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Rab3A Is Involved in Transport of Synaptic Vesicles to the Active Zone in Mouse Brain Nerve Terminals

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The rab family of GTP-binding proteins regulates membrane transport between intracellular compartments. The major rab protein in brain, rab3A, associates with synaptic vesicles. However, rab3A was shown to regulate the fusion probability of synaptic vesicles, rather than their transport and docking. We tested whether rab3A has a transport function by analyzing synaptic vesicle 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 synaptic vesicles to and their docking at the active zone.

INTRODUCTION

Synaptic vesicles of the mammalian brain take up fast-acting neurotransmitters, mostly glutamate and γ-aminobutyric acid (GABA), and release them, upon activation, into 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 synaptic vesicle 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/vesicle-associated membrane proteins, and the munc18/s1 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. Synaptic vesicles 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 (Geppert *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 soluble *N*-ethylmaleimide-sensitive factor attachment receptor (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 synaptic vesicles 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 synaptic vesicles again. Rab3 isoforms interact with different general rab-binding proteins and with at least two specific effector proteins, rabphilin-3A (Li *et al.*, 1994; Shirataki *et al.*, 1994) and Rim (Wang *et al.*,

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1997). Rab3A null-mutant mice (Geppert *et al.*, 1994) and rab3 null-mutant worms (Nonet *et al.*, 1997) are viable and have mild phenotypes, suggesting nonessential functions of rab3. *Caenorhabditis elegans* rab3 null mutants have fewer synaptic vesicles, especially near the active zone, but more at ectopic sites, suggesting that synaptic vesicle 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 knockout mice (Geppert *et al.*, 1994). 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 (Geppert *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 synaptic vesicles to their target. Stimulation of the terminals by chemical depolarization evokes a redistribution of synaptic vesicles 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 knockout mice. Concomitantly, recovery of the secretion capacity and replenishment of docked vesicles after exhaustive stimulation were impaired in the mutants. In contrast, synaptic vesicle recruitment and neurotransmitter release induced by hyperosmotic sucrose were not affected in rab3A-deficient nerve terminals.

MATERIALS AND METHODS

Rab3A Knockout Mice

Rab3A-deficient mice have been described previously (Geppert *et al.*, 1994). All experiments were performed with null-mutant and wild-type littermates from heterozygous matings by experimenters who were unaware of the genotype.

Synaptosomal Preparation

Synaptosomes were prepared from whole forebrain of 4–5-mo-old mice by Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) 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) that contained 132 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 10 mM HEPES, and 10 mM D-glucose + 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 h after isolation. Protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard.

Release Assay

K^+ Stimulations. To determine fast transmitter release, synaptosomes (40 μg of protein) were pelleted and resuspended in 20 μl of aCSF supplemented with 50 μM EGTA, preincubated at 37°C for 5 min, and depolarized for 100 ms by use of a rapid mixing device (Leenders *et al.*, 1999). In short, this mixing device consists of two syringes controlled by pneumatic dispensers. Syringe 1 (37°C) from this mixer device releases a small aliquot (100 μl) of high-K^+ medium (see below) to the synaptosomes and after a delay of 100 ms, controlled by a digital timer, stop medium (250 μl, see below) is added from syringe 2 (0–4°C) to terminate the depolarization. Depolarization medium: aCSF containing 50 mM KCl (which isoosmotically 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 predepolarization protocol synaptosomes (2 mg/ml) in aCSF with 2 mM $CaCl_2$ were preincubated at 37°C for 2 min and either depolarized, by increasing 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 before 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 an NaCl concentration that restored the isoosmolarity of the medium.

HPLC Analysis of Released Transmitters. Synaptosomal suspension (150 μl) was centrifugated through 75 μl of 50:50% (vol/vol) mixture of silicone oil (Dow Corning 550, Mavan, Alphen aan den Rijn, Netherlands) and dinonylphtalate for 2 min in a Sigma table centrifuge at 15,000 $\times$ *g*. From the supernatant a 90-μl aliquot was pipetted onto 10 μl ice-cold trichloroacetic acid (10%)/homoserine (5 μM). Amino acid levels (glutamate and GABA) were determined by reversed phase high-performance liquid chromatography (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% glutaraldehyde. Synaptosomes were embedded in Epon, and 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.*, 1997). For each experimental condition, 25 sections with a clearly visible active zone were selected for analysis of synaptic vesicle distribution. The minimal distance between the active zone and the center of each synaptic vesicle 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. Synaptic vesicles 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 synaptic vesicles, which was tested by one-way analysis of variance 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 synaptic vesicle transport, we analyzed the distribution of synaptic vesicles and their neurotransmitter release in nerve terminals isolated from rab3A null mutant mice and their wild-type littermates. Electron microscopy revealed no differences between the synaptosomal preparations from the two groups (Figure 1, A and B). Morphometric analysis indicated that the diameter of the terminals, the active zone length, and the

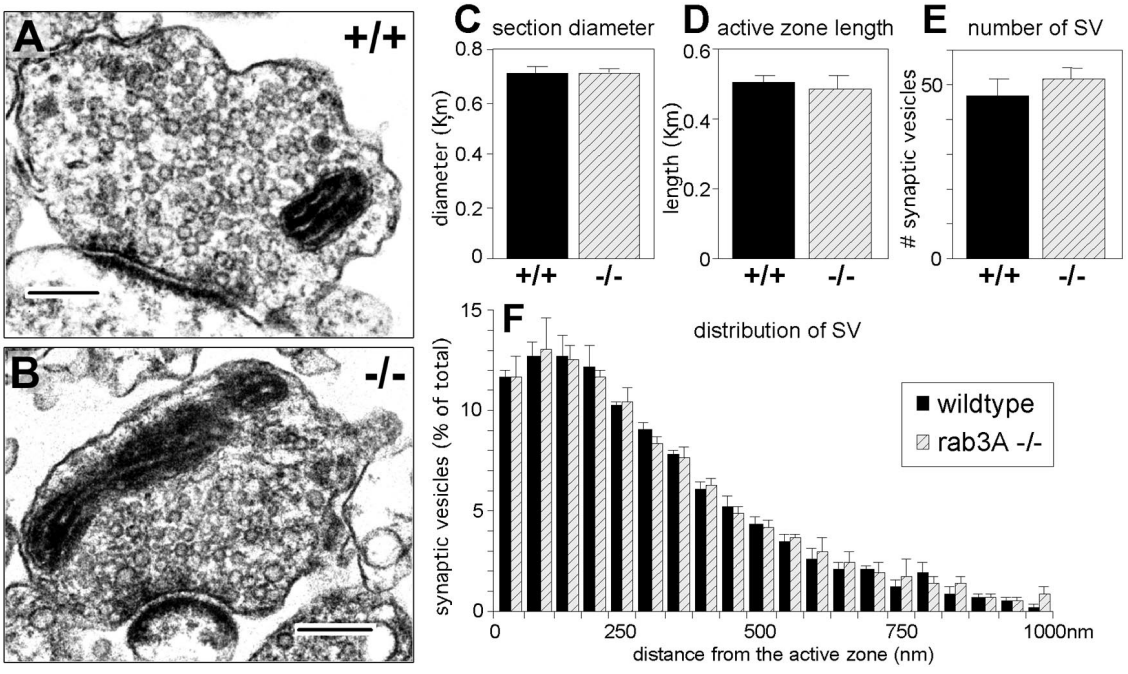

Figure 1. Ultrastructure and morphometric analysis of mouse brain nerve terminals from control and rab3A null-mutant littermates. Typical electron micrographs of synaptosomal preparations from wild-type (A) and rab3A-deficient (B) littermates. Micrographs were selected when the active zone with attached (part of the) postsynaptic density was present in the section and used for morphometric analysis of synaptic vesicle distribution and docking by an observer unaware of the genotype. Bar, 0.2 μm. Average diameter of the nerve terminals (C), active zone length (D), and total number of synaptic vesicles (E) were not different in resting nerve terminals of wild-type and rab3A mutants (n = 4 for both groups). Also, the intrasynaptic distribution of synaptic vesicles relative to the active zone was not different (F; see MATERIALS AND METHODS for details). The average diameter of the nerve terminals was determined by measuring from the active zone membrane the largest distance across the section.

total amount of synaptic vesicles per terminal were similar in the two groups (Figure 1, C–E). The distribution of synaptic vesicles 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 synaptic vesicle diameter). In resting nerve terminals, the synaptic vesicle distribution was not different between the two groups (Figure 1F).

Evoked Vesicle Recruitment Is Abolished in rab3A Null Mutants

A short episode of depolarization (0.1–15 s) evokes a redistribution of synaptic vesicles 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). Because the total amount of synaptic vesicles does not change after depolarization, their redistribution within the terminal reflects a net transport toward the active zone. This redistribution is referred to as depolarization-evoked vesicle recruitment. In nerve terminals isolated from wild-type mice, we observed similar depolarization-evoked vesicle recruitment as in rat nerve terminals after 100-ms depolarization in the presence of Ca^{2+} (Figure 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 synaptic vesicles in these terminals (Figure 2B). The total amount of vesicles per synaptic section was similar in wild-type and rab3A mutant mice and did not change after stimulation (Figure 2C).

An important aspect of depolarization-evoked vesicle recruitment is an increased number of synaptic vesicles morphologically docked at the active zone. In wild-type terminals, the number of docked vesicles increased >50% after 100-ms depolarization (2.1 ± 0.1–3.3 ± 0.3 vesicles/section; n = 4, $p < 0.05$; Figure 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 (Figure 2D, not significant, $p = 0.12$). The reduction in docked vesicles in the absence of rab3A is based on quantifications in random sections of isolated nerve terminals. Serial reconstructions of nerve terminals (Verhage *et al.*, 1991; Schikorski and Stevens, 1997) indicated that the absolute reduction *per synapse* is approximately five vesicles.

Recovery of Secretion Capacity after Exhaustive Stimulation Is Impaired in rab3A Null Mutants

The loss of depolarization-evoked synaptic vesicle recruitment in rab3A null mutants may compromise their ability to restore the secretion capacity after exhaustive stimulation. To test this, nerve terminals isolated from wild-type and

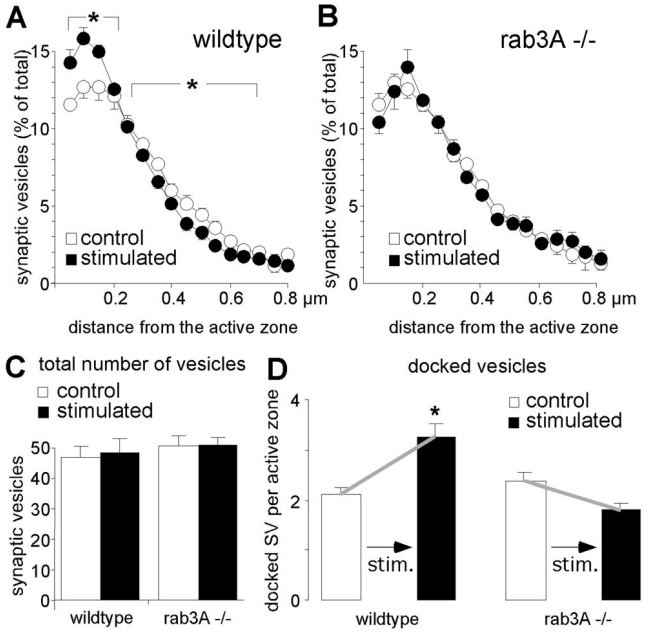

Figure 2. Redistribution of synaptic vesicles induced by 100-ms depolarization in nerve terminals from wild-type and rab3A-deficient littermates. Distribution of synaptic vesicles relative to the active zone in wild-type (A) and rab3A-deficient synaptosomal sections (B) with (filled symbols) and without (open symbols) 100-ms depolarization. Morphometric analysis as in Figure 1F. The total amount of synaptic vesicles (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 4 animals of each genotype. Data are means ± SEM *$p < 0.05$.

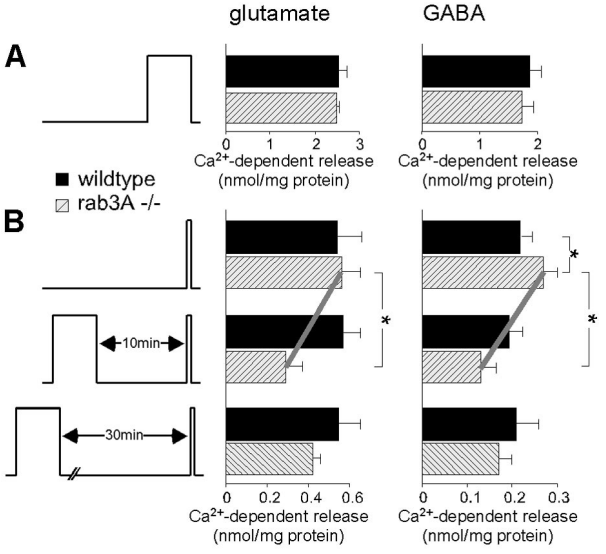

Figure 3. Neurotransmitter release during single depolarizations and after exhaustive predepolarization in nerve terminals from wild-type and rab3A-deficient littermates. Isolated nerve terminals from wild-type and rab3A-deficient mice were depolarized for 3 min (A) or for 100 ms (B) with or without 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 total content of the terminals (glutamate: 45.1 ± 1.4 in controls vs. 46.6 ± 1.6 in mutants; GABA: 37.1 ± 2.7 in controls vs. 37.6 ± 2.6 in mutants) and in the Ca^{2+}-independent component of release (glutamate: 2.6 ± 0.4 in controls vs. 2.5 ± 0.4 in mutants; GABA: 0.8 ± 0.1 in controls vs. 0.8 ± 0.1 in mutants). Data represent means ± SEM of 6–11 independent experiments. *$p < 0.05$.

rab3A null mutant littermates were first stimulated exhaustively to deplete releasable vesicles (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.

On exhaustive stimulation, the total amount of Ca^{2+}-dependent release of the major, endogenous neurotransmitters in brain, glutamate, and GABA was similar in wild-type and rab3A-deficient nerve terminals (Figure 3A). A single test pulse of 100-ms depolarization also led to a comparable Ca^{2+}-dependent release in the two groups (Figure 3B), although GABA release during 100-ms depolarization was slightly higher in rab3A-deficient terminals (0.22 ± 0.02 nmol/mg of protein in wild types versus 0.27 ± 0.04 nmol/mg of protein in rab3A null mutants, $n = 11$, $p < 0.05$). The Ca^{2+}-independent component of release was also similar between the two groups (glutamate: 2.6 ± 0.4 in controls vs. 2.5 ± 0.4 in mutants; GABA: 0.8 ± 0.1 in controls vs. 0.8 ± 0.1 in mutants). These similar release responses upon short or long stimulation are consistent with the similar distribution of synaptic vesicles observed in resting nerve terminals from the two groups (Figure 1).

After exhaustive depolarization, intracellular vesicle transport in wild-type mice had completely replenished the releasable pool within a 10-min recovery phase (Figure 3B). However, in rab3A-deficient terminals the replenishment was not complete, and neurotransmitter release was decreased by ~50% after 10-min recovery (Figure 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, Figure 3B). This indicates that the vesicle pool can be largely replenished in the absence of rab3A, but only with a considerable delay (>30 min, more than threefold slower than in wild-type terminals). Even after 30 min the recovery tended to be lower in the absence of rab3A (not significant, $p < 0.08$ for glutamate and $p < 0.18$ for GABA).

Replenishment of Docked Vesicles after Exhaustive Stimulation Is Impaired in rab3A Null Mutants

To investigate morphological correlates of the impaired replenishment in the rab3A mutants, we analyzed vesicle distribution in isolated nerve terminals at the ultrastructural level with the use of the same stimulation protocol. Nerve terminals were mixed with fixative instead of depolarization buffer at the start of the second stimulus (Figure 3). Subse-

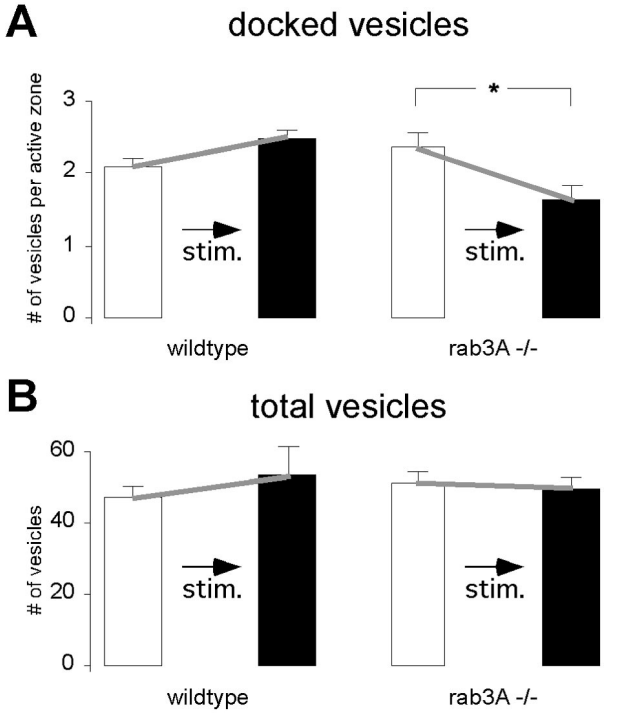

Figure 4. Total number of synaptic vesicles and the number of morphologically docked vesicles in nerve terminals from wild-type and rab3A-deficient littermates after a 3-min depolarization and 10-min recovery. Number of docked synaptic vesicles (A) and total number of synaptic vesicles (B) per section were analyzed in terminals fixated before (□) and after (■) 3-min depolarization and 10-min recovery (see Figure 3). Data represent means + SEM of four independent experiments. *$p < 0.05$.

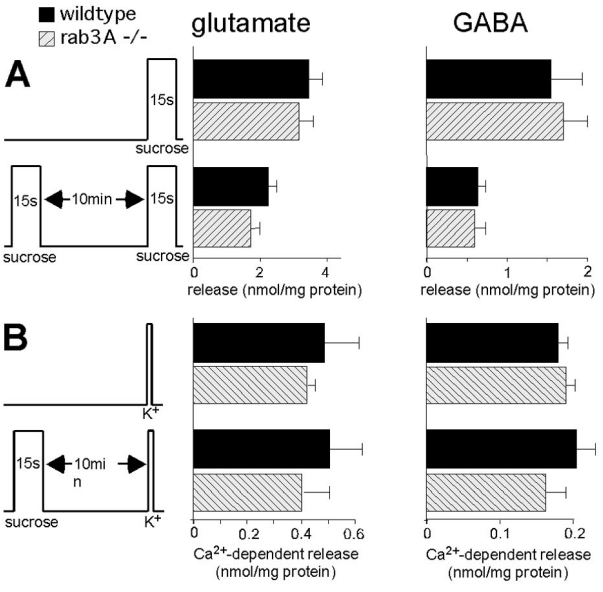

Figure 5. Neurotransmitter release after application of hyperosmotic sucrose solutions and chemical depolarization to nerve terminals from wild-type and rab3A-deficient littermates. Isolated nerve terminals from wild-type and rab3A-deficient mice were stimulated either once or twice with 0.5 M sucrose for 15 s (A) or with 100-ms chemical depolarization with or without previous sucrose stimulation (B) and the release of endogenous glutamate and GABA was measured. The stimulation protocols are outlined on the left. (A) Total release is plotted. (B) 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+}). Data represent means ± SEM of five independent experiments.

quent morphometric analysis showed that in wild-type terminals the number of docked vesicles was comparable with unstimulated nerve terminals. However, in rab3A-deficient terminals the pool of docked synaptic vesicles was decreased compared with unstimulated rab3A-deficient terminals (Figure 4A, $n = 4$, $p < 0.05$). The total number of vesicles was not altered in any of the conditions and in both groups (Figure 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 morphometric 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 artificial stimulus widely used to analyze the size and the replenishment of the releasable synaptic vesicle pool, especially in electrophysiological analyses of hippocampal autapses (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). We confirmed that also in the nerve terminal preparation, hyperosmotic sucrose induced vesicular release from the same vesicle pool as membrane depolarization, although in a Ca^{2+}-independent manner (our unpublished results; Lonart *et al.*, 1998). We applied hypertonic (0.5 M) sucrose solutions for 15 s (Geppert *et al.*, 1997). This yielded approximately six- to sevenfold more glutamate and GABA release than the Ca^{2+}-dependent component after 100-ms depolarization (Figure 5). The amounts released by either stimulus did not differ in the presence or absence of rab3A (Figure 5). Concomitantly, paired applications of hyperosmotic sucrose solutions separated by a 10-min period of recovery revealed no differences in vesicle replenishment between mutant and control nerve terminals, but the second sucrose application was less effective for both groups (Figure 5A). Finally, 100-ms depolarization after sucrose stimulation and 10-min recovery also revealed no differences in vesicle replenishment between control and rab3A-deficient nerve terminals (Figure 5B).

Application of 0.5 M sucrose for 15 s to wild-type nerve terminals produced an increase in the amount of synaptic vesicles within 150 nm from the active zone (Figure 6A; $p < 0.05$, $n = 6$) and a decrease in the amount of synaptic vesicles at 200-1000-nm distance from the active zone (our unpublished results). This redistribution was similar to the redistribution observed after 0.1 (Figure 2A) or 15-s chemical depolarization (Figure 6B). Unlike depolarization, redistribution of synaptic vesicles after hyperosmotic sucrose ap-

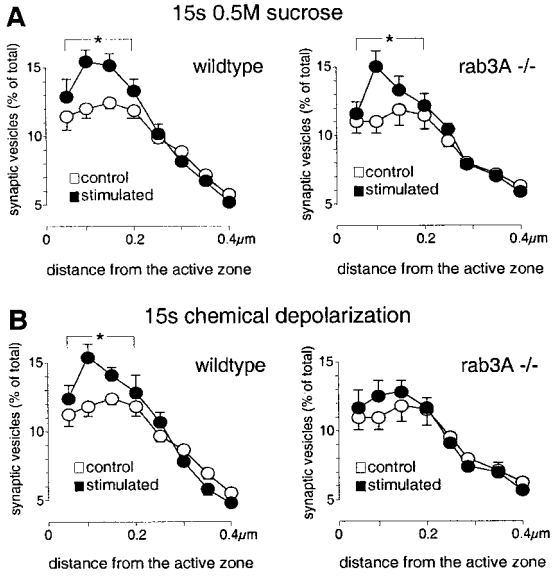

Figure 6. Redistribution of synaptic vesicles after application of hyperosmotic sucrose solutions and chemical depolarization to nerve terminals from wild-type and rab3A-deficient littermates. Distribution of synaptic vesicles in synaptosomal sections was analyzed as in Figure 1F with (filled symbols) and without (open symbols) stimulation, i.e., application of 0.5 M sucrose for 15 s (A) or 40 mM K^+ in presence of 2 mM Ca^{2+} (B). 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 six independent experiments.

plication was also observed in the absence of Ca^{2+} (our unpublished results). Furthermore, unlike depolarization, 0.5 M sucrose also induced redistribution in rab3A-deficient terminals as in wild types (Figure 6, A and B; $p < 0.05$, $n = 6$). 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 with the use of hyperosmotic sucrose.

DISCUSSION

We have analyzed the role of rab3A in synaptic vesicle trafficking in nerve terminals isolated from mouse brain. Deletion of rab3A expression did not affect the number of synaptic vesicles 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 complexes that drive vesicle fusion (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 synaptic vesicle (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 synaptic vesicles 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. First, 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 synaptic vesicles 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). Second, 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 was 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 predocked 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, because it was evident in the mixed population of isolated nerve terminals, i.e., from all forebrain 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.*, 1994; 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 synaptic vesicle pool. Because at least two specific rab3 effectors have been characterized (Shirataki *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 synaptic vesicle cycle (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 (Novick and Zerial, 1997). Nevertheless, the enhanced rundown of responsiveness in CA1 neurons of the hippocampus at 14 Hz, which was previously observed in the rab3A null mutants (Geppert *et al.*, 1994), and the loss of long-term potentiation in the CA3 area but not of regular transmission of these mutants (Castillo *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 synaptic vesicles and paired sucrose applications have been used to monitor the refilling rate of this pool (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). Although 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 knockout 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 have a facilitatory effect on vesicle recruitment (Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). The reduced expression of rabphillin-3A in rab3A knockout mice (Geppert *et al.* 1994) would be in agreement with a regulatory role of the former protein in vesicle recruitment. However, no phenotype has been observed in rabphillin-3A knockout animals (Schluter *et al.*, 1999).

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