Vesicle transport and neurotransmitter release in central nerve terminals
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Chapter 4

Rab3a is involved in the transport of synaptic vesicles to the active zone in mouse brain nerve terminals

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ABSTRACT

The Rab family of GTP-binding proteins regulates membrane transport between intracellular compartments. The major rab protein in brain, rab3A associates with SVs. However rab3A was shown to regulate the fusion-probability of SVs, rather than their transport and docking. We tested if rab3A has a transport function by analyzing SV distribution and exocytosis in rab3A null-mutant mice. Rab3A deletion did not affect the number of vesicles and their distribution in resting nerve terminals. The secretion response upon a single depolarization was also unaffected. In normal mice, a depolarization-pulse in the presence of Ca\(^{2+}\) induces an accumulation of vesicles close to and docked at the active zone (recruitment). Rab3A deletion completely abolished this activity-dependent recruitment, without affecting the total number of vesicles. Concomitantly, the secretion response in the rab3A-deficient terminals recovered slowly and incompletely after exhaustive stimulation and the replenishment of docked vesicles after exhaustive stimulation was also impaired in the absence of rab3A. These data indicate that rab3A has a function upstream of vesicle fusion in the activity-dependent transport of SVs to and their docking at the active zone.
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INTRODUCTION

SVs of the mammalian brain take up fast-acting neurotransmitters, mostly glutamate and GABA, and release them, upon activation, in a specialized area of the presynaptic membrane, the active zone. Subsequently, these vesicles recycle locally in preparation for a new round of transmitter release. Although SV recycling is a highly specialized form of vesicle trafficking, several molecular principles have been recognized that appear to be similar to vesicle trafficking in other compartments of neurons, in other cells and in other species. Different members of protein families, such as the syntaxins, the synaptobrevins/VAMPs and the munc18/SEC1 proteins, appear to have similar functions in different systems (Bennett and Scheller, 1993; Söllner et al., 1993; Jahn and Südhof, 1999). The rab protein family appears to be an exception. SVs contain three isoforms, rab3A is present in most if not all synapses in the rodent brain, and rab3B and rab3C are present in a subset of synapses (Fischer von Mollard et al., 1994, Jahn and Südhof, 1999). Recent evidence (Gepper et al., 1997) suggests that the function of rab3A may be different from other rab-proteins in other systems.

Rab proteins are a large family of small GTP-binding proteins. Its members are localized in distinct cellular compartments in mammals but also in yeast (Novick and Zerial, 1997; Olkkonen and Stenmark, 1997). Rab proteins are thought to act as a GTP-dependent molecular switch to improve the fidelity of protein-protein interactions at the targets of a transport step, i.e., the pairing of SNARE-proteins that drive vesicle fusion (Schimmöller et al., 1998; Gonzalez and Scheller, 1999). Hence, rab proteins generally act as facilitators in transport steps, i.e., upstream of SNARE-complexes and fusion.

Rab3A and rab3C are associated with SVs in their GTP-bound form and dissociate from the vesicle upon GTP hydrolysis or depolarization of the nerve terminal (Fischer von Mollard et al., 1994). After GDP-GTP exchange, rab3A can associate with SVs again. Rab3 isoforms interact with different general rab-binding proteins and with at least two specific effector proteins, rabphilin3A (Li et al., 1994; Shirataki et al., 1994) and RIM (Wang et al., 1997). Rab3A null-mutant mice (Geppert et al., 1994a) and rab3 null-mutant worms (Nonet et al., 1997) are viable and have mild phenotypes, suggesting non-essential functions of rab3. C. elegans rab3 null-mutants have fewer
SVs especially near the active zone, but more at ectopic sites, suggesting that SV transport in the nerve terminal is impaired in the absence of rab3 (Nonet et al., 1997). In contrast, the synapse morphology appeared to be normal in rab3A knock-out mice (Gepper et al., 1994a). Instead, rab3A was proposed to act as a negative regulator of vesicle fusion. Hence, it was concluded that rab3, unlike other rab proteins and rab3 in *C. elegans*, acts downstream of vesicle transport and vesicle docking at the active zone (Gepper et al., 1997). However, in a review of these findings, it has been suggested that this action is probably not the only function of rab3A (Bean and Scheller, 1997).

Here we show that in nerve terminals isolated from mouse brain, rab3A also has a classical transport role in the trafficking of SVs to their target. Stimulation of the terminals by chemical depolarization evokes a re-distribution of SVs such that more vesicles get close to and docked at the active zone (Leenders et al., 1999). This evoked vesicle recruitment is abolished in nerve terminals isolated from rab3A knock-out mice. Concomitantly, recovery of the secretion capacity and replenishment of docked vesicles after exhaustive stimulation were impaired in the mutants. In contrast, SV recruitment and neurotransmitter release induced by hyperosmotic sucrose were not affected in rab3A deficient nerve terminals.

**MATERIALS and METHODS**

*Rab3A knock-out mice*

Rab3A-deficient mice have been described previously (Gepper et al., 1994a). All experiments were performed with null-mutant and wildtype litter mates from heterozygous matings by experimenters who were unaware of the genotype.

**Synaptosomal preparation**

Synaptosomes were prepared from whole forebrain of 4 to 5 months old mice by Percoll (Pharmacia Biotech) density gradient centrifugation as described (Dunkley et al., 1988). The synaptosomal fractions in the 10-15 % and the 15-23 % Percoll interfaces were pooled and washed twice in artificial cerebrospinal fluid (aCSF) which contained (in mM): NaCl (132), KCl (3), MgSO₄ (2), NaH₂PO₄ (1.2), HEPES (10) and
D-Glucose (10) + 2 mM CaCl\(_2\). Synaptosomes were kept on ice in aCSF + 2 mM CaCl\(_2\) at a protein concentration of 2 mg/ml until use in the assay within 4 hours after isolation. Protein concentration was determined according to Bradford (1976) with Bovine Serum Albumine as a standard.

**Release assay**

*K\(^+\) stimulations*: To determine fast transmitter release, synaptosomes (40 µg protein) were pelleted and resuspended in 20 µl aCSF supplemented with 50 µM EGTA, preincubated at 37°C for 5 min and depolarized for 100 ms as described (Leenders et al., 1999). Depolarization media: aCSF containing 50 mM KCl (which isosmotically replaced NaCl) in the presence of 2 mM CaCl\(_2\) (for total release) or 50 µM EGTA (for the Ca\(^{2+}\)-independent release); Stop medium: aCSF containing 0.8 mM EGTA. For the pre-depolarization protocol synaptosomes (2mg/ml) in aCSF with 2 mM CaCl\(_2\) were preincubated at 37°C for 2 min and either depolarized, by raising K\(^+\) to 30 mM, or kept in control medium. After 3 min depolarization synaptosomes were pelleted and resuspended in aCSF with 2 mM CaCl\(_2\) and incubated at 37°C for 10-30 min. Thereafter synaptosomes were resuspended in aCSF + 50 µM EGTA prior to 100 ms depolarization.

*Sucrose stimulations*: Synaptosomes (2 mg/ml) in aCSF with 2 mM CaCl\(_2\) were preincubated at 37°C for 2 min. Synaptosomes where then stimulated with 0.5 M Sucrose in aCSF either with 2 mM CaCl\(_2\) or 50 µM EGTA. After 15 s stimulation was stopped by addition of aCSF medium with a NaCl concentration that restored the isosmolarity of the medium.

**HPLC analysis of released transmitters**: 150 µl of the synaptosomal suspension was centrifugated through 75 µl of 50:50 % (vol.: vol.) mixture of silicone oil (Dow Corning 550) and dinonylphthalate for 2 min in a Sigma table centrifuge at 15000g. From the supernatant a 90 µl aliquot was pipetted onto 10 µl ice-cold TCA (10 %)/Homoserine (5 µM). glutamate and GABA levels were determined by reversed phase HPLC (Verhage et al., 1989).

**Electron Microscopical analysis**

Synaptosomes (40 µg/20 µl) were stimulated as described above for release assay and fixed by rapid addition of ice-cold 2 % paraformaldehyde and 2.5 %
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glutaraldehyde. Synaptosomes were embedded in Epon, ultrathin coupes (80 nm) were stained with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope, essentially as described previously (Breukel et al., 1997a). For each experimental condition, 25 sections with a clearly visible active zone were selected for analysis of SV distribution. The minimal distance between the active zone and the centre of each SV was determined for all vesicles, vesicles were collected in 50 nm bins and the percentage of vesicles per bin was plotted against the distance to the active zone. SVs within 25 nm from the active zone were counted as morphologically docked vesicles.

Statistical analysis
The data were analyzed by paired or unpaired Student's *t*-test, except for difference in distribution of the SVs, which was tested by one-way ANOVA with repeated measures. The rejection of the null hypothesis was accepted as significant if *p*<0.05.

RESULTS

Vesicle distribution is normal in resting nerve terminals from rab3A null-mutants.
To identify a potential role of rab3A in SV transport, we analyzed the distribution of SVs and their neurotransmitter release in nerve terminals isolated from rab3A null-mutant mice and their wildtype littermates. Electronmicroscopy revealed no differences between the synaptosomal preparations from the two groups (Fig.1A,B). Morphometric analysis indicated that the diameter of the terminals, the active zone length, and the total amount of SVs per terminal were similar in the two groups (Fig. 1C-E). The distribution of SVs in the terminals was analyzed by measuring the shortest distance between the active zone and the Center of each vesicle and collecting these distances in bins of 50 nm (approximately the SV diameter). In resting nerve terminals, the SV distribution was not different between the two groups (Fig. 1F).
Fig. 1. Ultrastructure and morphometrical analysis of mouse brain nerve terminals from control and rab3A null-mutant littermates

Typical electron micrographs of synaptosomal preparations from wild-type (A) and rab3A-deficient (B) littermates. Micrographs were selected when the active zone with attached (part of the) postsynaptic density was present in the section and used for morphometrical analysis of SV distribution and -docking by an observer unaware of the genotype. Scale bar 0.2 μm. Average diameter of the sections (C), active zone length (D) and total number of SVs (E) were not different in resting nerve terminals of wildtype and rab3A mutants (n=4 for both groups). Also the intrasynaptic distribution of SVs relative to the active zone was not different (F, see Experimental Procedures for details).

**Evoked vesicle-recruitment is abolished in rab3A null-mutants**

A short episode of depolarization (0.1-15s) evokes a re-distribution of SVs in isolated rat brain nerve terminals such that more vesicles accumulate close to and docked at the active zone, whereas less vesicles remain at distant sites (Leenders et al., 1999). Since the total amount of SVs does not change after depolarization, their re-distribution within the terminal reflects a net transport towards the active zone. This re-distribution is referred to as depolarization-evoked vesicle recruitment. In nerve terminals isolated from wildtype mice, we observed similar depolarization-evoked vesicle recruitment as in rat nerve terminals after 100ms depolarization in the
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presence of Ca$^{2+}$ (Fig. 2A). However, this depolarization-evoked vesicle recruitment was completely abolished in nerve terminals isolated from rab3A-mutant littermates. Depolarization did not change the distribution of SVs in these terminals (Fig. 2B). The total amount of vesicles per synaptic section was similar in wild-type and rab3A-mutant mice and did not change after stimulation (Fig. 2C).

An important aspect of depolarization-evoked vesicle recruitment is an increased number of SVs morphologically docked at the active zone. In wildtype terminals, the number of docked vesicles increased more than 50% after 100 ms depolarization.

Fig. 2. Re-distribution of SVs induced by 100 ms depolarization in nerve terminals from wildtype and rab3A-deficient littermates.

Distribution of SVs relative to the active zone in wildtype (A) and rab3A-deficient synaptosomal sections (B) with (filled symbols) and without (open symbols) 100 ms depolarization. Morphometrical analysis as in Fig. 1F. The total amount of SVs (C) and the number of vesicles morphologically docked at the active zone (D) were calculated separately. For each condition (stimulated and unstimulated) 25 sections were analyzed per animal and four animals of each genotype. Data are means ± SEM. * p<0.05.
(2.1 ± 0.1 to 3.3 ± 0.3 vesicles per section; n = 4, p < 0.05; Fig. 2D). This increase in docked vesicles was also absent in rab3A-deficient terminals. Instead, the number of docked vesicles tended to decrease in the mutant nerve terminals after 100 ms depolarization (Fig. 2D, not significant, p = 0.12).

Recovery of the secretion capacity after exhaustive stimulation is impaired in rab3A null-mutants

The loss of depolarization-evoked SV recruitment in rab3A null-mutants may compromise their ability to restore the secretion capacity after exhaustive stimulation. To test this, nerve terminals isolated from wildtype and rab3A null-mutant littermates were first stimulated exhaustively to deplete releasable vesicles (see McMahon and Nicholls, 1991; Verhage et al., 1991). Synaptosomes were then repolarized to allow the replenishment of their secretion capacity. This replenishment was tested by measuring neurotransmitter release upon a brief depolarization (100 ms). In control experiments, the first, exhaustive depolarization was omitted.

Upon exhaustive stimulation, the total amount of Ca\(^{2+}\)-dependent release of the major, endogenous neurotransmitters in brain, glutamate and GABA, was similar in wildtype and rab3A-deficient nerve terminals (Fig. 3A). A single test-pulse of 100 ms depolarization also led to a comparable, Ca\(^{2+}\)-dependent release in the two groups (Fig. 3B), although GABA release during 100 ms depolarization was slightly higher in rab3A-deficient terminals (0.22 ± 0.02 nmol/mg protein in wildtypes versus 0.27 ± 0.04 nmol/mg protein in rab3A null-mutants, n = 11, p<0.05). These similar release responses upon short or long stimulation are consistent with the similar distribution of SVs observed in resting nerve terminals from the two groups (Fig. 1).

After exhaustive depolarization, intracellular vesicle transport in wildtype mice had completely replenished the releasable pool within a 10 min recovery phase (Fig. 3B). However, in rab3A-deficient terminals the replenishment was not complete, and neurotransmitter release was decreased by approximately 50% after 10 min. recovery (Fig. 3B, p<0.05). Only after 30 min recovery, the response of rab3A-deficient nerve terminals approached control levels (i.e., the response to a 100 ms depolarization without preceding exhaustive depolarization, Fig. 3B). This indicates that the vesicle pool can be largely replenished in the absence of rab3A, but only with a considerable delay (>30min, more than 3 fold slower than in wildtype terminals).
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Fig. 3. Neurotransmitter release during single depolarizations and after exhaustive pre-depolarization in nerve terminals from wildtype and rab3A-deficient littermates.

Isolated nerve terminals from wildtype and rab3A-deficient mice were depolarized for 3 min (A) or for 100 ms (B) with or without prior depolarization (3 min) and the release of endogenous glutamate and GABA was measured. In each protocol (outlined on the left) the Ca\(^{2+}\)-dependent (exocytotic) release of endogenous transmitters was calculated by subtracting the Ca\(^{2+}\)-independent release (40 mM K\(^+\) + 50 μM EGTA) from the total release (40 mM K\(^+\) + 2 mM Ca\(^{2+}\)). No differences were observed in the Ca\(^{2+}\)-independent component of release (data not shown). Data represent means ± SEM of 6-11 independent experiments. *p<0.05.

Replenishment of docked vesicles after exhaustive stimulation is impaired in rab3A null-mutants

In order to investigate morphological correlates of the impaired replenishment in the rab3A mutants, we analyzed vesicle distribution in isolated nerve terminals at the ultrastructural level using the same stimulation protocol. Nerve terminals were mixed with fixative instead of depolarization buffer at the start of the second stimulus (see
Fig. 4. The total number of SVs and the number of morphologically docked vesicles in nerve terminals from wildtype and rab3A-deficient littermates after a 3 min depolarization and 10 min recovery.

Number of docked SVs (A) and total number of SVs (B) per section were analyzed in terminals fixated before (open bars) and after (filled bars) 3 min depolarization and 10 min recovery (see Fig 3). Data represent means ± SEM of 4 independent experiments. *p<0.05.

Subsequent morphometric analysis showed that in wild type terminals the number of docked vesicles was comparable to unstimulated nerve terminals. However, in rab3A-deficient terminals the pool of docked SVs was decreased as compared to unstimulated rab3A-deficient terminals (Fig 4A, n = 4, p < 0.05). The total number of vesicles was not altered in any of the conditions and in both groups (Fig. 4B), indicating that the reduced number of docked vesicles in the rab3A mutants reflects an impaired transport of vesicles to their release-site. Hence, both morphometrical analysis at the ultrastructural level and biochemical analysis of neurotransmitter release indicated that the recovery of vesicular release after exhaustive stimulation was compromised upon deletion of rab3A expression.

**Hypertonic sucrose application does not reveal a transport phenotype in rab3A null-mutants**

Application of hyperosmotic sucrose solutions is an established tool to analyze the size and the replenishment of the releasable SV pool, especially in electrophysiological analyses of hippocampal autapses (Rosenmund and Stevens, 1996; Stevens and Tsujimoto, 1995). We confirmed that also in the nerve terminal preparation, hyperosmotic sucrose induced vesicular release from the same vesicle pool as membrane depolarization, though in a Ca²⁺-independent manner (data not shown, see also Lonart and Südhof, 1998).
Application of 0.5 M sucrose for 15 s to wildtype nerve terminals produced an increase in the amount of SVs within 150 nm from the active zone (Fig. 5A; p<0.05, n = 6) and a decrease in the amount of SVs at 200-1000 nm distance from the active zone (results not shown). This re-distribution was similar to the re-distribution observed after 0.1 (Fig. 2A) or 15 s chemical depolarization (Fig. 5B). Unlike depolarization, re-distribution of SVs after hyperosmotic sucrose application was also observed in the absence of Ca\(^{2+}\) (data not shown). Furthermore, unlike depolarization, 0.5 M Sucrose also induced re-distribution in rab3A-deficient terminals as in wildtypes (Fig. 5A + B; p<0.05, n = 6). Application of hypertonic sucrose also evoked comparable amounts of glutamate and GABA release in the presence or absence of rab3A (Fig. 5C). Concomitantly, paired applications of hyperosmotic sucrose solutions (15s, 0.5 M), separated by a 10 min period of recovery, revealed no differences in vesicle replenishment between mutant and control nerve terminals as indicated by transmitter release (data not shown). Hence, whereas a defect in evoked vesicle recruitment was evident in rab3A null-mutants upon membrane depolarization in the presence of Ca\(^{2+}\), no defects were observed using hyperosmotic sucrose.

**DISCUSSION**

We have analyzed the role of rab3A in SV trafficking in nerve terminals isolated from mouse brain. Deletion of rab3A expression did not affect the number of SVs or their distribution in resting nerve terminals, but depolarization-evoked recruitment of these vesicles was completely abolished in rab3A null-mutant mice. Concomitantly, the recovery of the secretion capacity and the replenishment of docked vesicles after exhaustive stimulation were reduced by 50% in the mutants. Application of hypertonic sucrose did not reveal this transport phenotype of the rab3A null-mutants. Electrophysiological analysis of synaptic transmission in hippocampal neurons in culture showed that rab3A-deletion altered the synaptic efficacy and suggested that rab3A limits vesicle fusion, i.e., may act as a negative regulator, downstream of vesicle docking at its target (Geppert et al., 1997). Hence, rab3A, unlike other rab proteins, appeared to act downstream of SNARE complex formation, the protein
Fig. 5. Re-distribution of SVs and transmitter release after application of hyperosmotic sucrose solutions and chemical depolarization to nerve terminals from wildtype and rab3A-deficient littermates.

Distribution of SVs in synaptosomal sections was analyzed as in Fig. 1F with (filled symbols) and without (open symbols) stimulation, i.e., application of 0.5 M sucrose for 15 sec. (A) or 40 mM K+ in presence of 2 mM Ca2+ (B). Ca2+-dependent release of glutamate and GABA induced by hyperosmotic sucrose was quantified in separate samples under exactly the same conditions (C). Vesicle distribution after hyperosmotic sucrose stimulation was significantly different from control (*p<0.05) for both wild-type and rab3A-deficient terminals. Vesicle distribution after 15 s depolarization was significantly different from control in wild-type (*p<0.05) but not in rab3A-deficient terminals. Data are means ± SEM of 6 independent experiments.
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complexes that drive vesicle fusion (see Jahn and Hanson, 1998). We have uncovered a separate function of rab3A, upstream of SNARE-complex formation between vesicle and target membrane, exploiting the fact that populations of isolated nerve terminals can be stimulated synchronously and repeatedly. Subsequent morphometric and functional assays allowed a direct analysis of SV (re-)distribution and their exocytosis, and revealed the role of rab3A in vesicle transport. This transport role of rab3A is a rate-limiting function. The total number of SVs and their distribution was unaltered in resting nerve terminals, but when their transport capacity was challenged by maximal activation of exocytosis, the transport role of rab3A was uncovered in two ways. Firstly, the depolarization-evoked vesicle recruitment to the active zone did not occur at all in the absence of rab3A, notably without major effects on neurotransmitter release at this point. This suggests that rab3A deletion affects the transport of SVs that do not (yet) take part in transmitter release. The slight increase in GABA release may be interpreted as an effect on release-probability and points in the same direction as previous observations in hippocampal autapses (Geppert et al., 1997). Secondly, the relevance of the impaired vesicle recruitment in the absence of rab3A was revealed during repeated stimulation: A second depolarization showed that the capacity to secrete transmitters as well as vesicle docking at the active zone were compromised by rab3A deletion. At this point, the reduction in the number of docked vesicles occurred in parallel with a reduction in neurotransmitter release. This indicates that the reduced vesicle pool at the active zone resulted from the reduced recruitment of vesicles and not from a faster vesicle depletion due to enhanced release. Hence, in isolated nerve terminals, rab3A deletion did not have major effects on the ongoing exocytosis of pre-docked vesicles even during maximal activation, but primarily affects the replenishment of vesicles for a new round of secretion. Even very long depolarizations did not reveal differences in transmitter release between mutants and controls. Such a role of rab3A is universal, since it was evident in the mixed population of isolated nerve terminals, i.e. from all fore-brain areas and representing all transmitter systems in the brain. Apparently, other rab3 isoforms cannot compensate for this function, although some isoforms are expressed at low levels in several areas of the brain (Geppert et al., 1994a; Li et al., 1994; Jahn and Südhof, 1999). Potentially, a more drastic transport phenotype will be obtained upon deletion of the other three rab3 genes,
similar to the clear phenotype in resting nerve terminals of *C. elegans* rab3 mutants (Nonet et al., 1997).

Our data do not exclude a separate role of rab3A as a negative regulator of release probability. The reported short-term enhancement in paired pulse facilitation after rab3A deletion (Geppert et al., 1997) applies to a small subset of vesicles released upon a pair of single action potentials. Hence, a change in the release of this small number of vesicles will not be detected in our morphological and functional analyses of the total SV pool. Since at least two specific rab3 effectors have been characterized (Shirakata et al., 1994; Wang et al., 1997) and additional effectors may be relevant, it is conceivable that rab3A exerts multiple functions at distinct steps in the SV cycle (see also Gonzalez and Scheller, 1999).

Apparently, a normal vesicle distribution can be maintained during low activity also in the absence of rab3A. Consequently, synaptosomal preparations from rab3A mutants and controls have similar vesicle numbers and -distribution. And after exhaustive stimulation, an extended recovery time will finally restore a normal vesicle distribution also in the absence of rab3A. Such a facilitatory role is in line with the proposed function for other members of the rab family, i.e., as timer devices that control protein-protein interactions (Aridor and Balch, 1996). In yeast, however, several rab proteins appear to have essential functions (see Novick and Zerial, 1997). Nevertheless, the enhanced run-down of responsiveness in CA1 neurons of the hippocampus at 14Hz, which was previously observed in the rab3A null-mutants (Geppert et al., 1994a), and the loss of LTP in the CA3 area but not of regular transmission of these mutants (Castillio et al., 1997) can all be explained by this facilitatory role of rab3A in vesicle transport. Such a role is also compatible with the altered vesicle distribution in the viable rab3 mutant of *C. elegans* (Nonet et al., 1997), and the effects of mutant rab proteins and introduction of rab antibodies (Olkkonen and Stenmark, 1997).

Application of hypertonic sucrose solutions is an established, Ca$^{2+}$-independent method to probe the releasable pool of SVs and paired sucrose applications have been used to monitor the refilling rate of this pool (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). While rab3A deletion had a clear effect on depolarization-evoked vesicle recruitment, no effects were observed during single or paired sucrose applications. This is in agreement with the unaltered responses to single or paired sucrose applications in cultured hippocampal neurons from rab3A
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knock-out mice (Geppert et al., 1997). Apparently, the transport role of rab3A that we have characterized here is specific for the depolarization-induced, Ca\(^{2+}\)-dependent recruitment. Application of hyperosmotic solutions produced similar release and vesicle recruitment in the presence or absence of rab3A and the response to hyperosmotic sucrose in the mutants was similar to depolarization-evoked recruitment in normal terminals. Hence, hyperosmotic shock appears to bypass the natural rab3A regulated vesicle transport. It is conceivable that the transport role of rab3A relates specifically to Ca\(^{2+}\)-dependent mechanisms of vesicle recruitment, especially because two of its downstream effectors are Ca\(^{2+}\)-binding proteins, i.e. rabphilin3A and RIM (Shirataki et al., 1994; Wang et al., 1997) and Ca\(^{2+}\)-influx has a facilitatory effect on vesicle recruitment (Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998)). The reduced expression of rabphilin3A in rab3A knock-out mice (Geppert et al. 1994a) would be in agreement with a regulatory role of the former protein in vesicle recruitment. However, no phenotype has been observed in rabphilin3A knock-out animals (Schlüter et al, 1999).

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