Quantitative analysis of synaptic vesicle release and readily releasable pool size in hippocampal neurons

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Abstract: In central nervous system only a limited number of vesicles exist in the presynaptic terminals. The size and fusion modes of the vesicles were particularly important because of their potential impact on neuronal communications. Efficient methods were needed to analyze the recycling kinetics of synaptic vesicle and the size of readily releasable pool (RRP). In this study, fluorescent dyes with different affinity for membranes (FM1-43 with high affinity and FM2-10 with low affinity) were used to stain the functional synaptic vesicles of cultured hippocampal neurons and the kinetics of vesicle recycling was measured. The results showed that the destaining proportion was larger for FM2-10 than that for FM1-43 during the first trial, while it was greater for FM1-43 than FM2-10 during the second and third trials (first round, 93.0%±5.9% versus 57.9%±3.5% for FM2-10 and FM1-43, respectively, P<0.0001; second round, 1.4%±3.8% versus 24.0%±2.3%, P<0.0001; third round, 2.3%±1.6% versus 8.6%±1.5%, P<0.0005). The results indicated that rapid endocytosis existed not only in the first round but also occurred when the vesicles were reused. Moreover, both high-frequency stimuli and hypertonic sucrose stimuli were used to estimate the RRP sizes in the mix cultured hippocampal inhibitory neurons at 13-14 days in vitro (DIV). We found that the RRP size estimated by hypertonic sucrose stimuli [(200±23.0) pC] was much larger than that estimated by high-frequency stimuli [(51.1±10.5) pC]. One possible reason for the discrepancies in RRP estimates is that in mix cultured conditions, one neuron may receive inputs from several neurons and hypertonic sucrose stimuli will cause RRP of all those neurons release, while using dual patch recording, only the connection between two neurons was analyzed. Thus, to exclude out the impacts of inputs numbers on RRP sizes, it is more reasonable to use high-frequency stimuli to estimate the RRP size in mix cultured neurons.

Key words: endocytosis; synaptic vesicles; whole cell recording; cultured cell; hippocampus

海马神经元突触囊泡释放特征及可释放囊泡含量定量分析

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摘 要：中枢神经系统突触前的神经末梢只有少量的突触囊泡存在，突触囊泡数目的多少和融合模式将影响突触传递的效率。对突触囊泡数目的多少和释放模式的研究依赖于有效的研究方法。在本研究中，与膜亲和力不同的荧光染料用于标记体外培养的海马神经元的功能性突触囊泡。通过场电位和高钾刺激，动态观察荧光强度的变化，结果显示在第一轮刺激中，与膜亲和力低的染料FM2-10脱色的比例(93.0%±5.9%)显著大于与膜亲和力高的染料FM1-43(57.9%±3.5%)，但是，第二和第三轮刺激中FM1-43脱色的比例分别为(24.0±2.3%)和(8.6±1.5%)，显著大于FM2-10的脱色比例[(1.4±3.8)%和(2.3±1.6)%。这个结果提示快速吞噬模式不仅存在于囊泡的第一次释放，同时还存在于囊泡回收后的再次释放。另一方面，高频刺激和高渗蔗糖溶液这两种方法同时用于检测体外混合培养13~14天的抑制性神经元的可释放囊泡池(readily releasable pool, RRP)的大小。
显示，用高渗蔗糖溶液估计的 RRP 的大小[(200±23.0) pC]显著大于用高频刺激估计的 RRP 的大小[(51.1±10.5) pC]。分析其可能的原因是用双patch 的方法分析的是两个神经元之间的联系，而在混合培养的系统中，一个神经元有可能受多个神经元的支配，用高渗溶液刺激则使所有的突触前 RRP 都释放，所以用这种方法计算的 RRP 值要大的多。因此为了排除混合培养的神经元中突触联系的多少对 RRP 值的影响，用高频刺激的方法来估计 RRP 值的大小更合理。

关键词：内吞；突触囊泡；全细胞记录；培养细胞；海马
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Synaptic transmission plays key roles of intercellular communications of neurons in the central nervous system (CNS). The release of neurotransmitters from presynaptic nerve terminals and their subsequent binding to specific postsynaptic receptors are the fundamental events leading to excitatory or inhibitory functions of the CNS. In CNS only a limited number of vesicles exist in presynaptic terminal [1]. The fusion modes of synaptic vesicle are particularly important because of their potential impact on neuronal communications [2]. There are two forms of fusion modes that may take place in CNS [3]. The classical mode is called full-collapse fusion. Vesicles flatten completely into the plasma membrane, lose their identity, and must be replaced eventually by newly generated vesicles. The non-classical mode of fusion is generically termed “kiss-and-run”, in which vesicles fuse transiently with the plasma membrane without complete loss of identity and vesicles retrieve rapidly [4]. The full-collapse fusion requires 8-21 s [5], and the means of vesicles fusion remain controversial for the small synapse containing only ~30 recycling vesicles [6]. Kiss-and-run offers a way out of this kinetic dilemma. Some questions remained unanswered as follows: Can recycled vesicles be reused? Does kiss-and-run exist exclusively in the first round or also exist when the vesicles are reused?

Besides the importance of fusion mode, the size of readily releasable pool (RRP) that immediately available under stimulation also plays important physiological roles [1]. Decreased RRP size contributes to defects of many diseases [7-9], for example, decreased RRP size of inhibitory input from basket cells to granule cells was found in temporal lobe epilepsy [9]. In previous studies, microisland cultured cells were often used to analyze the RRP size [10-12], while aspects of synaptic transmission differ between autaptic and dissociated cultures, autaptic cultures might not constitute an ideal model system to study fusion clamps because of lacking of connectivity [13]. So in our study, both high frequency stimuli and hypertonic sucrose stimuli were used to estimate the RRP sizes in mix cultured hippocampal neurons, and to find out whether there are any differences in the two methods used to estimate RRP sizes.

1 MATERIALS AND METHODS

1.1 Cell culture
Primary cultures of hippocampal neurons were prepared from 1-day-old mice as previously reported [14,15]. Briefly, after careful dissection from diencephalic structures, the hippocampi were chopped and digested in 0.25 % trypsin (Sigma, St. Louis, MO) for 15 min at 37 °C with gentle shaking. Dissociated cells were plated at a density of 2×10⁵ cells/cm² in a 35 mm dish with poly-D-lysine coated coverslips in Dulbecco’s Modified Eagle Media (Invitrogen, Carlsbad, CA) containing 10% FBS, 2 mmol/L glutamine, and maintained at 37 °C in 5% CO₂. After culturing in vitro for 24 h, the medium was replaced with a half and half mixture of DMEM and Neurobasal medium containing 2% B27 supplement, 1% antibiotic, and 0.25% glutamine (Invitrogen). At 5 days in vitro (DIV5), cytosine arabinofuranoside was added with a final concentration of 10 μmol/L. Thereafter, half of the medium was replaced twice a week with Neurobasal medium. Cells were plated on coverslips for fluorescent dye staining and paired cell recordings.

1.2 Dye loading and destaining
To label active synapses, the cultured neurons were loaded with fluorescent marker FM1-43 (5 μmol/L) and FM2-10 (200 μmol/L) (Molecular Probes) in high K+ solution containing (in mmol/L): 58 NaCl, 90 KCl, 10 HEPES, 3 CaCl₂·2H₂O, 8 glucose, and 2 MgCl₂·6H₂O (pH 7.3) for 90 s followed by washing in calcium-free solution for 10 min to reduce nonspecific staining. Time-lapse imaging of fluorescence destaining was recorded. After 60 s of steady-state recording, individual boutons were destained by 20 Hz field stimulation for 120 s. Field stimulation was applied through parallel platinum electrodes immersed into the perfusion chamber, delivering 5 V, 2 ms pulses. After a 120 s rest, two rounds of 90 mmol/L K+ solution were applied in the absence of FM dyes for 120 s with 120 s intervals. During the time-lapse imaging of fluorescence
changes, consecutive frames were acquired at 2 s intervals. All data were acquired and analyzed by Fluoview 500 software. All experiments were done at room temperature.

1.3 Electrophysiological recordings

Dual patch recordings from cultured hippocampal neurons were made as described previously[16]. Neurons cultured for 13-14 days were recorded at room temperature using an EPC-9 patch-clamp amplifier and corresponding Patchmaster software from Heka Electroniks (Germany). Postsynaptic currents (PSC) were recorded with a patch electrode (3-7 MΩ tip resistance) in whole-cell recording mode. Pipette solution contained (in mmol/L) 130.00 K-Glucuronate, 10.00 KCl, 9.00 NaCl, 1.00 MgCl2, 10.00 HEPES, 0.20 EGTA, 2.00 Mg-ATP, 0.30 Na-GTP, adjusted to 300-310 mOsm/L with sucrose. The extracellular solution was a HEPES-buffered saline containing (in mmol/L): 148.00 NaCl, 3.00 KCl, 3.00 CaCl2, 10.00 HEPES, and 8.00 glucose, pH 7.3. Depolarizing pulses (+100 mV, 2 ms) were delivered through the recording pipette to elicit synaptic responses. To control the timing for PSC measurements, synaptic contact between neurons was confirmed by 3 test stimuli, each separated by a 30 s interval. Following the test stimuli, a 3-min rest period was applied before data collection began to ensure that all fusion and recycling machinery had reset to resting levels. For RRP measurements, trains of 40 stimuli delivered at 20 Hz were used to depress the synaptic vesicles. On the other hand, hypertonic sucrose stimulation was also used for RRP measurements. Pulses (4 s) of hypertonic (500 mOsm/L) sucrose were applied near neuronal perikarya in the presence of tetrodotoxin (TTX, 1 μmol/L), 6,7-dinitroquinoxaline-2,3(1H, 4H)-dione (DNQX, 20 μmol/L), and α-amino-5-phosphonopentanoic acid (APV, 25 μmol/L).

The total charge transfer (corresponding to the total area under the current) was analyzed using the Patchmaster software.

1.4 Statistical analysis

Data were expressed as the mean±SEM and statistical significance (P<0.05) was determined by two-tailed Student’s t-test.

2 RESULTS

2.1 FM1-43 was retained in synaptic vesicles even after first fusion

Styryl dye FM1-43 was used to mark the recycling vesicles. 20 Hz field stimulation was used to evoke vesicle exocytosis. The fluorescence loss would fall short of completion (57.9%±3.5%), it is possible that dye retained by rapid vesicular recapture, so after 120 s rest, another two round of stimuli were given with 120 s interval. We found that fluorescence signal hardly changed during the 120 s quiescence between stimuli, but dropped immediately when depolarization was resumed (Fig. 1).

Parallel, FM2-10, another styryl compound with weaker affinity for membranes than FM1-43, was used. It can depart membrane faster[17]. Although destaining was larger for FM2-10 than that for FM1-43 during the first trial, it was greater for FM1-43 than FM2-10 during the second and third trials (first round, 93.0%±5.9% versus 57.9%±3.5% for FM2-10 (n=79) and FM1-43 (n=88), respectively, P<0.0001; second round, 1.4%±3.8% versus 24.0%±2.3%,

![Fig. 1. FM1-43 was retained in synaptic vesicles even after first fusion. A: Smaller ratio of initial destaining for FM1-43 compared to FM2-10 indicated re-internalization of FM1-43. B: Comparison of ratio of destaining. Destaining was larger for FM2-10 than that for FM1-43 during the first trial, while it was greater for FM1-43 (n = 88) than that for FM2-10 (n = 79) during the second and third trials. **P < 0.01 vs FM2-10.](image-url)
P<0.0001; third round, 2.3%±1.6% versus 8.6%±1.5%, P=0.005, Fig. 1). The different destaining kinetics indicated the existence of the non-classical mode of fusion, generically termed “kiss-and-run” in which vesicles fuse transiently with the plasma membrane without complete loss of identity and vesicle endocytosis rapidly.

2.2 Excitatory and inhibitory connections were analyzed by paired recording
To find out whether it is excitatory or inhibitory connection between two neurons, paired recordings were used. Both excitatory and inhibitory connections caused inward currents for the high chloride intracellular solution. The properties of glutamatergic and GABAergic synaptic connections were determined by the time course, reversal potential, and sensitivity of synaptic currents to specific antagonists for non-NMDA receptors and GABA_A receptors, i.e., DNQX and bicuculline, respectively (Fig. 2).

![Image](https://via.placeholder.com/150)

Fig. 2. Excitatory and inhibitory connections were analyzed by paired recording. A: Phase-contrast image showing dual patch recording from cultured neurons for detection of functional synaptic transmissions. Scale bar, 50 μm. B: Sample responses of glutamatergic (excitatory) and GABAergic (inhibitory) synapse. C: The difference of the properties of glutamatergic and GABAergic synaptic connections.

2.3 Development changes of inhibitory connections between neuronal pairs
In this study, we mainly focused on GABAergic synaptic connections. We analyzed inhibitory synaptic connections between neurons from DIV11 to DIV16. No significant change in the ratio of GABAergic connection was found between neurons at DIV11 (0.17±0.10) and DIV12 (0.20 ± 0.14). However, from DIV13 on, significant increase in connections occurred. As shown in Fig. 3, both ratios of inhibitory connections at DIV13-14 (0.53±0.09) and at DIV15-16 (0.53±0.01) were significantly higher than that at DIV11-12 (0.19±0.08). No significant change was found in ratio of inhibitory connections between DIV13-14 and DIV15-16. Thus, DIV13-14 was chosen as the time window to analyze the RRP size of inhibitory neurons.

2.4 Measurement of the RRP of GABAergic synapses
High-frequency stimuli were used to estimate the size of RRP of inhibitory neurons at DIV13-14. A train of 40 stimuli delivered at 20 Hz produced a rapid depression of the response that was followed by a nearly steady state where further depression occurred only very slowly (Fig. 4A). The cumulative area profile of the first 2 s during high-frequency trains of stimuli was analyzed. The cumulative profile of repeated evoked inhibitory postsynaptic currents (eIPSCs) showed a rapid rise followed by a slower linear increase of different steepness at later pulses (Fig. 4B). Assuming that the slow linear rise was attributable to the equilibrium between the release and the constant replenishment of vesicles, back-extrapolation of the linear portion to time 0 yielded the total release minus the total replenishment, corresponding to the RRP [18,19]. Thirty inhibitory neuronal pairs were recorded. The average RRP size was (51.1±10.5) pC. The distribution of the RRP size was shown in Fig. 4C. In cultured neurons, the RRP can also be released in a Ca\(^{2+}\)-independent fashion in response to local application of a hypertonic solution [20]. So, brief (4 s) pulses of hypertonic sucrose (500 mOsm/L) were also applied to measure the RRP size. The average RRP size estimated by hypertonic sucrose stimuli was (200±23.0)
Fig. 4. Measurement of the RRP of GABAergic synapses. A: The first trace illustrates the stimulation pattern. A 20-Hz train on the presynaptic neuron was used to deplete the vesicle recycling pool. The second and third traces show representative inhibitory postsynaptic currents (IPSCs) recorded from the neuron under this stimulation protocol. B: Mean cumulative IPSC area values from trains of 40 stimuli at 20 Hz in thirty neurons. Data points in the range of 1.5-2 s were fitted by linear regression and back-extrapolated to time 0 to estimate the cumulative eIPSC (evoked IPSC) area before steady-state depression. C: Distribution of the inhibitory hippocampal neurons’ RRP at DIV13-14. D: The total RRP charge, as determined with sucrose applications (n = 16) was much larger than that determined with trains of high-frequency stimuli (n = 30). *P < 0.05 vs train.

pC, which was much larger than that estimated by trains of high-frequency stimuli (Fig. 4D).

3 DISCUSSION

Our results demonstrate that rapid endocytosis of vesicles occurs at synapses between hippocampal neurons. The retrieval was swift enough to prevent complete loss of FM1-43 during an initial round of exocytosis. Dye retained by rapid vesicular recapture would continue to mark recycling vesicles but would eventually escape in later exocytotic events, supporting delayed destaining. The incompleteness of the initial dye loss suggests that free diffusion of dye from vesicle to plasmalemma may be restricted by a barrier, as might arise from a fusion pore [21,22]. Moreover, about 24% and 8.6% of FM1-43 intensity was destained during the second and third trials respectively, which are much larger than destaining ratio of FM2-10, indicating that the rapid vesicular recapture may exist not only in the first round but also when the vesicles are reused.

Besides the fusion mode, the RRP size was also analyzed in this study. Two methods were used to analyze the RRP size in mixed cultured hippocampal neurons. One interesting phenomenon was that the RRP size estimated by hypertonic sucrose stimuli was much larger than that estimated by high-frequency stimuli. One possible reason for the discrepancies in RRP estimates is that in mixed cultured neurons, one neuron may receive inputs from several neurons and hypertonic sucrose stimuli will cause RRP of all those neurons release [13], while using dual patch recording, only the connection between two neurons was analyzed. Thus, to exclude out the effects of inputs numbers on RRP sizes, it is more reasonable to use high-frequency stimuli to estimate the RRP sizes in mixed cultured cells.

Another potential advantage of paired recording is that it is easy to combine with molecular biological method, for example to transfect different molecules and analyze the effects of those molecules on the size of RRP. What’s more, it is also easy to find out whether the effect of those molecules is presynaptic or postsynaptic, because we can chose the transfection cell as stimulated cell or response cell.

In conclusion, methods used for quantitative analysis of
the kinetics of synaptic vesicle release and the size of RRP in mix cultured hippocampal neurons were established and rapid endocytosis of vesicles was found not only in the first round but also when the vesicles were reused. Those methods are useful for further analysis of the mechanism of synaptic transmission.

REFERENCES