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Surface immobilized cholera toxin B subunit (CTB) facilitates vesicle docking, trafficking and exocytosis†

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The subunit B of cholera toxin (CTB), which specifically binds with ganglioside GM1 enriched in membrane lipid rafts, is known to interfere with multiple cell functions. However, the specific, stable and spatially defined membrane signaling induced by CTB binding is often difficult to investigate by applying CTB molecules in bulk solution due to quick internalization, elicited intracellular reactions, and homogeneous interaction with the entire cell membrane. Here, we interfaced the neuroendocrine PC12 cells with surface immobilized and patterned CTB molecules, and interrogated the effects of CTB binding on vesicular exocytosis using integrative single-cell study methods. It was discovered that CTB binding facilitates vesicle trafficking, docking and exocytosis in a cholesterol dependent manner. And these effects are probably attributable to the increased membrane GM1 and cholesterol, and enhanced Ca^{2+} signaling.

1 Introduction

The subunit B of cholera toxin (CTB) specifically binds to gangliosides on the cell membrane, particularly to monosialotetrahexosylganglioside (GM1) with high affinity.¹ GM1 is predominately clustered in lipid rafts which are believed to be relay points for various cellular signaling events by selectively recruiting certain proteins or lipids while excluding others.^{1,2} Upon binding with GM1, CTB is quickly endocytosed and enters a retrograde trafficking pathway through endosomes to the Golgi apparatus.³ In addition to triggering endocytosis,⁴ crosslinking between GM1 and CTB can elicit other cellular responses, *e.g.*, enrichment of $\beta 1$ integrins in lipid rafts,⁵ increase or inhibition of cell proliferation,^{6–8} increase of cytoplasmic free Ca^{2+} ,^{9,10} inhibition of T cells

activation,⁸ phosphorylation of various signaling molecules.¹¹ As endocytosis and exocytosis are intimately linked¹² and GM1 enriched lipid rafts are critically involved in docking and exocytosis of secretory vesicles,^{13,14} we conceive that CTB binding on the cell surface may modulate the exocytotic pathways.

Despite that CTB has been widely used as an instrumental membrane probe with multiple-engagement of cell functions, elucidation of the molecular transductions induced by crosslinking between GM1 and CTB is inevitably complicated by several problems. Firstly, CTB molecules are rapidly internalized after binding due to triggered endocytosis. Thus, the effects induced by stable interaction of CTB with the cell membrane cannot be examined. In addition, trace contamination of the subunit A of cholera toxin (CTA) in the commercially prepared CTB samples will be uptaken into the cells and potentially trigger cAMP pathways by activating adenylate cyclase. Some of the reported CTB effects can be attributed to the influences of CTA.¹⁵ Furthermore, when membrane probes are applied uniformly in the bulk aqueous solution, it is not possible to investigate the effects due to their localized interaction with heterogeneous membrane domains. To avoid these problems, we studied the effects of surface immobilized CTB on vesicle docking, trafficking and

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Insight, innovation, integration

Cell membrane functions are spatially mediated by orchestrated actions of membrane proteins/lipids and their associated functional domains. Conventionally, membrane functions are studied by homogeneously applying membrane probes targeting on specific membrane components in bulk solution. Herein, we demonstrate that surface immobilized and patterned membrane probes provide unique advantages

over dissolved probes in order to specifically study stable membrane signaling and its spatial regulations. Using this strategy and the integrative experimental tools applied at the single-cell level, we discovered that crosslinking of CTB with membrane ganglioside GM1 facilitates the vesicular exocytosis in multiple ways, postulating CTB as a valuable probe to study the exocytotic pathways.

exocytosis of neuroendocrine PC12 cells using integrative approaches including surface functionalization, micro-contact printing, total internal reflection fluorescence microscopy (TIRFM), amperometric detection of exocytosis using carbon fiber microelectrodes, and single cell photometry.

2 Results

2.1 Surface functionalization and micro-contact printing

As illustrated in Fig. 1a, CTB molecules were immobilized onto a glass coverslip through a series of functionalization steps.¹⁶ The glass coverslip was first silanized with 3-aminopropyltriethoxysilane (APTES) which reacts with hydroxyl groups on the surface. The silanized coverslip was then covalently modified with glutaraldehyde (GA) which, in turn, reacts with the primary amine group on CTB to form the covalent linkage. The success and uniformity of CTB conjugation were confirmed by the binding of green fluorescent BODIPY[®] FL C5-GM1 (BODIPY-GM1) as shown in Fig. 1b.

The CTB coated surface which interfaces with the entire membrane of the adherent cell ensures the stable and homogeneous transmembrane signaling triggered by the crosslinking of CTB with GM1. To elucidate the spatially heterogeneous signaling, micro-patterns of CTB molecules were non-covalently created on the surface by micro-contact printing as schematically shown in Fig. 2a. Red fluorescent Alexa Fluor[®] 594 conjugated CTB (CTB-594) was printed on

Fig. 1 (a) Illustration of covalent conjugation of CTB molecules on glass surface. (b) BODIPY-GM1 binds to the CTB coating (left) but not the glutaraldehyde coating (right), indicating the effectiveness of CTB functionalization and preserved functionalities of immobilized CTB. The scale bars = 10 μm.

Fig. 2 (a) Schematic illustration of micro-contact printing of CTB patterns on poly-L-lysine (PLL) coated glass coverslip, using an elastomer stamp made of polydimethylsiloxane (PDMS). (b) Printed micro-line-patterns of CTB-594 (top) and binding of BODIPY-GM1 with CTB-594 patterns (bottom), indicating the effectiveness of micro-contact printing and the preserved functionality of immobilized CTB-594. The scale bars = 5 μm.

a poly-L-lysine (PLL) coated glass coverslip using a PDMS stamp to produce line patterns with the nominal width and spacing distance of 2 μm. As shown in Fig. 2b, BODIPY-GM1 selectively binds to the CTB patterns but not to the PLL patterns between the CTB-594 micro-lines, indicating the effectiveness of the micro-patterning and that CTB-594 is able to interact normally with GM1.

2.2 Surface immobilized CTB does not affect cell morphology, proliferation and adhesion

The chemical properties of the cell growth substrate could cause alterations in morphology, proliferation and adhesion of the cells, which, in turn, may profoundly affect cell functions in intricate ways.¹⁷ To assess the CTB effects on these phenotypes of PC12 cells, we have compared the surface coating of CTB with the coating of PLL which is a cell adhesion promoting poly-peptide commonly used for cell culturing. As shown in Fig. 3a (left), cells can grow well on the coverslips which were covalently and uniformly decorated by CTB molecules. The morphology of PC12 cells grown on the CTB substrates appeared normal and was indistinguishable to that of the cells cultured in parallel on the PLL coated substrates. In addition, the rate of cell proliferation was nearly identical on both types of substrates (Fig. 3b). When cells grew on the substrates with alternating CTB and PLL micro-patterns, the cells did not exhibit any preference to either pattern. In other words, cells spread across the two kinds of patterns, and orientation or polarization of the cell body correlated with the orientation of the patterns was not observed (Fig. 3c). Vinculin, a protein which links the actin cytoskeleton to the

transmembrane adhesion molecule integrin in focal adhesion plaques, was fluorescently stained to indicate the focal adhesion points of the cells. As seen in Fig. 3c, the focal points were not preferentially colocalized with either pattern, indicating that CTB and PLL similarly facilitate cell adhesion. Consistently, the distributions of focal adhesion points of the cells on the substrates with homogeneous CTB or PLL coatings were similar (data not shown).

2.3 Immobilized CTB facilitates vesicle docking and trafficking in a cholesterol dependent manner

Exocytosis of hormones, neurotransmitters or other signaling molecules is a dynamic process highly regulated by the combined actions of various proteins and lipids.¹⁸ The exocytotic process involves sequential steps of secretory vesicles, including their arrival to the subplasmalemmal region, docking and priming to become readily releasable, and final fusion with plasma membrane upon triggering. The recently emerged total internal reflection fluorescence microscopy (TIRFM), which evanescently and selectively illuminate the thin subplasmalemmal region (<200 nm thick) just above the

interface between the coverslip and the cell membrane (Fig. 4a, left top), is instrumental in revealing the vesicle dynamics near the cell membrane.^{19,20}

The large dense core vesicles (LDCV) inside PC12 cells were enlightened by overexpressing EGFP tagged neuropeptide Y (NPY-EGFP) which is a specific LDCV marker.²¹ The fluorescently labeled secretory vesicles in the subplasmalemmal region can be individually resolved under TIRFM. Fig. 4a (right) presents a typical TIRFM image of a PC12 cell grown on the CTB functionalized coverslip. As revealed by TIRFM

Fig. 3 (a) Optical images of PC12 cells grown on CTB-coated (left) and PLL-coated (right) substrates. The scale bars = 50 μm . (b) Growth curves of cells grown on CTB- or PLL-coated surfaces. Each data is normalized to the initial seeding density ($\sim 180 \text{ cells mm}^{-2}$) at day 0, and averaged from 30 areas ($\sim 1.1 \text{ mm}^2$) in 3 different samples. The error bar indicates standard error (SE). (c) Confocal image showing a PC12 cell grown on a surface with dual patterns of CTB-594 (red) and PLL (dark). The green spots indicate the cell focal adhesion points stained by FITC conjugated antibodies against vinculin. The scale bar = 5 μm .

Fig. 4 (a) Total internal reflection fluorescence microscopy (TIRFM) to reveal vesicle trafficking. Left top: the schematics of evanescent illumination of the thin subplasmalemmal region (<200 nm). Right: A typical TIRFM image of the footprints of secretory vesicles in PC12 cells. The vesicles (bright dots, highlighted in circles) are enlightened by overexpression of NPY-EGFP. The cell contour is outlined by the dashed line. The scale bar = 5 μm . Left bottom: A typical trajectory of vesicle lateral movement. The rectangle (dotted line) that just encases all the footprints of the vesicle gives the first estimation of the coverage area of vesicle lateral motion. The scale bars = 100 nm. (b)–(e) The statistics of the average vesicle number in the subplasmalemmal region (b), the vesicles arrived from the cytosol (c), the diffusion constant (d), and the motion coverage area (e) for cells grown on PLL- (open columns) or CTB-coated (dark columns) coverslips without (w.o. MβCD) or with MβCD treatment (w. MβCD). Each data is analyzed from the time-lapse images with a duration of 2 min and an interval of 0.5 s, from 11 cells with 618 vesicles (PLL, w.o. MβCD), 11 cells with 960 vesicles (CTB, w.o. MβCD), 10 cells with 329 vesicles (PLL, w. MβCD) and 10 cells with 300 vesicles (CTB, w. MβCD). The error bars indicate SE. *** $p < 0.001$ in Student's t-test.

studies,^{14,19,20} secretory vesicles undertake constant lateral movement (parallel to the cell membrane) and vertical movement (transition between inner cytosol and subplasmalemmal region). The trafficking dynamics of the secretory vesicles are highly relevant to the vesicle fusion competence and exocytotic kinetics.^{20,22,23} The LDCV vesicles in PC12 cells were individually tracked at 2 Hz for 2 min to obtain the trajectory of their lateral movement, diffusion constant, motion area, and the vertical transport. Fig. 4a (left bottom) depicts a typical trajectory of a vesicle's lateral movement, whose coverage area can be approximated by the rectangular region that just encases all the vesicle footprints.

Vesicles constantly arrive from the inner cytosol to the subplasmalemmal region and retreat back after some dwell time. However, the total number of the subplasmalemmal vesicles stays nearly constant as the arrival and retrieval rates are balanced. It was found that the mean total number of the subplasmalemmal vesicles in the cells grown on CTB coated coverslips was significantly greater than that in the cells on PLL coated coverslips, implying that CTB interaction facilitates vesicle docking (Fig. 4b). In addition, CTB coating enhanced the vertical trafficking as evidenced by the increase in vesicle arrival from the inner cytosol (Fig. 4c) and enhanced the lateral motion as evidenced by the increased average diffusion constant (Fig. 4d) and motion coverage area (Fig. 4e).

GM1 is largely clustered in cholesterol rich membrane domains (lipid rafts). It has been found that methyl- β -cyclodextrin (M β CD), a cholesterol extracting agent which disrupts lipid rafts, diminishes the clustering of GM1 on the cell membrane²⁴ and impairs CTB binding to the lipid rafts.²⁵ Here, we found that M β CD treatment (incubation with 5 mM M β CD for 30 min at 37 °C)¹⁴ completely abolished the CTB effects on vesicle docking and trafficking (Fig. 4), indicating that the observed CTB effects are cholesterol dependent. Membrane cholesterol was removed by $37.04 \pm 2.57\%$ (3 independent experiments) after M β CD treatment (measured by Amplex Red Cholesterol Assay Kit from Invitrogen).

The facilitating roles of CTB on vesicle docking and trafficking were corroborated by the TIRFM experiments on cells grown on dual micropatterns of CTB and PLL. As shown in Fig. 5a and b, more vesicles were localized on the CTB patterns rather than on the PLL patterns. Consistent with the results demonstrated in Fig. 4, vesicles on the CTB patterns exhibited enhanced vertical and lateral trafficking, and cholesterol depletion by M β CD completely eliminated the facilitating effects of the CTB patterns (Fig. 5). Deprivation of membrane cholesterol by culturing the cells in the lipoprotein deficient serum (LPDS, Sigma) for 5 days gave similar results as M β CD treatment (Fig. S1 in ESI†).

2.4 Immobilized CTBs enhance vesicular exocytosis in a cholesterol dependent manner

As the interaction between the cell membrane and the surface immobilized CTB molecules promotes vesicle docking and trafficking, it is thus likely that it would promote vesicular exocytosis. Single-cell amperometric measurement based on a carbon-fiber-microelectrode (Fig. 6a), which is able to electrochemically detect single vesicle release of catecholamine

Fig. 5 (a) Distribution of secretory vesicles in a PC12 cell grown on the CTB-594 and PLL dual patterns without (left) or with (right) M β CD treatment. The scale bars = 5 μm . (b)–(e) The statistics of the average vesicle number in the subplasmalemmal region (b), the vesicles arrived from the cytosol (c), the diffusion constant (d), and the motion coverage area (e) for cells localized on PLL- (open columns) or CTB-patterns (dark columns) without (w.o. M β CD) or with M β CD treatment (w. M β CD). Each data is analyzed from the time-lapse images with a 2 min duration and a 0.5 s interval, from 22 cells (w.o. M β CD) with 514 vesicles on PLL patterns and 770 vesicles on CTB patterns, 22 cells (w. M β CD) with 457 vesicles on PLL patterns and 424 vesicles on CTB patterns. The error bars indicate SE. *** $p < 0.001$, ** $p < 0.01$ in Student's t-test.

molecules from PC12 cells,²⁶ was employed to test this hypothesis. Fig. 6 presents amperometric recordings from a cell on a PLL coated surface and from a cell on a CTB coated surface in response to administration of high K^+ solution by an application pipette. High K^+ solution induces Ca^{2+} influx which, in turn, triggers vesicular exocytosis. Each amperometric current spike corresponds to single vesicle release of catecholamines.

Fig. 6 (a) Left: Illustration of single-cell amperometric measurement using a carbon fiber microelectrode (CFE). Right: two typical amperometry recordings from a PC12 cell grown on PLL- or CTB-coated coverslip, in response to a 2 min stimulation of high K^+ solution delivered by an application pipette. Each spike corresponds to single vesicle release of catecholamines. (b) The average total number of amperometric spikes from cells grown on PLL coating (open columns) or CTB coating (dark columns) without (w.o. M β CD) or with M β CD treatment (w. M β CD). The error bars indicate SE. ** $p < 0.01$ in Student's t-test. The statistics were obtained from 18 cells (CTB, w.o. M β CD), 19 cells (PLL, w.o. M β CD), 16 cells (CTB, w. M β CD) and 16 cells (PLL, w. M β CD), respectively. (c) Averaged amperometric spikes based on the experiments shown in (b) from 18 cells on CTB coating without M β CD treatment (2165 spikes, dark curve on the left), 19 cells on PLL coating without M β CD treatment (1041 spikes, gray curve on the left), 16 cells on CTB coating with M β CD treatment (357 spikes, dark curve on the right), 16 cells on PLL coating with M β CD treatment (394 spikes, gray curve on the right). (d) The average quantal size (total charge per amperometric spike) under the four conditions. *** $p < 0.001$ in Student's t-test.

As demonstrated in Fig. 6a and b, the extent of exocytosis was significantly augmented when the cells grew on CTB substrates as compared to those grown on PLL substrates. Such increase in the frequency of exocytotic response is likely attributable, at least in part, to the enhanced vesicle docking and trafficking (Fig. 4 and 5). The total charge (quantal size) of each amperometric spike, which reflects the total number of released molecules, was also significantly increased by CTB coating (Fig. 6c and d). The CTB-enhanced quantal vesicle release was completely abolished by deprivation of membrane cholesterol using M β CD. Interestingly, although crosslinking of CTB occurs only at the adherent cell membrane, the enhanced vesicular exocytosis was detected by the carbon fiber electrode on the upper cell membrane. It implies that local crosslinking induces global effects.

2.5 CTB effects may be attributable to increase of GM1, cholesterol and Ca^{2+} signaling

Fig. 7a demonstrates that the stable interaction between the surface immobilized CTB molecules and the adherent cell membrane resulted in a drastic increase of overall membrane GM1 which was reported by the increased fluorescence staining of membrane GM1 by Alexa Fluor[®] 488 conjugated CTB molecules (CTB-488). GM1 molecules are segregated in the cholesterol enriched membrane domains that are believed

to serve as the functional domains for vesicular exocytosis.¹³ Interestingly, increase of GM1 induced by CTB was accompanied by increase of membrane cholesterol which was assayed using the increased fluorescence staining with filipin. Therefore, it is conceivable that CTB binding to GM1 on the adherent membrane leads to overall increase of GM1-cholesterol enriched functional domains, and thus enhances vesicle docking, trafficking and exocytosis.

We measured the basal intracellular Ca^{2+} concentration and its increase upon high K^+ stimulation which excites the cell membrane and induces Ca^{2+} influxes through voltage gated Ca^{2+} channels, using single cell Ca^{2+} ratiometric photometry. As demonstrated in Fig. 7c, the basal Ca^{2+} level was higher in the cells grown on CTB substrates as compared to that in the cells grown on PLL substrates. In addition, the elicited Ca^{2+} signal by high K^+ solution was stronger in the cells on the CTB substrates. It has been shown that application of CTB in the aqueous phase leads to Ca^{2+} influx¹⁰ through voltage gated Ca^{2+} channels which are likely intrinsically modulated by GM1.^{27,28} Our observation is consistent with the previous findings and suggests that the enhanced Ca^{2+} signaling is achieved through transmembrane signaling of CTB binding instead of through formation of CTB channels or through intracellular signaling triggered by internalized CTB molecules. The CTB facilitated Ca^{2+} signal likely in part accounts for the enhanced Ca^{2+} dependent vesicular exocytosis in PC12 cells.

Fig. 7 (a) Intensity of fluorescent staining of membrane GM1 using CTB-488 from cells grown on PLL coating ($n = 200$) or CTB coating ($n = 200$). The error bars indicate SE. *** $p < 0.001$ in Student's t -test. (b) Intensity of fluorescent staining of membrane cholesterol using fluorescent filipin from cells grown on PLL coating ($n = 200$) or CTB coating ($n = 200$). The error bars indicate SE. *** $p < 0.001$ in Student's t -test. (c) Intracellular free Ca^{2+} reported by emission ratio of Ca^{2+} indicator Fura2-AM excited at 340 and 380 nm, from 16 cells on PLL coating and 16 cells on CTB coating. The error bars indicate SE.

3 Discussion

Membrane signaling is conventionally studied by applying membrane probes to bulk solution. Stable, long-term, and specific effects induced by the binding probes, however, are often difficult to be examined as the probes may be quickly internalized into the cells due to triggered endocytosis or constant constitutive membrane turnover. Once internalized, the probes may trigger additional intracellular reactions. In addition, some membrane probes require organic solvent, *e.g.* DMSO, as a vehicle which may affect cell behaviors. Furthermore, homogenous aqueous application of membrane probes does not allow investigation of the spatial regulation of membrane signaling. Here, we demonstrated that surface immobilized membrane probes, with possibility of engineered micro/nano patterns, provide a novel alternative to study membrane signaling. Using this strategy and the integrative functional study platform at single cell level, we discovered that membrane binding of CTB, which is known to trigger endocytosis, modulates vesicular exocytosis which is intimately linked with endocytosis to ensure balanced membrane turnover.¹²

Gangliosides, particularly GM1 with which CTB binds with high affinity, are critical membrane lipid species involved in micro-organization of cell membrane and various cellular processes *via* interactions with other membrane lipids and membrane proteins such as ion channels, receptors, and

membrane associating kinases.^{29–31} Crosslinking of CTB with GM1 is known to elicit multiple cellular signaling.^{11,32} In this study, it was found that CTB binding facilitates vesicle docking, trafficking and exocytosis in a cholesterol dependent manner. This study implies the important involvement of GM1 in exocytosis, and lends further support to the widely recognized notion that GM1-cholesterol enriched membrane microdomains (lipid rafts) play critical roles in spatially defined vesicle docking and trafficking, and final vesicle fusion.^{13,14}

GM1 and cholesterol enriched lipid rafts recruit secretory proteins such as SNARE proteins and are believed to serve as vesicle docking and fusion sites. Disruption of lipid rafts by depleting membrane cholesterol impairs vesicle docking, trafficking and exocytosis.^{14,33} As membrane molecules and structures are highly mobile within the lipid bilayer, the CTB patterns underlying the cell membrane presumably recruit GM1 and GM1 associated membrane structures, creating GM1 patterns on the cell membrane. Consistent with the raft theory, we show that secretory vesicles preferentially colocalize with CTB patterns (thus GM1 patterns on the cell membrane) with enhanced trafficking in a cholesterol dependent way, indicating the preferential interactions between the vesicles and the GM1-cholesterol enriched functional domains.

The exact molecular mechanisms underlying the CTB enhanced exocytosis require further investigations. It is conceivable that the interaction between the adherent membrane and immobilized CTB molecules increases the number of GM1-cholesterol enriched functional domains on the entire cell membrane based on the observations that both membrane GM1 and cholesterol were increased and that increased exocytosis was detected on the non-adherent upper membrane. And as vesicle docking, the kinetics and extent of exocytosis, quantal vesicle fusion, and replenishment of releasable vesicles are Ca^{2+} dependent,³⁴ the CTB induced Ca^{2+} signal may be partly responsible for the observed facilitation of vesicle docking, trafficking and exocytosis. Furthermore, gangliosides are known to interact and inhibit exocytosis promoting enzymes such as protein kinase C³⁵ and phospholipase C.³⁶ Binding of CTB may relieve such inhibitions and consequently facilitates exocytosis.

Using immobilized CTB molecules as the probes can avoid effects that are not directly caused by membrane binding of CTB with GM1, and make it possible to reveal phenomena that are exclusive when membrane binding of CTB is only transient.

We showed that immobilized CTB did not affect the morphology, proliferation, and focal adhesion of PC12 cells. Such phenotypes may directly or indirectly influence exocytosis. For example, it was reported that cell adhesion is directly involved in spatial regulation of vesicle exocytosis.³⁷ Therefore, it is likely that the observed CTB effects are directly and specifically related to membrane binding of CTB. In contrast to effects induced by immobilized CTB, applying CTB molecules in the solution did not cause enhanced vesicle docking or trafficking (Fig. S2 in ESI†) or increase vesicle exocytosis (Fig. S3 in ESI†). Also in contrast to our observations, it has been shown that applying CTB molecules in the

solution promotes cell proliferation of 3T3 fibroblasts¹⁰ and neuritogenesis of PC12 cells.¹⁵ The discrepancy between the effects induced by immobilized and dissolved CTB molecules may be attributable to the transient binding of CTB due to internalization, or intracellular reactions caused by internalized CTB molecules or CTA contaminants. Clearly, immobilizing CTB is a novel or better alternative to interrogate the functions of GM1.

Surface functionalization and patterning have been utilized to study how cell anchoring affects cell proliferation, migration, polarization and differentiation.^{38–41} Our study postulates that surface immobilized and patterned probes can be used to study membrane signaling mediated by membrane functional domains. Using patterning techniques such as dip-pen lithography,^{42–44} chemically functionalized nanopatterns with various geometries (*e.g.*, dots, meshes) can be readily created. With appropriate chemistry, membrane probes targeting on other membrane lipid species or membrane proteins can be attached to the surface. This strategy promises many applications that would be instrumental to fundamental biology.

4 Materials and methods

4.1 Covalent functionalization

Glass coverslips were cleaned in piranha solution, and repetitively washed with ethanol and DI water followed by blow drying with nitrogen. The cleaned coverslips were then silanized by soaking in 5% 3-aminopropyltriethoxysilane (APTES, Sigma) in ethanol for 1 h with agitation. The silanized coverslips were vigorously washed with ethanol and heated at 120 °C for 1 h. Subsequently, the coverslips were treated with 5% glutaraldehyde in water for 1 h followed by repetitive washing with DI water. Cholera toxin subunit B (CTB, Sigma) was dissolved (100 µg ml⁻¹) in phosphate buffered saline (PBS) titrated to pH 9.0. The coverslips were incubated with the CTB solution for 6 h to allow crosslinking, followed by extensive washing with DI water to remove CTB residues. The whole process was conducted in a sterile environment.

4.2 Micro-contact printing

Silicon masters with line trenches (with width and spacing distance of 2 µm) were fabricated by photolithography and silanized with octadecyltrichlorosilane (OTS) to facilitate the releasing of PDMS stamps. PDMS stamps were made by casting Sylgard 184 (Dow Corning) on the silicon masters followed by curing at 70 °C overnight. The elastomeric stamps bearing the negative pattern of the masters were peeled off from the masters, washed with ethanol, and dried under nitrogen.

Sterilized coverslips were incubated in 0.01% poly-L-lysine (PLL, Sigma) aqueous solution for 1 h, followed by rinsing with DI water and drying with nitrogen. One drop of CTB or Alexa Fluor[®] 594 conjugated CTB (100 µg ml⁻¹ in PBS, Invitrogen) was pipetted onto the PDMS stamp (pre-sterilized with 70% ethanol) to ensure full coverage of the stamp. After inking for 30 min, the stamp was washed with DI water and

blown with a mild stream of nitrogen. It was then brought into contact with the PLL coated coverslip for 1 min. The coverslip with contact-printed proteins was finally rinsed with DI water and dried with nitrogen.

4.3 Cell culture and solutions

PC12 cells (American Type Culture Collection) were cultured in Advanced RPMI medium 1640 (Gibco) supplemented with 5% (v/v) heat-inactivated horse serum (Gibco), 10% heat inactivated fetal bovine serum (Gibco), and 1% penicillin-streptomycin (Gibco), and incubated at 37 °C in 5% CO₂/air. Cells were planted on the functionalized coverslips 3–4 days before the experiments. The bath solution used for imaging and recording contained (in mM, titrated to pH 7.2): 150 NaCl, 2.4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES. High K⁺ solution to stimulate Ca²⁺ influx and exocytosis contained (in mM, titrated to pH 7.2): 40 NaCl, 105 KCl, 1 MgCl₂, 6 CaCl₂ and 10 HEPES.

4.4 Imaging focal adhesion

PC12 cells were fixed in 3.7% (w/v) formaldehyde (Sigma) in PBS for 20 min and permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min. Non-specific binding sites were blocked with 1.5% Bovine Serum Albumin (BSA, Sigma) in PBS for 1 h. Focal adhesion complexes were stained with FITC conjugated mouse monoclonal anti-vinculin antibodies in PBS (40 µg ml⁻¹, Sigma) for 40 min and subsequently imaged by a LSM510 Meta confocal laser scanning microscope equipped with 63× oil-immersion objective (1.4 NA) (Carl Zeiss).

4.5 Total internal reflection fluorescence microscopy (TIRFM)

PC12 cells were transfected with NPY-EGFP plasmid (a kind gift from Dr Wolfhard Almers, Vollum Institute, Oregon Health & Science University) to label large dense core vesicles (LDCV) using FuGENE 6 Transfection Reagent (Roche Diagnostics GmbH), 1–2 days before being imaged by an inverted TIRFM microscope (Axiovert200, Carl Zeiss) equipped with a 100× oil-immersed object (1.45 NA), EMCCD camera, and a 488 nm laser excitation. Time-lapse images were taken at 2 Hz and the motion of individual vesicles was tracked using ImageJ (National Institute of Health, Wayne Rasband) and analyzed by Igor (WaveMetrics) routines.²⁰ The diffusion constant of vesicle lateral motion was calculated as previously reported.⁴⁵

4.6 Amperometric measurement

A 5 µm carbon fiber electrode (ALA Scientific Instruments) was biased at 700 mV and held closely adjacent to the cell membrane for amperometric recording by an EPC-10 double patch-clamp amplifier (HEKA Elektronik) while exocytosis was stimulated by superfusion of high K⁺ solution *via* an application micropipette. The amperometric signals were sampled at 4 kHz and filtered at 1 kHz, and analyzed by an Igor program, Amperometric Spike Analysis 8.15, developed by Dr Eugene Mosharov at Columbia University. Only spikes with amplitude greater than 2 pA (10 times that of background noise) were considered as true signals and used for analysis.

4.7 Single cell photometry

A photometry system (TILL Photonics, GmbH) was used to assess the GM1 and cholesterol content on the cell membrane and the intracellular Ca^{2+} concentration. The fluorescent probes were excited at desired wavelength using a monochromator and the emission fluorescence was collected by a photodiode.

To assess membrane GM1, PC12 cells were fixed and stained with Alexa Fluor[®] 488 conjugated CTB ($1 \mu\text{g ml}^{-1}$ in PBS, Invitrogen) followed by extensive washing with PBS. The fluorescent CTB was excited at 488 nm. For measuring membrane cholesterol, PC12 cells were fixed and stained with filipin (0.05 mg ml^{-1} in PBS, Sigma) and washed with PBS. Filipin was excited at 340 nm. The emitted fluorescence intensity was subtracted from background intensity to indicate the amount of GM1 or cholesterol on the cell membrane.

For Ca^{2+} imaging, PC12 cells were first incubated with a $5 \mu\text{M}$ membrane permeable Ca^{2+} dye, Fura2 acetoxymethyl ester (Fura2-AM, Molecular Probes), in a serum-free medium for 45 min. The cells were incubated in fresh medium for > 30 min to allow hydrolysis of the ester and retaining of the dye before imaging in the bath solution. The fluorescence intensity ratio at 340 and 380 nm excitation (F340/F380) was measured to indicate the intracellular Ca^{2+} concentration and its change upon stimulation by high K^{+} solution.

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