Chapter 11
Dynamin Is a Key Molecule to Decode Action Potential Firing

Shota Tanifuji and Sumiko Mochida

Abstract The maintenance of neurotransmission relies on the replenishment of synaptic vesicles (SVs) adapted to wide variations in the number and frequency of incoming action potentials (APs). A candidate mechanism for SV recycling indexed to AP firing activity could involve a protein-initiating endocytosis. Dynamin is a GTPase, which mediates fission of SVs from the presynaptic terminal membrane. There are three dynamin isoforms, dynamin 1, 2 and 3, in mammalian neurons. Knockout of dynamin 1 and 3 in central neurons suggests a role of each dynamin isoform in neuronal activity. We have carefully accessed the SV replenishment into the release site (readily releasable pool, RRP) in relation to AP firing activity and dynamin 1, 2 and 3 mediation. The three isoforms in sympathetic superior cervical ganglion neurons, an ideal model for direct physiological measurement of synaptic transmission combined with genetic knockdown, mediate the RRP replenishment, having distinct rate and time constants. Individual isoforms regulate distinct SV recycling pathways that cover the full range of physiological AP frequency. Thus, dynamin 1, 2 and 3 decode AP firing for SV recycling in sympathetic neurons. In this chapter, we review dynamin, in mammalian central and peripheral neurons, that is a key molecule for the selection and regulation of distinct SV recycling pathways in sensing of presynaptic AP firing patterns.

Keywords Dynamin • Action potential firing • Membrane recycling • Synaptic vesicle replenishment

11.1 Introduction

Neurons in both central and peripheral nervous systems encounter a wide range of activities in the form of action potential (AP) firing patterns. In accord, membrane transport systems, in particular, synaptic vesicle (SV) recycling pathways in synapses that mediate fast neurotransmission, must maintain the ability to rapidly react to ongoing changes in AP firing and adjust membrane trafficking to provide sufficient membrane replenishment. SV recycling through repetitive cycles of

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exocytosis and endocytosis is well established to operate under different conditions of AP firing at presynaptic terminals.

AP firing triggers exocytosis of SVs. The SV membrane, fused with the presynaptic nerve terminal membrane, is retrieved by various morphological forms of endocytosis (Ceccarelli et al. 1973; Heuser and Reese 1973; Wu 2004; Wu et al. 2009). For endocytosis, dynamin is an essential protein that mediates fission of SVs from the presynaptic nerve terminal membrane (Shpetner and Vallee 1989; Obar et al. 1990; Takei et al. 1995; Roux et al. 2006). The functional role of dynamin was revealed by *Drosophila* mutant flies carrying a temperature-sensitive allele of dynamin (*Drosophila shibire*) at the nonpermissive temperature (Koenig and Ikeda 1989; Chen et al. 1991; Kuromi and Kidokoro 2002). Since then, numerous evidences have demonstrated for dynamin as a key molecule in the SV endocytosis (Ferguson and De Camilli 2012; Saheki and De Camilli 2012).

In mammals, three isoforms, dynamin 1, 2 and 3, are present (Sontag et al. 1994; Cook et al. 1996; Cao et al. 1998). Each dynamin isoform has distinct expression pattern; dynamin 1 is expressed primarily in brain, whereas dynamin 2 is expressed ubiquitously (Cook et al. 1994, 1996; Cao et al. 1998; Ferguson et al. 2007). Dynamin 3 is also highly expressed in brain and testis (Nakata et al. 1993; Cook et al. 1996; Cao et al. 1998). In the central nervous system (CNS), knockout of dynamin 1 in the mice cortical neurons demonstrated a selective activity-dependent requirement for dynamin 1 in SV endocytosis (Ferguson et al. 2007). Together with following study of dynamin 1 and 3, double knockout suggests that dynamin 1 is required for SV recycling during high-frequency stimulation, and dynamin 3 cooperates with dynamin 1 to support optimal rates of SV recycling (Ferguson et al. 2007; Raimondi et al. 2011). The evidence of dynamin 1 knockout also suggested that dynamin 2 is required for SV recycling after cessation of stimulation (Ferguson et al. 2007).

In the mammalian peripheral nervous system, we have shown roles of dynamin in the fast cholinergic synaptic transmission between rat superior cervical ganglion (SCG) neurons applying the inhibitors (Lu et al. 2009) and genetic knockdown (Tanifuji et al. 2013) combined with direct physiological measurements of the readily releasable pool (RRP) in the active zone. The effects of dynamin inhibitor, dynasore, and peptide perturbing dynamin interaction with amphiphysin suggest that dynamin and synaptic activity regulate SV recycling into the RRP through two distinct pathways (Lu et al. 2009). Moreover, knockdown of each dynamin isoform confirmed the specific selection of SV recycling pathways by activation of individual dynamin in response to various neuronal firing patterns (Tanifuji et al. 2013). With dynamin isoform mediation, a presynaptic terminal covers synaptic transmission in response to full range of physiological AP firing.

This chapter reviews the role of dynamin and its isoforms in SV recycling, especially the molecular mechanisms linking variation in presynaptic neuronal activity to SV recycling. We focus on progressed functional studies of dynamin and its isoforms at presynaptic terminals in the mammalian CNS, at the calyx of Held, hippocampal and cortical neurons and peripheral sympathetic neurons.

11.2 Dynamin Activation in the CNS

Ca^{2+} influx after APs arrived at presynaptic nerve terminal triggers SV exocytosis and also initiates various forms of endocytosis (Gad et al. 1998; Hosoi et al. 2009; Wu et al. 2009). Recent studies at the calyx of Held nerve terminal using Ca^{2+} chelators EGTA and BAPTA suggest the essential role of Ca^{2+} in triggering endocytosis both at the active zone and the peri-active zone (Yamashita et al. 2010). In addition, numerous studies have revealed a modulatory role for Ca^{2+} in endocytosis at nerve terminals in different synaptic preparations (Balaji et al. 2008; Wu et al. 2009; Yao et al. 2009; Yamashita et al. 2010). Interestingly, the rate of SV exocytosis is controlled by Ca^{2+} accumulation following consecutive AP firing (Armbruster et al. 2013). High-sensitivity vGlut 1-pHluorin assays of SV endocytosis in cultured hippocampal and cortical neurons demonstrated that acceleration of endocytosis is followed by the stalling during AP bursts and that the biphasic dependence on electrical activity is blocked by mutation of dynamin at the phosphorylation site (Armbruster et al. 2013). These observations suggest that the rate of endocytosis varies with number and frequency of APs due to dephosphorylation of dynamin following Ca^{2+} influx with repeated AP firing. Thus, dynamin controls optimization of the SVs recycling rate for varying synaptic activity.

Most forms of endocytosis in the physiological range require the protein dynamin, a large GTPase that oligomerizes around the neck of clathrin-coated pits and catalyzes SV fission triggered by GTP hydrolysis (Takei et al. 1995; Marks et al. 2001; Praefcke and McMahon 2004). With the use of the nonhydrolyzable GTP analogue, GTPγS, to inhibit endocytosis (Takei et al. 1995; Song et al. 2004), it reduced synaptic transmission in an activity-dependent manner at the calyx of Held (Yamashita et al. 2005; Xu et al. 2008); however, endocytosis independent on dynamin GTP hydrolysis was also suggested (Xu et al. 2008).

11.2.1 Selective Requirement of Dynamin Isoforms in Activity-Dependent and -Independent SV Recycling

Dynamin 1 is present at high concentrations in presynaptic nerve terminals of the CNS. Ferguson et al. (2007) demonstrated a selective requirement of dynamin 1 in the SV endocytosis only during high-frequency stimulation, but not after cessation of the stimulus train. Genetically engineered mice that lack dynamin 1 are able to form functional synapses, even though their postnatal viability is limited. Dynamin 1 knockout mice appear normal at birth, with near-normal numbers of neurons and SVs. However, they exhibit failure to thrive within several hours after birth and die within 2 weeks of birth. At inhibitory presynaptic nerve terminals of dynamin 1 knockout cortical neurons, electron microscopy analysis revealed massive increase in clathrin-coated pit abundance under spontaneous network activity. Their higher levels of tonic activity lead to a build-up of clathrin-coated

intermediates with perturbation of endocytic protein functions (Hayashi et al. 2008). Under intense stimulation with high-potassium buffer, larger-branched tubular networks connected to the plasma membrane capped by clathrin-coated buds were increased (Ferguson et al. 2007). Visualization of endocytosis by expressing synapto-pHluorin in cultured dynamin 1 knockout cortical neurons shows completely blocked endocytosis during stimulation with 300 APs at 10 Hz, yet resumes at a normal rate after termination of the stimulus. This endocytic blockade during stimulation is fully rescued by dynamin 1 transfection. Synaptic vesicle endocytosis is also rescued by the overexpression of dynamin 3 in dynamin 1 knockout neurons, whereas partially rescued by dynamin 2. Thus, gene knockout studies with the cortical neurons demonstrate that dynamin 1 is required during stimulation, but not indispensable after cessation of stimulation, and that dynamin 3 has a greater functional similarity with dynamin 1. The efficient formation of endosome-like structures in dynamin 1 KO synapses during the high-potassium stimulation, whereas SV recycling in these mutant neurons detected profound endocytic defect during the field stimulation (300 APs at 10 Hz) suggests that different endocytic pathways are activated by dynamin 1/3 and 2 in response to different modes of stimulation (Hayashi et al. 2008).

Furthermore, dynamin 3 plays a role in supporting optimal rates of SV endocytosis by cooperating with dynamin 1 (Raimondi et al. 2011). Dynamin 3 knockout mice do not show any obvious pathological phenotype observed in dynamin 1 knockout mice; however, dynamin 1 and dynamin 3 double-knockout mice have a more severe phenotype than dynamin 1 single-knockout mice. They exhibit lack of milk in the stomach and hunched posture 2 h after birth and die within several hours after birth. Compared to dynamin 1 knockout synapses, the double-knockout nerve terminals in cultured cortical neurons show more severe endocytic defect, confirmed by a massive accumulation of clathrin-coated pits at presynaptic nerve terminals and by a delay in compensatory endocytosis in response to a stimulus monitored with vGlut 1-pHluorin, a pH-sensitive probe that is very efficiently targeted to SVs (Balaji and Ryan 2007). However, consecutive rounds of stimulation at 10 Hz with the interval of 10 min show that, given sufficient time, vGlut 1-pHluorin signals recover in dynamin 1- and dynamin 3 double-knockout neurons albeit at a much reduced rate, indicating a contribution of dynamin 2 to SV recycling. Overall, in the CNS, overlapping roles of dynamin 1 and dynamin 3 in SV endocytosis related to high synaptic activity and a role of dynamin 2 and/or dynamin-independent mechanisms in basic SV endocytosis have been suggested.

11.2.2 Dynamin-Mediated Ultrafast Endocytosis

SVs are thought to be regenerated from plasma membrane approximately 20 s after fusion by the classic clathrin-mediated endocytosis (Heuser and Reese 1973) or within 1 s by kiss-and-run endocytosis that retrieves fusing vesicles by reversing their neck (Ceccarelli et al. 1973; Zhang et al. 2009). However, combination of

optogenetics and rapid high-pressure freezing, 'flash-and-freeze' approach, allows for visualizing synaptic ultrastructure with electron microscopy following a single physiological stimulation. This approach revealed an ultrafast mode of endocytosis, which occurs within 50 to 100 ms after the onset of the stimulus, requiring actin and dynamin (Watanabe et al. 2013a). In cultured mouse hippocampal neurons expressing channelrhodopsin-2, a light-activated channel protein, a 10-ms pulse of blue light elicits an influx of sodium ions, leading to an AP. Within 30 ms of the stimulus, docked vesicles fuse and collapse into the membrane. These fusions are observed within the active zone, and vesicle-depleted active zones are repopulated with a full complement of docked vesicles with a time constant of 3.8 s. This value is consistent with that for the recovery of the RRP previously measured electrophysiologically in mouse hippocampal neurons (Pyott and Rosenmund 2002). Following exocytosis, the shallow invaginated pits occur at locations outside the active zone within 50 ms after stimulation. In addition, within 50–100 ms, large vesicles which have about fourfold greater surface area than normal SVs are generated. This compensatory endocytosis seems not to be mediated by clathrin coats and is inhibited by latrunculin A which disrupts polymerization of actin and dynasore which interferes with the GTPase activity of dynamin. Thus, the ultrafast endocytosis appears to compensate the membrane added to the plasma membrane immediately after exocytosis, in clathrin-independent but actin- and dynamin-dependent manner.

11.2.3 Role for Dynamin in Exocytic-Endocytic Coupling

Several recent studies interfering with the function of endocytic proteins have reported inhibitory effects on SV exocytosis, indicating a role for endocytic proteins in regulation of SV exocytosis (Saheki and De Camilli 2012). Dynamin dysfunction with *Drosophila shibire* mutant (Kawasaki et al. 2000) and that in the calyx of Held (Hosoi et al. 2009) demonstrated that rapid dynamin action is required for synaptic transmission in response to repetitive firing of APs, indicating that clearance of used SVs including the cargo proteins from the active zone is triggered by dynamin activation. This step is the rate limiting for transmitter release, and delayed clearance leads to the short-term synaptic depression (i.e. gradual decrease in transmitter release in response to each AP firing) (Hosoi et al. 2009; Neher 2010). Dynamin dysfunction by dynasore, anti-dynamin antibody or dynamin inhibitory peptide which perturbs dynamin binding to the SH3 domain of amphiphysin delayed SV recruitment to docking sites and enhanced short-term synaptic depression at the calyx of Held. Another study with measurements of capacitance, which is proportional to net changes in neuron surface area and thus constitute measurement of synaptic exocytosis and endocytosis, also showed that dynasore slows down the RRP replenishment 100 ms after the first pulse (Wu et al. 2009). Clearance of the active zone could be achieved by rapid lateral diffusion of SV proteins towards the peri-active zone, an area that surrounds the

active zone and into which SV membranes are recycled following exocytosis (Roos and Kelly 1999; Haucke et al. 2011). In consistent, ultrafast endocytic invaginations are outside the active zone and are often observed flanking the active zone (Watanabe et al. 2013a). Thus, exocytic-endocytic coupling could be achieved by the direct dynamin-mediated retrieval of SVs near the exocytic site.

11.3 Dynamin Activation in SCG Neurons

Evidence in a large presynaptic terminal of the calyx of Held (Yamashita et al. 2005, 2010; Hosoi et al. 2009; Neher 2010) and other central neurons (Newton et al. 2006; Ferguson et al. 2007; Raimondi et al. 2011; Hayashi et al. 2008) provides a general role for dynamin and its isoforms in SV recycling to regulate synaptic transmission in the CNS. We have used a model system for the genetic analysis of fast cholinergic transmission between rat SCG neurons to examine the role of dynamin isoforms in activity sensing (Tanifuji et al. 2013). The SCG neurons are a useful model to study presynaptic terminal proteins because of the large cell body and nucleus for the manipulation of gene expression and function via acute microinjection of siRNA, peptides and antibodies (Mochida et al. 1994, 2008; Ma et al. 2009), an approach not technically feasible for cultured neurons from the central nervous system. Dynamin function was disrupted by inhibitors (Sect. 11.3.1), or each isoform was knocked down by the specific siRNAs (Sects. 11.3.2, 11.3.3, 11.3.4, and 11.3.5) injected into presynaptic neurons. These neurons were challenged with various AP firing patterns, and the resultant changes in the replenishment of the RRP were monitored by recording evoked excitatory postsynaptic potentials (EPSPs).

11.3.1 Sympathetic Neurons Maintain Synaptic Transmission via the SV Recycling Through Dynamin-Mediated Pathways

In SCG neurons, dynamin dysfunction by either P4 peptide (QVPSRPNRAP) which interferes linkage of dynamin to clathrin coats through interaction with amphiphysin or dynasore, a specific cell-permeable dynamin inhibitor, impaired synaptic transmission with various presynaptic AP firing patterns, such as paired AP and consecutive APs at low or high frequency (Lu et al. 2009). These electrophysiological recordings from P4- or dynasore-treated SCG neurons demonstrated that sympathetic neurons have two dynamin-mediated SV replenishment pathways, one activity dependent and the other activity independent (Lu et al. 2009). In addition, the differential mechanisms of P4 and dynasore for dysfunction of dynamin suggest that the RRP refills at two time constants, fast and slow, with

the RRP refilling through distinct dynamin-mediated pathways. The fast refilling rate reflects a rapid SV replenishment into the RRP from the reserve pool (RP) in an amphiphysin-dependent endocytic pathway, whereas the slow refilling rate involves a gradual SV replenishment through an amphiphysin-independent endocytic pathway (Lu et al. 2009). Together, these data provide evidence for physiological and molecular heterogeneity in endocytosis.

11.3.2 Dynamin 1, 2 and 3 Differentially Mediate Rapid SV Recycling in Sympathetic Neurons

All three isoforms of dynamin are expressed in peripheral SCG neurons (Tanifuji et al. 2013), similarly to central neurons (Cao et al. 1998; Ferguson et al. 2007). Dynamin isoforms were specifically knocked down in presynaptic neurons by microinjection of siRNA, and the transfected neurons were challenged with various AP firing patterns. Applying a paired-pulse protocol to assess rapid replenishment of the RRP, changes in EPSP amplitude after an AP-evoked transmitter release were monitored by eliciting two consecutive APs at various interstimulus intervals (ISIs) in the siRNA-injected neuron (Fig. 11.1a, left). Under control conditions, at the synapses injected with control siRNA (control), paired-pulse ratio (2nd/1st EPSP ratio) values were less than 1.0 (paired-pulse depression) at short ISIs ($\leq$100 ms), whereas at longer ISIs paired-pulse ratio values were ~1.0, in agreement with our previous studies (Lu et al. 2009; Ma et al. 2009). Shown in Fig. 11.1a, right, dynamin 1 knockdown (KD) increased paired-pulse depression at short ISIs (50–100 ms), but did not increase it at ISIs of 20 or 30 ms. On the other hand, dynamin 2 or 3 KD increased paired-pulse depression at short ISIs (20–100 ms). At longer ISIs (100 ms $\leq$), however, paired-pulse ratio values at dynamin 1, 2 or 3 KD synapses were 60–70 % of that for control. It is unlikely that basal release probability is affected by dynamin loss of function because the mean EPSP amplitude of the first recording was unchanged. Therefore, in contrast to findings in central neurons of dynamin knockout mouse, each dynamin isoform differently contributes to rapid SV recycling.

The rate of endocytosis is generally thought to range from hundreds of milliseconds to hundreds of seconds (Royle and Lagnado 2003; Wu 2004), estimated by capacity measurements (Sun et al. 2002) and synapto-pHluorin imaging (Fernandez-Alfonso and Ryan 2004). However, recent studies in cultured hippocampal neurons (Watanabe et al. 2013a) and at *Caenorhabditis elegans* neuromuscular junctions (Watanabe et al. 2013b) have provided evidence that, by optogenetics coupled with high-pressure freezing, dynamin-mediated SV endocytosis occurs on a millisecond timescale following a single physiological stimulus (see Sect. 11.2.2). The 'flash-and-freeze' electron microscopy revealed that the ultrafast mode of endocytosis occurs within 50–100 ms after a single pulse of blue light, consistent with rapid temporal windows of dynamin-mediated SV recycling in SCG neurons.

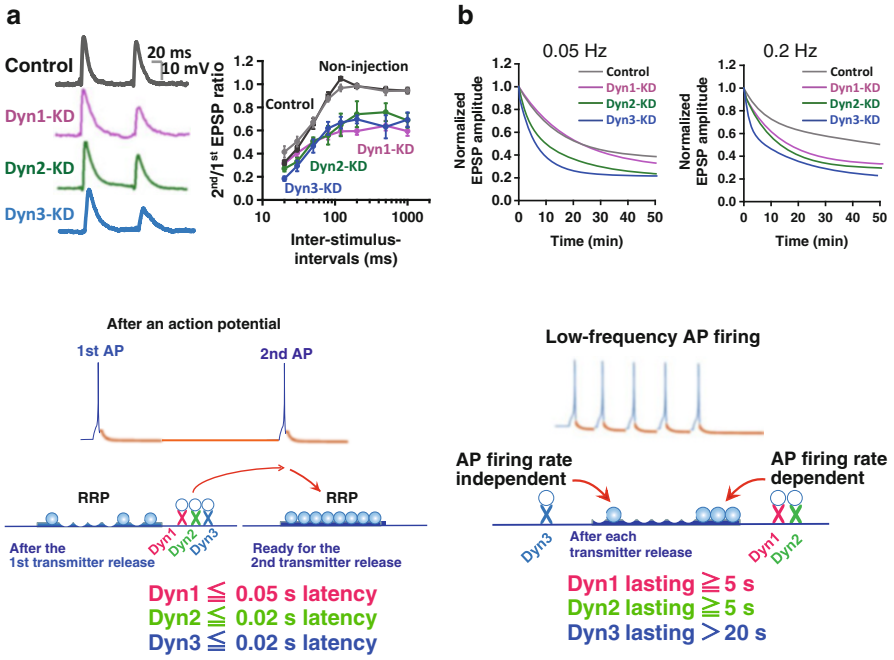

Fig. 11.1 The RRP replenishment mediated by dynamin 1, 2 or 3 under low-frequency AP firing. Presynaptic neurons were injected with control or dynamin siRNA (control, Dyn1-KD, Dyn2-KD or Dyn3-KD). (**a**) Changes in EPSP amplitude after an AP-evoked transmitter release were monitored by eliciting two consecutive APs at various ISIs (20–1,000 ms), every 1 min, in the siRNA-injected neuron. (*left*) EPSPs from a representative recording with an interstimulus interval of 120 ms. (*right*) The averaged paired-pulse ratio (2nd/1st EPSP ratio) is plotted against the ISIs. Dyn1-KD increased paired-pulse depression (PPD) at ISIs of 50–1,000 ms, whereas Dyn2- or 3-KD increased PPD at ISIs of 20–1,000 ms. (*Scheme*) Results of paired-pulse ratio demonstrate an asymmetry in the involvement of dynamin in SV recycling with a distinct latency after AP generation. After the first neurotransmitter release, dynamin 1, 2 or 3 is activated with a latency of $\leq$0.05, $\leq$0.02 and $\leq$0.02 s, respectively, to replenish the RRP ready for the second transmitter release. (**b**) Changes in the EPSP amplitude elicited by consecutive presynaptic APs at 0.05 or 0.2 Hz were monitored over 50 min. (*left*) Single exponential decay curves fitted with the averaged EPSP amplitudes at 0.05 Hz were reduced more rapidly with Dyn3-KD than Dyn1- or 2-KD. (*right*) Single (control, Dyn1-KD or Dyn2-KD) or double (Dyn3-KD) exponential decay curves fitted with averaged EPSP amplitudes at 0.2 Hz were reduced more rapidly with each dynamin-KD. (*Scheme*) During low-frequency AP firing, dynamin 1 and 2 are activated dependent on AP firing rate $\geq$0.2 Hz, while dynamin 3 is activated independent on the AP firing rate. The RRP replenishment process mediated by dynamin 1 and 2 lasts 5 s, while that mediated by dynamin 3 lasts more than 20 s

11.3.3 Distinct Roles of Dynamin 1, 2 and 3 During High- or Low-Frequency AP Firing in Sympathetic Neurons

Endocytosis is essential for maintaining transmitter release during repetitive AP firing (Wu 2004). At cultured hippocampal synapses expressing synapto-pHluorin

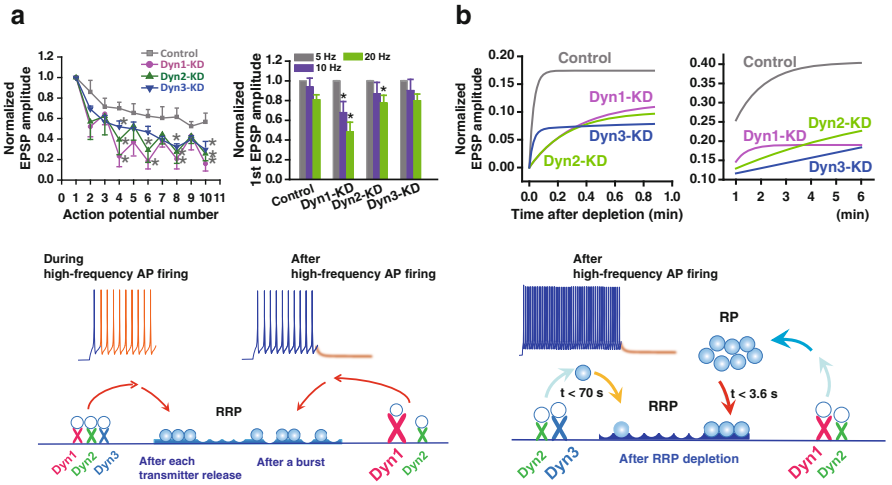

Fig. 11.2 The RRP replenishment mediated by dynamin 1, 2 or 3 under high-frequency AP firing. (**a**) EPSPs elicited by 2-s AP trains at 5, 10 or 20 Hz were monitored every 2 min, 3 times for each frequency. (*left*) Normalized and averaged peak amplitudes from the end of the previous EPSP at 10 Hz were plotted against AP number, indicating that each dynamin isoform contributes to SV recycling during high-frequency AP firing. (*right*) Normalized 1st EPSP amplitudes of each train to that of the 5-Hz train decreased 2 min after a 5- or 10-Hz AP train in Dyn1-KD neurons and after 10-Hz AP train in Dyn2-KD neurons. (*scheme*) During high-frequency AP firing, each dynamin isoform contributes to SV recycling in the RRP, while dynamin 1 and 2 also mediate it after high-frequency AP firing. (**b**) The recovery of SVs in the RRP after full depletion due to a 4-min AP train at 5 Hz was monitored by measuring the EPSP amplitude every 1 s. EPSP amplitudes normalized to the mean value before the depletion were averaged and fitted with single exponential growth curves to estimate the recovery rate and the time constant. (*left*) Dyn1- or 2-KD delayed the fast recovery phase. (*right*) Dyn2- or 3-KD delayed the slow recovery phase. (*Scheme*) After high-frequency AP firing dynamin 1 drives the fast RRP replenishment pathway via RP, whereas dynamin 3 drives the slow replenishment pathway. Dynamin 2 contributes to driving both fast and slow replenishment pathways. Time constant for the fast replenishment of empty RRP with SVs from the RP is <3.6 s, while that of the slow RRP replenishment is <70 s in presynaptic SCG neurons

(Fernandez-Alfonso and Ryan 2004), fast endocytosis occurs during AP firing, although it is slowed down after repetitive stimulation (Sun et al. 2002), suggesting that AP firing frequency at nerve terminal determines the time course of endocytosis. In SCG neurons, dynamin 1, 2 or 3 KD induced severe synaptic depression at 5 or 10 Hz (Fig. 11.2a, left) AP train, in contrast to control synapses that only induced synaptic depression at higher frequency of 20 Hz (Tanifuji et al. 2013). In addition, dynamin 1 KD increased the number of EPSP failures during 2-s AP trains at high-frequency AP firing. Therefore, our results suggest that each dynamin isoform contributes to maintain SV replenishment during high-frequency AP firing. At 2 min after 5 or 10-Hz AP train, the recovery of first EPSP amplitude of each train from depression was impaired with dynamin 1 KD and weakly with dynamin

2 KD (Fig. 11.2a, right), suggesting that dynamin 1 and 2 mediate longer-lasting, at least 2 min, SV recycling after high-frequency AP firing.

In addition to the effect of dynamin isoform KD on SV replenishment during high-frequency AP firing, we also found that at control synapses, synaptic transmission gradually depressed over the 50-min-recording session under low-frequency presynaptic AP firing at 0.05 or 0.2 Hz (Fig. 11.1b) but was reduced more rapidly with dynamin 1, 2 or 3 KD than control level of depression following repetitive APs at 0.2 Hz (Fig. 11.1b, right). Dynamin 3 KD also showed a rapid reduction of the EPSP amplitudes at a much lower AP firing frequency, 0.05 Hz (Fig. 11.1b, left). Together, these data suggest that dynamin 1- or 2-mediated SV replenishment is activated by AP firing at 0.2 Hz and completes within 5 s after AP generation, in an AP firing rate-dependent manner, whereas dynamin 3-mediated SV replenishment is activated by AP at lower than 0.05 Hz and takes more than 20 s, in an AP firing rate-independent manner.

11.3.4 Dynamin 1, 2 and 3 Have Distinct Roles in SV Recycling Pathways with Distinct Kinetics

Multiple recycling pathways are used by SVs with the selection of pathway depending on AP firing frequency (Granseth et al. 2006; Lu et al. 2009; Zhang et al. 2009; Zhu et al. 2009; Cheung et al. 2010). The classic clathrin-mediated endocytic pathway is responsible for the RRP refilling via an RP, whereas the fast mode of endocytosis involves rapid reuse pathway of SVs that bypass the RP (Lu et al. 2009). As discussed above, the fast SV replenishment of the RRP involves SV transport from the RP via dynamin-dependent and clathrin-mediated pathway or de novo sorting via an endosomal pool, whereas the slow SV replenishment of the RRP involves another mode of SV recycling, which bypasses the RP via dynamin-mediated pathway (Lu et al. 2009). The kinetics of refilling of the RRP with SVs following full RRP depletion due to a train of 4-min APs at 5 Hz was monitored by measuring the EPSP amplitude every 1 s. The refilling rate of the fast phase was delayed with dynamin 1 or 2 KD, whereas that of the slow phase was delayed with dynamin 2 or 3 KD, respectively (Fig. 11.2b). Thus, in sympathetic neurons, dynamin isoforms have distinct specificity for fast and slow recycling pathways. Taken together, dynamin 1 mainly regulates the RRP replenishment with a rapid rate for the selective recovery of SV depletion during and after high-frequency AP firing, whereas dynamin 3 mainly regulates the RRP replenishment at a slow rate, mediating a separate endocytic pathway in an AP firing frequency-independent manner. Dynamin 2 exhibits a contribution equally to both rapid and slow replenishment.

11.3.5 Dynamin 1, 2 and 3 Decode AP Firing for SV Replenishment into the RRP

How dynamin 1, 2 and 3 contribute to the RRP replenishment via distinct SV recycling pathways linking variation in neuronal activity is speculated in the schemes (Figs. 11.1 and 11.2). In SCG neurons, individual dynamin isoforms regulate distinct SV reuse pathway that covers the full range of physiological AP firing frequency patterns for the selection of specific vesicle reuse modes that replenish a shared RRP. Dynamin 1-mediated SV recycling pathway is primarily activated during and after high-frequency AP firing at >5 Hz with a latency of 0.05 s and lasting 5 s after AP generation. In contrast, dynamin 3-mediated SV recycling pathway supports a separate mode of the RRP replenishment that was independent of AP firing frequency and rapidly activated within 0.02 s after AP generation. Dynamin 2 mediates both SV recycling pathways, with hybrid properties between the other isoforms. Overall, fine-tuning mechanisms of SV recycling dependent on dynamin isoforms allow synapses to maintain stable neurotransmission during dynamic changes in AP firing properties.

11.4 Concluding Remarks

In mammals, dynamin is encoded by three different genes, whose products undergo further alternative splicing to generate a multiplicity of variants (Cao et al. 1998). Given the differences in the expression pattern between dynamin isoforms and the unique protein-protein interactions of each isoform, additional studies are required to fully address the issue of allocation between dynamin isoforms in the nervous systems, even though some studies in non-neuronal cells demonstrate that each dynamin isoform regulates distinct endocytic pathways (Shpetner and Vallee 1989; Artalejo et al. 2002; Lu et al. 2008). At central synapses, knockout studies of dynamin isoforms revealed that clathrin-mediated endocytosis is tailored to work well under different conditions of synaptic activity which activates specific dynamin (Ferguson et al. 2007; Raimondi et al. 2011). However, while these studies collectively establish a general role for dynamin and its isoforms in membrane recycling, several important questions remain unanswered. First, how do the three dynamin isoforms collaborate in single synapses to maintain stable SV recycling pools in response to AP firing properties? To address this question would require synaptic electrophysiological methods to measure ongoing synaptic transmission and quantitate the recovery of the RRP after stimulation. Second, how do individual dynamin isoforms allocate specific time windows and rate constants to allow a cell to respond to variable patterns of electrical stimulation? To address this question would necessitate an acute dynamin knockdown rather than a mouse knockout approach, for which genetic compensation may occur. Moreover, to address the role of dynamin isoforms in RRP maintenance, the study should be done in a system in

which dynamin loss-of-function specifically occurs in the presynaptic but not postsynaptic cell to avoid complications from dynamin-dependent postsynaptic regulation. In SCG neuron, dynamin isoforms were specifically knocked down in presynaptic neurons by microinjection of siRNA. The transfected neurons were challenged with various AP firing patterns, and the resultant changes in the recovery of readily releasable SVs were monitored by recording evoked EPSPs. The studies revealed distinct dynamin isoforms controlling unique vesicle pools may allow synapses to dynamically respond to rapid or complex APs burst into EPSPs that reflect a cell's history of synaptic firing, while simultaneously maintaining the general capability for fast and stable SV recycling and maintenance after sparse single APs. Whether there is a spatial synaptic organization to dynamin isoform-dependent recycling pathways remains a challenging future issue.

Ultrastructually, the recent study using 'flash-and-freeze' microscopy (Watanabe et al. 2013a) supports the proposal that dynamin mediates exocytic-endocytic coupling to clear the vesicle release site for the next set of exocytosis (Hosoi et al. 2009). Dynasore used for the study on exocytic-endocytic coupling is reported to inhibit the GTPase activity of dynamin 1, dynamin 2 and Drp 1, the mitochondrial dynamin (Macia et al. 2006), but not dynamin 3. Thus, which dynamin isoform mediates the clearance pathway remains to be determined. The new 'flash-and-freeze' microscopy technique (Watanabe et al. 2013a) also supports our findings that dynamin isoforms differently drive SV recycling with a more rapid time window than that estimated by membrane capacity measurements (Sun et al. 2002) and vesicle trafficking with fluorescence imaging (Fernandez-Alfonso and Ryan 2004). The rapid activation of dynamin isoforms covers the range of AP frequency patterns for the selection of specific vesicle reuse modes; however, the molecular mechanism underlying the relationship of dynamin isoforms and sensing cell activity will require further investigation.

Increase in intracellular Ca^{2+} accompanying AP firing at presynaptic nerve terminals plays a critical role in SV endocytosis and efficient replenishment of the RRP (Wu et al. 2009; Denker and Rizzoli 2010; Yamashita 2012). Thus, identification of Ca^{2+} sensor proteins is essential to understand the molecular mechanism for the selection of dynamin isoforms, sensing cell activity, to drive the specific SV recycling pathway. In this regard, synaptotagmin is one of the candidate molecules to play a role in fast endocytosis (Poskanzer et al. 2003; Nicholson-Tomishima and Ryan 2004; Watanabe et al. 2010), which forms complexes with presynaptic proteins such as AP2 (Takei et al. 1999), a clathrin coat protein that interacts with dynamin. On the other hand, endocytic proteins including dynamin are phosphoproteins. Upon AP firing at presynaptic nerve terminals in the brain, dynamin is dephosphorylated by the Ca^{2+}/calmodulin (CaM)-dependent phosphatase calcineurin (Cousin and Robinson 2001; Sun et al. 2010), suggesting that CaM, calcineurin and endocytic phosphoproteins represent Ca^{2+} sensors, mediators, and effectors, respectively, for slow endocytosis. The low- and high-affinity Ca^{2+} sensors seem to be used to drive endocytosis at different synapses or different developmental stages. At the calyx of Held synapses, Ca^{2+} elevation in the Ca^{2+} nano-domain around Ca^{2+} channels triggers dynamin-mediated endocytosis

via low-affinity Ca^{2+} binding molecules, whereas bulk Ca^{2+} elevation outside of the Ca^{2+} nano-domain, peri-active zone triggers clathrin-mediated endocytosis via Ca^{2+}/CaM/calcineurin mediation at immature synapses, but not in mature synapses (Yamashita et al. 2010). In summary, given that specific interactions between dynamin isoforms and Ca^{2+} sensor proteins must initiate SV endocytosis dependent on presynaptic activity, it remains a challenging future issue to elucidate the molecular mechanisms.

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