under TIRFM. After high K^+-stimulation, multiple bright spots appear and the total fluorescence intensity of the cell footprint increases abruptly (Fig. 1B). To verify that the fluorescence change was due to the fusion of NPY-pHluorin-containing LDCVs, the same cell was perfused with pH 5.5 external solution (ES) in the presence of high K^+. We observed that the fluorescence was readily quenched (Fig. 2B). Furthermore, the application of high K^+ stimulation in pH 5.5 ES produced no fluorescence increase (Fig. 2C). These results suggest that the brightening of fluorescence under high K^+-stimulation is due to exposure of NPY-pHluorin to the extracellular medium. By screening for sudden brightening spots in TIRFM images, we can easily identify fusion events. We noticed that the average fusion rate ($5.60 \pm 0.30\ \mu m^{-2}\ s^{-1}$) by using NPY-pHluorin is 3.7-fold higher than that reported by NPY-EGFP ($1.50 \pm 0.30\ \mu m^{-2}\ s^{-1}$) [4]. Hence, NPY-pHluorin is much more efficient and sensitive to report LDCV fusion events than NPY-EGFP.

Armed with this new probe, we then checked which Syt isoform(s) is/are involved in LDCV exocytosis. We verified that the shRNA sequences specific for Syt I or Syt IX reduced the protein levels by 70% and 76% in PC12 cells by RNAi, respectively (data not shown). As shown in Fig. 1D, silencing of either Syt I or Syt IX alone produced only a slight reduction in the fusion rate which was not statistically significant. Only when both Syt I and Syt IX were down-regulated did we observe a significant inhibition by 60% as compared with that of control. Together with the high degree of co-localization, these data clearly demonstrate that Syt I and Syt IX function redundantly as the Ca^{2+}-sensors for LDCV exocytosis.

Three fusion modes identified for LDCV exocytosis

By scrutinizing the dynamics of single fusion events reported by NPY-pHluorin, we have noticed different modes of fusion. One example is shown in Fig. 2A where we saw a sudden brightening followed by a gradually expanding 'cloud' of fluorescence, suggesting significant

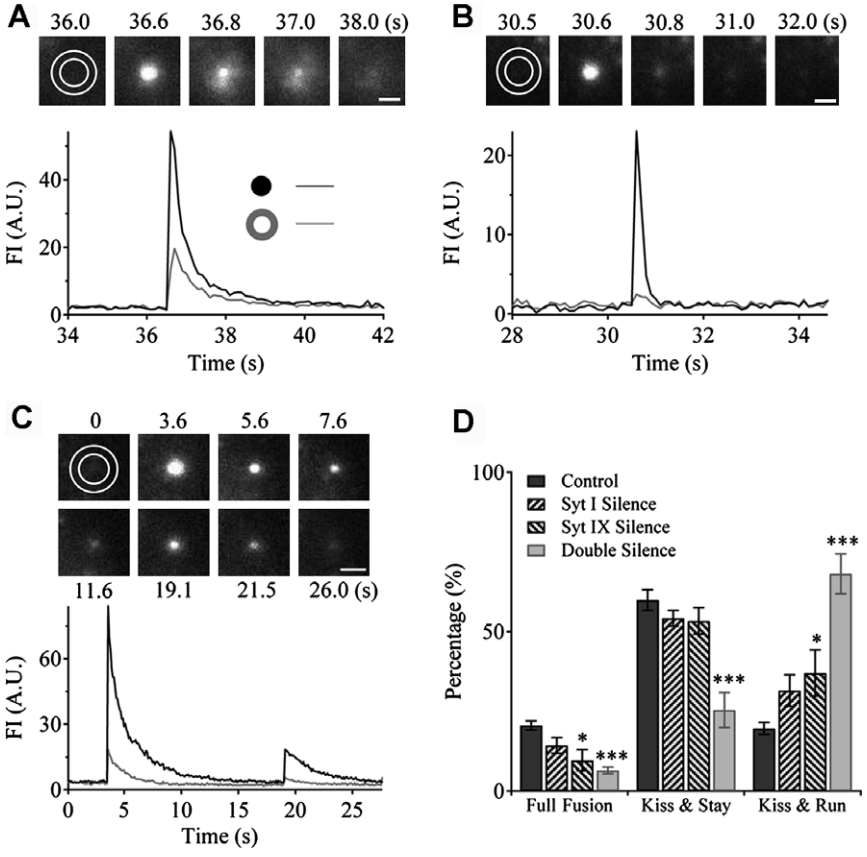

Fig. 2. Three different fusion modes of LDCV triggered by high K^+. (A) An example shows a full fusion event. Sequential images of a single LDCV labeled with NPY-pHluorin undergoing complete exocytosis (upper) and its corresponding fluorescence kinetics and released content were displayed (lower). (B) Sequential images of another LDCV undergoing kiss-and-run. (C) A vesicle undergoing kiss-and-stay. The fluorescence of NPY-pHluorin dimmed without absolute disappearance after the first exocytosis at 3.6 s. At 19.1 s, the same vesicle underwent the second round of exocytosis. The black and gray lines represent the time courses of mean fluorescence intensity in inner circle and concentric annulus, respectively. All scale bars indicate 1 μm. (D) Comparison of the fractions of three fusion modes among the control, single and double silence groups. *, $p < 0.05$; ***, $p < 0.001$ compared to the control.

release and diffusion of LDCV cargo. By placing two concentric circles centered at the fusion site, we could observe the diffusion of NPY-pHluorin into the outer annulus, which results in a significant increase of fluorescence inside the annulus followed by an exponential decay within several seconds (Fig. 2A). We categorized this kind of fusion as full fusion mode.

Sometimes we observed sudden brightening of fluorescent spot with no apparent diffusion. The brightened spot rapidly vanished within 1 s as shown in Fig. 2B, which is also evident by the rapid decay of fluorescence within the inner circle ($\tau = 0.13$ s). The fluorescence within the annulus displays little increase, manifesting no significant cargo release. This kind of fusion is reminiscent of the kiss-and-run mode [4,5,22]. Often we observed another distinct mode of fusion (Fig. 2C). After fusion, the vesicle remains visible for a fairly long time without a complete diffusion. There is a small increase in the annulus, suggesting partial release. This kind of fusion is consistent with previously reported "kiss-and-stay" or "cavicapture" type [4,5], which means that vesicle remains intact after fusion pore formation without collapsing into the plasma membrane. After partial release, the vesicle reseals and remains attached with the plasmalemma for some time (15.5 s in this case). The fluorescence of the vesicle diminishes much slower ($\tau_1 = 0.36$ s; $\tau_2 = 2.60$ s). Occasionally, we observed multiple rounds of fusion for the same vesicle in the case of kiss-and-stay mode as shown in Fig. 2C.

Based on the diffusion pattern and the kinetics of fluorescence decay after fusion, we classified all the fusion events as illustrated in Fig. 2 into three modes: full fusion, kiss-and-run, and kiss-and-stay. In control PC12 cells, most fusion events ($59.9\% \pm 3.2\%$) are the kiss-and-stay type, which is consistent with previous study [4]. Full fusion and kiss-and-run constitute minor fractions with percentages of $20.5\% \pm 1.4\%$ and $19.6\% \pm 1.8\%$, respectively. We asked whether Syt I and Syt IX could participate in the decision of fusion mode. As shown in Fig. 2D, down-regulation of either Syt I or Syt IX alone seems to reduce the fraction of full fusion and increase the fraction of kiss-and-run, but the effect is not significant. By contrast, double silencing of both Syt I and Syt IX renders most fusion events in the kiss-and-run mode ($68.1\% \pm 6.3\%$) with a concurrent reduction in the fraction of full fusion and kiss-and-stay. To verify the effect of depleting Syt I and Syt IX on the dynamics of fusion, we compared the averaged time course of fluorescence change during fusion between control and double silenced cells. The fluorescence intensities of the central circle and the concentric annulus were normalized as $\Delta F/F_0$ (Fig. 3A). The averaged fluorescence dynamics reveals striking differences. Firstly, the fluorescence increase upon fusion in control group is much larger as compared with that from double silenced group. Secondly, the fluorescence increase in the annulus is much smaller under double silenced condition, in contrast to the remarkable increase in the control group. These differences are consistent with our above observation that double

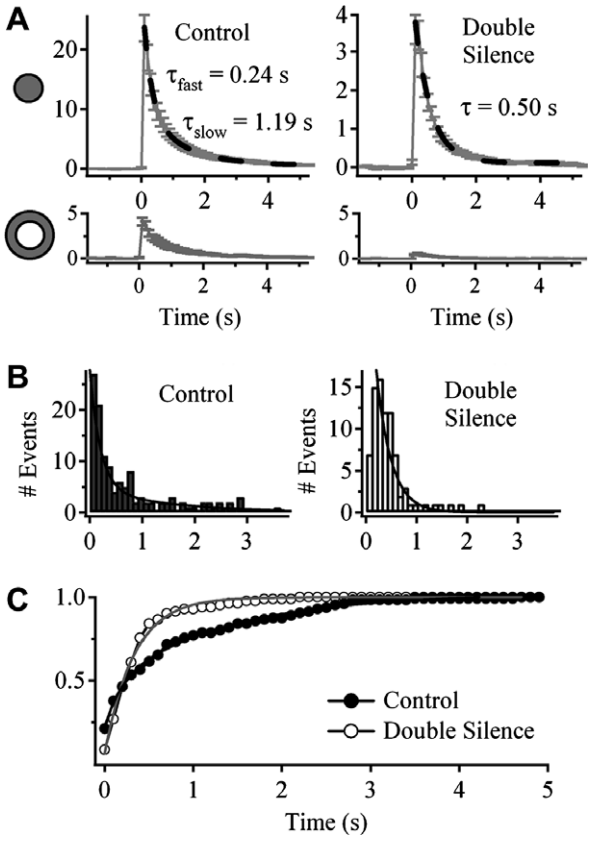

Fig. 3. Knockdown of both Syt I and Syt IX changes the kinetics of LDCV fusion. (A) Average fluorescence intensity of LDCV fusion stimulated by high K^+ are compared between control (left; 61 events) and double silenced cells (right; 58 events). Both the fluorescence intensity from the inner circle (upper) and the concentric annuli (lower) were temporally aligned to the moment of fusion. The fluorescence intensity was normalized as $\Delta F/F_0$. Best exponential fits to the averaged fluorescence decay are superimposed as dashed lines with time constants indicated. (B) Histograms of fluorescence decay time constants of LDCV fusion events from control (left) and double silenced cells (right). (C) Comparison of the cumulative distribution of decay time constants between control (filled circle) and double silence group (open circle). Superimposed smooth lines represent the exponential fits.

silencing of Syt I and IX results in more kiss-and-run mode of fusion, where the fusion pore opening is transient and the release of peptide cargo is limited.

We further compared the kinetics of fluorescence decay because different modes of fusion have distinct kinetics of decay (see Fig. 2). Among three fusion modes, kiss-and-run has the fastest decay and can be best fitted by mono-exponential function, whereas kiss-and-stay has the slowest decay that is normally best described by double exponentials. We fitted every fluorescence decay and plotted the distribution of all the time constants in Fig. 3B. The distribution of time constants is much broader in the control group with more in the longer range. Instead, more time constants from the double silenced group are within the shorter range. The cumulative histogram (Fig. 3C) further emphasizes this difference. Taken together, our analysis of fusion dynamics using NPY-pHluorin suggests that the level of Syt I and Syt IX controls the choice among different fusion modes.

Amperometric recording of catecholamine release in PC12 cells

To further validate our above observation, we analyzed single catecholamine release events from PC12 cells employing amperometry. As shown in Fig. 4A, high K^+ application induces multiple amperometric spikes at irregular intervals in PC12 cells. Silencing neither Syt I nor Syt IX alone produced significant reduction of the spike frequency (Fig. 4B and C). By contrast, double silencing of Syt I and Syt IX together significantly reduced the amperometric spike frequency (Fig. 4D and E), further supporting the notion that Syt I and Syt IX act redundantly in LDCV exocytosis.

In the amperometric measurement, full fusion displays as a spike with or without a prespike foot (PSF). Whereas kiss-and-run events are revealed as "stand-alone-foot" (SAF), in which fusion pores open and close without dilating so that the event terminates without a spike [24]. In agreement with our above conclusion, we found that double silencing of Syt I and IX significantly increased the frequency of SAF in amperometric recording. The percentage of SAF was increased from 16% in control cells to 52% in the double silenced cells (Fig. 4F).

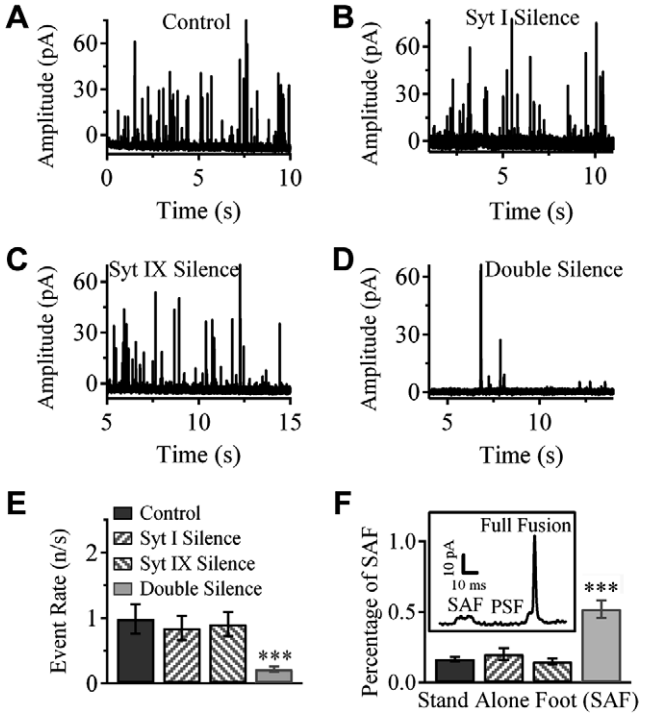

Fig. 4. Double silence of Syt I and Syt IX reduces catecholamine release from PC12 cells. (A–D) Sample traces of amperometry within 10 s in control, Syt I-silenced, Syt IX-silenced, and double silenced cells, respectively. (E) Averaged rate of amperometric events. (F) Comparison of the averaged percentage of SAF events. A typical example of SAF and full fusion with a pre-spike foot (PSF) was shown in the inset. ***, $p < 0.001$ compared to the control.

Discussion

TIRFM imaging has the advantage of monitoring the behavior of single vesicles provided they are specifically labeled with fluorescent marker. Previous studies have suggested that the fusion events detected using TIRFM imaging are considerable low [22]. This is probably due to the fact that previous fusion identification relies on the 2-D diffusion assay [25], which is usually hard to catch and quantify. We have now come up with a new probe, NPY-pHluorin, to monitor LDCV fusion. By using NPY-pHluorin, the criterion for fusion identification is simplified to one dimensional fluorescence change. As a consequence, we showed that 3.7-fold more fusion events were detected using NPY-pHluorin than using NPY-EGFP. Hence, NPY-pHluorin represents a new sensitive probe specific for LDCV fusion assay. We have also demonstrated that NPY-pHluorin is valuable in assessing the fusion pore dynamics.

In many instances, multiple Syts are co-expressed and it is unclear whether different Syt isoforms could function redundantly in Ca^{2+}-triggered LDCV exocytosis. Syt I is recognized as a major Ca^{2+} sensor for the fast synchronous release of neurotransmitters in neurons [9,10] and for exocytosis in chromaffin cells [11]. However, Syt I-deficient PC12 cells exhibit normal Ca^{2+}-dependent exocytosis [26]. A previous report [12] concludes that Syt IX but not Syt I mediates Ca^{2+}-dependent LDCV exocytosis in PC12 cells. Taking advantage of LDCV exocytosis assay, in combination with the specific gene knockdown technique, we now show that down-regulation of either Syt I or Syt IX alone didn't affect LDCV exocytosis. However, when both Syt I and Syt IX were knocked-down, we observed a large inhibition on the LDCV exocytosis assessed by different methods. Therefore, we propose that these two Syt isoforms function redundantly in Ca^{2+}-dependent LDCV exocytosis in PC12 cells.

Understanding how the fusion pore is formed and controlled emerges as a central goal in elucidating the mechanisms of membrane fusion. Syts also have been suggested to be intimately associated with fusion pores. Overexpression of different Syt isoforms has been shown to modulate the fusion pore dilation [16]. Moreover, different domains of Syts are proposed to control the choice between kiss-and-run and full fusion [17]. Nevertheless, as increased protein/lipid ratio tends to destabilize the vesicle membrane [18], the exact role of Syts in fusion control is controversial [19]. This needs to be verified by intervening endogenous levels of Syts. By employing NPY-pHluorin to monitor fusion kinetics, we now show that down-regulation of both Syt I and Syt IX, but not alone, renders more fusion events in the kiss-and-run mode. This result is further confirmed by amperometry. Thus, we provide direct evidence that the level of Syt I and Syt IX on the LDCVs control the choice between different fusion modes. How the fusion pores are regulated is still in controversy. It has been proposed that multiple Syt proteins arranged in a ring

structure participate in the fusion pore formation upon Ca^{2+}-binding. Hence, the reduction in Syt numbers could favour a transient fusion pore formation without full dilation. Further work will be necessary to determine how different Syts function in the initiation and dilation of fusion pore.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.083.

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