Cellular/Molecular

Rab3 Superprimes Synaptic Vesicles for Release: Implications for Short-Term Synaptic Plasticity

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Presynaptic vesicle trafficking and priming are important steps in regulating synaptic transmission and plasticity. The four closely related small GTP-binding proteins Rab3A, Rab3B, Rab3C, and Rab3D are believed to be important for these steps. In mice, the complete absence of all Rab3s leads to perinatal lethality accompanied by a 30% reduction of probability of Ca^{2+}-triggered synaptic release. This study examines the role of Rab3 during Ca^{2+}-triggered release in more detail and identifies its impact on short-term plasticity. Using patch-clamp electrophysiology of autaptic neuronal cultures from Rab3-deficient mouse hippocampus, we show that excitatory Rab3-deficient neurons display unique time- and frequency-dependent short-term plasticity characteristics in response to spike trains. Analysis of vesicle release and repriming kinetics as well as Ca^{2+} sensitivity of release indicate that Rab3 acts on a subset of primed, fusion competent vesicles. They lower the amount of Ca^{2+} required for action potential-triggered release, which leads to a boosting of release probability, but their action also introduces a significant delay in the supply of these modified vesicles. As a result, Rab3-induced modifications to primed vesicles causes a transient increase in the transduction efficacy of synaptic action potential trains and optimizes the encoding of synaptic information at an intermediate spike frequency range.

Key words: **synaptic transmission; hippocampus; exocytosis; vesicle trafficking; release probability; GTP binding proteins**

Introduction

Rab proteins are the largest family of ras-related GTPases (Pereira-Leal et al., 2001). These are known to act as directional molecular switches at all membrane trafficking events in eukaryotic cells. They are localized on the donor membrane and mediate contact with the target acceptor membrane by recruiting their effectors. Identified functions include the recruitment of SNARE complexes (Rab1), tethering factors (Rab5), or motor proteins (Rab6). The most abundant Rab in brain, localized on synaptic vesicles, is Rab3a. Of all mammalian Rab proteins, it shows the highest degree of simililarity to SEC4, which in yeast is essential for exocytosis. Four Rab3 paralogs (Rab3A, Rab3B, Rab3C, and Rab3D) are expressed in the brain (Touchot et al., 1987; Zahraoui et al., 1989; Baldini et al., 1995; Schluter et al., 2002) and are apparently functionally redundant. Although removal of individual Rab3 isoforms does not affect viability or fertility, genetic deletion of all four rab3 isoforms in mice causes perinatal lethality possibly by respiratory failure (Schluter et al., 2004). The function of Rab3 at the synapse is to regulate vesicular release probability. In excitatory hippocampal neurons, removal of all Rab3s lead to a 30 and 40% reduction of the mean vesicular and synaptic release probability without affecting the number of primed vesicles (Schluter et al., 2004). The documented reduction in the efficiency of synaptic output for single action potentials will likely affect also the dynamics of postsynaptic response amplitudes during spike trains. Although short-term plasticity (STP) characteristics are governed by multiple factors, synaptic release probability P_r has the greatest impact, because synapses with high P_r tend to depress during spike trains, because readily available vesicles are more quickly consumed as they are replenished. Conversely, synapses with low initial release probability tend to facilitate, because the increase of intraterminal Ca^{2+} helps to release vesicles that were not as easily released during previous action potentials. Other factors that affect STP include feedback activation of presynaptic receptors (von Gersdorff et al., 1997), presynaptic Ca^{2+} handling (Edmonds et al., 2000; Rozov et al., 2001; Blatow et al., 2003; Sippy et al., 2003), heterogeneous release probability (Trommershauser et al., 2003), Ca^{2+}-dependent Ca^{2+} channel inactivation (Xu and Wu, 2005), and postsynaptic receptor desensitization (Otis et al., 1996). In addition, spike train-dependent increases of intraterminal Ca^{2+} can accelerate vesicle priming (Stevens and Wesseling, 1998). To identify the particular phase of Rab3 action on the vesicular release process and to gain insights into the exact mechanism of Rab3-dependent STP via modulation of vesicular release probability, we used quadruple Rab3A, Rab3B, Rab3C, and Rab3D mice and analyzed synaptic transmission in autaptic neuronal cultures derived from

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the hippocampus. We found that Rab3 proteins play a major and unexpected role in short-term synaptic plasticity. Rab3 proteins act to "boost" the apparent Ca^{2+} sensitivity of a subset of vesicles in the readily releasable pool (RRP), thereby introducing a functional heterogeneity among the primed vesicles. As a result, the reliability of synaptic responses for low-frequency presynaptic spiking activity is particularly heightened. Among many possible consequences, this may help to increase accuracy of sensory information coding.

Materials and Methods

Mouse breeding and genotyping. The Rab3A, Rab3B, Rab3C, and Rab3D knock-out (KO) mice were bred and genotyped as described previously (Schluter et al., 2004).

Cell culture. Mouse embryos of embryonic day 19 (E19) Rab3A $^{+/-}$ or Rab3A $^{+/-}$BCD $^{-/-}$ timed pregnancies were delivered by cesarean section. Hippocampi from the entire litter were isolated and cultured in a chemically defined media (Neurobasal medium A; Invitrogen, San Diego, CA) supplemented with B27 (Invitrogen) on glial microdot islands (Bekkers and Stevens, 1991; Pyott and Rosenmund, 2002). Islands containing single neurons forming recurrent synapses (autapses) were used after 9–16 d in culture. In all analyses, cultures from the respective KO mice and their littermate control mice were initially established and later analyzed on the same days to avoid culture artifacts (Fernandez-Chacon et al., 2001).

Electrophysiology. Synaptic transmission was recorded in whole-cell configuration under voltage-clamp at −75 to −85 mV (Axopatch 200B; Molecular Devices, Union City, CA) at room temperature (~22–24°C). Synaptic transmission was induced by depolarization to 0 mV for 2 ms, and the triggered synaptic responses were recorded with a sampling rate of 10 kHz filtered at 2 kHz. The standard extracellular recording solution contained the following (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 $CaCl_2$, 4 $MgCl_2$, pH 7.3, with an adjusted osmotic strength of 300 mOsm. Patch pipettes (2.5–3.5 mΩ) were filled with internal solution containing the following (in mM): 125 K-gluconate, 10 NaCl, 4.6 $MgCl_2$, 4 ATP-Na_2, 0.3 GTP-Na_2, 15 creatine phosphate, 20 U/ml phosphocreatine kinase, 1 EGTA, pH 7.3, also adjusted to 300 mOsm. Solutions were applied using a fast flow application system (Pyott and Rosenmund, 2002). Sucrose (500 mM) was added to the standard extracellular recording solution for the measurement of size and refilling kinetics of the readily releasable vesicle pool. Relative changes in the Ca^{2+} dependence of EPSC amplitudes were determined by applying an external solution with an initial high release probability (12 mM $CaCl_2$, 1 mM $MgCl_2$) and the effect of subsequent reduction of Ca^{2+} influx on EPSC amplitude was determined by alternating between control solution and solutions containing variable Cd^{2+} concentrations (1–100 μM). In this manner, EPSC amplitude changes caused by possible divalent concentration-dependent alteration in axonal excitability was excluded. All chemicals were purchased from Sigma with the exception of EGTA-AM (Calbiochem, La Jolla, CA).

Data analysis. The synchronous and asynchronous component of release during high-frequency stimulation was determined as described, without normalizing to the first EPSC (Hagler and Goda, 2001; Pyott and Rosenmund, 2002). Statistical analyses were performed using Student's *t* test, and significance was considered when $p < 0.05$. For data fitted with exponential curves, the goodness of fit for each curve fitting was determined by comparing both the χ^2 and sum of squared errors.

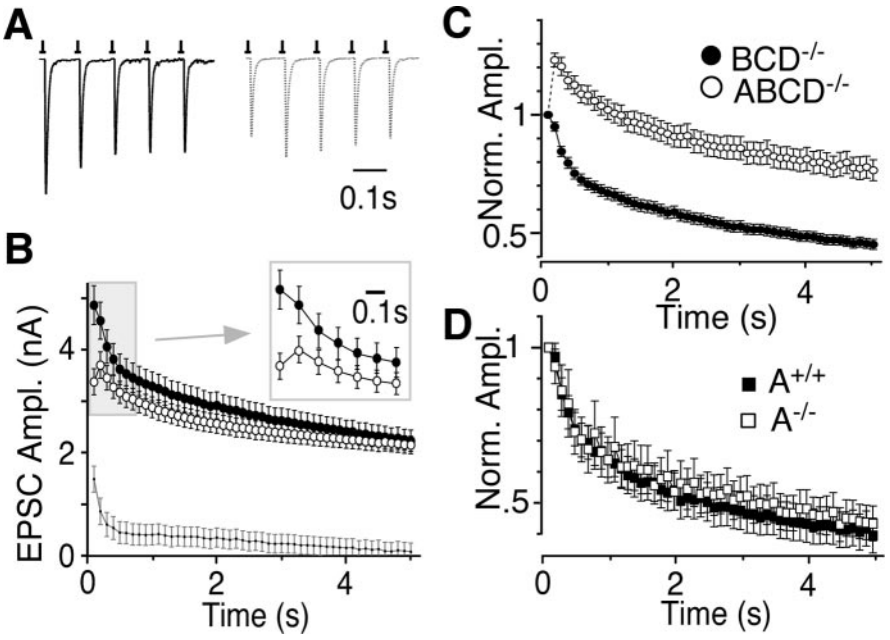

Figure 1. Synaptic responses to repetitive stimulation. ***A***, Sample traces for Rab3a-containing (left) and Rab3-deficient excitatory hippocampal neurons (right) showing that the Rab3-deficient synapses facilitates in response to the first few stimuli in a 10 Hz spike train compared with the typical depression seen in the wild-type-like Rab3a-containing synapses. ***B***, Absolute EPSC amplitude (Ampl.) synaptic responses at 10 Hz stimulation frequency recorded in 4 mM Ca^{2+} in neurons from Rab3ABCD $^{-/-}$ quadruple KO mice ($n = 182$) and as a control, Rab3BCD $^{-/-}$ triple KO mice ($n = 166$). The gray curve at the bottom represents the subtraction curve between the two genotypes to reveal the Rab3-dependent component. The inset shows the first seven responses at higher time resolution. ***C***, Normalized EPSC amplitudes from the data set of ***B***. ***D***, Normalized synaptic responses to 10 Hz stimulation in Rab3A $^{-/-}$ KO ($n = 20$) and wild-type control ($n = 30$) mice.

Results

To study the role of the Rab3-dependent component of release in short-term synaptic plasticity, we used cultured autaptic excitatory hippocampal neurons from Rab3-deficient mice. The triple Rab3BCD KO (BCD $^{-/-}$) neurons (hence forward named Rab3a containing) were used as controls for the quadruple KO Rab3ABCD KO (ABCD $^{-/-}$) neurons (Rab3 deficient) in these experiments, because previous studies have shown that Rab3a (which is the only Rab3 isoform expressed in the triple KO neurons) is fully capable of maintaining a wild-type like synaptic behavior, and showed no signs of morbidity or impaired survival (Schluter et al., 2004).

Synaptic responses facilitate during action potential trains in Rab3-deficient neurons

We applied trains of action potentials at a frequency of 10 Hz and compared the absolute and normalized EPSCs elicited by these action potentials. We found that the EPSC exhibited strong initial facilitation in the quadruple KO, whereas the triple KO neurons showed normal moderate depression (Fig. 1*A*,*C*). This change in short-term plasticity was not caused by the removal of Rab3a alone, because we observed no facilitation in Rab3A KO neurons compared with their wild-type littermates (Fig. 1*D*). To estimate the overall effect of Rab3 deficiency on synaptic output, we plotted the mean absolute EPSC amplitudes of Rab3-deficient and Rab3a-containing neurons (Fig. 1*B*). Although the initial synaptic response in Rab3a-containing synapses was ~35% larger, the responses of the two groups converged to similar amplitudes after approximately five synaptic responses. To reveal the Rab3-dependent synaptic component, we subtracted the absolute mean amplitudes at each time point of the Rab3-deficient neurons from the amplitudes of individual Rab3a-containing ones

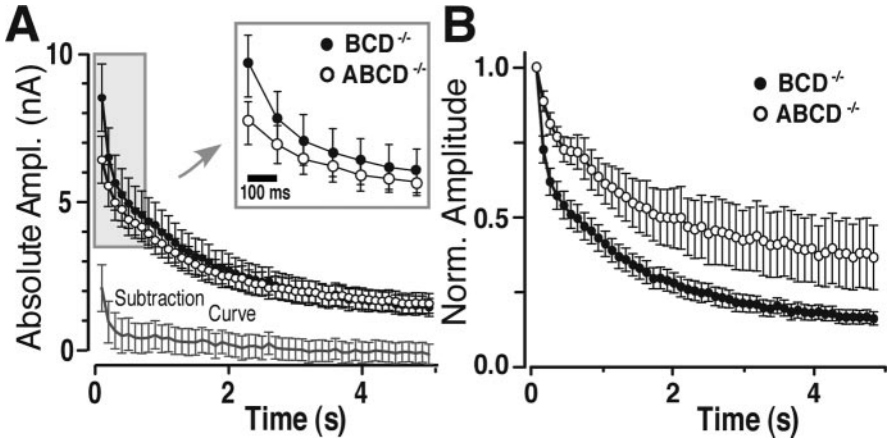

Figure 2. Synaptic responses to repetitive stimulation at increased release probability. ***A***, Absolute mean EPSC amplitudes (Ampl.) during 10 Hz stimulation recorded in 12 mM extracellular Ca^{2+} to increase the release probability. The gray curve at the bottom represents the difference in absolute amplitudes between the two genotypes to reveal the Rab3-dependent component. The inset shows the first seven responses at higher time resolution (BCD $^{-/-}$, $n = 20$; ABCD $^{-/-}$, $n = 21$). ***B***, Same data as in ***A***, except that synaptic responses are plotted in normalized (Norm.) terms.

(Fig. 1*B*). These data show that Rab3 has a pronounced impact on boosting the initial EPSC amplitude; however, this effect fades quickly after the first five spikes in a train of presynaptic action potentials.

Multiple mechanisms can contribute to changes in STP, including changes in the presynaptic Ca^{2+} handling (Edmonds et al., 2000; Rozov et al., 2001; Blatow et al., 2003; Sippy et al., 2003). Although we consider this an unlikely scenario in the case of Rab3 based on its structure and its known binding partners, we wanted to verify that the effect of Rab3 removal is independent of intraterminal Ca^{2+} buffering. We compared 10 Hz EPSC trains before and after incubation of the neurons with the membrane-permeable Ca^{2+} buffer EGTA (100 μM EGTA-AM; 5 min). As expected, the increase in exogenous Ca^{2+} buffer in the nerve terminal caused a reduction of the initial EPSC amplitude in both groups (~20–30%; $n = 7$), we also observed enhanced depression in both Rab3a-containing ($n = 4$) and Rab3a-deficient synapses ($n = 3$) (Hagler and Goda, 2001; Rozov et al., 2001), supporting the fact that enhanced Ca^{2+} buffering affects short-term plasticity. However, although both groups showed more depression under increased buffering, the relative differences between the two groups were maintained (data not shown). This supports the idea that the phenotype associated with the lack of Rab3 is not an exponent of changes in handling of residual Ca^{2+} but rather a direct effect of Rab3 on the release machinery itself.

Does Rab3 boost release probability in a subset of primed vesicles?

How is the effect of Rab3 limited to only the first few action potentials during a spike train? From previous experiments, we know that Rab3 removal does not affect the availability of primed vesicles. Among two possibilities, Rab3 may boost release probability by acting on the entire pool of readily releasable vesicles on a temporary time scale or, alternatively, by modifying only a subset of vesicles within the pool. If the entire pool is equally affected, we would not expect that the amplitudes of the two groups would merge within a few action potentials (Fig. 1*B*), unless significant depletion had occurred. However, the mean vesicular release probability is only 4.5% in the Rab3-deficient and 6% in Rab3a-containing control neurons (Schluter et al., 2004). Therefore, the convergence of the amplitudes within five action potentials is unlikely because of pool depletion. If, however, Rab3 acts on a subset of vesicles and boosts their release probability, then they would preferentially fuse and be depleted during the first few action potentials of the spike train. This would explain the observed transient effect of Rab3. If heterogeneity is an intrinsic property of the vesicles within their subpools, it should persist when the initial release probability is altered.

To test this hypothesis, we increased release probability by elevating extracellular Ca^{2+} in the external medium (from the regular 4 mM Ca^{2+}/4 mM Mg^{2+} to 12 mM Ca^{2+}/1 mM Mg^{2+}) and analyzed the relative changes in short-term plasticity characteristics during 10 Hz trains in the Rab3-deficient synapses versus the Rab3a-containing synapses. Plots of absolute responses confirmed that the Rab3-deficient synapses exhibited a selective loss of synaptic strength during the initial stimuli of the 10 Hz train, leveling out with the control within a few stimuli (Fig. 2*A*). As expected (Zucker and Regehr, 2002), synaptic responses depressed much faster and stronger under conditions of increased release probability. However, we still observed that responses in the quadruple KO neurons show relatively lesser depression than the triple KO (Fig. 2*B*). Thus, the patterns of changes in short-term plasticity in the Rab3-deficient neurons are insensitive to elevations of release probability. Therefore, a global increase of release probability does not lead to a disappearance of the Rab3-deficient phenotype. This observation inclines us to favor the idea of heterogeneity of intrinsic vesicle release properties.

Paired-pulse facilitation reveals additional heterogeneity of Rab3 action

Because Rab3 may regulate release probability in a subset of vesicles, it would inherently introduce a use-dependent component to synaptic output. To elucidate which time window is most affected by Rab3, we measured short-term plasticity as a function of the frequency of synaptic responses. Most simply, this can be analyzed by varying the interval between two synaptic stimuli and computing the ratio of the second response to the first (Fig. 3*A*). We also extended the analysis to a comparison of the 10th and the first response (Fig. 3*B*).

Interestingly, the loss of Rab3 showed its greatest impact on responses to paired-pulse stimulation when they were applied at intermediate frequencies. Although at interpulse intervals of 20 and 33 ms, factors other than Rab3 dominate the use dependency of the synapse, interpulse intervals of 100 ms caused an unusually strong increase in the second response in the Rab3-deficient synapses. In contrast, Rab3a containing synapses show at intermediate and long interstimulus intervals more stable responses with only a moderate depression of the synaptic responses, thereby implicating this molecule in stabilizing synaptic output in this frequency range.

Paired-pulse facilitation is inversely proportional to the initial release probability (Zucker and Regehr, 2002). We asked whether the increased facilitation in quadruple KO neurons is solely attributable to the decrease in release probability, affecting all vesicles equally. This would explain the observed phenotype by a simple shift in vesicular release probability. In contrast, if coex-

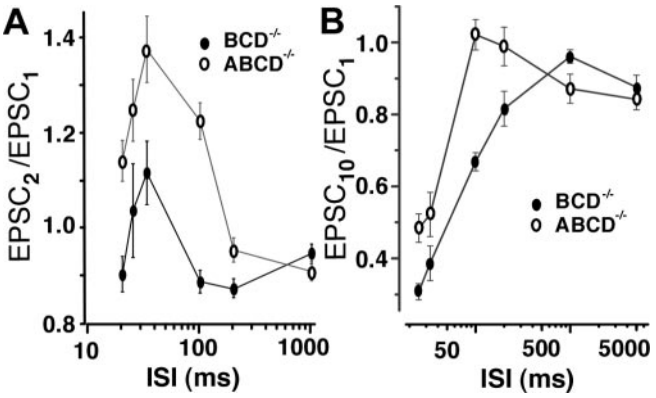

Figure 3. Paired-pulse ratio in Rab3-deficient synapses. ***A***, Paired-pulse ratio (ratio of the second to the first synaptic response to two closely spaced action potentials) plotted as a function of the stimulus interval. Experiments were performed in standard extracellular Ca^{2+} solution (4 mM Ca^{2+}/4 mM Mg^{2+}; $n = 16–182$). ***B***, Ratio of the 10th synaptic response to the first synaptic response recorded in stimulus trains applied between 0.2 and 50 Hz. Responses are plotted as a function of the interstimulus interval (ISI) based on the stimulation frequency.

isting Rab3a-dependent and Rab3-independent components of release have intrinsically different release probabilities, we would expect that lowering the release probability in all wild-type synapses would not induce the same changes in paired-pulse ratios seen in the Rab3-deficient neurons. To address this issue, we systematically lowered release probability in wild-type neurons to levels equivalent to or lower than that in the Rab3-deficient synapses and compared paired-pulse behavior under these conditions. Release probability was reduced by lowering Ca^{2+} influx, either by reducing external Ca^{2+} concentrations from 4 mM to 2 or 3 mM or by adding 3 or 5 μM Cd^{2+} to block Ca^{2+} channels. These means of affecting Ca^{2+} influx have been shown previously to affect presynaptic release in a similar manner (Mintz et al., 1995).

We found that reduction of release probability in wild-type synapses to 54–72% of the control value barely changed the paired-pulse behavior at 100 ms interval (Fig. 4). This is in stark contrast to the Rab3-deficient synapses that display at 30% reduced release probability strong paired-pulse facilitation. At higher frequencies, the difference between Rab3-containing and Rab3-deficient neurons were mostly occluded, most likely because of the prominent effect of classical short-lived facilitation. The discrete effect of Rab3 removal on paired-pulse behavior at intermediate frequencies indicate that the underlying mechanism of Rab3 action cannot be explained by an increase in mean release probability across all vesicles; rather, this effect compliments the idea that a change in the release properties of a subpopulation of vesicles is the underlying cause. As a result, addition of Rab3 causes a preferential boost of synaptic activity encoded by intermediate spike frequencies.

Rab3 deletion lowers the Ca^{2+} sensitivity of release

To determine whether the decrease in synaptic responses in Rab3-deficient neurons is associated with a relative change in the apparent Ca^{2+} affinity of release, we titrated synaptic responses with increasing concentrations of extracellular Cd^{2+} (1–100 μM). If the lack of Rab3 increases the amount of Ca^{2+} required for release (i.e., decreases the apparent Ca^{2+} affinity of release), the apparent Cd^{2+} concentrations needed to inhibit release should be lower. Conversely, if a mutation decreases the amount of Ca^{2+} required for release (i.e., increases the apparent Ca^{2+} affinity of release), higher Cd^{2+} concentrations will be needed to

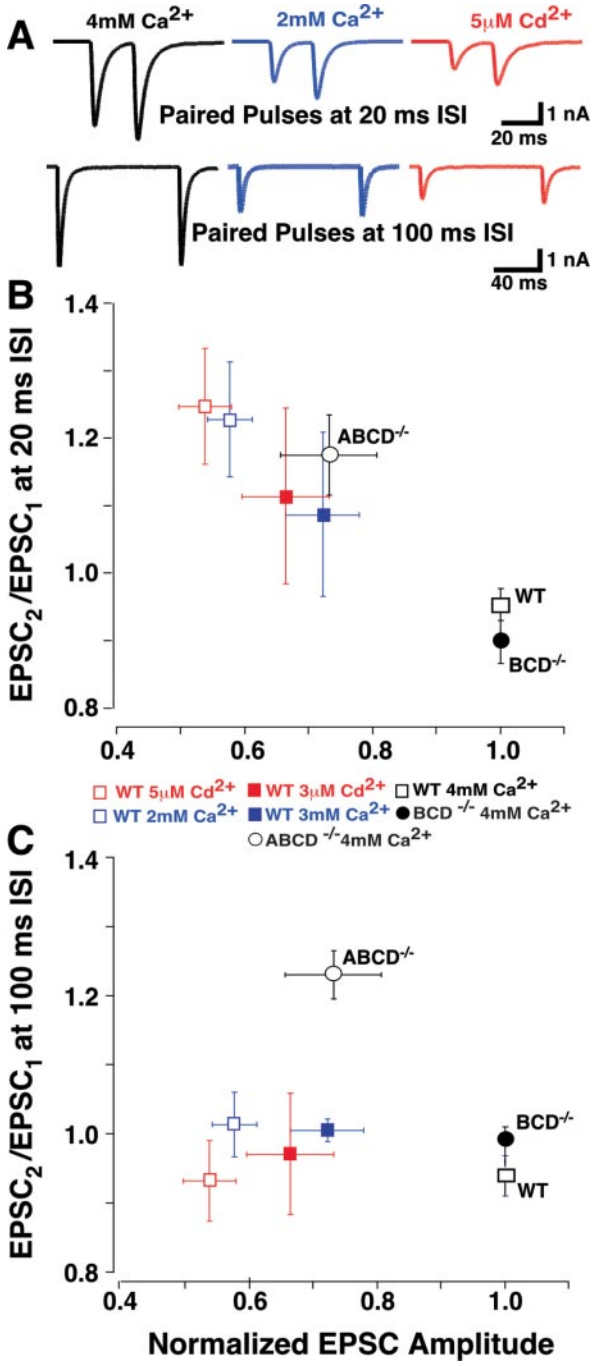

Figure 4. Rab3-dependent uncoupling of release probability and paired pulse behavior at intermediate interpulse intervals. ***A***, Exemplary traces of a wild-type neuron in response to paired pulses given at the interstimulus intervals (ISI) of 20 ms (top) and 100 ms (bottom) under conditions of varying Ca^{2+} influx: 4 mM Ca^{2+} (left, black traces), 3 mM Ca^{2+} (middle, blue traces), and 4 mM Ca^{2+}/5 μM Cd^{2+} (right, red traces). Mg^{2+} was 4 mM in all cases. Correlation of paired-pulse behavior at 20 ms (***B***) and 100 ms (***C***) interstimulus intervals and release probability in WT and Rab3-deficient mice. Release probabilities, as estimated from relative changes of EPSC amplitudes compared with control conditions (4 mM Ca^{2+}, 4 mM Mg^{2+}; black open squares), were reduced by changing external Ca^{2+} concentrations (2–3 mM; blue symbols) or by addition of Cd^{2+} (3 or 5 μM; red symbols) to the external solution. For comparison, the paired-pulse behavior of the Rab3-deficient (72% of Rab3BCD $^{-/-}$ synaptic amplitude; black filled circle) and Rab3a-containing (black open circle) synapses at control conditions are overlaid. Although at the 20 ms interval synaptic responses from wild-type neurons and in the Rab3 groups showed generally an increase in paired-pulse ratio with decreased release probability, little change of paired-pulse ratio was observed at 100 ms interstimulus intervals over a wide range of release probabilities in wild-type, whereas the Rab3-deficient synapses showed unusually strong facilitation at a 30% reduced release probability.

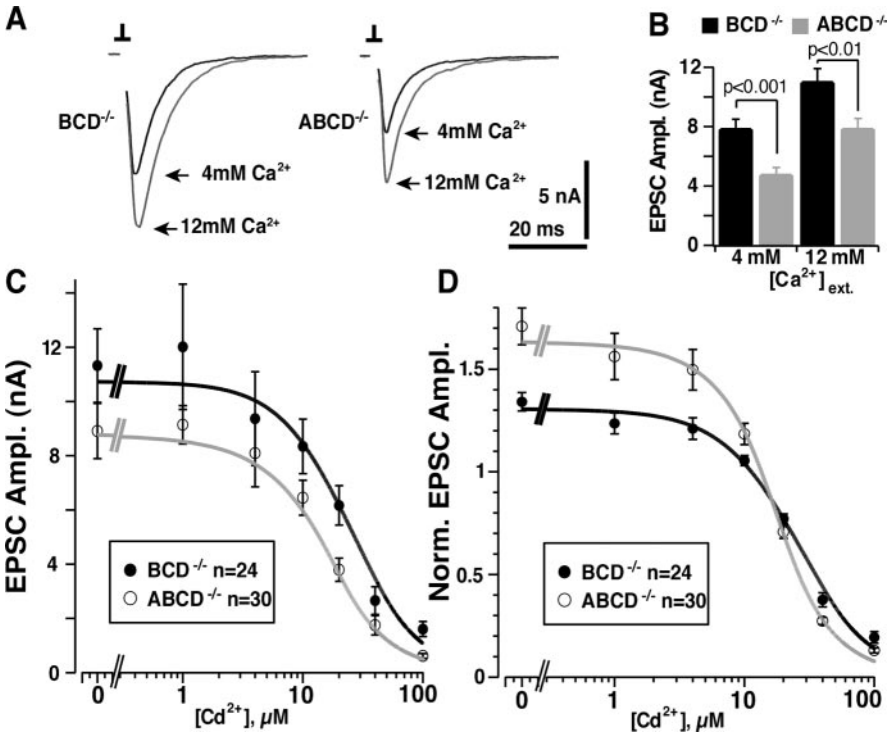

Figure 5. Decreased Ca^{2+} sensitivity in Rab3 KO neurons. ***A***, Sample traces of EPSCs from Rab3a-containing (top) and Rab3-deficient (below) neurons evoked at 4 mM (black trace) and 12 mM (gray trace) Ca^{2+} concentrations. ***B***, Bar graph of mean absolute EPSC amplitudes (Ampl.) of Rab3a containing (Rab3BCD $^{-/-}$; black bars) and Rab3-deficient (Rab3ABCD $^{-/-}$; gray bars) at 4 mM Ca^{2+}/4 mM Mg^{2+} and at 12 mM Ca^{2+}/1 mM Mg^{2+}. ***C***, Titration of synaptic responses with increasing concentrations of extracellular Cd^{2+} (0–100 μM) to progressively block Ca^{2+} influx. Data shown are absolute EPSC sizes recorded in 12 mM Ca^{2+} and 1 mM Mg^{2+}. ***D***, Same data as in ***C*** but normalized to alternating control responses (4 mM Ca^{2+}, 4 mM Mg^{2+}). The Ca^{2+} cooperativity in the two groups was not significantly different (Rab3ABCD $^{-/-}$, 2.03 ± 0.20; Rab3BCD $^{-/-}$, 1.84 ± 0.19).

inhibit release. To cover a wide range of release probabilities, the background Ca^{2+} concentration was elevated to near saturating levels (12 mM). Generally, at all Cd^{2+} concentrations tested, we observed smaller absolute EPSC amplitudes in the Rab3-deficient neurons than in the Rab3A-containing neurons (Fig. 5*B*,*C*); this shows that elevation of Ca^{2+} does not rescue the lack of Rab3. However, when we fitted the data points with a Hill function to estimate the Cd^{2+} concentration required for half-maximal inhibition of release, significantly lower Cd^{2+} concentrations were required for inhibition of the Rab3-deficient neuronal release compared with Rab3a-containing neurons (ABCD $^{-/-}$, 17.3 ± 1.0 μM Cd^{2+}, $n = 30$; BCD $^{-/-}$, 29.1 ± 2.8 μM Cd^{2+}, $n = 24$; $p < 0.001$) (Fig. 5*D*).

This suggests that deletion of Rab3 decreases the overall apparent Ca^{2+} affinity of release. To confirm this, we measured the enhancement of synaptic responses induced by increased extracellular Ca^{2+}concentration (12 mM Ca^{2+}/1 mM Mg^{2+} vs 4 mM Ca^{2+}/4 mM Mg^{2+}). We found that the relative potentiation of release in high Ca^{2+} was significantly larger in the Rab3-deficient than in Rab3a-containing neurons (BCD $^{-/-}$, 1.3 ± 0.05-fold, $n = 24$; ABCD $^{-/-}$, 1.7 ± 0.09-fold, $n = 30$; $p < 0.001$), consistent with the notion that deletion of all Rab3 isoforms decreases the apparent Ca^{2+} affinity of release. The increased Ca^{2+} sensitivity of release in the presence of Rab3 would move the efficiency of release to a more shallow region of the Ca^{2+}-EPSC amplitude function and, in turn, lead to a smaller potentiation of the evoked response when Ca^{2+} is elevated.

Rab3 does not contribute to the release kinetics during evoked release

The data so far show that deletion of Rab3 effectively dampens the release probability of a subset of vesicles in the readily releasable pool, resulting in an unexpected alteration in short-term synaptic plasticity for a particular frequency domain. How is this Rab3-dependent vesicle subpool defined? Vesicles may be characterized by displaying subpool-specific kinetics of release. At room temperature, synaptic vesicles at hippocampal synapses are released with two time courses: a fast synchronous component, accounting for ~80% of the charge of an EPSC, and a slow asynchronous component (Goda and Stevens, 1994). During repetitive stimulation the ratio is shifted progressively toward asynchronous release (Hagler and Goda, 2001; Pyott and Rosenmund, 2002). We tested whether Rab3 has any influence on the time course of release during repetitive stimulation by analyzing the relative size of synchronous and asynchronous release (Fig. 6). We detected no difference between Rab3-deficient and Rab3a-containing neurons, indicating that complete loss of Rab3 did not selectively affect one or the other component of release. This indicates that the proposed Rab3-induced heterogeneity within the RRP cannot be correlated with heterogeneity of vesicular release kinetics. Therefore, Rab3 action may not be associated with processes regulating the kinetics of the vesicular release.

Vesicle priming in Rab3-deficient synapses

We then focused on the well documented function of Rab proteins in the docking and tethering of vesicles to target membranes (for review, see Jahn et al., 2003). In principle, the parameter that would most likely be affected by lack of the tethering function is a change in vesicle-priming rates. Indeed, heterogeneity within the RRP has been demonstrated through dual time course of response recovery after activity-induced depletion of synaptic responses at the calyx of Held and at the cerebellar climbing fiber synapse (Silver et al., 1998; Sakaba and Neher, 2001a). We analyzed both Ca^{2+}-dependent and Ca^{2+}-independent pool refilling after depletion. Two consecutive pulses of hypertonic sucrose, separated by a variable interval, were used to measure Ca^{2+}-independent refilling. The first pulse depletes the pool, and the second pulse monitors its reformation. We then plotted the amplitude of the second response as a percentage of the first response. Rab3-deficient neurons and Rab3a-containing neurons were indistinguishable in this assay, with both exhibiting a recovery time constant of ~6 s, similar to wild-type synapses (Rosenmund and Stevens, 1996) (Fig. 7*A*). This observation shows that vesicle priming at rest is not impaired after loss of Rab3.

In a second set of experiments, we investigated priming rates under conditions of elevated Ca^{2+}, for example, after high-frequency stimulation. When the readily releasable pool of vesicles is depleted by an action potential train, the rate of pool refilling is faster than at resting Ca^{2+} concentrations (Stevens and Sullivan, 1998; Rhee et al., 2002). We depleted the primed vesicle

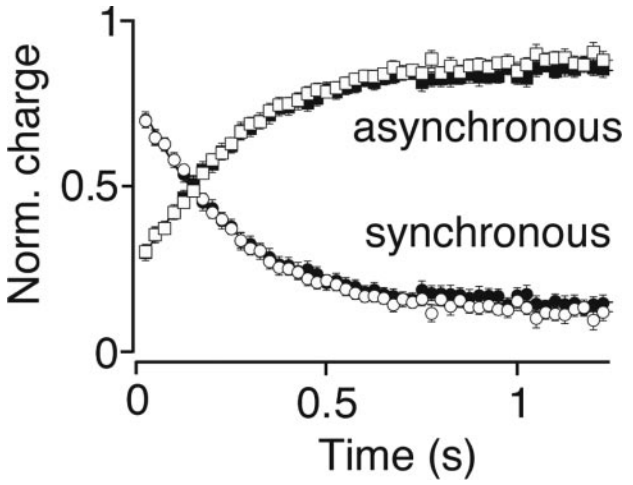

Figure 6. Loss of Rab3s does not affect timing of release. The plot shows the mean fraction of synchronous (circle symbols) and asynchronous (square symbols) release during an action potential train (40 Hz) in the Rab3a-containing (closed symbols) and Rab3-deficient (open symbols) neurons as a function of the stimulus number. The asynchronous component of the EPSC was extrapolated from the initial slope of the following integrated EPSC time course. The synchronous component was calculated by subtraction of the asynchronous component from the total charge transfer of an EPSC [for details, see Pyott and Rosenmund (2002)]. Norm., Normalized to sum of synchronous and asynchronous component.

pool with a 40 Hz train of action potentials in Rab3-deficient neurons and measured the recovery rate of synaptic responses by recording EPSCs induced by isolated action potentials (Fig. 7*B*). As expected, we found that synaptic responses of both Rab3a-containing and Rab3-deficient neurons recovered faster after action potential-driven depletion of the readily releasable pool (time constant, ~1 s) than after sucrose-induced depletion of this pool (time constant, ~6 s).

We then compared the recovery rate of the absolute amplitudes of each individual Rab3-deficient neuron (tau, 0.75 ± 0.1 s) and in Rab3a-containing neurons (tau, 1.4 ± 0.3 s; $p < 0.05$) using initially a single exponential fit. We also calculated the Rab3-dependent component by subtraction of the Rab3-deficient response from the control response (Fig. 7*B*). The time constant of the Rab3-dependent component was significantly larger (tau, 1.5 s) than the time constant of refilling measured in the absence of Rab3. These data suggest that the Rab3-dependent component of release recovers at least twofold slower after depletion than the Rab3-independent component. Using previously published data from other synapses as a yard stick (Silver et al., 1998; Sakaba and Neher, 2001a), we probed whether this shift is based on a modification of one of the two kinetic components of pool recovery by replotting the normalized recovery phase of the EPSC (Fig. 7*C*). The recovery rate of the Rab3-deficient synapses was well fitted with a single exponential, resulting in a time constant of 0.48 s, comparable with the recovery rate of the reluctant vesicles in the calyx and the fast recovery in the climbing fiber terminals. The recovery rate of the control neurons was then fitted with a double exponential function, setting one time constant fixed to 0.48 s. This resulted in a second time constant of 2.2 s. The weight of the fast recovery was ~40%, whereas the slow recovery contributed ~60%. Thus, in the absence of Rab3, EPSCs recover fast, whereas Rab3 is required for the presence of an additional slowly recovering (tau, ~2 s) response component. These data are consistent with the idea of an overall functional heterogeneity of primed vesicles and implicates the essential role of Rab3 in this heterogeneity.

Discussion

Rab3 proteins are abundant GTP-binding proteins of synaptic vesicles (Sudhof, 2004). In a recent study, we showed that deletion of all four Rab3 isoforms, but not of any combination of three Rab3 isoforms, causes lethality in mice, results in a ~30% decrease in Ca^{2+}-evoked neurotransmitter release, and causes an equivalent decline in the synaptic and vesicular release probabilities P_r and P_{vr} (Schluter et al., 2004). This simultaneous lethality of the quadruple Rab3 KO with a surprisingly discrete impairment in presynaptic function raised two questions: which step in the synaptic vesicle cycle does Rab3 deletion affect so as to account for the discrete modification in neurotransmitter release, and why is such a relatively small decrease in release lethal in consequence?

Although we can only speculate about the origin of the lethal consequences (see below), the present study allows for quite a precise determination of the role of Rab3 on the dynamics of vesicle release. Our data suggest that Rab3 acts on a subset of vesicles in the RRP to increase their release probability, a function that we call "superpriming." The evidence of heterogeneity of synaptic vesicle function is based on the following arguments. We see an initial reduction in the EPSC amplitude but no change in vesicle pool size in Rab3-deficient neurons (Schluter et al., 2004). Therefore, the average release probability is reduced, and Rab3 targets its effect within the RRP. Second, we measured a higher paired-pulse ratio at AP frequencies of 10–50 Hz than predicted from a mean reduction of release probability, suggesting that lack of Rab3 reduces release probability unequally within the RRP (Figs. 1, 3, 4). Third, we see different repriming rates for the Rab3-dependent and -independent components, suggesting that Rab3 modulation introduces a rate-limiting step in priming (Fig. 7). Fourth, we noticed a significant shift in the apparent Ca^{2+} sensitivity of the Rab3-dependent versus -independent components (Fig. 5). This suggests that Rab3 helps to increase the efficiency of release. All of these features imply that the vesicles "superprimed" by Rab3 are the first to undergo exocytosis when a nerve terminal is invaded by trains of action potentials. However, because they are reprimed relatively slowly after exocytosis, their effect on the response size ceases rather quickly during ongoing synaptic use.

What are the characteristics of the Rab3-dependent and -independent pools of vesicles? The time course of release is similar in both vesicle populations, because neither the time course of individual EPSCs nor the shift from more synchronous to asynchronous release during repetitive stimulation is changed after removal of Rab3 (Schluter et al., 2004). This finding is consistent with a previous study showing that release probabilities of the asynchronous and synchronous components are similar (Otsu et al., 2004). However, the superprimed pool shares important similarities with the fast-depleting vesicles of the calyx of Held. Both the superprimed pool and the fast-depleting vesicles constitute 40–50% of the RRP, they have a relatively high release probability, and are replenished with a time constant of 2–5 s (Sakaba and Neher, 2001a,b). In contrast, the Rab3-independent vesicles and the slowly depleted vesicles of the calyx of Held have a reduced release probability but are replenished with a time constant of 200–500 ms. Significantly, the time course of an EPSC does not change regardless of whether the synapse is stimulated in a naive state or after partial replenishment of the reluctant pool [referred to as slowly depleting pool in the study by Sakaba and Neher (2001a,b)]. This states that pool heterogeneity based on differences in release probability are not correlated with

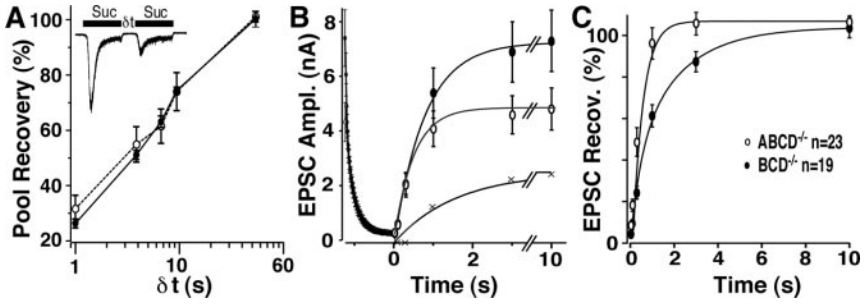

Figure 7. Slow recovery of the Rab3-dependent response component from action potential train-induced pool depletion. ***A***, Recovery of the readily releasable pool measured as the release triggered by hypertonic sucrose (Suc). Two successive sucrose pulses (4 s) were applied separated by an interval of 1–100 s, and the size of the second response is measured as a percentage of the first (note the semilogarithmic representation; $n = 10–13$ for each time point and group). ***B***, Recovery of the readily releasable pool measured as the release triggered by action potentials. The readily releasable pool was first depleted by a 1.25 s stimulation at 40 Hz, and then the recovery was followed by measuring synaptic responses after 0.1, 0.3, 1, 3, and 10 s. Responses are displayed as absolute amplitudes (Ampl.). Data shown are means ± SEMs. ***C***, Normalized (to initial EPSC) pool recovery of ABCD −/− (open symbols) and BCD −/− (closed symbols) neurons. Exponential fitting of recovery were performed as described in Results.

heterogeneity in the time course of triggered release (Wu and Borst, 1999).

This study introduces a new function for Rab proteins, namely the boosting of the efficiency of the Ca^{2+}-triggered release by modifying already primed vesicles, thereby introducing heterogeneity in the primed vesicle pool. We can think of two alternatives but not exclusive mechanisms for this Rab3-induced superpriming effect. First, Rab3 may serve to direct vesicles to release sites with intrinsically high release probability, for example next to Ca^{2+} channels, because close proximity of vesicles to Ca^{2+} channels increases their release probability (Meinrenken et al., 2002). Second,, Rab3 may function to recruit or enhance binding of additional proteins to the release machinery that make Ca^{2+}-triggered release more efficient. Based on the observed phenotype, we think that the two best described Rab3 effector molecules rabphilin and Rim1 (Shirataki et al., 1993; Wang et al., 1997) are unlikely to be involved in the Rab3 function described above. Removal of rabphilin in mice has no detectable influence on synaptic transmission (Schluter et al., 1999), and deletion of Rim1 in mice causes a change of other parameters compared with Rab3-deficient mice (Schoch et al., 2002; Calakos et al., 2004). Comparable with the Rab3 quadruple KOs, the Rim1 KO neurons display a 50% decrease in EPSC size (Calakos et al., 2004), but this is accompanied by a parallel reduction in RRP size and protein levels of the priming factor Munc13–1, which are not observed in the Rab3-deficient mice. Furthermore, a shift in the release probability and apparent Ca^{2+} affinity was only seen in Rab3 quadruple KOs, whereas only the RIM1 KOs showed a prominent decrease in the asynchronous component of release. Although both Rim1 KO and Rab3 quadruple KO neurons display increased paired-pulse facilitation, close inspection reveals that the effect is evident at different frequencies in the two KO mouse models (Rim1 short interpulse intervals, Rab3 intermediate interpulse intervals). These phenotypical differences between Rim1 KO, rabphilin KO, and Rab3 quadruple KO suggests that Rab3 acts on yet undefined target(s). Furthermore, a comparison of the Rab3 and Rim1 KO models suggests that Rim1 acts during initial priming to globally increase synaptic release probability, whereas Rab3 enters in a secondary priming step to cause an increased efficiency of triggered release by targeting a subset of primed vesicles. A possible additional effector of Rab3 could be Synapsin I, which was shown to bind to Rab3a and modulate its function *in vitro* (Giovedi et al., 2004). Indeed, the paired-pulse ratio at a CA1 pyramidal cell synapse is increased in the synapsin I KO mice (Rosahl et al., 1993). Despite the lack of change of the number of docked vesicles, the absence of synapsin I causes a reduction of the immediately releasable pool (Li et al., 1995; Ryan et al., 1996). In addition to its function in regulating the reserve pool of vesicles, these results suggest a potential function of synapsin I in regulating release probability, potentially via Rab3.

How does the function of Rab3 in boosting of a subset of vesicles translate in synaptic encoding pattern? The 30% decrease in initial synaptic responses in the Rab3-deficient neurons can probably be compensated by a vertebrate nervous system. It seems feasible that moderate and uniform change in synaptic strength can readily be adjusted by homeostatic mechanisms (Panzeri et al., 2001; Turrigiano and Nelson, 2004).

What is likely to be of impact in synaptic coding is not the 30% decrease as such but the fact that the relative weakness of Rab3-deficient synapses is dependent on the frequency and the history of synaptic activity. Removal of Rab3 mainly affects the first few spikes of a train of action potentials, and this cannot simply be compensated by adding more synapses or adjustment of synaptic strength of individual synapses. A change in synaptic strength during spike trains may have strong impact on many aspects of synaptic encoding, one of such examples may be the precision of timing of neuronal excitation (Silberberg et al., 2004) or the cortical processing of sensory information. Studies correlating sensory inputs with firing patterns in the processing cortical neuron show that the delay between a sensory stimulus (hence synaptic input) and the first postsynaptic spike carried 80–90% of the total sensory information (Panzeri et al., 2001). Based on this, the action of Rab3 would make first spike delay much more precise. A third example is the role in auditory processing: precise regulation of short-tem plasticity features participates in distinguishing static from dynamic qualities of the auditory signal process in the primary auditory cortex (Atzori et al., 2001).

How can these changes in short-tem plasticity be of importance for the survival of mice? One obvious speculation is that the short-term plasticity profoundly disturb the neuronal network responsible for respiratory rhythmogenesis (Schluter et al., 2004) or, alternatively, the lack of Rab3 function in peripheral nervous system, directly at the neuromuscular junction of the diaphragm might cause perinatal lethality in the Rab3-deficient mice. Importantly, Rab3a is abundant at the neuromuscular junction (Mizoguchi et al., 1992).

Although our analysis revealed that Rab3 functions in a much more discreet manner than is expected from its general role in vesicle trafficking, the deleterious impact of loss of Rab3 function, however, is powerful evidence for the general importance of this function in synaptic encoding and for precise short-term plasticity pattern in shaping temporal properties in neuronal networks in general.

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