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碩士論文

Institute of Zoology
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Master Thesis

牛腎上腺嗜鉻細胞中 NCS-1 維持了正常的胞吐活動

NCS-1 Maintains the Normal Exocytotic Activities in Bovine Chromaffin Cells

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求學生涯在此暫告一段落，回顧當研究生的日子，最先要感謝的是我的指導教授潘建源老師，謝謝您兩年半前給了我機會進入動物所，並加入 730 實驗室學習怎麼做研究。您有親切的態度並樂於指導學生，減輕我進實驗室時候的緊張感。論文寫作的過程頗為煎熬，謝謝老師付出了極大的耐心指導我、幫助我，這段日子讓您操心真是對不起；謝謝口試委員戴晶瑩老師、王致恬老師、陳示國老師細心的指教，讓我通過口試也幫助我改善論文。終於論文完成，有一個自己的作品，事實上還有很大的改進空間，我想作為一個經驗，往後在其他方面都要注意充實自己和調整心態。

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摘要

鈣離子結合蛋白的家族種類眾多，其中包含具有 EF hands 結構，可與鈣離子結合的鈣離子感應蛋白，如 neuronal calcium semsor-1 (NCS-1)，它的結構含有 N 端 myristoylation 及三個有功能的 EF hands。前人的研究指出，NCS-1 會影響分泌膜囊的回收再利用，但詳細機制尚未釐清。我們在牛腎上腺髓質細胞中表現 NCS-1，運用安培法測量分泌出的神經傳遞物質以及影像技術來偵測胞內鈣離子變化，以研究高鉀刺激所造成突觸小泡釋放的機轉。我們發現，相較於對照組，表現 NCS-1 的細胞，其胞吐作用沒有顯著變化；表現 NCS-1^{G2A} (無法結合細胞膜)或 NCS-1^{R102Q} (發現於自閉症患者)的細胞，其胞吐程度降低，氧化電流和電量均變小，顯示有抑制胞吐作用的現象及突觸小泡內的傳導物質可能減少；而 NCS-1^{E120Q} (無法結合鈣離子) 則增加胞吐電量。胞內鈣離子濃度變化的結果顯示，過度表現 NCS-1^{G2A} 的細胞有顯著較低的鈣離子反應，而表現 NCS-1 與其他變異的實驗組，則與控制組細胞相近。這些結果顯示，調節胞吐作用的功能與結合到膜上能力有很大相關；而失去鈣離子結合能力，並不影響 NCS-1 調節的作用。因此 NCS-1 可能參與在調控分泌膜囊的回收再利用機轉中，並在神經突觸的長期變化，扮演重要的角色。

關鍵字: NCS-1、腎上腺髓質細胞、胞吐作用、胞吞作用、分泌膜囊的回收再利用

Abstract

Many proteins have a Ca^{2+} binding capability and involve in various physiological activities. Calcium sensor protein family belongs to the calcium binding protein group and has EF hands for Ca^{2+} binding. Neuronal calcium sensor-1 (NCS-1) has an N-terminal myristoylation site, one cryptic EF hand and 3 functional EF hands. NCS-1 overexpression affects the vesicle recycling, however, the mechanism is not clear. In this report, we monitor the catecholamine release and $[Ca^{2+}]_i$ evoked by high K^+ depolarization to characterize the roles of NCS-1 in the stimulus-secretion coupling in bovine chromaffin cells. We found that cells overexpressing NCS-1 had similar exocytosis level as the control group expressing GFP; cells overexpressing NCS-1^{G2A} (no plasma membrane anchoring capability) or NCS-1^{R102Q} (identified in an autism patient) had less exocytosis events, and smaller oxidation currents; in contrast, cells overexpressing NCS-1^{E120Q} (loses Ca^{2+}-binding ability) increased the exocytosis level. For the intracellular Ca^{2+} response, all constructs caused similar responses as the control group except NCS-1^{G2A} which had a smaller elevation than the control group. These results indicated that the plasma membrane targeting capability is important for the regulatory role of the NCS-1 in the stimulus-secretion coupling; however, losing the Ca^{2+}-binding ability does not affect the regulatory role of the NCS-1. In summary, NCS-1 may be involved in regulating the synaptic vesicle recycling and playing an important role in long term synapse plasticity.

Key words: NCS-1, chromaffin cell, exocytosis, endocytosis, synaptic vesicle recycling

Contents

1.Introduction

Nervous system is the decision center that regulates the physiological activities of an animal body. Neurons connect with one another, through transmitting signals to coordinate the voluntary and involuntary actions of an individual. In addition, the human brain is responsible for learning, memory and emotion.

One of the transmission pathways is that neurons and neuroendocrine cells secrete chemical signals via vesicles. The process that vesicles fuse with the plasma membrane and release neurotransmitters is called exocytosis. As cells need to stay the same size, the membrane added by the vesicle in exocytosis must be internalized by endocytosis. Scientists described that the behavior of vesicle fusion is like “kissing” (Scheme 2), giving the studies of vesicle recycling some lovely imagination.

The operation of nervous system is so fascinating that leads scientists to invest efforts to do the endless research. In my thesis, what I want to seek is the secret of secretion.

1.1 Synaptic Transmission

A neuron is an electrical excitable cell which has a soma, and branched extensions called dendrites and axon. The thin and branched fibers extended from the

soma are dendrites, which receive the signal inputs from other neurons; soma is the cell body which processes the information; and an axon is often long and extended from the soma. The axon is responsible for the signal outputs and limited one per soma.

The transmission takes place at the synapse which could be electrical or chemical connections. The electrical transmission occurs by gap junctions formed at the synapse where a cell directly exchanges ions and small molecules. Its response is the same signal as the source and the transmission is fast. The chemical transmission occurs by releasing neurotransmitter from the axon terminal. The presynaptic terminal contains synaptic vesicles which store neurotransmitters, and the released transmitters activate the receptors of postsynaptic cell.

The cell membrane of a neuron contains various ion channels with different ion permeability; the membrane potential and intracellular ion concentration are varied according to the changes in the permeability. The influx of cations depolarizes the membrane potentials; when the membrane potential reaches the threshold, it results in the occurrence of an action potential. The action potential opens the voltage-gated Ca^{2+} channels and let Ca^{2+} enter into cytosol. Furthermore, the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) leads to the release of neurotransmitters from the synaptic vesicles to the synaptic cleft to active the postsynaptic neurons.

1.2 Vesicle Cycle

Neurons and neuroendocrine cells secrete neurotransmitters through synaptic vesicles. The vesicles in the recycling pool fused with the plasma membrane to transmit signals, and then the fused membrane vesicles are retrieved into the recycling pool. The endocytosed synaptic vesicles become the parts of early endosome; after packaged with neurotransmitters, they will be transported to the reserved pool and then moves to the readily releasable pool (RRP) near the membrane. Also, the vesicles perform docking and priming at active zone on the plasma membrane. When the cell is activated, the vesicles in the RRP will be fuse with the plasma membrane. Then the vesicles release the neurotransmitters and increase the membrane surface area; that is exocytosis. After that, to maintain the membrane homeostasis, the membrane invaginates with the help of vesicle-associated proteins, it reforms into a vesicle and is transported to the recycling pool; that is endocytosis (Scheme 1).

The behavior of vesicle fusing with the plasma membrane is similar to kissing (Scheme 2). The vesicle fusion modes include two non-completed fusions and the general fully fusion. The vesicles transiently open a fusion pore and release neurotransmitters at high $[Ca^{2+}]_i$. The fastest mode, that vesicle locally recycling with undocking is “kiss-and-run”; the moderate mode, that vesicles locally refill with neurotransmitters without undocking is “kiss-and-stay”. The slow mode, vesicles are

retrieved by clathrin-mediated endocytosis at low $[Ca^{2+}]_i$ (LoGiudice and Matthews, 2006).

1.3 Calcium

Differential $[Ca2^{+}]_i$ regulates various cell functions, that is, Ca^{2+} plays an important role in neuron transmission. The $[Ca2^{+}]_i$ elevation is required for exocytosis. It is mediated by the opening of the Ca^{2+} channels on the plasma membrane or by the Ca^{2+} release from the ER Ca^{2+} stores (Baker and Knight, 1978). We could infer the steps of vesicle recycling by detecting the Ca^{2+} response.

It is generally agreed that Ca^{2+} achieves exocytosis via an interaction with some molecular targets located at or near to the site of membrane fusion. At the synapse the Ca^{2+}-binding proteins bind Ca^{2+} via the C2-domain motif, include synaptotagmin, Munc13, RIM, Piccolo, Rabphilin and Doc2. Another family, EF-hand domain proteins bind calcium in conformational changes that exposes a hydrophobic surface which can bind to a target protein. Calmodulin and NCS-1 are two EF-hand domain proteins which are related to exocytosis. Besides, two major calcium-activated kinases, CaMKII and PKC, which phosphorylate a number of key exocytotic proteins (Barclay et al., 2005).

1.4 Neuronal Calcium Sensor-1 (NCS-1)

Neuronal calcium sensors belong to a large family of proteins, which have a calcium-dependent molecular switch, includes members like Frequenin (NCS-1), recoverin, GCAP, neurocalcin, visinin etc. All the members carry 4 EF hands, helix-loop-helix calcium binding motifs, and a myristoylated N-terminal group. The discovery of frequenin is a mutant from the *Drosophila melanogaster*. The homologous gene of frequenin in mammalian is neuronal calcium sensor-1 (NCS-1). It has three functional EF hands and one cryptic EF hand (Aravind et al., 2008) and locates near the cell membrane. The EF hands bind to Ca^{2+} to activate the cell functions. It is believed that the function is mediated by the Ca^{2+} channel and related to neuronal transmission (Dason et al., 2012); but the mechanism is still unclear.

Recently, it have been found that the binding partners of NCS-1 include VAMP2, a vesicle-associated membrane protein; PI4Kβ (Phosphatidylinositol 4-Kinase), an enzyme related to signal transduction pathways; the inositol 1, 4, 5 triphosphate receptor (IP3R), a receptor-operated Ca^{2+} channel, and TRPC (transient receptor potential channel) subtypes 1 and 5 (Petko et al., 2009) etc. These findings further evidence that NCS-1 may involve in neuronal transmission.

In resonance assignments, Chandra showed that both myristoylated and non-myristoylated structures of NCS-1 are in the plasma membrane; he found that the

myristoylated form had chemical shift perturbations in artificial membrane, while the non-myristoylated form had not (Chandra et al., 2010). This result suggests the interaction between membrane and the myristoylated NCS-1. That is consistent with Ames which also showed that Ca^{2+}-induced extrusion of the myristoyl group with an exposed hydrophobic residues for target binding (Ames and Lim, 2012) and the myristoylated form is thought able to activate some target proteins like the Ca^{2+} channels (Dason et al., 2012). These evidences suggest that NCS-1 is involved in the calcium signaling pathways. Pan showed that the overexpression of NCS-1 led to faster rundown in exocytosis, and the intracellular $[Ca^{2+}]_i$ was similar to control cells (Pan et al., 2002). Weiss suggested NCS-1 was able to increase PIP2 levels, which directly facilitated exocytosis and stabilized voltage gated-Ca^{2+} channel function (Weiss et al., 2010). The general results, which has reflected NCS-1 targets the Ca^{2+}-dependent activator proteins for secretion. The effects of NCS-1 are affecting exocytosis and regulating the activities of neural cells.

1.5 Approaches

The basic apparatus in visualizing the vesicle movement and recycling in single cell is microscopy. In the study of exocytosis, it is unique that to detect the actual chemical contents of the release from the vesicles by electrochemical approach. On

the other hand, it is helpful to measure the electric properties and predict of the ionic channels involved in exocytosis by patch clamp techniques. Patch clamp recording detects cell's capacitance changes that reflect the changes in the surface area of cell membrane which is caused by exocytosis and endocytosis. In fact, the amplifiers used in patch clamp experiments have extremely low noise so that the apparatus are usually used cooperatively in exocytosis research such as electrochemical experiments (Mundorf et al. 2002).

Amperometry is an electrochemical technique to detect electric current with excellent spatial-temporal resolution. It is used to study the release of catecholamine from single cells by the oxidization reaction (Mundorf et al. 2002). I measured the current, and analyzed the amplitude (pA), rise time (ms), decay time (ms), area (fC) and half-width (ms) of the elicited spikes. The amplitude is the maximal current change of a spike; the rise time is 10 % - 90 % of the period which a spike climbed from the baseline to the peak; the decay time is the period which a spike fell from the peak to the 37 % of peak height; the area under single spike is the quantity of electric charge; the half width is the period at the 50% of peak height (Fig. 3).

Calcium imaging is a technique using calcium sensitive fluorescent dye to detect the concentration of calcium ($[Ca2^{+}]_i$). From the variation of the fluorescence intensity, we can know the changes of in $[Ca2^{+}]_i$. In my study, fura-2, the

Ca^{2+}-sensitive dye, was used as a marker. It was excited alternatively with 340 and 380 nm with or without Ca^{2+} binding. The emission at 510 nm was collected as the signals.

1.6 Aims

The aim of my research is to study the effect of NCS-1. In previous studies, the evidences of membrane capacitance recording and Ca^{2+} current recording have shown that the NCS-1 affect the neurotransmission. Three mutant forms have been prepared in our lab to study the roles of NCS-1. The first one, a G2A mutant, losw the membrane-bound capability caused by the defect in myristoylation (Chandra et al., 2010). The second mutant, E120Q, shows an impaired calcium-dependent conformational change resulting from inactivation in the 3rd EF hand. It is a dominant negative inhibitor (Weiss et al., 2000). The last one, R102Q mutant was identified in an autism patient (Piton et al., 2008) but does not show changes in its affinity for Ca^{2+} (Handley et al., 2010).(Fig. 3)

I propose: (1) To characterize the effects of NCS-1 on exocytosis. (2) To detect the effects of NCS-1 on $[Ca^{2+}]_i$.

2. Materials and Methods

2.1 Solutions

Lock's solution: 154 mM NsCl, 2.15 mM $Na_2PO_4 \cdot 2H_2O$, 10 mM Glucose, 10mM HEPES, pH = 7.2, 303 mOsm/kg

Sucrose solution: 0.3 M sucrose, 3 mM HEPES, pH = 7.2, 303 mOsm/kg

BCCM (Bovine Chromaffin Complete Medium): Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad CA, USA), penicillin/streptomycin (100 IU/ml-100 mg/ml, Invitrogen, Carlsbad CA, USA) and Ara-C (10 μM)(Sigma-Aldrich)

DMEM: Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad CA, USA) supplemented with 15 mM HEPES, 26 mM $NaHCO_3$, pH = 7.0-7.3, 300 mOsm/kg

PBS: 142 mM NaCl, 2 mM KCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, pH = 7.3, 305 mOsm/kg

Loading buffer: 150 mM NaCl, 5 mM Glucose, 10 mM HEPES, 1mM $MgCl_2 \cdot 6H_2O$, 5 mM KCl, 2.2 mM $CaCl_2 \cdot 2H_2O$, pH = 7.3, 305 mOsm/kg

High K^+ solution: 5 mM NaCl, 5 mM Glucose, 10 mM HEPES, 1mM $MgCl_2 \cdot 6H_2O$, 150 mM KCl, 2.2 mM $CaCl_2 \cdot 2H_2O$, pH = 7.3, 305 mOsm/kg

Opti-MEM® I Reduced Serum Medium (Gibco®)

All other chemicals were reagent grade and were purchased from Sigma-Aldrich, unless indicated specially.

2.2 Primary bovine chromaffin cell culture

I acquired chromaffin cells from bovine adrenal medulla. First, the adipose and connective tissue surrounding the adrenal gland were removed and the gland was washed by Lock's solution. The gland was digested by type I collagenase (2 mg/ml dissolved in Lock's solution; Invitrogen) and incubated for 40 min at 37°C. Then the gland was opened with surgical scissors and scraped out the medulla with knife. The dissected medulla was filtrated with gauze and the liquid of medulla was centrifuged (800 G, 5 min). The suspension was discarded and the precipitation was filtrated with 40 μm cell strainer. The precipitation was diluted with Lock's solution. Again, the liquid of medulla was centrifuged (800 G, 5 min) and also discard the suspension. The precipitation of medulla cells was collected and mixed with Lock's solution. To do density gradient centrifuge, the mixture was loaded on the top of an equal volume sucrose solution carefully. The solution was centrifuged (600G, 5 min). The density gradient centrifuge step was repeated twice. Finally, the cell pellets was resuspended in BCCM, and they were plated at a density of 5×10^5 cell/ml on 24 mm

collagen-coated coverslips. 3 hours later, when cells adhered to the coverslips better, the dish was added 0.5 ml medium till the total volume was 1.5 ml. I replaced the medium every 2 days.

2.3 Plasmid of NCS-1 and its mutations

The *rattus* NCS-1 plasmid was obtained from primary cortical neuron in the previous study in our lab. Previous researchers in our lab utilized point mutation to generate NCS-1^{G2A}, NCS-1^{E120Q} and NCS-1^{R102Q}.

Transformation

The 50 ng plasmid was gently mixed with 100 μl competent cell (*E. coli*, DH5α, ECOS^{TM} 101, Yeastern Biotech, Taiwan) and sat on ice for 30 min. The mixture was incubated at 42℃ for 90 sec and then chilled on ice for 2 min. Next, the mixture was added with 100 μl LB broth and incubated at 37℃ and 180 rpm for 45 min. Finally, the mixture was spread on agarose plate containing Ampicillin (100 μg/ml) or Kanamycin (50 μg/ml.) and incubated at 37°C overnight.

Plasmid extraction

Single colonies on agarose plate were picked separately and incubated in 50-ml

centrifuge tube which had 5 ml LB broth containing 50 μg/ml of Ampicillin or Kanamycin. The cultures were incubated at 37℃ and 180 rpm overnight. To extract plasmids from the bacteria suspensions, I used QIAprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacture's manual.

2.4 LTX transfection

I did transfection on day 1-7 after the chromaffin cells were isolated. The protocol was based on the manual of Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad CA, USA) with some modifications. First, I mixed 150 μl Opti-MEM®, 2 μl Plus Reagent, and 2 μg plasmid in 1.5-ml eppendorf tube which wasmarked A;let it stand for 10 min under room temperature. In another 1.5-ml tube, I mixed 150 μl Opti-MEM® with 4μl LTX and marked it as B. The contents of A and B were mixed and stand for 30 min under room temperature. The medium in the cell culture dish was replaced with Opti-MEM® , and the mixture of plasmid was gently added into the dish. The cells were incubated about 1 day, after that, we replaced the medium with BCCM.

2.5 Stimulation and Amperometry

I used 150 mM High K^+ solution to stimulate single chromaffin cell and measured the released catecholamine by amperometry using ProCFE carbon fiber (Qty-96, Dagan Co., Minneapolis, Minnesota, USA). The puff pipettes were made by Flaming/brown micropipette puller Mode P-97 (Sutter Instrument Co.,Novato, CA, USA) using glass capillary (Catalog 617000, A-M Systems Inc., WA, USA). I utilized PM2000B 4 channel pressure injector (MicroDataInstrument, USA) to puff High K^+ solution onto single cells, and utilized HEKA Electronic EPC-10 amplifier (Lambrecht/Pfalz, Germany) and Pulse software (HEKA Electronic, Germany) to measure current. The setting of recording: Gain, 10 mv / pA; Filter 1, Bessel 10 kHz; Filter 2, Bessel 2.9 kHz.

I put the cells in a recording chamber containing loading buffer. The puff pipette tip was positioned at about 30 μm from the cell and the carbon fiber electrode was right above the cell with gentle touch (Fig. 1). The High K^+ solution in the puff pipette was pressure-puff onto the cells for 3 sec repetitively with an interval of 2 min. To oxidized the catecholamine, I set the electrode at 650 mV and measured the oxidation current.

2.6 Calcium imaging technique

To measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)_changes, I loaded the cells with Fura-2 AM (0.4 μM, Teflabs, Austin, TX, USA) for 40 min and measured the changes in the fluorescence intensities. I excited the cells with 340 and 380 nm wavelengths alternatively provided by the DG4 (Sutter) and detected the emission by Cool Snap CCD camera (Princeton, US). The whole system was under the control of MetaFlour software (Molecular Devices, Inc., USA). Then it record the emission of the excitation at both 340 nm and 380 nm and created a 340/380 ratio. The High K^+ solution in the puff pipette was pressure-puff onto the cells for 3 sec repetitively with an interval of 2 min.

2.7 Data Analysis

The data of amperometry were record by Pulse software and analyzed by MiniAnalysis (Synaptosoft Inc., Chapel Hill, NC, USA). There were the characteristics: amplitude (pA), rise time (ms), decay time (ms), area (fC) and halfwidth (ms) of the elicited spikes to be analyzed. The settings of the analysis: LoPass filter, 200 Hz; Threshold, 25 pA; Period to search a maximum, 50 ms; Time before a peak for baseline, 75 ms; Period to search a decay time, 200 ms; Fraction of

peak to find a decay time, 0.37; Period to average a baseline, 20 ms; Area threshold ,60 fC; Number of point to average for peak, 3.

All data were calculated by Excel (Microsoft, USA) and OriginPro (OriginLab, USA). The value of average of the spike characteristic were counted and be compared among groups of cells. Data were presented as mean ± SEM. and analyzed by Kruskal-Wallis ANOVA with Mann-Whitney post hoc test (*: $p < 0.05$ when compared with the GFP group).

3. Results

To study the effect of NCS-1 in exocytosis and Ca^{2+} response, I performed amperometry recording and Ca^{2+} imaging on the untransfected cell and a cell expressing GFP; NCS-1; NCS-1^{G2A}; NCS-1^{E120Q}; NCS-1^{R102Q}. The untransfected cells are normal chromaffin cells. I used the group as a comparison for transfection effect. The GFP group was tagged by green fluorescence as a control group which had no effect on function. The data mentioned below were presented as the mean ± SEM and analyzed by Kruskal-Wallis ANOVA with Mann-Whitney post hoc test (*: $p < 0.05$ when compared with the GFP group).

3.1 The traces of amperometry

Amperometry is an electrochemical technique using carbon fiber electrode to oxidize released chemicals during exocytosis. To determine release contents, chromaffin cells which have vigorous secretory function were used for the catecholamine oxidation. Fig.1 shows the experimental set up and the carbon fiber electrode. I stimulated cells for 3 s with high K^+ starting at the 1s and record the spikes of exocytosis in one sweep for 30 s. The protocol repeated 3 times with an interval of 2 min. Fig. 2 A-F show the representative raw traces in one sweep of different treatments. Consider the occurrence time, numbers and size of the spike,

these groups present different characteristics. In general, the GFP group is similar to the NCS-1 group, and the other 3 mutant groups are 3 types respectively. The details of statistics were mentioned in the following sections. Fig.3 shows the spike analysis. The amplitude (pA) is the maximal current change of a spike; the rise time (ms) is 10 % - 90 % of the period which a spike climbed from the baseline to the peak; the decay time (ms) is the period which a spike fell from the peak to the 37% of peak height; the area (fC) under single spike is the quantity of electric charge; the half width (ms) is the period at the 50% of peak height. The amplitude and area are determined as the contents of neurotransmitters; the rise time, decay time and half width are related to the fusion kinetics of vesicles.

3.2 Cells overexpressing NCS-1 maintains the exocytosis; cells overexpressing NCS-1^{G2A} or NCS-1^{R102Q} down-regulate the exocytosis; cells overexpressing NCS-1^{E120Q} have an elevated exocytosis level.

To find out the exocytosis level, I analyzed the data of amplitude, area and spike numbers. Fig. 4 A and B show the averaged amplitude and area in 3 sweeps per spike per cell in different groups. The amplitude (pA): untransfected, 59.5 ± 6.3*; GFP, 75.0 ± 6.7; NCS-1, 88.8 ± 9.1; NCS-1^{G2A}, 48.0 ± 5.2*; NCS-1^{E120Q}, 69.8 ± 6.8; NCS-1^{R102Q}, 36.1 ± 2.1*. The area (fC): untransfected, 845.1 ± 78.4; GFP, 929.3 ± 162.7; NCS-1,

1137.4 ± 167.7; NCS-1^{G2A}, 671.3 ± 105.5; NCS-1^{E120Q}, 1035.8 ± 88.6*; NCS-1^{R102Q}, 462.8 ± 79.6*. Compared with the GFP group, cells overexpressing NCS-1 have no significant change; NCS-1^{G2A} and NCS-1^{R102Q} have smaller amplitude or area, which represent the inactive release; cells overexpressing NCS-1^{E120Q} have larger area, which represent the dynamic release. Fig. 4 C and D shows the average of spike numbers and total area in 3 sweeps per cell in different groups. The spike numbers: untransfected, 11.3 ± 2.6*; GFP, 24.6 ± 4.2; NCS-1, 29.0 ± 10.9; NCS-1^{G2A}, 9.1± 3.7*; NCS-1^{E120Q}, 32.1 ± 7.1; NCS-1^{R102Q}, 7.7 ± 2.6*. The total area (fC): untransfected, 10790.9 ± 2796.5*; GFP, 20084.6 ± 3902.2; NCS-1, 30591.3 ± 10612.5; NCS-1^{G2A}, 6041.9 ± 2676.8*; NCS-1^{E120Q}, 35578.7 ± 8734.5; NCS-1^{R102Q}, 5587.7 ± 2807.0*. The spike numbers represent the exocytosis events; the total area represents the release contents of a cell. Cells overexpressing NCS-1^{G2A} or NCS-1^{R102Q} have fewer spikes and total area; that suggest the inhibition of exocytosis. The sample size of spike numbers: untransfected, 519; GFP, 788; NCS-1, 465; NCS-1^{G2A}, 118; NCS-1^{E120Q}, 642; NCS-1^{R102Q}, 100. The sample size of cell numbers: untransfected, 46; GFP, 32; NCS-1, 16; NCS-1^{G2A}, 13; NCS-1^{E120Q}, 20; NCS-1^{R102Q}, 13.

3.3 Cells overexpressing NCS-1^{E120Q} have long vesicle fusion time.

To detect the fusion kinetics of vesicles, I analyzed the rise time, decay time and

half width. Fig. 5 A-C shows the averaged rise time, decay time and half width in 3 sweeps per spike per cell in different groups. The rise time (ms) : untransfected, 17.4 ± 1.4; GFP, 16.4 ± 2.3; NCS-1, 15.5 ± 1.1 ; NCS-1^{G2A}, 21.5 ± 4.1 ; NCS-1^{E120Q}, 19.3 ± 1.4; NCS-1^{R102Q}, 13.0 ± 1.8. The decay time (ms) : untransfected, 17.5 ± 2.0*; GFP, 15.5 ± 4.5; NCS-1, 14.5 ± 2.7; NCS-1^{G2A}, 15.6 ± 5.2; NCS-1^{E120Q}, 17.1 ± 2.2*; NCS-1^{R102Q}, 12.1 ± 2.0. The half width (ms): untransfected, 17.3 ± 1.9; GFP, 15.2 ± 4.0; NCS-1, 14.2 ± 2.2; NCS-1^{G2A}, 16.5 ± 4.2; NCS-1^{E120Q}, 17.6 ± 2.1; NCS-1^{R102Q}, 11.9 ± 1.5. The rise time means the fusion time before maximal release, these experiment groups have no significant when compare with the GFP group. The decay time means the fusion time after maximal release. Cells overexpressing NCS-1^{E120Q} have longer decay time. The half width is the combined changes of rise time and decay time, or it is related to the size of a spike. These experiment groups have no significant change when compare with the GFP group. The above results indicate the fusion mode change in cells overexpressing NCS-1^{E120Q}. The sample size of spike numbers: untransfected, 519; GFP, 788; NCS-1, 465; NCS-1^{G2A}, 118; NCS-1^{E120Q}, 642; NCS-1^{R102Q}, 100. The sample size of cell numbers: untransfected, 46; GFP, 32; NCS-1, 16; NCS-1^{G2A}, 13; NCS-1^{E120Q}, 20; NCS-1^{R102Q}, 13.

3.4 Cells overexpressing NCS-1^{G2A} or NCS-1^{R102Q} have relative infrequent exocytosis in the 3rd sweep.

To separate the contribution to exocytosis in different sweeps, I presented spike numbers and total area by different sweeps. Fig. 6 A shows the average of spike numbers per sweep per cell in different groups. The 1st sweep: untransfected, 4.3 ± 1.0 ; GFP, 7.6 ± 1.8; NCS-1, 9.6 ± 4.4; NCS-1^{G2A}, 4.5 ± 1.9; NCS-1^{E120Q}, 12.2 ± 3.3; NCS-1^{R102Q}, 3.8 ± 1.6. The 2nd sweep: untransfected, 3.7 ± 1.0*; GFP, 8.8 ± 2.2; NCS-1, 8.1 ± 4.7; NCS-1^{G2A}, 2.5 ± 0.9; NCS-1^{E120Q}, 11.2 ± 2.5; NCS-1^{R102Q}, 2.8 ± 1.0. The 3rd sweep: untransfected, 3.3 ± 0.8*; GFP, 8.2 ± 1.6; NCS-1, 11.3 ± 3.7; NCS-1^{G2A}, 2.1 ± 1.0*; NCS-1^{E120Q}, 8.8 ± 2.3; NCS-1^{R102Q}, 1.1 ± 0.5*. Fig. 6 B shows the average of total area (fC) per sweep per cell in different groups. The 1st sweep: untransfected, 3739.4 ± 1134.2; GFP, 4407.5 ± 960.5; NCS-1, 8915.2 ± 3640.2; NCS-1^{G2A}, 2784.9 ±1305.4; NCS-1^{E120Q}, 10248.9 ± 2975.4; NCS-1^{R102Q}, 2908.6 ± 1894.0. The 2nd sweep: untransfected, 3690.0 ± 1006.5*; GFP, 8107.0 ± 2207.2; NCS-1, 9240.1 ± 4909.8; NCS-1^{G2A}, 1554.2 ± 614.6; NCS-1^{E120Q}, 13435.4 ± 3128.5; NCS-1^{R102Q}, 2078.6 ± 933.5. The 3rd sweep: untransfected, 3361.5 ± 924.6*; GFP, 7570.0 ± 1604.3; NCS-1, 12436.1 ± 4836.9; NCS-1^{G2A}, 1702.8 ± 860.9*; NCS-1^{E120Q}, 11894.4 ± 4029.7; NCS-1^{R102Q}, 600.6 ± 314.9*. These experiment groups have no significant change when compare with the GFP group in the 1st sweep and the 2nd

sweep. Cells overexpressing NCS-1^{G2A} or NCS-1^{R102Q} have relative fewer spikes and total area in the 3rd sweep. The sample size of spike numbers: untransfected, 519; GFP, 788; NCS-1, 465; NCS-1^{G2A}, 118; NCS-1^{E120Q}, 642; NCS-1^{R102Q}, 100. The sample size of cell numbers: untransfected, 46; GFP, 32; NCS-1, 16; NCS-1^{G2A}, 13; NCS-1^{E120Q}, 20; NCS-1^{R102Q}, 13.

3.5 Cells overexpressing NCS-1^{G2A} have a relative uniform distribution on the exocytosis events

To know the occurring timing of spikes, I draw a spikes accumulation figure. The sample size of spike numbers: untransfected, 519; GFP, 788; NCS-1, 465; NCS-1^{G2A}, 118; NCS-1^{E120Q}, 642; NCS-1^{R102Q}, 100. Fig.7. shows that in untransfected group, GFP group, NCS-1group, NCS-1^{E120Q} group and NCS-1^{R102Q} group, there are about 80 % spikes occurred during 0 s-12 s in the recoding which last for 30 s; the curves are exponential distribution. But the curve of NCS-1^{G2A} group is near linear; it has a relative uniform distribution of spikes.

3.6 The traces of calcium imaging

To measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes, the cell was loaded Ca^{2+} sensitive dye, Fura-2, and repetitively stimulated with High K^+ for 3 s

with an interval of 2 min. The cells were record the fluorescent emission of the excitation at both 340 nm and 380 nm and the measurements were created a 340 / 380 ratio. Fig. 8 A - F show the representative traces of fluorescence intensity ratio from the different treatments for 3 stimulations during 380 s. In this thesis, I focused on the fluorescence intensity changes which were measured from baseline to the peak height; generally the NCS-1^{G2A} group has smallest changes. The details of statistics were mentioned in the following section.

3.7 Cells overexpressing NCS-1^{G2A} reduces calcium response

Fig. 9 shows the average of fluorescence intensity change (ΔF) per stimulation per cell in different groups. The 1^{st} stimulation: untransfected, 2.4 ± 0.2; GFP, 1.6 ± 0.4; NCS-1, 1.4 ± 0.2; NCS-1^{G2A}, 0.6 ± 0.2*; NCS-1^{E120Q}, 1.6 ± 0.3; NCS-1^{R102Q}, 1.6 ± 0.3. The 2^{nd} stimulation: untransfected, 1.8 ± 0.2; GFP, 1.5 ± 0.3; NCS-1, 1.1 ± 0.2; NCS-1^{G2A}, 0.4 ± 0.2*; NCS-1^{E120Q}, 1.3 ± 0.2; NCS-1^{R102Q}, 1.2 ± 0.3. The 3^{rd} stimulation: untransfected, 1.6 ± 0.2; GFP, 1.3 ± 0.3; NCS-1, 0.9 ± 0.2; NCS-1^{G2A}, 0.3 ± 0.1*; NCS-1^{E120Q}, 1.2 ± 0.3 ; NCS-1^{R102Q}, 0.91 ± 0.2. The sample size of cell numbers: untransfected, 19; GFP, 9; NCS-1, 23; NCS-1^{G2A}, 11; NCS-1^{E120Q}, 10; NCS-1^{R102Q}, 13. As can be seen in the graph, all of these groups have the similar fluorescence intensity changes except the NCS-1^{G2A} group. Cells expressing

NCS-1^{G2A} have significant low calcium response every stimulation.

3.8 The relationship between Ca^{2+} response and exocytosis

Fig. 10 A and B plots the changes in the Ca^{2+} against the total spike numbers and summarized area, respectively, during the first stimulation. The arrangement of the 6 points is not linear, and there are a main group includes GFP, NCS-1and NCS-1^{E120Q}. The relationship between exocytosis and Ca^{2+} response is indirect in the regulation of NCS-1 and the other mutants.

4. Discussion

It has been show that endogenous NCS-1 expresses in the bovine chromaffin cells by previous studies (McFerran et al., 1998) including immunostaining in our lab (Wang, 2010; Chen. 2012). Therefore, I examine the influence of NCS-1 by overexpressed NCS-1 and its mutant forms in bovine chromaffin cell and compared with the GFP group in exocytosis and Ca^{2+} response. The untransfected cells are normal chromaffin cells; I used the group as a comparison for transfection effect. The results show that the cells with transfection have more releases; one possible reason is that the LTX reagents altered the vesicle pool. However, consider the functions of proteins, there are significant differences among the GFP group, NCS-1 group and other mutant groups. That is worthwhile to debate the functions of NCS-1. Consequently, I focusd the discussions on these cells overexpressing constructs in the following sections.

4.1 Cells overexpressing NCS-1 maintains the exocytosis activities

Cells overexpressing NCS-1 have no significant change when comparing with GFP in amplitude, area, spike numbers and total area. That indicates the contents of neurotransmitters per vesicle, the exocytosis events per cell and the total release contents per cell are similar to the normal level; the uptaking of transmitters and the

vesicle recruitment are taken as usual. NCS-1 makes moderate functions in the process of vesicle recycling. In addition, there is also no significant change between NCS-1 and GFP in the features of vesicle fusion kinetics: rise time, decay time and half width. That means the size and shape of a spike in NCS-1are matching with GFP. At the results of spike numbers or total area per stimulation, the contribution to exocytosis in different sweeps in NCS-1 is identical to GFP. It presents that the vesicle pool supplied sufficient vesicles to release as primary; the vesicle recycling works stably. In summary, NCS-1 maintains the exocytosis activities.

4.2 Cells overexpressing NCS-1^{G2A} or NCS-1^{R102Q} down-regulate the exocytosis

The results of amplitude, area, spike numbers and total area show that NCS-1^{G2A} or NCS-1^{R102Q} reduces exocytosis. The contents of neurotransmitters per vesicle, the exocytosis events per cell and the total release contents per cell are smaller to the normal level; the uptaking of transmitters and the vesicle recruitment may be attenuated. The mutant proteins have deficient structures and weaken the primary function of NCS-1 in vesicle recycling. However, in the results of rise time, decay time and half width, there is no significant change when comparing with GFP. These mutants do not change the fusion kinetics of vesicle. At the results of spike numbers or total area per stimulation, the contribution to exocytosis in the 3rd sweep in

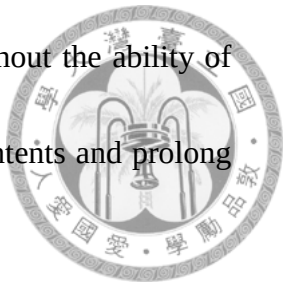
NCS-1^{G2A} or NCS-1^{R102Q} is smaller to GFP. Because the evoked exocytosis is vigorous at the beginning, repetitive stimulations let cell not recover completely. Therefore the difference in exocytosis events occurs in later stimulations. It presents that NCS-1^{G2A} and NCS-1^{R102Q} affect the vesicle recycling during the repetitive stimulations.

The NCS-1^{G2A} mutant has no plasma membrane anchoring capability and it distributes over the cytosol. It indicates that the capability of membrane targeting is important for the regulation of the normal exocytosis activities. The NCS-1^{R102Q} mutant has a structural deficit but it still can bind to Ca^{2+} and target the membrane; it may affect the interaction with some proteins to reduce exocytosis.

4.3 Cells overexpressing NCS-1^{E120Q} have an elevated exocytosis level

Cells overexpressing NCS-1^{E120Q} have significantly large area when comparing with GFP. It represents that NCS-1^{E120Q} may increases the uptaking of transmitters. The results of the fusion kinetics indicate cells overexpressing NCS-1^{E120Q} have the significantly long decay time which expresses the long duration of vesicle fusion after the maximum release. Besides, the results of spike numbers or total area per stimulation, the contribution to exocytosis in different sweeps in NCS-1^{E120Q} is identical to GFP. It presents that NCS-1^{E120Q} keeps the primary vesicle pool; the

vesicle recycling works stably. These results suggest that even without the ability of Ca^{2+} binding, NCS-1^{E120Q} may target some proteins to raise the contents and prolong the fusion time of the vesicle.



4.4 The timing of exocytosis events

Through the accumulation spikes figure (Fig. 7), it can be known the precise occurring timing of spikes. Except the NCS-1^{G2A} group, the accumulation spikes curves are exponential distribution in other groups. In general, the evoked exocytosis is vigorous after stimulation and then becomes weak gradually over time. But the curve of NCS-1^{G2A} group is near linear, it has a relative uniform distribution of spikes. The possible explanation is that NCS-1^{G2A} down- regulates the exocytosis therefore spike numbers are low in all the timings and there are no obvious accumulation in the beginning.

4.5 NCS-1^{G2A} overexpression reduces Ca^{2+} response

The calcium imaging by Fura-2 reflect the $[Ca^{2+}]_i$ change. The results show only the NCS-1^{G2A} group has a significantly small calcium response. This is possible that NCS-1^{G2A} affecting the vesicle recycling and reducing the contents of the synaptic vesicle through the reduction of Ca^{2+} response. In contrast, NCS-1 and other mutant

forms have similar Ca^{2+} response when comparing with the GFP group. Therefore the Ca^{2+} response may be related to the membrane binding capability. Ca^{2+} sources include extracellular Ca^{2+} influx and internal Ca^{2+} stored in ER; NCS-1 and the mutant forms may not only influence the vesicle release, but also mediate the ER release.

4.6 NCS-1 keeps the machinery between exocytosis and Ca^{2+} response.

It has been known that Ca^{2+} induces the exocytosis, and NCS-1 also takes part in exocytosis. Therefore I combined the results of my two experiments to draw a relation figure and consider the effect of NCS-1and other mutants. Fig. 10 A and B plots the changes in the Ca^{2+} response against the total spike number and the summarized area. The mutant forms change the primary machinery between exocytosis and Ca^{2+} response. As it shows that the arrangement of the 6 points is not linear. The relationship between exocytosis and Ca^{2+} response may be indirect through the regulation of the other mutants. These results show that there are a main group includes GFP, NCS-1and NCS-1^{E120Q}; it means there are similar machinery between exocytosis and Ca^{2+} response in these treatments. It is could be infer that the machinery is similar in the main group. However, it is need to be support by more clear evidences.

4.7 Internal Ca^{2+} store may mediate the function of NCS-1

To clarify the role of NCS-1 in vesicle recycling, I summarized my results to previous studies of capacitance recording and Ca^{2+} current in our lab (Wang, 2010; Chen, 2012). It shows that there are no significant changes in Ca^{2+} current and $[Ca^{2+}]_i$ in NCS-1, and it maintains the exocytosis level, but the membrane capacitance (C_m) is smaller. It could be that NCS-1 promotes more endocytosis event of vesicle. The results of NCS-1^{G2A} show that it down-regulate the Ca^{2+} current, $[Ca^{2+}]_i$, C_m and the exocytosis activities. The results of NCS-1^{E120Q} show that it down-regulates the Ca^{2+} current and C_m, but it maintains $[Ca^{2+}]_i$ and have elevated exocytosis level. The Ca^{2+} sources include the influx of extracellular Ca^{2+} and the release of internal Ca^{2+} store. It may be that the ER store increases the Ca^{2+} release in cytosol to conserve $[Ca^{2+}]_i$; NCS-1^{E120Q} raises the endocytosis level leads to the decrease of C_m. Last, the results of NCS-1^{R102Q} show that it down-regulates the Ca^{2+} current, C_m and the exocytosis activities and maintains $[Ca^{2+}]_i$. It may be caused by the increase of Ca^{2+} store from ER.

In conclusion, it is possible that NCS-1 is also related to the internal Ca^{2+} store according to the studies of our lab. Internal Ca^{2+} store may mediate the function of NCS-1. The examination of future works in Ca^{2+} store could be taken by the inhibition of Ca^{2+} channel on the ER membrane.

4.8 Suggestions for improvements

The results of amperometry recording are different from different methods of analysis. It has been reported that when comparing amperometric spike characteristics between groups of cells, it is more appropriate to compare samples of mean spike values (Colliver et al., 2000). So I presented the mean of spike characteristics per spike per cell as results. I found that there were certain variances in the spike numbers every cell in the same group; the variances were due to the conditions of detection. The detection of exocytosis events are not only related to the release of vesicle but also the recording of carbon fiber electrode. The skill of manipulation would affect the recoding. Hence the increase of sample numbers could have more data with adequate spike numbers per cell and eliminate the inaccuracy of data. The future researchers might process the data of amperometry severely.

5. Conclusion

The results of amperometry recording suggest that the cells overexpressing NCS-1 maintain the exocytosis; cells overexpressing NCS-1^{G2A} and NCS-1^{R102Q} have down-regulated the exocytosis; cells overexpressed NCS-1^{E120Q} release more neurotransmitters and has longer fusion time. The results of Ca^{2+} imaging indicate that NCS-1^{G2A} reduce Ca^{2+} response; whereas NCS-1 and other mutants have similar Ca^{2+} response when comparing to the GFP group. These results suggest that NCS-1 may interact with some proteins at the membrane to mediate the exocytotic activities, and NCS-1 keep the machinery between exocytosis and Ca^{2+} response. Also, from the results of NCS-1^{E120Q}, Ca^{2+} plays an indirect role in the effect of exocytosis.

Therefore, NCS-1 targets the plasma membrane to regulate the synaptic vesicle recycling and maintains the Ca^{2+} response. NCS-1 may play an important role in long term synapse plasticity changes.

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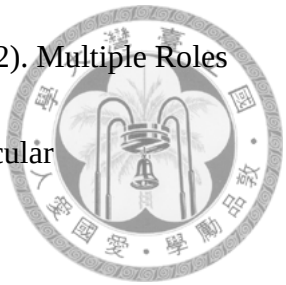

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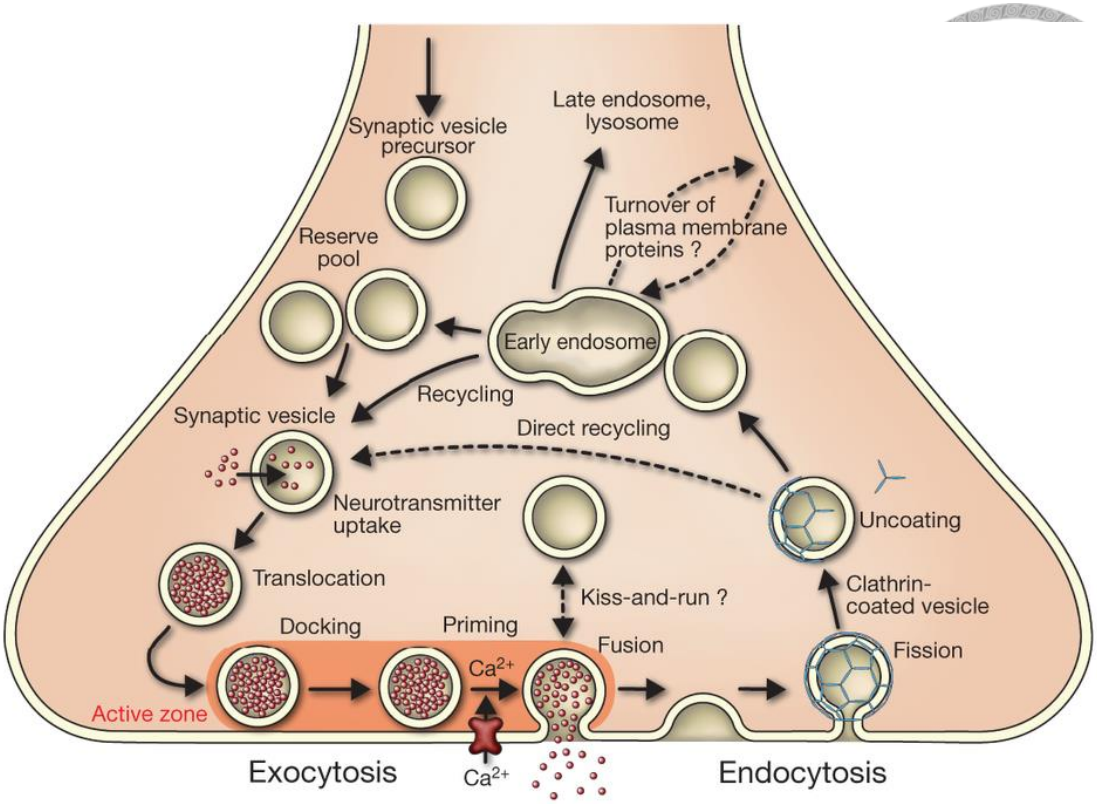
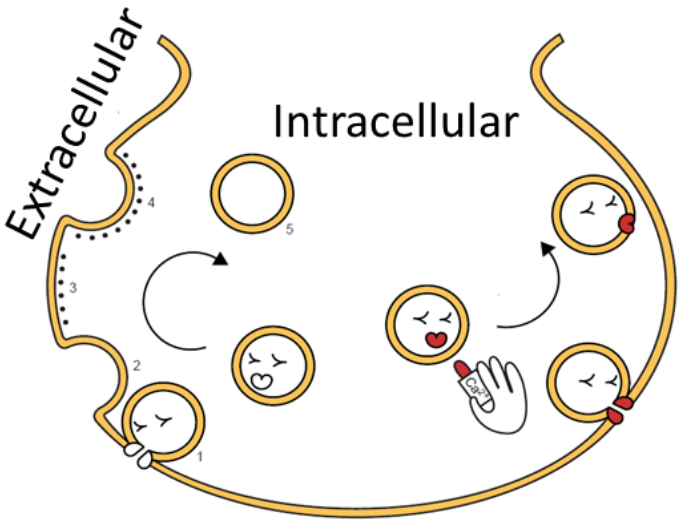


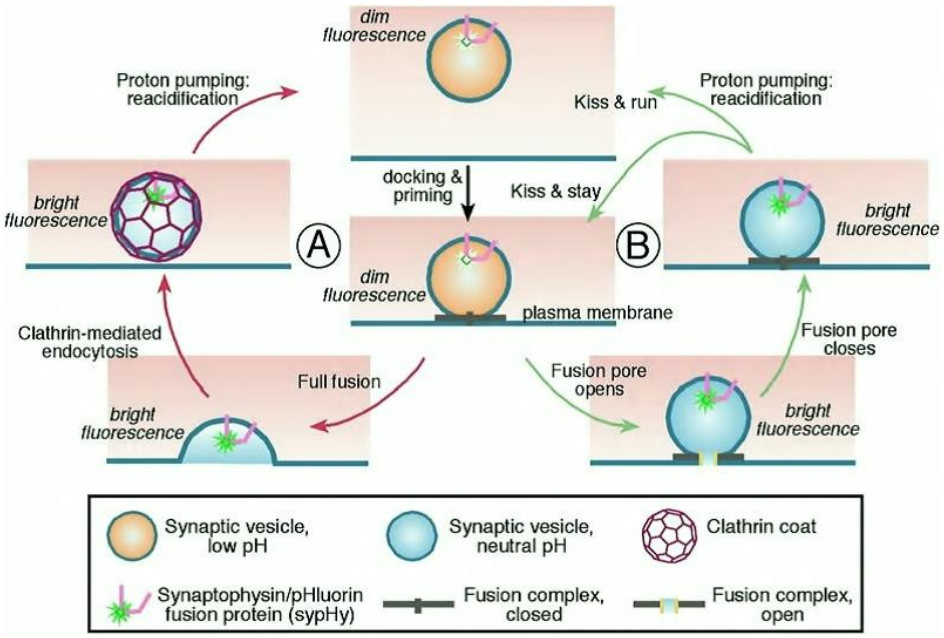
Scheme 1. The synaptic vesicle cycle

Initially, synaptic vesicles are isolated from the early endosome and move to the reserve pool (RP). After vesicles have completed the neurotransmitter loading, they translocate to the active zone on the membrane. Vesicles perform docking and priming there and accumulate in the readily release pool (RRP). When voltage-gated Ca^{2+} channel open, the increase in the intracellular Ca^{2+} concentration leads to the fusion of vesicle with the plasma membrane by exocytosis. After releasing the neurotransmitters, the vesicle retrieval is generally mediated by clathrin-mediated endocytosis. The uncoating of clathrin leads vesicles to require in recycling pool and refill the neurotransmitters (Jahn and Fasshauer, 2012).

(A)



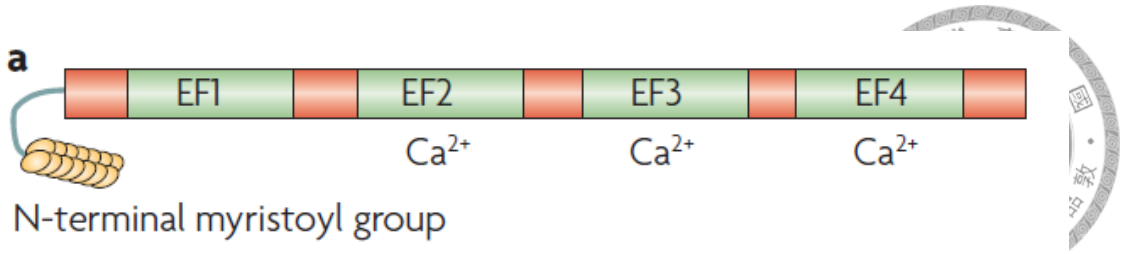
(B)



Scheme 2. The kiss-and-run and full fusion models

(A) The cartoon model of vesicle fusion in coupled exocytosis–endocytosis. Ca^{2+} influx induces vesicle fusion. The left is general full fusion process. The right is kiss-and-run fusion. (modified from Fesce and Meldolesi, 1999). (B) Two alternative

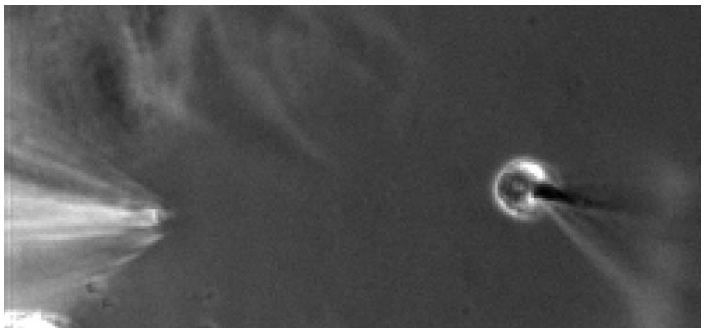
schemes for the synaptic vesicle cycle at synapses of cultured hippocampal neurons. Ⓐ The vesicle membrane fully fuses with the plasma membrane, and vesicles are then retrieved by clathrin-mediated endocytosis. Ⓑ The neurotransmitters are released by a transient opening of a fusion pore, and the vesicle membrane remains separate from the plasma membrane. One of the two cases is local refilling with neurotransmitters without undocking (“kiss-and-stay”), and the other is local recycling with undocking (“kiss-and-run”), and then full recycling of vesicles. In either case, vesicles undergoing exocytosis lose their high concentration of protons. Exocytosis and subsequent reacidification after endocytosis can be tracked by an sensor, sypHy, which localizes in synaptic vesicles. In the low-pH environment inside synaptic vesicles, the pH-sensitive GFP of sypHy is quenched. Upon exposure to the pH-neutral extracellular space during exocytosis, sypHy increases its fluorescence, and this state is maintained until the intravesicular H^+ gradient is restored by proton pumping after endocytosis(LoGiudice and Matthews, 2006).



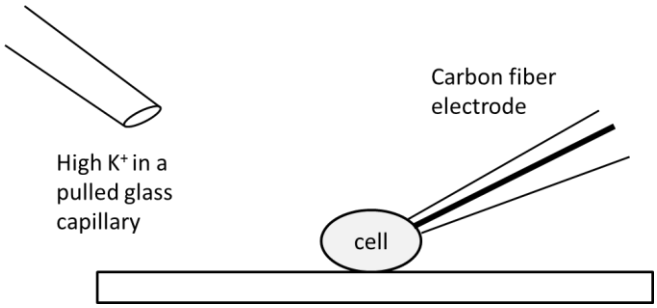
Scheme 3. A schematic representation of the general domain structure of NCS-1

NCS-1 contains an N-terminal myristoyl group and 4 EF-hands, the EF-1 can not bind Ca^{2+}. The N-terminal in the G2A mutant is non-myristoylated; the 3rd EF hand in the E120Q mutant is inactivated; the R102Q mutant is identified in an autism patient (Burgoyne, 2007).

(A)



(B)



(C)

Fig.1. The experimental setup and the carbon fiber electrode

(A) and (B) The photo view and the cartoon of the experimental setup. The puff pipette tip was positioned at about 30 μm from the cell and the carbon fiber electrode was right above the cell with gentle touch. (C) The size of carbon fiber electrode is approximate as a 10 μl pipette tip.

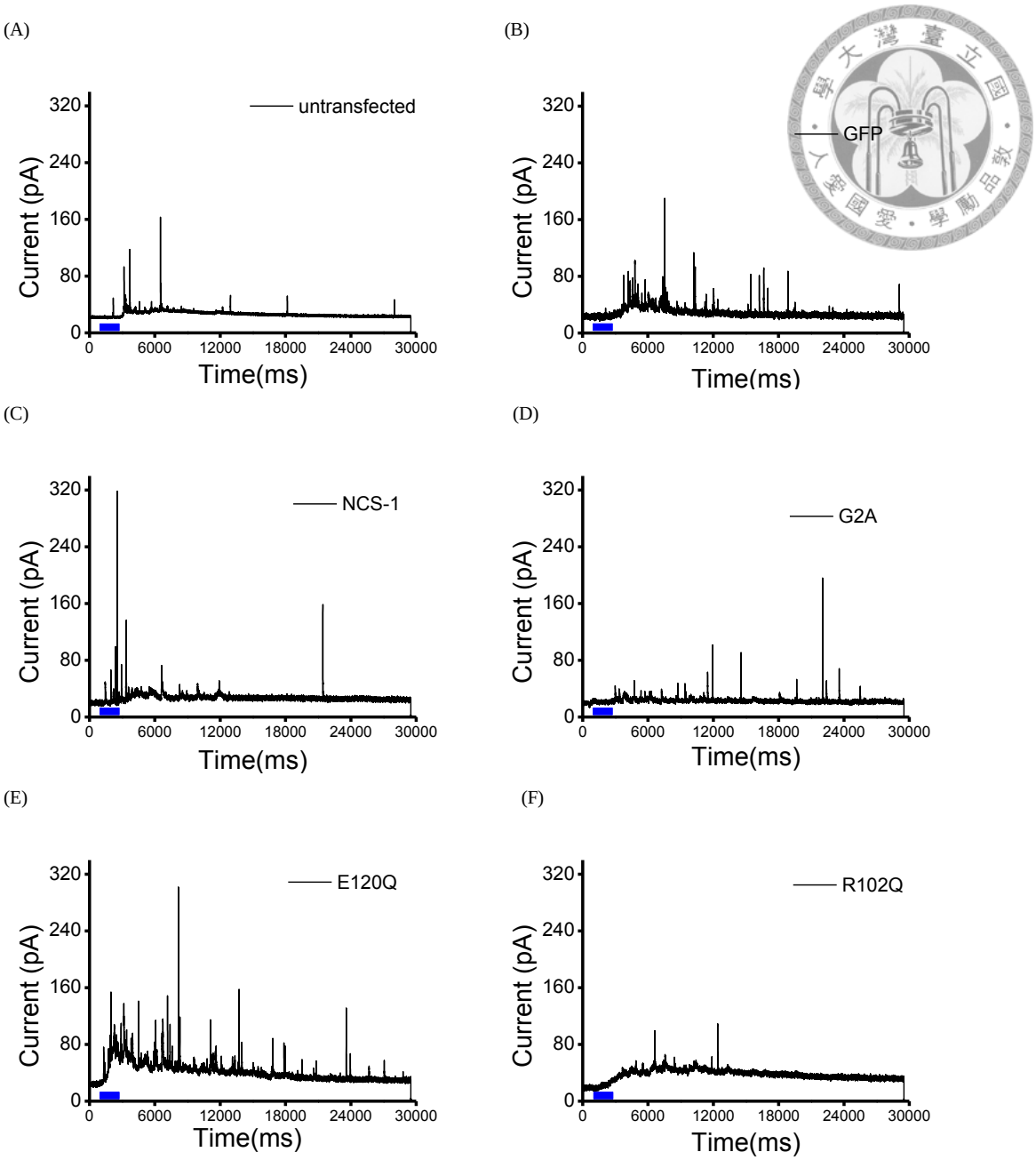


Fig.2. Representative amperometry recordings

One of 3 recordings of the oxidation currents for 30 s with the high K^+ stimulation. (A)-(F) The representative oxidation current traces from a untransfected cell or cells expressing GFP, NCS-1,NCS-1^{G2A},NCS-1^{E120Q}, and NCS-1^{R102Q}. The blue bar at the bottom is the period of the high K^+ stimulation (3 s).

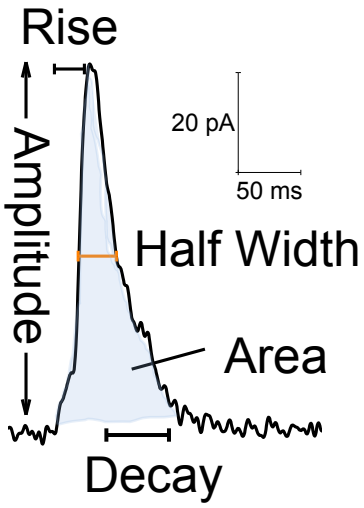


Fig.3. Spike analysis

A recording of oxidation currents includes numerous spikes. The amplitude, rise time, decay time, area and half width of a spike were analyzed. The amplitude is the maximal current change of a spike; the rise time is 10 % - 90 % of the period which a spike climbed from the baseline to the peak; the decay time is the period which a spike fell from the peak to the 37 % of peak height; the area under single spike is the quantity of electric charge; the half width is the period at the 50% of peak height.

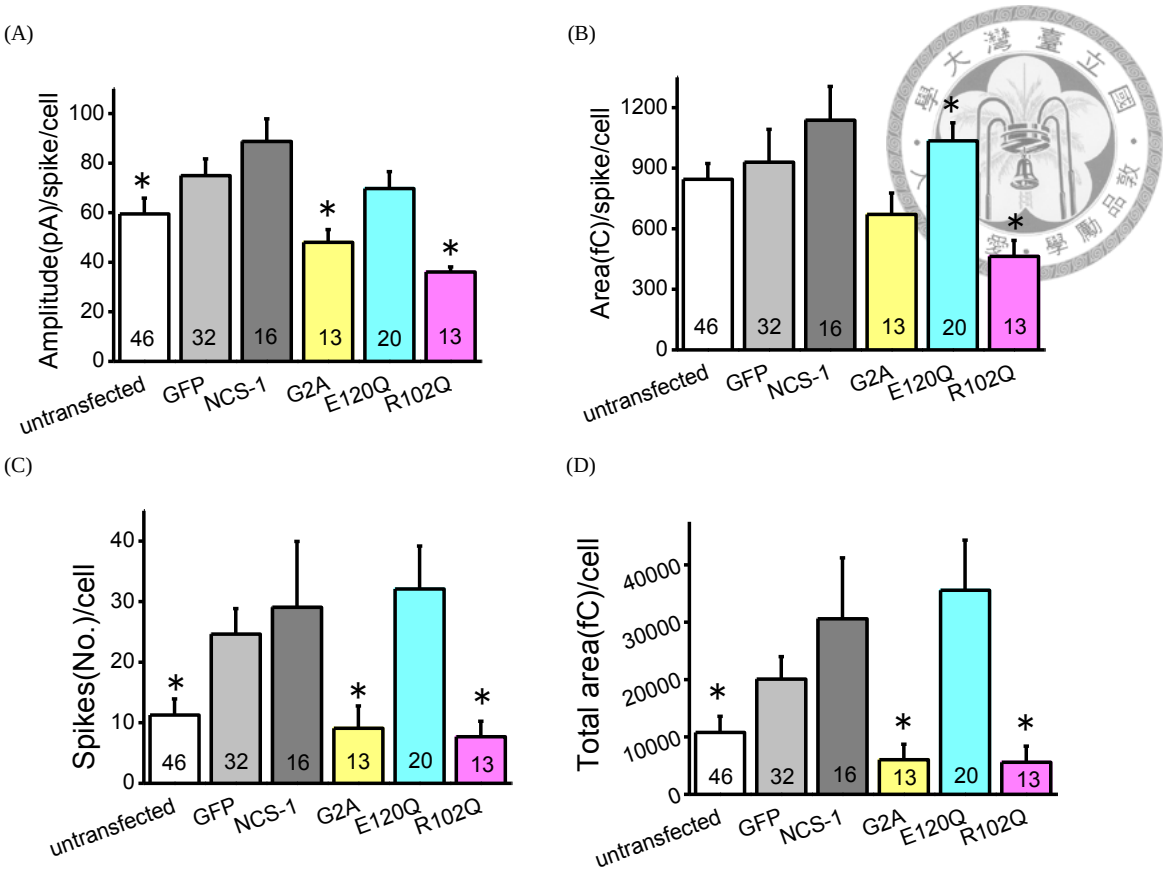


Fig. 4. Cells overexpressing NCS-1 maintains the exocytosis; cells overexpressing NCS-1^{G2A} or NCS-1^{R102Q} down-regulate the exocytosis; cells overexpressing NCS-1^{E120Q} have an elevated exocytosis level.

I stimulated the cell with high K^+ and record the oxidation current for 30 s. A cell was stimulated for 3 times and the interval between the two stimulations is 2 min. (A) and (B) The averaged amplitude and area in 3 sweeps per spike per cell in different groups. (C) and (D) The averaged spike numbers and total area in 3 sweeps per cell in different groups. Data presented are as the mean ± SEM and analyzed by a Kruskal-Wallis ANOVA with Mann-Whitney post hoc test (*: $p < 0.05$ when compared with the GFP group). The number on the bar is sample size.

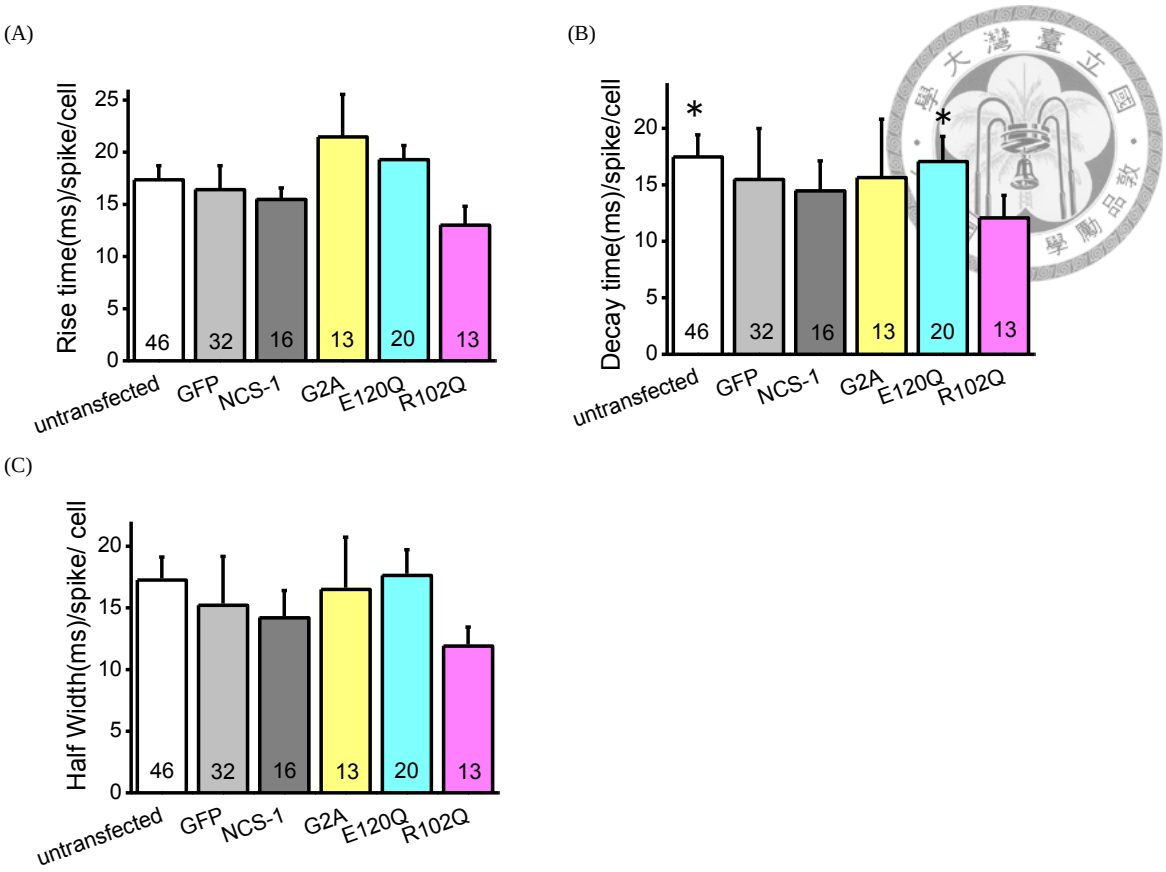
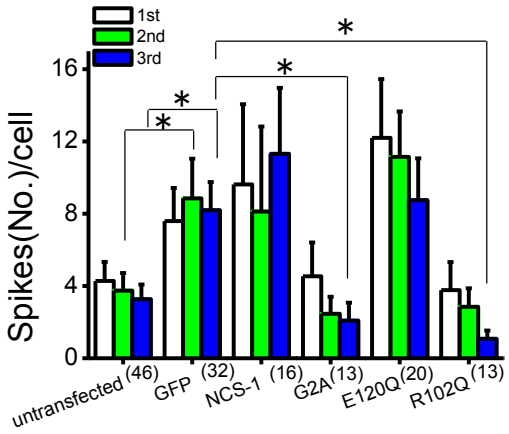


Fig. 5. Cells overexpressing NCS-1^{E120Q} have long vesicle fusion time.

I stimulated the cell with high K^+ and record the oxidation current for 30 s. A cell was stimulated for 3 times and the interval between the two stimulations is 2 min. (A) - (C) The averaged rise time, decay time and half width in 3 sweeps per spike per cell in different groups. Data presented are as the mean ± SEM and analyzed by a Kruskal-Wallis ANOVA with Mann-Whitney post hoc test (*: $p < 0.05$ when compared with the GFP group). The number on the bar is sample size.

(A)



(B)

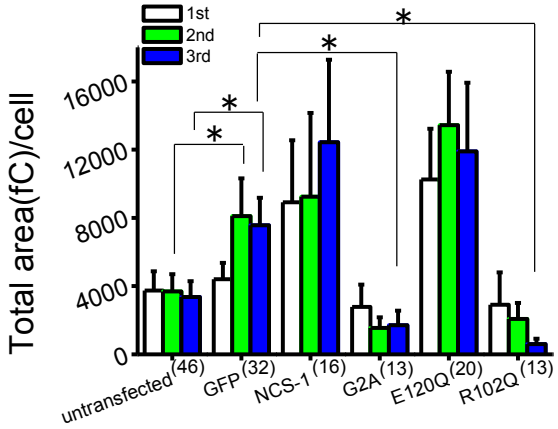


Fig. 6. Cells overexpressing NCS-1^{G2A} or NCS-1^{R102Q} have relative infrequent exocytosis in the 3rd sweep.

(A) The mean of spike numbers per sweep per cell (B) The mean of total spike area per sweep per cell. Data presented are as the mean ± SEM and analyzed by a Kruskal-Wallis ANOVA with Mann-Whitney post hoc test (*: $p < 0.05$ when compared with the GFP group separately by different stimulations). The number in parentheses is the sample size.

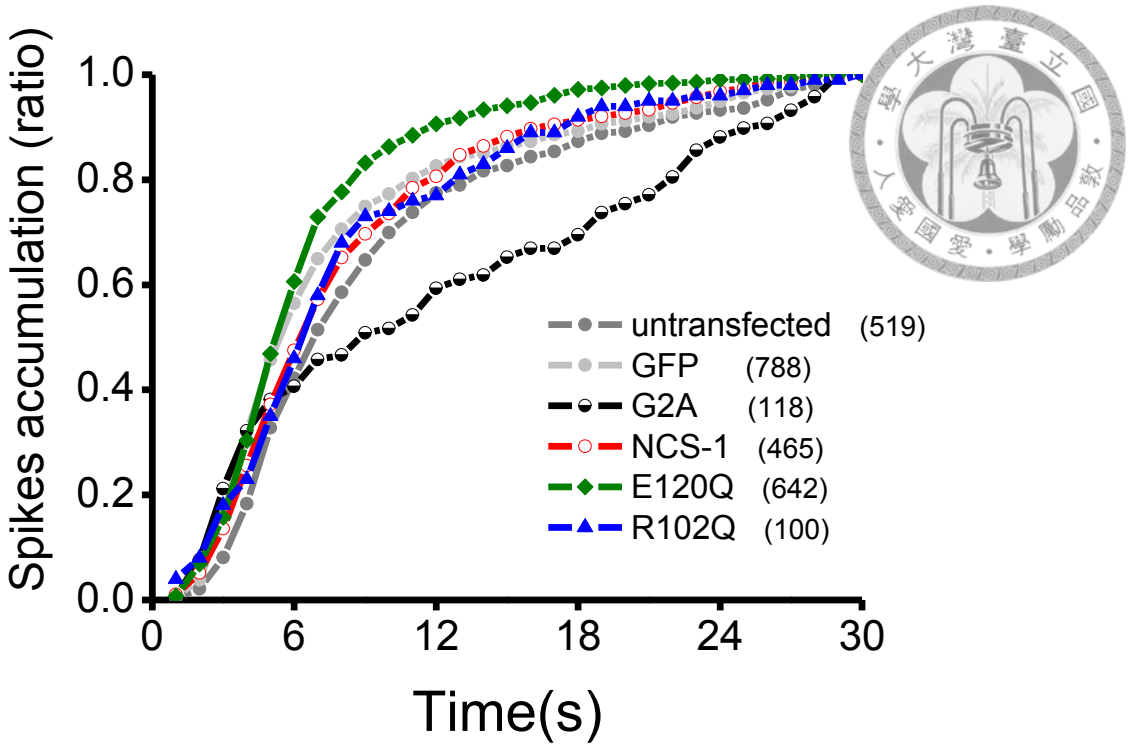


Fig. 7. Cells overexpressing NCS-1^{G2A} have a relative uniform distribution on the exocytosis events

The accumulation distribution of all spikes during recording (30 s) in different groups.

The number in parentheses is the total number of spike.

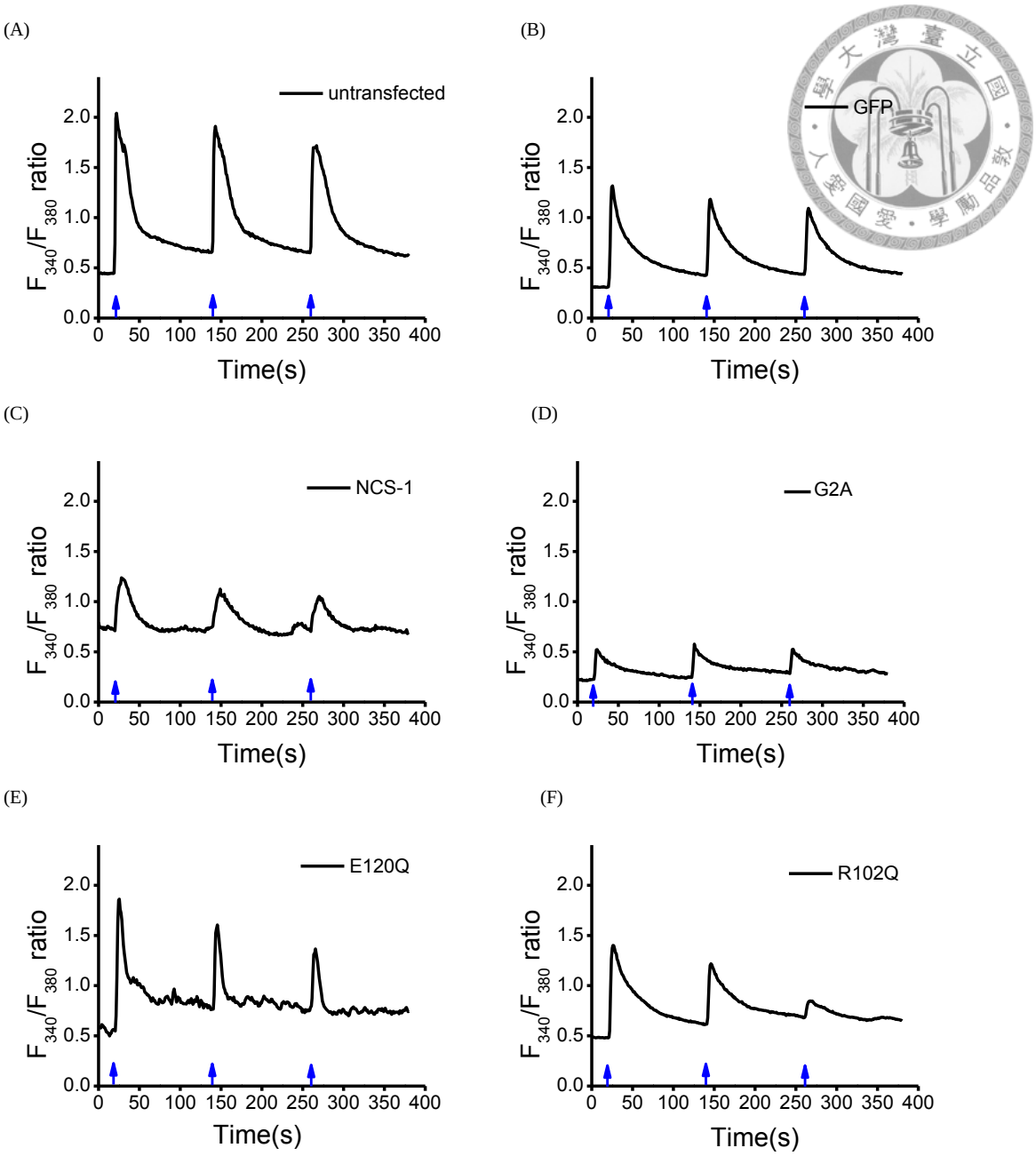


Fig.8 Representative Ca^{2+} imaging

The cell was loaded Ca^{2+} sensitive dye, Fura-2, and repetitively stimulated by High K^{+} for 3 s with an interval of 2 min. (A)-(F) A untransfected cell or cells expressing GFP, NCS-1, NCS-1^{G2A}, NCS-1^{E120Q}, and NCS-1^{R102Q}. The 3 stimulations are indicated by the arrows.

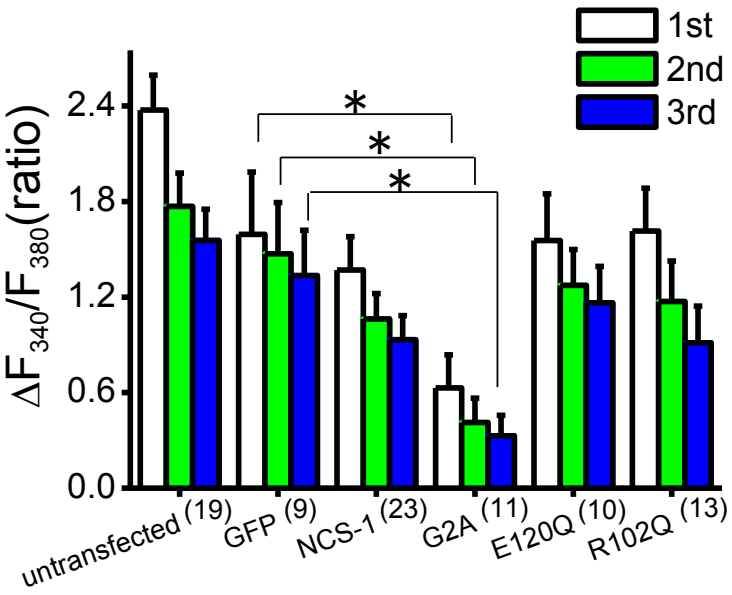
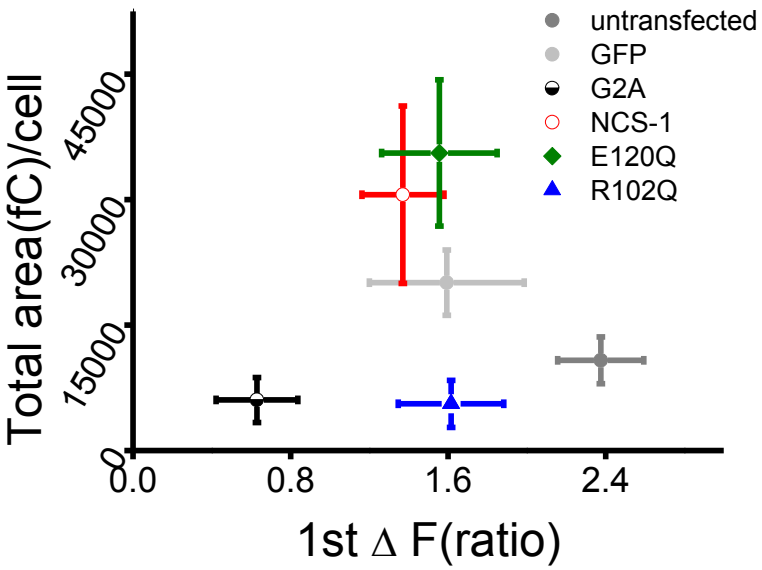


Fig. 9. Cells overexpressing NCS-1^{G2A} reduces calcium response

The average changes in the ratio (ΔF_{340} / F_{380}) by every stimulation per cell in different groups. Data presented are as the mean ± SEM and analyzed by a Kruskal-Wallis ANOVA with Mann-Whitney post hoc test (*: $p < 0.05$ when compared with the GFP group separately by different stimulations). The number in parentheses is the sample size.

(A)



(B)

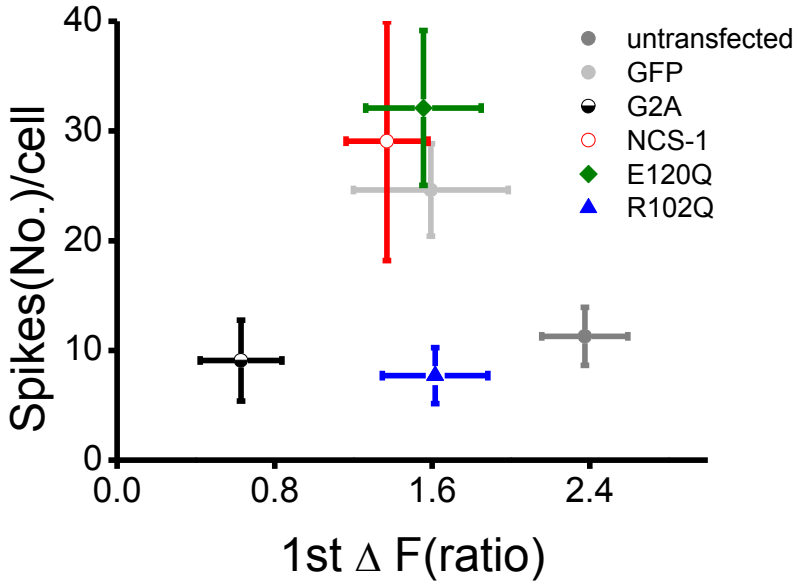


Fig. 10. The relationship between Ca^{2+} response and exocytosis

(A) and (B) plots the changes in the Ca^{2+} against the total spike number and summarized area, respectively, during the first stimulation.