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Signaling for Vesicle Mobilization and Synaptic Plasticity

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Abstract The hypothesis that release of classical neurotransmitters and neuropeptides is facilitated by increasing the mobility of small synaptic vesicles (SSVs) and dense core vesicles (DCVs) could not be tested until the advent of methods for visualizing these secretory vesicles in living nerve terminals. In fact, fluorescence imaging studies have only since 2005 established that activity increases secretory vesicle mobility in motoneuron terminals and chromaffin cells. Mobilization of DCVs and SSVs appears to be due to liberation of hindered vesicles to promote quicker diffusion. However, F-actin and synapsin, which have been featured in mobilization models, are not required for activity-dependent increases in the mobility of DCVs or SSVs. Most recently, the signaling required for sustained mobilization has been identified for *Drosophila* motoneuron DCVs and shown to increase synaptic transmission. Specifically, presynaptic endoplasmic reticulum ryanodine receptor-mediated Ca^{2+} release activates Ca^{2+}/calmodulin-dependent kinase II to mobilize DCVs and induce post-tetanic potentiation (PTP) of neuropeptide release in the *Drosophila* neuromuscular junction. The shared signaling for increasing vesicle mobility and PTP links vesicle mobilization and synaptic plasticity.

Keywords Synaptic vesicle · Dense core vesicle · Release · Exocytosis · Neuropeptide · Mobility

It has long been speculated that neurotransmission is facilitated by increasing synaptic vesicle mobility. For example, it was hypothesized that liberation of small synaptic vesicles (SSVs) tethered to F-actin by synapsin regulates transmitter release [1]. However, without direct observation of regulated vesicle mobility in nerve terminals, the meaning of the term "mobilization" broadened to encompass any process that increases release. This vagueness arose because an activity-dependent increase in vesicle mobility (i.e., what is meant by "mobilization" for the rest of this review) was not detected directly until the advent of approaches for labeling vesicles with green fluorescent protein (GFP) and FM1–43 (a styryl dye) so that vesicle motion could be monitored directly in living cells with fluorescence microscopy. Here we describe the recent insights made with live cell imaging that link the signaling that increases vesicle mobility in the nerve terminal to synaptic plasticity.

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Vesicle Mobilization by Liberation

Activity-dependent vesicle mobilization in a native nerve terminal was first demonstrated with neuropeptide-containing dense core vesicles (DCVs) in the *Drosophila* neuromuscular junction (NMJ) [2]. In that case, DCV mobility is increased for minutes following seconds of electrical activity. A year and a half later, stimulation was found to mobilize chromaffin cell DCVs and frog NMJ SSVs [3, 4]. Mobilized secretory vesicles appear to move randomly with little sensitivity to temperature [2–4]. These observations are in accord with the earlier demonstration that releasable and reserve vesicles move by diffusion, but at different rates [5]. Of course, such movement is not necessarily simple because vesicle diffusion is affected by heterogeneities in the cytoplasm (e.g., caging by the cytoskeleton). Nevertheless, the simplest interpretation of vesicle mobility experiments is

that activity-induced mobilization liberates hindered vesicles to quicken their local diffusion.

F-actin and Synapsin are not Involved in Mobilization

Vesicle mobility measurements also ruled out two players in popular models of mobilization: F-actin and synapsin. F-actin had been speculated to either hinder vesicles directly, to act as a scaffold for tethering vesicles, or to provide a track for regulated motors. However, depolymerizing F-actin does not mimic or prevent activity-induced mobilization of DCVs or SSVs [2–4]. The first indication that synapsin is also not essential came with the observation of DCV mobilization in type III *Drosophila* NMJ boutons [2], which do not have detectable synapsin [6]. More recently, SSV mobilization at the mouse NMJ was found to be intact even when all three synapsin genes are knocked out [7]. The lack of involvement of F-actin and synapsin shows that a new model is required to explain activity-dependent secretory vesicle mobilization. The shared features of mobilization in diverse cell types with different types of secretory vesicles (i.e., DCVs and SSVs) suggest that this mechanism is conserved for all secretory vesicles in presynaptic terminals.

Variation in Vesicle Mobility Affects the Size of the Reserve and Releasable Pools

The relevance of vesicle mobility in release was first established by in vitro imaging experiments. Specifically, it was shown that most cytoplasmic DCVs are held in reserve by being immobilized in the cytoplasm [8]. Even if such vesicles are close to the cell surface, they do not undergo exocytosis because they do not move to the plasma membrane [5]. Thus, mobility, rather than initial location, determines whether vesicles are relegated to the reserve or releasable pools.

This conclusion was strengthened by the finding that the most mobile cytoplasmic DCVs were most prone to undergo exocytosis [5]. Subsequently, similar conclusions were reached for SSVs: releasable SSVs are mobile and widely distributed, while reserve SSVs hardly move [4, 9]. The importance of vesicle mobility for release was further bolstered by the demonstration that the rate at which mobile vesicles can encounter the cell surface can be slower than docking and fusion [10–12]. Indeed, the kinetics of neuropeptide release is affected by an unusual wide distribution of vesicle diffusion coefficients, which can shift in response to facilitators of release [10, 13]. Hence, the time course and extent of release, which are often interpreted to be due to releasable and reserve vesicle pools, are affected by differences in vesicle mobility at release sites.

In addition to mobilization that promotes cytoplasmic vesicle movement to the membrane, a recent study detected an increase in vesicle motion associated with membrane fusion itself [14]. This could reflect the operation of exocytotic machinery or be a specialized version of the Ca^{2+}-induced liberation that occurs with cytoplasmic vesicles that are not in contact with the membrane. Regardless of its basis, the increase in vesicle mobility just prior to release reiterates that the emphasis on immobilization in a long-lived docked state before exocytosis, which was formulated based on static electron microscopy images, should be replaced by the realization that vesicle motion is beneficial for release.

The Importance of Signaling for Vesicle Mobilization

Past studies raised the possibility that physiological regulation of vesicle mobility could contribute to facilitation of release. However, because there was no method for perturbing activity-dependent vesicle mobilization (e.g., it was resistant to inhibitors of motors and actin polymerization), this hypothesis could not be tested experimentally. Without knowing the molecular basis of mobilization, how could its relevance be probed? Seizing on signal transduction, Shakiryanova et al. focused on the trigger for prolonged mobilization [15]. Their strategy was to identify signaling steps that produce activity-dependent DCV mobilization and then to test whether disrupting this pathway affects a form of synaptic plasticity that accompanies mobilization. Post-tetanic potentiation (PTP) of neuropeptide release was a natural candidate for inquiry because seconds of activity had been shown to produce minutes of DCV mobilization and potentiated neuropeptide release [2].

Presynaptic ER Ca^{2+} Release is Necessary for Activity-induced DCV Mobilization

The many minutes of increased DCV mobility following seconds of activity and the requirement for Ca^{2+} influx for mobilization suggested that Ca^{2+} triggered signaling to induce prolonged mobilization. Shakiryanova et al. began their signaling studies with the hypothesis that a brief influx of Ca^{2+} could trigger a long-lasting effect by evoking Ca^{2+}-induced Ca^{2+} release via ryanodine receptors (RyRs) in the presynaptic endoplasmic reticulum (ER). First, RyR-mediated Ca^{2+} release was shown to be sufficient to increase DCV mobility in type Ib boutons in the absence of extracellular Ca^{2+}. Specifically, when bath Ca^{2+} was replaced with the a Ca^{2+} chelator EGTA, (a) a Ca^{2+} ionophore increased DCV mobility; (b) application of the RyR channel opener

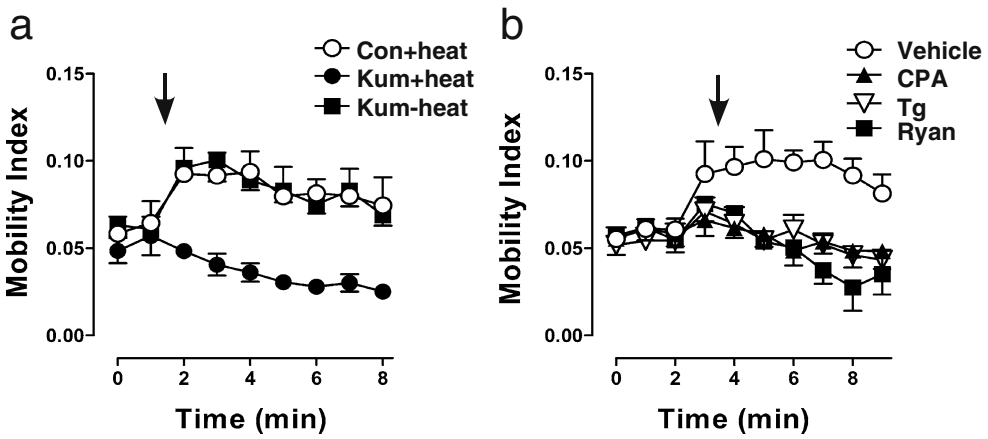

Fig. 1 ER Ca^{2+} release via RyRs is necessary for DCV mobilization evoked by electrical activity. Emptying the ER Ca^{2+} stores with genetics (**a**) or SERCA inhibitors (**b**) blocks mobilization by a 15-s tetanus (*arrow*). Ryanodine also inhibits mobilization (**b**). From [15]

caffeine evoked Ca^{2+} release from internal stores and increased DCV mobility; (c) ryanodine at a concentration known to block RyRs prevented DCV mobilization in response to caffeine; and (d) genetic depletion of ER Ca^{2+} stores blocked caffeine-evoked vesicle mobilization. For the latter experiments, the *Kum170* mutant was used, which results in temperature-sensitive, long-lasting loss of function of the Ca^{2+} pump that maintains ER Ca^{2+} storage, the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). Together, the above results established that ER Ca^{2+} release via RyRs is sufficient to induce DCV mobilization in synaptic boutons.

ER Ca^{2+} release was then shown to be necessary for activity-induced mobilization (Fig. 1). In these experiments, vesicle mobility was quantified in terms of the decrease in the pixel-by-pixel correlation coefficient (CC) between sequential images in a time lapse experiment: mobility index=1−CC [2, 15, 16]. This assay has the benefit that dynamic and reversible changes in vesicle mobility can be followed continually during an experiment. Prolonged mobilization of DCVs induced by brief tetanic stimulation of motor neurons was evident in *Kum170* larvae at permissive temperatures (Fig. 1a, Kum−heat) and in control animals briefly heated to 40°C (Con+heat). However, the tetanus failed to evoke DCV mobilization in *Kum170* mutant synaptic boutons following the temperature-dependent inactivation of SERCA (Kum+heat). ER Ca^{2+} stores were also implicated with the SERCA inhibitors thapsigargin and cyclopiazonic acid, which also inhibited activity-induced DCV mobilization (Fig. 1b). Inhibition of RyRs with ryanodine (Ryan) also blocked mobilization induced in the presence of extracellular Ca^{2+} (Fig. 1b). Finally, a genetically targeted dominant negative SERCA subunit was used to demonstrate that presynaptic ER Ca^{2+} release was required. Thus, genetic and pharmacological experiments showed that Ca^{2+} influx from the extracellular medium induces Ca^{2+} release via presynaptic ER RyRs that is necessary for activity-induced vesicle mobilization.

Transient Ca^{2+} Release Activates CamKII to Induce Mobilization

Shakiryanova et al. then addressed whether presynaptic Ca^{2+} sustains prolonged mobilization. Specifically, to test whether DCV mobilization is maintained by residual $[Ca^{2+}]_i$ following stimulation, $[Ca^{2+}]_i$ in synaptic boutons was measured with Ca^{2+} indicators. Motor neuron terminals were either induced transgenically to express the ratiometric Ca^{2+} indicator Cameleon 2.1 or boutons were loaded with dextran-conjugated OGB-1, a chemical single wavelength indicator. With either indicator, a 70-Hz, 15-s tetanus increased cytoplasmic Ca^{2+} in boutons only briefly (i.e., Ca^{2+} decayed back to baseline levels within seconds of halting the stimulus). Thus, the cytoplasmic Ca^{2+} increase is

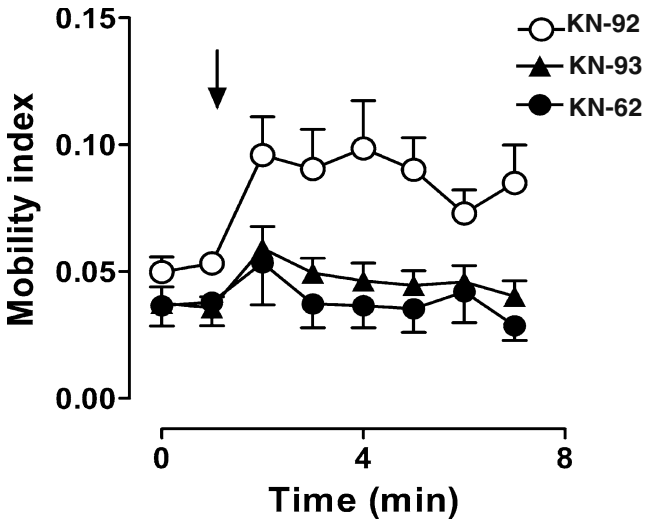

Fig. 2 DCV mobilization induced by a 70-Hz, 15-s tetanus (*arrow*) is blocked by the CamKII inhibitors KN-62 and K-93, but not by the inactive analog KN-92. KN compounds were applied at 10 μM. Similar results were obtained with AIP-II, a CamKII inhibitory peptide. From [15]

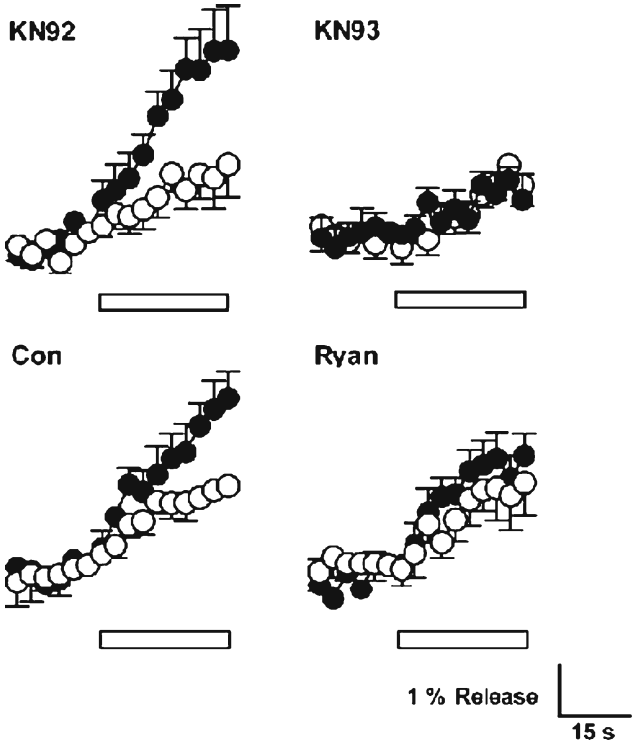

Fig. 3 PTP requires CamKII and RyRs. *Top*, PTP is evident with the inactive control (*KN92*, *left*), but abolished with the CamKII inhibitor (*KN93*, *right*). *Bottom*, PTP occurs with vehicle (*Con*, *left*), but is blocked with the RyR inhibitor ryanodine (*Ryr*, *right*). *Open bars* indicate 3-Hz stimulation. *Open circles* show response to first bout of 3-Hz stimulation. *Closed circles* show response to 3-Hz stimulation 2.5 min after 70-Hz, 15-s tetanus. From [15]

far too transient to sustain the many minutes of vesicle mobilization induced by this stimulus.

The mismatch in the kinetics of $[Ca^{2+}]_i$ and mobilization suggested that downstream signaling sustains activity-induced vesicle mobilization. Ca^{2+}/calmodulin-dependent kinase II (CamKII) is a potential long-acting mediator of transient Ca^{2+} changes that is activated by RyRs to facilitate SSV exocytosis and release of neurotrophins [17–19]. Therefore, Shakiryanova et al. probed the role of this plasticity-associated enzyme. Specifically, CamKII was acutely inhibited by treating boutons with the CamKII inhibitors KN-62 or KN-93, which bind to the calmodulin binding site (Fig. 2), or a membrane permeant inhibitory peptide (AIP-II), which binds to the active site. Each CamKII inhibitor abolished vesicle activity-induced mobilization, while inactive controls such as KN-92 did not prevent mobilization. Thus, CamKII is required for activity-induced DCV mobilization.

The next question was whether CamKII functions as an upstream activator of RyRs or a downstream effector of released ER Ca^{2+}. In fact, the latter proved to be the case. First, Ca^{2+} responses were unaffected by CamKII inhibition, showing that RyRs were not regulated by CamKII. Second, bypassing RyRs to evoke Ca^{2+} release in the absence of bath Ca^{2+} with an ionophore or veratridine (which was shown to act independently of the ER) evoked mobilization that still required CamKII [15]. Therefore,

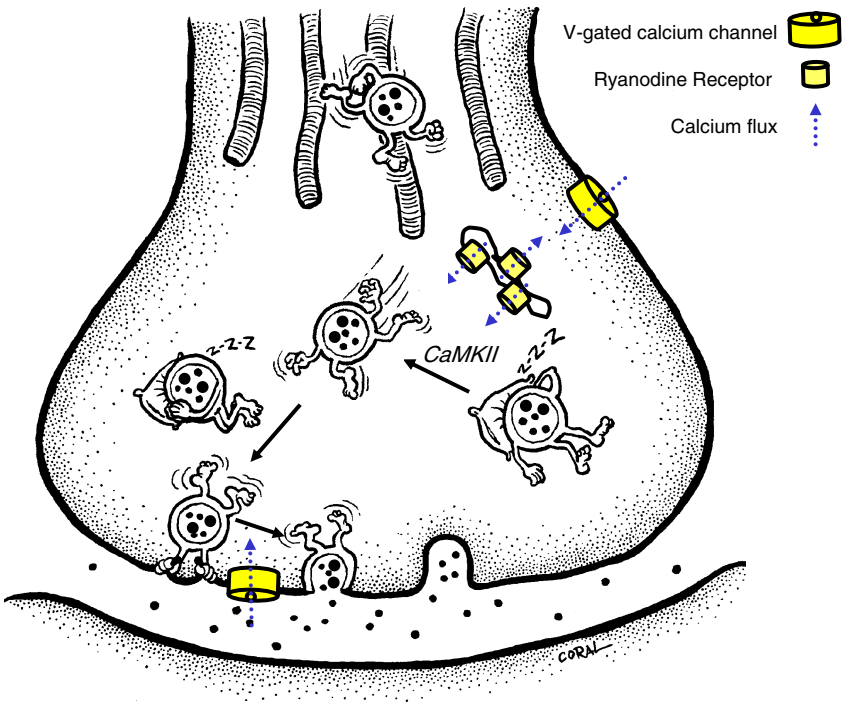

Fig. 4 Model of vesicle mobilization and facilitation of neuropeptide release. Calcium influx through voltage-gated channels elicits calcium release from endoplasmic reticulum via ryanodine receptors, which in turn activates CamKII. The kinase then increases the mobility of secretory vesicles so that they can encounter the plasma membrane more quickly to undergo calcium-evoked exocytosis

CamKII must be a downstream effector of the transiently released Ca^{2+}.

RyR-activated CamKII Induces Vesicle Mobilization and Synaptic Plasticity

As mentioned earlier, increased DCV mobility induced by a conditioning tetanus is correlated with PTP of neuropeptide secretion [2]. Are vesicle mobilization and synaptic plasticity coincidental? To test whether mobilization-inducing signaling has a role in PTP, nerve terminals were treated with inhibitors of CamKII (KN-93) and the RyR (ryanodine), or their associated controls (KN-92 or vehicle, respectively), and then stimulated to induce release. Release evoked by 3-Hz stimulation, which was measured as the decrease in GFP-tagged peptide fluorescence in boutons, was not affected by the inhibitors. This shows that RyR-activated CamKII is not relevant at a low firing rate. However, no PTP was induced after inhibiting CamKII (Fig. 3, KN93) or RyRs (Fig. 3, Ryan). Therefore, CamKII activated by presynaptic RyRs increases DCV mobility and evokes potentiation of neuropeptide release after a tetanus. These results are consistent with the conclusion that RyR-activated CamKII induces DCV mobilization, which in turn contributes to PTP of neuropeptide secretion (Fig. 4).

Conclusions

Shakiryanova et al. reasoned that identifying the signaling that controls vesicle motion would provide the opportunity to test whether inhibiting mobilization affects synaptic plasticity. Synaptic DCV motion and neuropeptide release were imaged in vivo to show that both activity-dependent increases in DCV mobility and PTP in *Drosophila* synaptic boutons require presynaptic ER Ca^{2+} release via RyRs leading to CamKII activation [15]. The use of a single signaling pathway for controlling DCV mobility and release establishes a mechanistic connection between vesicle mobilization and synaptic plasticity. Mobilization of DCVs and SSVs is similar (see "Vesicle Mobilization by Liberation" and "F-actin and Synapsin are not Involved in Mobilization" sections) and synaptic plasticity mechanisms are likely to be evolutionarily conserved. Hence, the RyR–CamKII–vesicle mobilization mechanism discovered in *Drosophila* may facilitate classical neurotransmission and DCV-mediated release of neuropeptides and neurotrophins in mammalian synapses.

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References

1. Benfenati F, Valtorta F, Bähler M, Greengard P (1989) Synapsin I, a neuron-specific phosphoprotein interacting with small synaptic vesicles and F-actin. Cell Biol Int Rep 13:1007–1021
2. Shakiryanova D, Tully A, Hewes RS, Deitcher DL, Levitan ES (2005) Activity-dependent liberation of synaptic neuropeptide vesicles. Nat Neurosci 8:173–178
3. Allersma MW, Bittner MA, Axelrod D, Holz RW (2006) Motion matters: granule motion adjacent to the plasma membrane and exocytosis. Mol Biol Cell 17:2424–2438
4. Gaffield MA, Rizzoli SO, Betz WJ (2006) Mobility of synaptic vesicles in different pools in resting and stimulated frog motor nerve terminals. Neuron 51:317–325
5. Han W, Ng YK, Axelrod D, Levitan ES (1999) Neuropeptide release by efficient recruitment of diffusing cytoplasmic secretory vesicles. Proc Natl Acad Sci USA 96:14577–14582
6. Vömel M, Wegener C (2007) Neurotransmitter-induced changes in the intracellular calcium concentration suggest a differential central modulation of CCAP neuron subsets in *Drosophila*. Dev Neurobiol 67:792–808
7. Gaffield MA, Betz WJ (2007) Synaptic vesicle mobility in mouse motor nerve terminals with and without synapsin. J Neurosci 27:13691–13700
8. Burke NV, Han W, Li D, Takimoto K, Watkins SC, Levitan ES (1997) Neuronal peptide release is limited by secretory granule mobility. Neuron 19:1095–1102
9. Rizzoli SO, Betz WJ (2004) The structural organization of the readily releasable pool of synaptic vesicles. Science 303:2037–2039
10. Ng YK, Lu X, Gulacsi A, Han W, Saxton MJ, Levitan ES (2003) Unexpected mobility variation among individual secretory vesicles produces an apparent refractory neuropeptide pool. Biophys J 84:4127–4134
11. Silverman MA, Johnson S, Gurkins D, Farmer M, Lochner JE, Rosa P, Scalettar BA (2005) Mechanisms of transport and exocytosis of dense-core granules containing tissue plasminogen activator in developing hippocampal neurons. J Neurosci 25:3095–3106
12. Scalettar BA (2006) How neurosecretory vesicles release their cargo. Neuroscientist 12:164–176
13. Ng YK, Lu X, Levitan ES (2002) Physical mobilization of secretory vesicles facilitates neuropeptide release by nerve growth factor-differentiated PC12 cells. J Physiol 542:395–402
14. Degtyar VE, Allersma MW, Axelrod D, Holz RW (2007) Increased motion and travel, rather than stable docking, characterize the last moments before secretory granule fusion. Proc Natl Acad Sci USA 104:15929–15934
15. Shakiryanova D, Klose MK, Zhou Y, Gu T, Deitcher DL, Atwood HL, Hewes RS, Levitan ES (2007) Presynaptic ryanodine receptor-activated calmodulin kinase II increases vesicle mobility and potentiates neuropeptide release. J Neurosci 27:7799–7806
16. Levitan ES, Lanni F, Shakiryanova D (2007) In vivo imaging of vesicle motion and release at the *Drosophila* neuromuscular junction. Nat Protoc 2:1117–1125
17. Jin I, Hawkins RD (2003) Presynaptic and postsynaptic mechanisms of a novel form of homosynaptic potentiation at *Aplysia* sensory motor neurons. J Neurosci 23:7288–7297
18. Yang F, He XP, Russell J, Lu B (2003) Ca^{2+} influx-independent synaptic potentiation mediated by mitochondrial Na^{+}–Ca^{2+} exchanger and protein kinase C. J Cell Biol 163:511–523
19. Kolarow R, Brigadski T, Lessmann V (2007) Postsynaptic secretion of BDNF and NT-3 from hippocampal neurons depends on calcium calmodulin kinase II signaling and proceeds via delayed fusion pore opening. J Neurosci 27:10350–10364