

# Redundant functions of RIM1 $\alpha$ and RIM2 $\alpha$ in Ca $^{2+}$ -triggered neurotransmitter release

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**$\alpha$ -RIMs (RIM1 $\alpha$  and RIM2 $\alpha$ ) are multidomain active zone proteins of presynaptic terminals.  $\alpha$ -RIMs bind to Rab3 on synaptic vesicles and to Munc13 on the active zone via their N-terminal region, and interact with other synaptic proteins via their central and C-terminal regions. Although RIM1 $\alpha$  has been well characterized, nothing is known about the function of RIM2 $\alpha$ . We now show that RIM1 $\alpha$  and RIM2 $\alpha$  are expressed in overlapping but distinct patterns throughout the brain. To examine and compare their functions, we generated knockout mice lacking RIM2 $\alpha$ , and crossed them with previously produced RIM1 $\alpha$  knockout mice. We found that deletion of either RIM1 $\alpha$  or RIM2 $\alpha$  is not lethal, but ablation of both  $\alpha$ -RIMs causes postnatal death. This lethality is not due to a loss of synapse structure or a developmental change, but to a defect in neurotransmitter release. Synapses without  $\alpha$ -RIMs still contain active zones and release neurotransmitters, but are unable to mediate normal Ca $^{2+}$ -triggered release. Our data thus demonstrate that  $\alpha$ -RIMs are not essential for synapse formation or synaptic exocytosis, but are required for normal Ca $^{2+}$ -triggering of exocytosis.**

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## Introduction

In presynaptic nerve terminals, exocytosis of transmitter-filled synaptic vesicles at the plasma membrane is under tight spatial and temporal control. Synaptic vesicle exocytosis is restricted to a specialized area of the plasma membrane, the active zone. The protein network that constitutes the active zone organizes the docking and priming of synaptic vesicles. In addition, the active zone mediates use-dependent changes in release during short- and long-term forms of presynaptic plasticity (reviewed in Dresbach *et al*, 2001; Rosenmund *et al*, 2003; Kaeser and Südhof, 2005).

The active zone is composed of a network of proteins that includes RIMs (for Rab3-interacting molecule; see Wang *et al*, 1997, 2000) as central components (Koushika *et al*, 2001; Castillo *et al*, 2002; Schoch *et al*, 2002). RIMs are multi-domain proteins that are expressed in variable forms: two  $\alpha$ -RIMs (RIM1 $\alpha$  and 2 $\alpha$ ) that include a full complement of RIM domains (an N-terminal region that comprises a Rab3-binding sequence and a Munc13-binding zinc-finger; a central PDZ-domain; and two C-terminal C<sub>2</sub>-domains that however do not bind Ca $^{2+}$ ), a single  $\beta$ -RIM (RIM2 $\beta$ ) that contains all of the domains of  $\alpha$ -RIMs except for the N-terminal Rab3- and Munc13-binding sequences; and three  $\gamma$ -RIMs (RIM2 $\gamma$ , 3 $\gamma$ , and 4 $\gamma$ ) that are composed of only the C-terminal C<sub>2</sub>-domain preceded by a short N-terminal flanking sequence (Wang *et al*, 2000). RIMs are encoded by four genes, of which the RIM1, RIM3, and RIM4 genes express only a single isoform (RIM1 $\alpha$ , 3 $\gamma$ , and 4 $\gamma$ , respectively), whereas the RIM2 gene expresses three isoforms (RIM2 $\alpha$ , 2 $\beta$ , and 2 $\gamma$ ; Wang and Südhof, 2003). Further variation is introduced into  $\alpha$ - and  $\beta$ -RIMs (but not  $\gamma$ -RIMs) by extensive alternative splicing (Wang *et al*, 2000; Johnson *et al*, 2003; Wang and Südhof, 2003).

In addition to interacting with Munc13 and Rab3,  $\alpha$ -RIMs bind to multiple other synaptic proteins: ELKS via the central PDZ-domain (Ohtsuka *et al*, 2002; Wang *et al*, 2002), RIM-BPs via an SH3-domain-binding sequence between the two C<sub>2</sub>-domains (Wang *et al*, 2000), and  $\alpha$ -liprins and synaptotagmin 1 via the C-terminal C<sub>2</sub>-domain (Coppola *et al*, 2001; Schoch *et al*, 2002). Furthermore, *in vitro* interactions with several proteins have been described, including cAMP-GEFII (guanine nucleotide-exchange factor II) (Ozaki *et al*, 2000), SNAP-25 (Coppola *et al*, 2001), N-type Ca $^{2+}$  channels (Coppola *et al*, 2001), and 14-3-3 adaptor proteins (Sun *et al*, 2003; Simsek-Duran *et al*, 2004). RIMs are connected indirectly with the active zone proteins Piccolo and Bassoon via ELKS (Takao-Rikitsu *et al*, 2004) and with receptor tyrosine phosphatases via liprins (Serra-Pages *et al*, 1998). Of these interactions, only RIM1 $\alpha$  and 2 $\alpha$  bind to Munc13 and Rab3, whereas  $\gamma$ -RIMs bind only to  $\alpha$ -liprins and synaptotagmin 1. The binding of the N-terminal region of  $\alpha$ -RIMs to Rab3 on synaptic vesicles and Munc13s is particularly interesting because a relatively short sequence (<150 residues) contains two nested subdomains, an  $\alpha$ -helical region that

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binds to Rab3 (Wang *et al*, 2001) and a zinc-finger that binds to Munc13 (Betz *et al*, 2001; Dulubova *et al*, 2005). This binding is mutually compatible with each other, resulting in a trimeric complex in which the  $\alpha$ -RIM/Munc13 dimer on the active zone is coupled to the synaptic vesicle protein Rab3 (Dulubova *et al*, 2005). Finally, RIMs are substrates for cAMP-dependent protein kinase (PKA) that phosphorylates RIM1 $\alpha$  and RIM2 $\alpha/\beta$  at two sites (Lonart *et al*, 2003).

Analysis of RIM1 $\alpha$  knockout (KO) mice showed that RIM1 $\alpha$  plays a key regulatory role in synaptic vesicle exocytosis at the active zone, from vesicle priming to short- and long-term synaptic plasticity (Castillo *et al*, 2002; Schoch *et al*, 2002; Calakos *et al*, 2004). RIM1 $\alpha$ -deficient synapses did not exhibit major changes in ultrastructure, suggesting that it is essential only for regulating exocytosis, and not for building an active zone architecture (Schoch *et al*, 2002). Although important, loss of this function does not impair mouse survival, as RIM1 $\alpha$  KO mice have a normal apparent life expectancy (Schoch *et al*, 2002). The importance of RIM1 $\alpha$  function nevertheless is apparent from the severe behavioral abnormalities observed in these mice, which include impairments in spatial learning and in fear conditioning as well as an increase in locomotor responses to novelty (Powell *et al*, 2004).

The currently available data confirm that RIM1 $\alpha$  is an active zone protein with a central role in regulating neurotransmitter release, and suggest that the other RIM isoforms may also be involved in the regulation of synaptic vesicle exocytosis. However, so far, only RIM1 $\alpha$  has been analyzed. Although the various RIM isoforms are coexpressed in brain, their relative expression patterns are unknown, and it is unclear how much potential redundancy may exist among RIM isoforms. Such redundancy could exist, for example, between RIM1 $\alpha$  and RIM2 $\alpha$  because both of these RIM

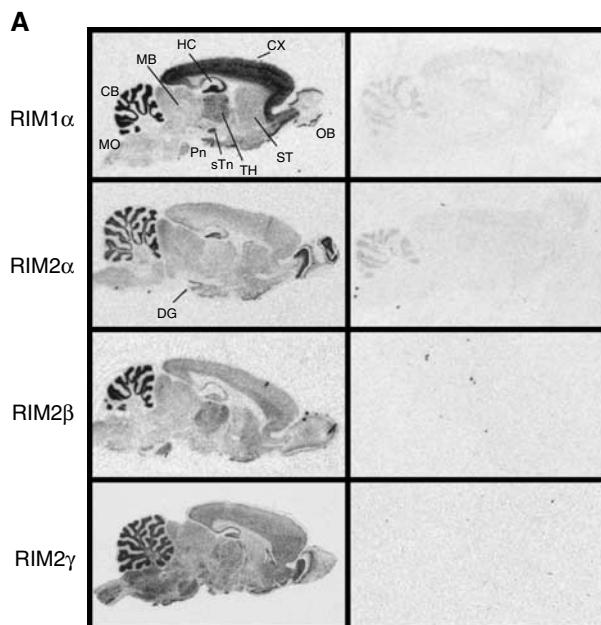
isoforms bind to Munc13 and to Rab3 (Dulubova *et al*, 2005), although they are the only isoforms that do so. Therefore, major questions remain unanswered: (1) in which cell types are the various RIM isoforms expressed? (2) Are RIM1 $\alpha$  and RIM2 $\alpha$  functionally redundant? (3) How do the two  $\alpha$ -RIMs relate to each other? (4) Does the deletion of both  $\alpha$ -RIMs lead to ultrastructural changes? To examine the role of the  $\alpha$ -RIMs in synaptic transmission, we generated single and double KO mice (DKO) lacking either or both  $\alpha$ -RIMs. Our data demonstrate that the RIM- $\alpha$ -isoforms are essential for survival and exhibit partially overlapping functions in the regulation of synaptic transmission, but are not required for building a normal synapse.

## Results

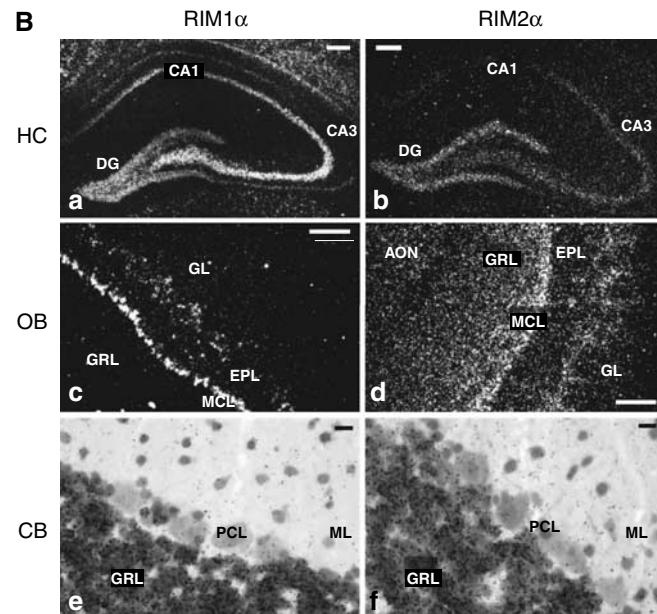
### Differential expression of RIM1 and RIM2 isoforms

To examine whether RIM1 $\alpha$ , RIM2 $\alpha$ , RIM2 $\beta$ , and RIM2 $\gamma$  are differentially expressed in brain, we performed *in situ* hybridizations on brain sections from adult rats (Figure 1A, left panels). Two oligonucleotides were used for each RIM isoform to ensure that the same labeling patterns were obtained (data not shown). This labeling was abolished when excess unlabeled oligonucleotides were added to the hybridization mix (Figure 1A, right panels).

Autoradiographs of hybridized rat brain sections revealed differential but overlapping expression patterns of RIM mRNAs. In each case, regions rich in glial cells (e.g., white matter of cerebral cortex and cerebellum) were unlabeled, indicating a neuron-specific expression of RIM isoforms (Figure 1A, left panel). RIM1 $\alpha$  mRNA is present throughout the brain, with the highest levels in the cortex, cerebellum, hippocampus and thalamus. RIM2 $\alpha$  mRNA is concentrated in the cerebellum, the olfactory bulb and the dentate gyrus of



**Figure 1** *In situ* hybridization of RIM mRNAs in the rat brain. (A) Film images showing the distribution of RIM mRNAs in the adult rat brain (CB, cerebellum; CX, cerebral cortex; DG, dentate gyrus; HC, hippocampus; MB, midbrain; OB, olfactory bulb; Pn, pontine nucleus; sTn, subthalamic nucleus; ST, striatum; TH, thalamus; MO, medulla oblongata). (B) Dark-field images of emulsion-dipped sections from rat hippocampus, olfactory bulb, and cerebellum (AON, anterior olfactory nucleus; DG, dentate gyrus; EPL, external plexiform layer; GL, glomerular layer; GRL, granule cell layer; MCL, mitral cell layer; ML, molecular layer; PCL, Purkinje cell layer; scale bar B, a-d = 100  $\mu$ m, B, e-f = 20  $\mu$ m).



the hippocampus. However, comparison of the signals obtained with the negative control indicates that RIM2 $\alpha$  is also ubiquitously expressed in brain, albeit at low levels. RIM2 $\beta$  and RIM2 $\gamma$  mRNAs are present in a pattern similar to RIM1 $\alpha$ , but both are probably expressed at lower levels based on the hybridization signal and exhibit regional differences (e.g., RIM2 $\beta$  is expressed more in the thalamus, and RIM2 $\gamma$  more in the brainstem; Figure 1A). These results show that the various RIM1- and RIM2-isoforms exhibit a differential, but highly overlapping expression pattern in rat brain.

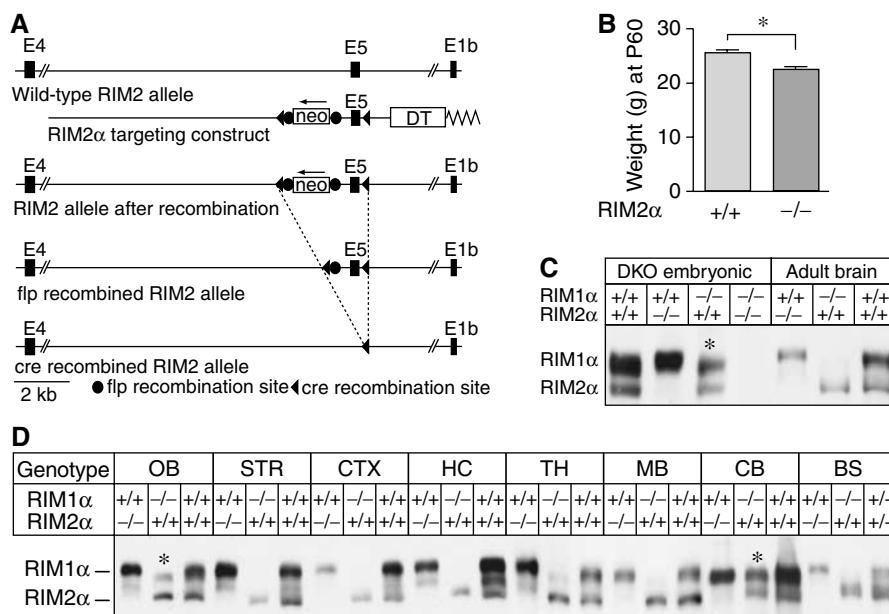
To determine the cellular distribution of RIM mRNA species, we analyzed emulsion-dipped sections. In the hippocampus, RIM1 $\alpha$  can be detected at high levels in the dentate gyrus and the CA3 region, and at lower levels in the CA1 region (Figure 1B). In contrast, strong labeling for RIM2 $\alpha$  was largely restricted to the dentate gyrus, with lower levels observed in the CA3 region. In the olfactory bulb, significant RIM1 $\alpha$  expression was detected in the mitral cell layer (MCL) and in some cells of the external plexiform layer (EPL), whereas the cells of the granule cell layer (GRL) and the glomerular layer (GL) were devoid of a RIM1 $\alpha$  signal (Figure 1B). In contrast, RIM2 $\alpha$  showed strong expression in the GL, the MCL, and the GRL. Within the cerebellum, RIM1 $\alpha$  and RIM2 $\alpha$  expression is highly concentrated in the GRL (Figure 1B). Clearly, most neurons express multiple isoforms of RIMs with distinct relative expression levels.

#### Characterization of RIM2 $\alpha$ KO mice

We generated mutant mice in which the fifth exon of the RIM2 $\alpha$  gene (which encodes part of the zinc-finger; Wang and Südhof, 2003) is flanked by loxP sites (Figure 2A). Mice

containing the targeted floxed RIM2 $\alpha$  gene synthesized RIM2 $\alpha$  at approximately wild-type levels (data not shown). We then crossed the floxed mice to transgenic mice that express cre recombinase in the male germ line to produce KO mice in which the floxed exon was deleted (Figure 2A) (O'Gorman et al, 1997). The conditional KO mice may prove to be useful in the analysis of the function of the  $\alpha$ -RIMs in specific brain regions. However, in the present study, we focused on the constitutive RIM2 $\alpha$  KO mice in order to establish the baseline function of the  $\alpha$ -RIM isoforms.

Homozygous RIM2 $\alpha$  KO mice were viable and fertile. However, systematic analysis of the offspring from heterozygous matings revealed that homozygous KO mice were slightly smaller than littermate wild-type controls (males,  $N=36$ ) (Figure 2B), and less frequent than would be expected based on Mendelian inheritance (24.1% wild type, 56.3% heterozygous, and 19.6% homozygous mutant mice in offspring at >1 month of age;  $N=591$ ;  $P<0.01$ ). Immunoblotting confirmed that the KO mice lacked RIM2 $\alpha$  (Figure 2C and D and Supplementary Figure 2). Similar to the RIM1 $\alpha$  KO mice (Schoch et al, 2002), RIM2 $\alpha$  mutant mice exhibited a deficit in maternal behavior, as they did not take care of their litters even after multiple pregnancies. Brains of single RIM2 $\alpha$  KO mice showed a normal cell density, cytoarchitecture, connectivity, distribution/density of synapses, and ultrastructural morphology as assessed by H&E staining, NeuN immunohistochemistry, and electron microscopy (Supplementary Figure 1). Taken together, these data demonstrate that although RIM2 $\alpha$  KO mice are viable and exhibit no apparent developmental abnormalities, the behavior and survival of these mice are slightly impaired.



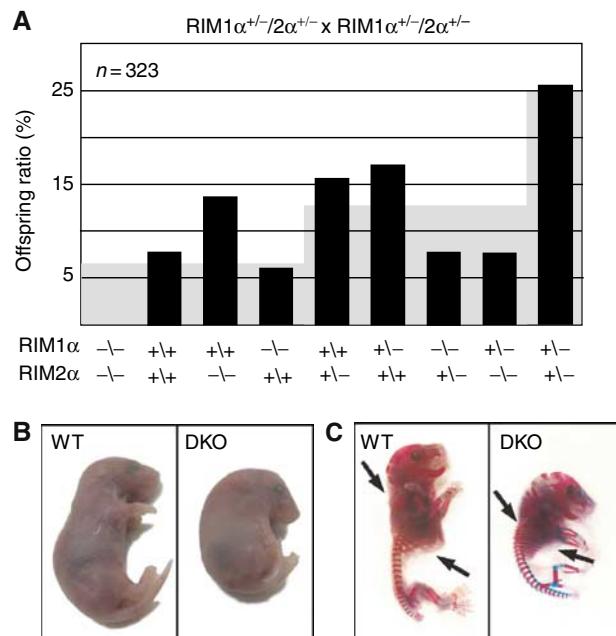
**Figure 2** Generation of RIM2 $\alpha$  KO mice. (A) Structures of the RIM2 wild-type gene (wild-type allele), of the targeting vector used for homologous recombination (targeting construct), and of the mutant RIM2 alleles after homologous recombination before and after further recombination of flp and cre recombinases. In the targeting vector, exon 5 is flanked by loxP sites (black triangles) and the neomycin resistance gene cassette (*neo*) that is flanked by flp recombination sites (black circles). A diphtheria toxin gene (DT) is included for negative selection. (B) Weights of male RIM2 $\alpha$  KO and littermate control mice ( $N=36$ ,  $*P<0.001$ ). (C) Immunoblots of E18.5 embryonic and adult wild-type, RIM1 $\alpha$  KO, RIM2 $\alpha$  KO and embryonic DKO whole brain homogenates. (D)  $\alpha$ -RIM immunoblots of proteins from different brain regions from adult RIM1 $\alpha$  KO, RIM2 $\alpha$  KO, and wild-type control mice; blots were probed with an antibody that recognizes both RIM1 $\alpha$  and RIM2 $\alpha$  (abbreviations of brain areas: OB, olfactory bulb; STR, striatum; CTX, cortex; HC, hippocampus; TH, thalamus; MB, midbrain, BS, brain stem; CB, cerebellum; \*, bands of alternatively spliced RIM2 $\alpha$  isoforms of higher molecular weight).

To search for compensatory changes in the expression of RIM1 $\alpha$  as the only other  $\alpha$ -RIM isoform in RIM2 $\alpha$  KO mice, we compared the relative expression of RIM1 $\alpha$  and RIM2 $\alpha$  in various brain regions in wild-type, RIM1 $\alpha$  KO, and RIM2 $\alpha$  KO mice (Figure 2D and Supplementary Figure 2). Antibodies to the zinc-finger region of RIM1 $\alpha$  and RIM2 $\alpha$  often crossreact because of their structural similarities (Schoch et al, 2002). Moreover, these antibodies detect multiple bands even when one of the two  $\alpha$ -RIM isoforms is deleted because of the extensive alternative splicing of RIMs (Wang and Südhof, 2003). Consistent with the *in situ* hybridization data, immunoblotting of different brain regions in single RIM1 $\alpha$  and RIM2 $\alpha$  KO mice showed that RIM1 $\alpha$  is the more abundant isoform, whereas RIM2 $\alpha$  is found at low levels in most rostral brain regions examined, but at high levels in the cerebellum and olfactory bulb (Figure 2D). However, we detected no region-specific compensatory changes in RIM1 $\alpha$  protein levels in RIM2 $\alpha$  KO mice, or conversely in RIM2 $\alpha$  protein levels in RIM1 $\alpha$  KO mice (Figure 2D and Supplementary Figure 2). Interestingly, alternative splicing of RIM2 $\alpha$  seems to be regulated in a region-specific manner. Although in most brain regions, a smaller isoform is detected, in cerebellum and olfactory bulb an additional larger protein could be observed (asterisks in Figure 2D and Supplementary Figure 2).

In RIM1 $\alpha$  KO mice, synaptic transmission in the hippocampus is severely impaired (Castillo et al, 2002; Schoch et al, 2002; Calakos et al, 2004). To test whether RIM2 $\alpha$  performs a similar fundamental role in synaptic transmission in the hippocampus, we recorded excitatory and inhibitory synaptic responses in acute hippocampal slices (Supplementary Figure 3). We first probed excitatory synaptic transmission in the CA1 region of the hippocampus, and compared synaptic responses of wild-type and KO mice in four paradigms that measure different types of short-term synaptic plasticity: paired-pulse facilitation (Supplementary Figure 3A), post-tetanic potentiation (Supplementary Figure 3B), and use-dependent depression (Supplementary Figure 3C). In addition, we examined the synaptic release probability by analyzing the progressive block of NMDA-dependent synaptic responses by the irreversible NMDA receptor antagonist MK801 (Supplementary Figure 3D). In all of these measurements, we observed no significant difference between wild-type and RIM2 $\alpha$  KO mice. Next, we tested whether RIM2 $\alpha$  KO mice exhibited a change in mossy fiber LTP in excitatory synapses of the CA3 region, which is abolished in RIM1 $\alpha$  KO mice (Castillo et al, 2002). Again, we found no change in RIM2 $\alpha$  KO mice (Supplementary Figure 3E). Finally, we examined inhibitory synaptic transmission at CA1 pyramidal cells, but failed to detect significant changes in paired-pulse-ratio (Supplementary Figure 3F). Thus, RIM2 $\alpha$ -deficient synapses, different from RIM1 $\alpha$ -deficient synapses, do not exhibit a major impairment of synaptic transmission in the hippocampus.

#### **Impaired survival of RIM1 $\alpha$ /2 $\alpha$ DKO mice**

To assess the redundant or divergent functions of the two  $\alpha$ -RIMs, we generated RIM1 $\alpha$ /2 $\alpha$  DKO mice. Analysis of the offspring from systematic breedings of double heterozygous RIM1 $\alpha$ /2 $\alpha$  KO mice revealed that the survival of RIM1 $\alpha$  or RIM2 $\alpha$  homozygous mutant mice exhibited no significant decrease as long as two RIM1 $\alpha$  or RIM2 $\alpha$  wild-type alleles



**Figure 3** (A) Survival analysis of the offspring from matings of double heterozygous RIM1 $\alpha$ /2 $\alpha$  mutant mice. The black bars plot the observed frequency of the indicated genotypes as percentage of the total, whereas the gray background indicates the expected frequency based on Mendelian inheritance ( $N=323$ ). (B, C) Images of E18.5 RIM1 $\alpha$ /2 $\alpha$  DKO mutant and control littermate mice overall morphology (B) and skeleton (C, bones are stained in blue and cartilage in pink; black arrows point to ribcage and cervical vertebrae).

were present (Figure 3A). However, in >100 crossings, no surviving mouse that was homozygous mutant for both RIM1 $\alpha$  and RIM2 $\alpha$  was observed, demonstrating that RIM1 $\alpha$  and RIM2 $\alpha$  are redundant in terms of survival. Moreover, even single homozygous RIM1 $\alpha$  or RIM2 $\alpha$  KO mice exhibited significantly impaired survival when one of the other two  $\alpha$ -RIM alleles was also deleted (i.e., in the RIM1 $\alpha$ <sup>-/-</sup>2 $\alpha$ <sup>+/+</sup> or RIM1 $\alpha$ <sup>+/+</sup>2 $\alpha$ <sup>-/-</sup> genotypes,  $P<0.001$ ; see Figure 3A).

RIM1 $\alpha$ /2 $\alpha$  DKO mice died immediately after birth because they could not breathe. The mutant mice did not respond to tactile stimuli, and exhibited an abnormal body posture similar to synaptobrevin 2 KO mice (Figure 3; Schoch et al, 2001), suggesting that they were paralyzed. Double KO mice could be recovered alive, however, at E18.5 as embryos by hysterectomy; at this age, DKO mice were present in a normal Mendelian ratio, suggesting that the deletion of both  $\alpha$ -RIMs does not impair survival *in utero* (data not shown). Thus, for all subsequent studies on DKO mice, we analyzed embryos at E18.5. Although whole-body staining for bone and cartilage revealed no major developmental defects in the DKO mice, their vertebrae in the KO skeleton seemed more compact at the cervical level, and the ribcage appeared enlarged (Figure 3C), possibly because of permanent paralysis of the embryo during development.

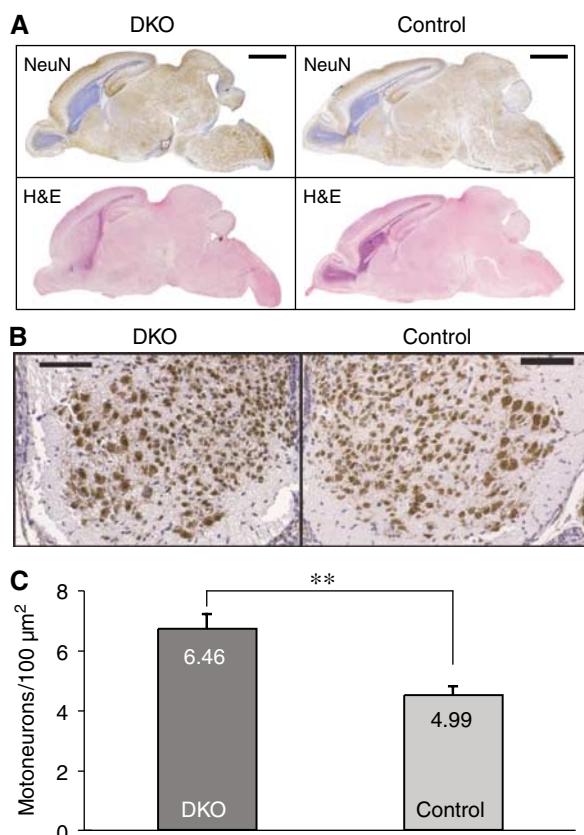
#### **Brain composition and synapse structure of RIM1 $\alpha$ /2 $\alpha$ double KO mice**

Histochemistry and immunocytochemistry of brain sections revealed that RIM1 $\alpha$ /2 $\alpha$  DKO mice displayed no obvious impairments in central nervous system development

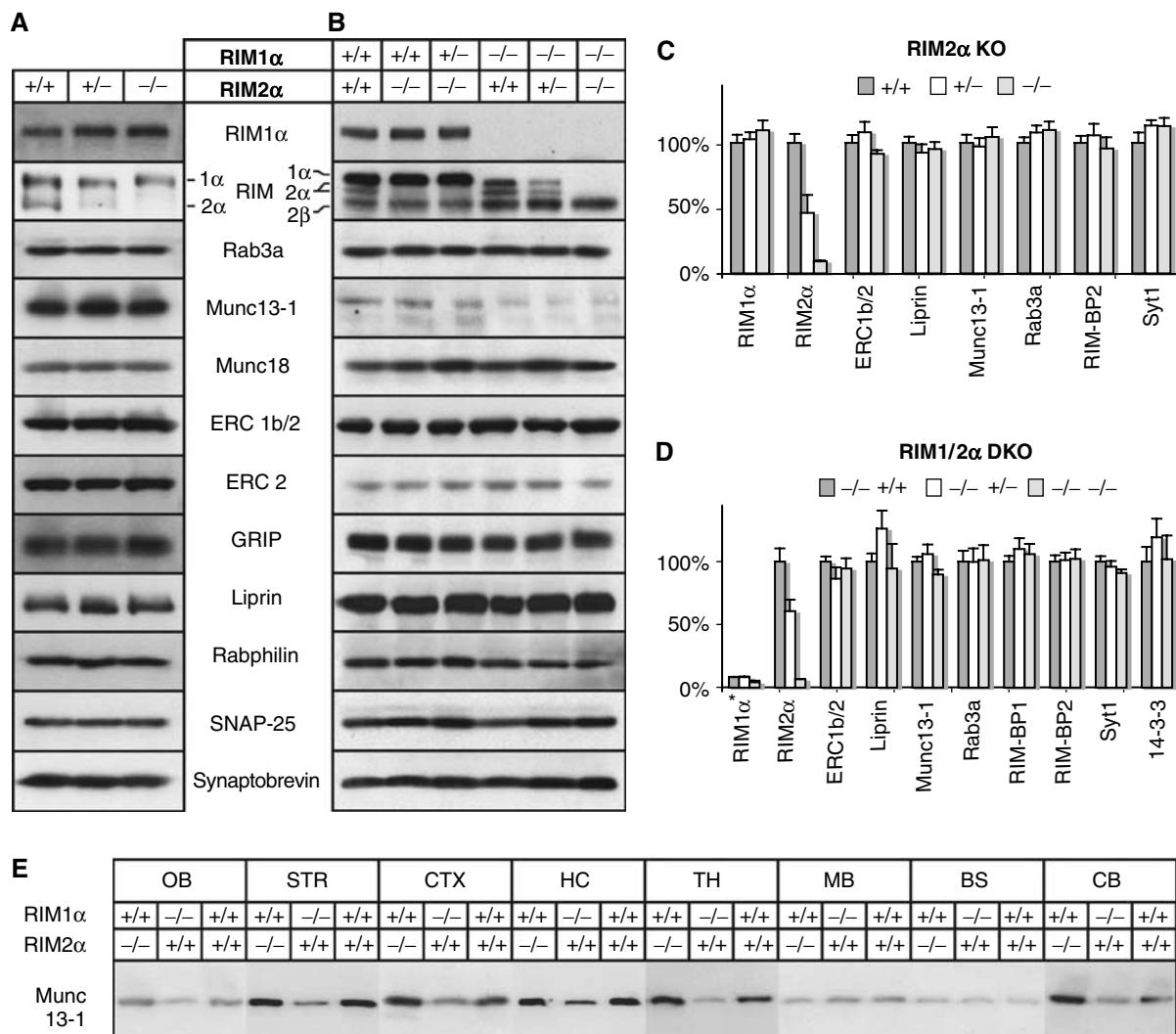
(Figure 4A and data not shown). However, H&E- and NeuN-stained vibratome sections of paraffin-embedded spinal cords showed an increased number of ventral horn motoneurons at all cervical levels in RIM1 $\alpha$ /2 $\alpha$  DKO mice as compared with control littermates (Figure 4B). A morphometric analysis of NeuN-immunopositive motoneurons revealed a significant increase ( $**P < 0.005$ ) in motoneuron density in E18.5 DKO spinal chords (Figure 4C). No sign of neurodegeneration was detectable in the spinal cord by TUNEL or Fluoro JadeB staining (data not shown). The increase in motoneurons may be due to decreased synaptic transmission at the neuromuscular junction (NMJ) (see below) (Harris and McCaig, 1984; Oppenheim, 1991).

To test whether deletion of  $\alpha$ -RIMs causes a major change in the protein composition of the brain, we quantified the levels of neuronal proteins in adult littermate wild-type, heterozygous, and homozygous mutant RIM2 $\alpha$  KO mice (Figure 5A and C and Supplementary Table I) and in E18.5 embryos that were homozygous for the RIM1 $\alpha$  KO allele and wild-type, heterozygous, or homozygous mutant for the RIM2 $\alpha$  KO allele (Figure 5B and D and Supplementary Table II). We detected no significant changes in any protein in adult RIM2 $\alpha$  KO mice compared with controls; in particular, no significant decrease in Munc13-1 in RIM1 $\alpha$ /2 $\alpha$  DKO embryos compared with RIM1 $\alpha$  KO embryos was observed. To rule out region-specific changes in Munc13-1 level, we investigated different brain areas, but detected no obvious alterations

(Figure 5E). These results were unexpected because previous studies showed that Munc13-1 is decreased ~50% in RIM1 $\alpha$  KO mice (Schoch et al, 2002), which prompted the hypothesis that the remaining Munc13-1 is stabilized by RIM2 $\alpha$ . As shown here, however, additional deletion of RIM2 $\alpha$  does not cause a major further decrease in Munc13-1 levels beyond that observed in RIM1 $\alpha$  KO mice. A recent report described a role for RIM1 $\alpha$  in the regulation of presynaptic recruitment of Munc13-1 and ubMunc13-2 (Andrews-Zwilling et al, 2006). Therefore, we examined if deletion of both  $\alpha$ -RIMs results in a redistribution of Munc13-1 from the membrane-bound to the soluble fraction (Supplementary Figure 4B and data not shown). Soluble and membrane-bound protein fractions from wild-type and RIM1 $\alpha$ /2 $\alpha$  DKO embryonic brains were separated by ultrathurrax homogenization in buffer or in the presence of detergents of different strength and high-speed centrifugation. In agreement with Andrews-Zwilling et al (2006) we detected an increase of Munc13-1 in the soluble fraction in the brains of DKO mice. However, the majority of Munc13 was still found in the insoluble fraction with buffer or nondenaturing detergents but was solubilized with SDS. Thus, consistent with the fact that only some of the Munc13 isoforms contain the N-terminal C<sub>2</sub>-domain that binds to  $\alpha$ -RIMs (Brose et al, 1995; Betz et al, 2001; Dulubova et al, 2005), only approximately half of the synaptic Munc13-1 depends on  $\alpha$ -RIMs, and  $\alpha$ -RIMs are not absolutely essential for Munc13 function.



**Figure 4** Morphology of RIM1 $\alpha$ /2 $\alpha$  DKO brains and spinal cord. (A) H&E- and NeuN-stained sagittal section of brains from E18.5 mice of the indicated genotype (scale bar = 2 mm). (B) NeuN-stained coronal sections of spinal cord from E18.5 mice of the indicated genotype (scale bar = 100  $\mu\text{m}$ ). (C) Morphometric analysis of NeuN-immunopositive spinal motoneurons ( $**P < 0.005$ ). (D) Electron micrographs of synapses in the spinal cord. The arrows in (c, f) point to presynaptic dense projections in active zones. Abbreviations: pr, presynaptic; po, postsynaptic; sv, synaptic vesicles; pm, presynaptic plasma membrane. Scale bars, a = 300 nm, d = 370 nm, b = 300 nm, e = 240 nm, c = 100 nm, f = 130 nm.



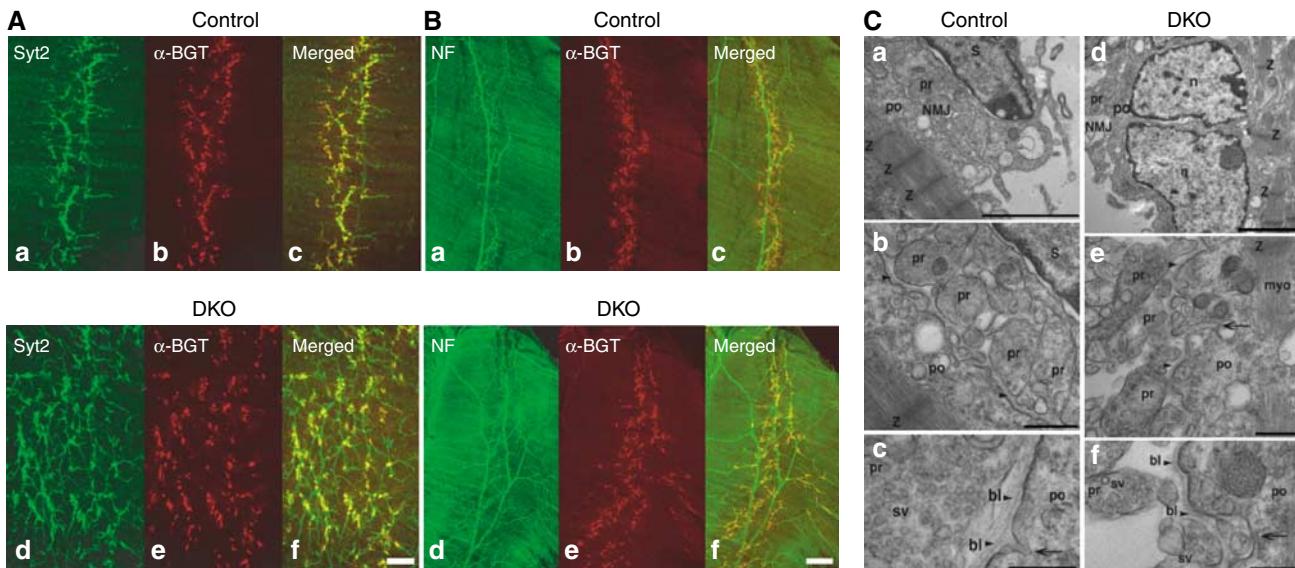
**Figure 5** Analysis of synaptic protein levels in brains from RIM2 $\alpha$  KO mice and RIM1 $\alpha$ /2 $\alpha$  DKO embryos. Brain homogenates of the indicated genotypes were analyzed by immunoblotting using antibodies to the indicated proteins (**A**, **B**), the blots were quantified and all data normalized to control level (**C**, **D**, RIM1 $\alpha$  level in **D**) normalized to wild type). (**A**, **C**) RIM2 $\alpha$ -KO (adult), (**B**, **D**) RIM1 $\alpha$ /RIM2 $\alpha$ -DKO (E18.5). (**E**) Proteins from different brain areas of adult RIM1 $\alpha$  KO, RIM2 $\alpha$  KO, and wild-type mice were analyzed with an antibody against Munc13-1 (abbreviations of brain areas: OB, olfactory bulb; STR, striatum; CTX, cortex; HC, hippocampus; TH, thalamus; MB, midbrain; BS, brain stem; CB, cerebellum).

To investigate whether the deletion of both  $\alpha$ -RIMs alters synapse assembly, we analyzed the ultrastructure of RIM1 $\alpha$ /2 $\alpha$  DKO synapses in the spinal cord by quantitative electron microscopy. We observed typical synaptic structures in RIM1 $\alpha$ /2 $\alpha$  DKO neurons (Figure 4D), and detected no obvious changes in synapse density (DKO =  $35.7 \pm 24.1$  per  $10^3 \mu\text{m}^2$ ; control [RIM1 $\alpha$ -/-, RIM2 $\alpha$ +/-] =  $25.1 \pm 21.3$  per  $10^3 \mu\text{m}^2$ ;  $N=3$  for both genotypes; 50 synapses for both genotypes), presynaptic bouton area (DKO =  $0.381 \pm 0.222 \mu\text{m}^2$ ; control =  $0.371 \pm 0.274 \mu\text{m}^2$ ;  $N=3$  and 100 synapses), density of synaptic vesicles (DKO =  $196 \pm 136$  synaptic vesicles per  $\mu\text{m}^2$  bouton area; control =  $202 \pm 150$  synaptic vesicles per  $\mu\text{m}^2$  bouton area;  $N=3$  and 50 synapses), active zone length (DKO =  $397 \pm 177 \text{ nm}$ ; control =  $354 \pm 165 \text{ nm}$ ;  $N=3$  and 50 synapses), or number of docked vesicles (DKO =  $7.8 \pm 4.2$  per  $\mu\text{m}$  active zone; control =  $8.3 \pm 5.4$  per  $\mu\text{m}$  active zone;  $N=3$  and 50 synapses). Statistical analysis was performed with Student's *t*-tests (two-sided, unpaired data with unequal variances). Thus, deletion

of  $\alpha$ -RIMs does not abolish the formation of synapses with docked vesicles and an ultrastructurally clearly visible active zone.

#### Structure of the NMJ in RIM1 $\alpha$ /2 $\alpha$ double KO mice

We next examined the morphology of the NMJ in the phrenic nerve/diaphragm muscle preparation, and performed immunostainings of whole-mount muscles at E18.5 with antibodies to presynaptic proteins (synaptotagmin 2, SV2B, synaptophysin, Rab3A, liprin- $\alpha$ , and ELKS1) and with Texas Red-conjugated  $\alpha$ -bungarotoxin that labels postsynaptic acetylcholine receptors (Figure 6A and Supplementary Figure 4A and data not shown). In wild-type mice, the left and right phrenic nerves form synapses on the diaphragm in a discrete spatially restricted endplate band within the central region of each hemidiaphragm. Strikingly, in RIM1 $\alpha$ /2 $\alpha$  DKO mice, synapses as visualized by synaptotagmin 2 and acetylcholine receptor staining were only poorly aligned along the midline of the hemidiaphragm but more



**Figure 6** Morphology of diaphragm NMJs in RIM1 $\alpha$ /2 $\alpha$  DKO mice. **(A, B)** Confocal micrographs of E18.5 RIM1 $\alpha$ /2 $\alpha$  DKO mutant and control littermate whole-mount diaphragm muscles labeled with Texas Red-conjugated  $\alpha$ -bungarotoxin ( $\alpha$ -BGT) and antibodies to synaptotagmin-2 (Syt2) (A, a–d) or to neurofilament (NF) (B, a–d). **(C)** Electron micrographs of NMJs in RIM1 $\alpha$ /2 $\alpha$  DKO mice and control littermates demonstrate normal ultrastructure of NMJs in control and DKO mice. (a, d) Low-magnification electron micrographs of the NMJ demonstrating normal overall architecture of the NMJ in control and DKO mice. (b, c) and (e, f) Normal ultrastructural appearance of the NMJs at higher magnifications. Arrows point to postsynaptic invaginations that are not yet fully developed at E18 in both control and DKO mice. Arrowheads point to basal lamina material in the synaptic cleft of the NMJ. Abbreviations: NMJ, neuromuscular junction; S, Schwann cell; n, nucleus of a postsynaptic muscle fiber; Z, Z stripes in sarcomeres of postsynaptic muscle fibers; myo, myofibrils; pr, presynaptic; po, postsynaptic; bl, basal lamina of neuromuscular junction between the presynaptic terminals and the postsynaptic plasma membrane; sv, synaptic vesicles. Scale bars, A = 100  $\mu$ m, B = 200  $\mu$ m, C, a, d = 3  $\mu$ m, b, e = 700 nm, c, f = 400 nm.

randomly distributed across a broader region of the muscle (Figure 6A). Similar results were obtained for other presynaptic markers, and in control as well as in RIM1 $\alpha$ /2 $\alpha$  DKO diaphragms, all axon terminals were always juxtaposed to acetylcholine receptors clusters. Furthermore, immunostainings of the whole-mount muscles with antibodies against the RIM-binding proteins liprin, ELKS, and Rab3 revealed a synaptic localization even in the absence of  $\alpha$ -RIMs (Figure 6A and Supplementary Figure 4A and data not shown).

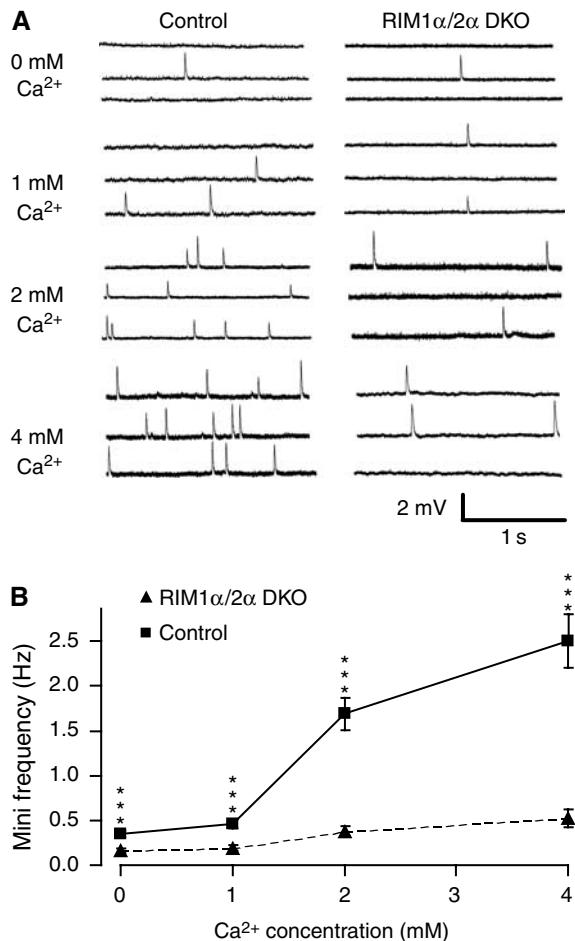
The observed pattern of synaptic contacts in the mutant NMJs suggests an increase in innervation or an expansion of innervation. To confirm this observation, we stained whole-mount diaphragm muscles with antibodies to the neurofilament protein or to syntaxin 1, both of which label axons. Costaining with Texas Red-conjugated  $\alpha$ -bungarotoxin showed that in control mice, synapses were distributed regularly (Figure 6B). In RIM1 $\alpha$ /2 $\alpha$  DKO mice, however, phrenic nerves exhibited abnormally extensive branching throughout the muscle, and their terminal arborizations covered a much broader surface of the muscle (Figure 6B).

To examine synapse ultrastructure of the NMJ, we performed electron microscopy at E18.5. Both in control and RIM1 $\alpha$ /2 $\alpha$  DKO mice, typical but immature synapses were observed with clusters of normal-sized small synaptic vesicles that were occasionally docked at the active zone, and with continuously aligned pre- and postsynaptic membranes that were separated by a well-developed basal lamina (Figure 6C). Thus, similar to central synapses (Figure 4), on a qualitative level no obvious ultrastructural changes were detectable in NMJs.

#### Electrophysiological properties of the NMJ in RIM1 $\alpha$ /2 $\alpha$ DKO mice

To examine synaptic transmission in the NMJ, we performed electrophysiological recordings in the phrenic nerve/diaphragm preparation at E18.5. Because of the impossibility of obtaining sufficient numbers of matched littermate wild-type control mice for these experiments, we used in these experiments control embryos that were from the same litter as the tested RIM1 $\alpha$ /2 $\alpha$  DKO embryos and that contained at least one wild-type RIM1 $\alpha$  allele, that is, embryos that could have up to three mutant  $\alpha$ -RIM alleles.

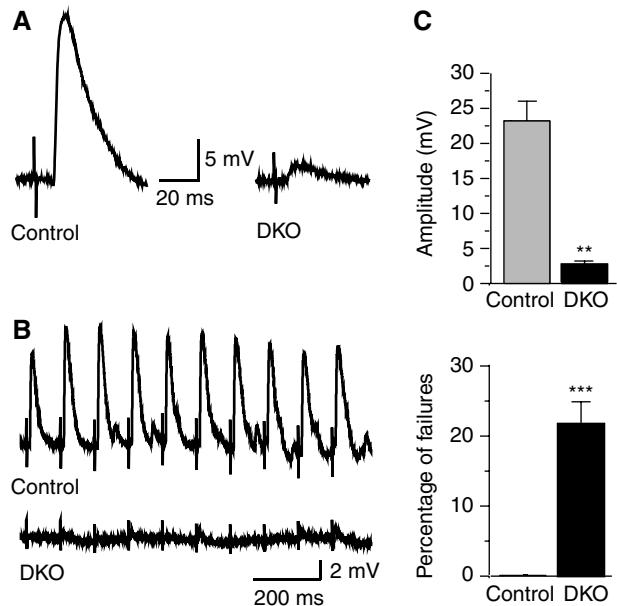
Recordings of spontaneous miniature endplate potentials (mEPPs) in normal Ringer solution revealed that the mEPP frequency and amplitudes were not significantly different between control samples and RIM1 $\alpha$ /2 $\alpha$  DKO mice (frequency: control,  $0.71 \text{ Hz} \pm 0.12$  ( $N=3$  embryos, 13 muscle fibers); DKO,  $0.65 \text{ Hz} \pm 0.07$  ( $N=4$  embryos, 25 muscle fibers); amplitudes: control,  $1.7 \text{ mV} \pm 0.16$  ( $N=3$  embryos, 13 muscle fibers); DKO,  $1.9 \text{ mV} \pm 0.16$  ( $N=4$  embryos, 25 muscle fibers)). The very low frequency of mEPPs at E18.5, a reflection of the immaturity of the NMJ at this stage in development, may have precluded observing a difference between control and RIM1 $\alpha$ /2 $\alpha$  DKO mice. We therefore increased the mEPP frequency by depolarizing the nerve terminals with  $40 \text{ mM K}^+$  in the presence of extracellular  $\text{Ca}^{2+}$  concentrations ranging from 0 to 4 mM (Figure 7). Quantitation of the mEPP frequency uncovered a significant difference between control and RIM1 $\alpha$ /2 $\alpha$  DKO mice at all examined extracellular  $\text{Ca}^{2+}$  concentrations. At 0 and 1 mM  $\text{Ca}^{2+}$ , the mEPP frequencies were low in control and RIM1 $\alpha$ /2 $\alpha$  double-deficient NMJs. However, in RIM1 $\alpha$ /2 $\alpha$  DKO NMJs, the mEPP frequency was reduced by half as compared with



**Figure 7** Spontaneous miniature endplate potentials (mEPPs) at the diaphragm NMJ from control and RIM1 $\alpha$ /2 $\alpha$  DKO mice recorded in 40 mM KCl in 0–4 mM extracellular Ca<sup>2+</sup>. (A) Representative traces from muscles from control (left panel) and double-mutant mice (right panel). (B) Average mEPP frequencies as a function of extracellular Ca<sup>2+</sup> concentration (data shown are means  $\pm$  s.e.m.;  $n$  (number of cells) for mutants/controls are 34/27 (0  $\mu$ M Ca<sup>2+</sup>), 27/26 (1  $\mu$ M Ca<sup>2+</sup>), 30/27 (2  $\mu$ M Ca<sup>2+</sup>), and 26/22 (4  $\mu$ M Ca<sup>2+</sup>), respectively, \*\*\* $P$ <0.0005, Student's *t*-test).

control NMJs ( $P$ <0.0005). At 2 and 4 mM Ca<sup>2+</sup>, the mEPP frequency increased >3-fold in control NMJs but remained almost unchanged in RIM1 $\alpha$ /2 $\alpha$ -deficient NMJs (Figure 7B) ( $P$ <5  $\times$  10<sup>-8</sup>).

The mEPP frequency data suggest that synaptic vesicle exocytosis is not abolished in RIM1 $\alpha$ /2 $\alpha$  DKO mice, but that Ca<sup>2+</sup> triggering of exocytosis may be impaired. To confirm this conclusion, we examined evoked EPPs elicited by a single stimulation or at 10 Hz (Figure 8). Deletion of both  $\alpha$ -RIMs did not abolish evoked responses, but severely impaired them. The EPP amplitudes were almost 10-fold smaller in RIM1 $\alpha$ /2 $\alpha$  DKO than in control mice (control: 23  $\pm$  3 mV ( $N$ =3 embryos, 16 muscle fibers); DKO: 2.7  $\pm$  0.5 mV ( $N$ =4 embryos, 19 muscle fibers);  $P$ <0.001; failures were excluded from this analysis), and the failure rates were dramatically increased—in three control embryos, there was only one failure in response to a total of 309 stimuli (0.32  $\pm$  0.15%,  $N$ =3 mice, 25 muscle fibers), whereas in four double mutants analyzed, there were 115 failures in response to a total of 518 stimuli (22.2  $\pm$  2.39%,  $N$ =4 mice,



**Figure 8** Impaired evoked neurotransmitter release at the NMJ of RIM1 $\alpha$ /2 $\alpha$  DKO mice. (A, B) Representative traces of EPPs evoked at low frequency (A) or at 10 Hz (B) in muscles from control (left trace in (A), top trace in (B)) or RIM1 $\alpha$ /2 $\alpha$  DKO embryos (right trace in (A); bottom trace in (B)). (C) Decreased amplitudes (upper panel) and increased failure rates (lower panel) of evoked EPPs in  $\alpha$ -RIM-deficient NMJs (for amplitudes (excludes failures), controls,  $n$ =3 embryos, 16 cells; DKO,  $n$ =4 embryos, 19 cells; for failure rates, controls = 1 failure in 309 stimuli,  $N$ =3 mice, 25 cells; DKO = 115 failures in 518 stimuli,  $N$ =4 mice, 39 cells, \*\* $P$ <0.001, \*\*\* $P$ <0.0005, Student's *t* test).

39 muscle fibers; Figure 8C). These measurements likely underestimate the degree of impairment as the controls are not wild-type mice but mice that contain a partial lack of  $\alpha$ -RIMs. Thus,  $\alpha$ -RIMs are not essential for exocytosis as such, but are required for normal Ca<sup>2+</sup>-triggering of exocytosis.

#### Insulin secretion in RIM1 $\alpha$ and RIM2 $\alpha$ knockout mice

Recent reports suggested that RIM2 $\alpha$  plays a central role in regulating insulin secretion (Iezzi *et al*, 2000; Kashima *et al*, 2001). Therefore, we examined the glucose metabolism in the single RIM1 $\alpha$  and RIM2 $\alpha$  KO mice with two standardized tests: body fat measurement by NMR and a glucose tolerance test. Deletion of either RIM1 $\alpha$  or RIM2 $\alpha$  had no significant effect on body fat levels in mice (Supplementary Figure 5). Intraperitoneal glucose tolerance tests were performed on the same mice after an overnight fast, and also did not reveal any significant alterations in the KO mice (Supplementary Figure 5).

#### Discussion

RIMs comprise a family of active zone proteins, with two full-length  $\alpha$ -isoforms (RIM1 $\alpha$  and RIM2 $\alpha$ ) that interact with a host of other active zone proteins in addition to synaptic vesicle proteins, and  $\beta$ - and  $\gamma$ -isoforms that exhibit more restricted interaction patterns. However, to date, only RIM1 $\alpha$  has been analyzed functionally (Castillo *et al*, 2002; Schoch *et al*, 2002; Lonart *et al*, 2003; Calakos *et al*, 2004; Powell *et al*, 2004). The currently available data demonstrate that RIM1 $\alpha$  performs a central role in regulating neurotransmitter release, but suggest that other RIM isoforms may also be

involved in the regulation of synaptic vesicle exocytosis. Although the various RIM isoforms are coexpressed in brain, their relative expression patterns were unknown, and it was unclear whether potential redundancy among RIM isoforms exists. Such redundancy could be present, for example, between RIM1 $\alpha$  and RIM2 $\alpha$  because these two RIM isoforms are the only RIMs that bind to Munc13 and Rab3 (Dulubova et al, 2005).

In the present study, we have examined the expression patterns of various RIM isoforms, generated RIM2 $\alpha$  KO mice to explore its functions, and tested the possibility that RIM1 $\alpha$  and RIM2 $\alpha$  are functionally redundant. Moreover, we have probed the extent of their functions in release by comparing the effect of a double RIM1 $\alpha$  and 2 $\alpha$  deletion on synapse structure and neurotransmitter release. Our data demonstrate that  $\alpha$ -RIMs are expressed in differential but largely overlapping expression patterns, they are functionally redundant, and they are selectively required to maintain  $\text{Ca}^{2+}$ -triggered neurotransmitter release, not synapse structure. Among others, our data demonstrate that the linkage of Munc13s to  $\alpha$ -RIMs in the active zone, and of Rab3 on synaptic vesicles to  $\alpha$ -RIMs, is not essential for synaptic vesicle docking, priming, or exocytosis because in the RIM1 $\alpha$ /2 $\alpha$  double-deficient neurons, these linkages are abolished whereas exocytosis continues. Our data demonstrate, however, that this linkage is required for  $\text{Ca}^{2+}$ -triggered release because the release is decreased approximately 10-fold in the double-deficient neurons.

### Distribution of RIMs

To analyze if RIM isoforms are coexpressed and could thus be redundant, we performed *in situ* hybridizations and immunoblotting. Our results show that RIM1 $\alpha$ , 2 $\alpha$ , 2 $\beta$ , and 2 $\gamma$  are ubiquitously present throughout the brain in overlapping, but non-identical expression patterns (Figure 1). RIM1 $\alpha$  is the most abundant isoform that appears to be expressed uniformly. RIM2 $\alpha$  is also present in all brain areas, but at lower levels; we observed high levels of RIM2 $\alpha$  in a small subset of neurons, such as olfactory bulb granule cells, hippocampal dentate gyrus granule cells, and cerebellar granule cells. RIM2 $\beta$  and RIM2 $\gamma$  are also expressed ubiquitously in the brain, again with high levels in a subset of neurons, for example the thalamus for RIM2 $\beta$  and the medulla oblongata for RIM2 $\gamma$ . Immunoblotting of protein homogenates from single RIM1 $\alpha$  and RIM2 $\alpha$  KO mice verified the differential distribution and divergent expression levels of RIM1 $\alpha$  and RIM2 $\alpha$  in the brain (Figure 2D). These findings suggest that in some neurons, all RIM isoforms appear to be present, most conspicuously cerebellar granule cells, whereas in other neurons, one isoform predominates, for example, hippocampal CA1 neurons that primarily express RIM1 $\alpha$ , or olfactory bulb granule cells that primarily express RIM2 $\alpha$  (Figure 1B).

### Essential functions of RIM2 $\alpha$

Whereas deletion of RIM1 $\alpha$  causes large changes in the amount and regulation of neurotransmitter release (Schoch et al, 2002; Calakos et al, 2004), we found that the single deletion of RIM2 $\alpha$ , the only other  $\alpha$ -RIM, has milder phenotypic consequences compared with the deletion of RIM1 $\alpha$ . RIM2 $\alpha$ -deficient mice are impaired, but their release properties in hippocampal synapses appear to be normal (Supplementary Figure 3). It is puzzling that RIM1 $\alpha$  but not

RIM2 $\alpha$  KO mice exhibit a loss of mossy fiber LTP in the CA3 region of the hippocampus (Castillo et al, 2002; Supplementary Figure 3E), because RIM1 $\alpha$  and RIM2 $\alpha$  are both highly expressed in the dentate granule cell neurons that supply the mossy fiber synapses, and both are phosphorylated by PKA at a site equivalent to serine 413 in RIM1 $\alpha$  (Lonart et al, 2003). Furthermore, it has been postulated that  $\alpha$ -RIMs play a role in regulating insulin release from pancreatic  $\beta$ -cells (Iezzi et al, 2000; Kashima et al, 2001). However, we were unable to detect any changes in body fat content or in blood glucose levels in RIM1 $\alpha$  and 2 $\alpha$  KO mice (Supplementary Figure 5).

### Redundant functions of RIM1 $\alpha$ and 2 $\alpha$

Although deletion of either RIM1 $\alpha$  or RIM2 $\alpha$  does not significantly impair survival of mice, deletion of both  $\alpha$ -RIMs causes complete lethality at birth, whereas the absence of only three of the four  $\alpha$ -RIM alleles—in either combination—induces an ~40% lethality (Figure 3A). We detected no obvious developmental abnormalities, changes in synapse structure, or alterations in brain composition in the RIM1 $\alpha$ /2 $\alpha$  DKO mice (Figures 4 and 5), suggesting that these mice perished because of a functional and not a developmental defect.

Because of the severe loss of motor control in RIM1 $\alpha$ /2 $\alpha$  DKO mice, we studied the NMJ as a model system to understand the consequences of deleting both  $\alpha$ -RIMs. As indirect indicators of defective synaptic transmission at the neuromuscular junction, we found an increased density of motoneurons in the ventral horn of the spinal cord, increased branching of presynaptic motor nerves, and increased numbers of irregularly distributed motor synapses on skeletal muscle fibers. These effects are considered to be consequences of an impaired function at the neuromuscular junction (Harris and McCaig, 1984; Dahm and Landmesser, 1991; Oppenheim, 1991). Direct measurement of synaptic transmission at the neuromuscular junction revealed profound defects. We found that spontaneous release was unchanged in frequency, but evoked synaptic transmission was strongly reduced (Figures 7 and 8). The miniature frequency could not be enhanced in the double mutants by increasing the  $\text{Ca}^{2+}$  concentration under depolarizing conditions (Figure 7), whereas the amplitudes and failure rate of evoked release were massively decreased and increased, respectively (Figure 8). This phenotype demonstrates that deletion of  $\alpha$ -RIMs does not abolish spontaneous or  $\text{Ca}^{2+}$ -dependent exocytosis, but severely and specifically impairs  $\text{Ca}^{2+}$ -triggering of exocytosis. Interestingly, a recent study at the NMJ of *Caenorhabditis elegans* has shown that Unc-10/RIM localization is restricted to dense projections and that the number of membrane-contacting vesicles is reduced in unc-10-deficient animals (Weimer et al, 2006). It was therefore hypothesized that the Rab3-unc10/RIM-unc13/Munc13 tripartite complex localizes primed synaptic vesicles in close proximity of calcium channel containing dense projections. Consistent with a loss of synaptic transmission, the pattern of synapses in the NMJs of DKO mice exhibited morphological changes that are typically observed upon complete block of synaptic transmission in NMJs (Figure 6; Misgeld et al, 2002; Brandon et al, 2003).

Munc13-deficient NMJs also display an impairment of evoked transmitter release similar to NMJs lacking  $\alpha$ -RIMs.

But different from NMJs lacking  $\alpha$ -RIMs, Munc13-deficient NMJs exhibit an increased frequency of spontaneous transmitter release (Varoqueaux *et al*, 2005). Moreover, NMJs deficient for the SNARE protein SNAP-25 also exhibit increased spontaneous transmitter release but lack evoked release (Washbourne *et al*, 2002). Based on the observation of decreased evoked and increased spontaneous release in Munc13 and SNAP-25 KO mice, it was hypothesized that Munc13-mediated priming via SNARE proteins is essential for the majority of synaptic vesicles at the NMJ, whereas a small subpopulation of vesicles undergo spontaneous Munc13-independent priming (Varoqueaux *et al*, 2005). The increase in mini-frequency observed in Munc13 and SNAP-25 KO mice was then explained by an increase in the number of synapses in mutant NMJs. However, NMJs lacking  $\alpha$ -RIMs exhibited the same morphological changes (increase in number of motoneurons, in branching of presynaptic nerve fibers, and in neuromuscular synapse formation) and the same loss of evoked release as Munc13-deficient synapses, but the mini-frequency is not increased. These results thus suggest that the increase in mini-frequency in Munc13- and SNAP-25-deficient synapses is not due to an increased number of synaptic contacts in the NMJ, but could be a homeostatic response to the decrease in neurotransmitter release. Thus,  $\alpha$ -RIMs may act as downstream effectors of Munc13s, presumably via the N-terminal interaction between these two proteins (Betz *et al*, 2001; Dulubova *et al*, 2005).

Our study indicates that in some synapses as at the NMJ, loss of either RIM1 $\alpha$  or RIM2 $\alpha$  can be compensated by the respective other, whereas in hippocampal excitatory and inhibitory neurons, RIM1 $\alpha$  can compensate for RIM2 $\alpha$  but not vice versa. A similar result has been obtained in the analysis of Munc13-1/-2-deficient neurons, which show a complex pattern of redundancy (Varoqueaux *et al*, 2002). In GABAergic hippocampal neurons, both isoforms are redundant; however, in glutamatergic neurons, the presence of Munc13-1 is required. Furthermore, Munc13-1- or Munc13-2-equipped synapses exhibit diverging short-term plasticity characteristics in spite of their high structural homology (Rosenmund *et al*, 2002). Nevertheless, the molecular mechanisms underlying this phenotype are still unclear. As RIM1 $\alpha$  and RIM2 $\alpha$  are also structurally highly homologous, interact with the same set of proteins, and are both PKA substrates, a detailed gain-of-function and loss-of-function analysis in RIM1 $\alpha$ /2 $\alpha$ -deficient neurons can now address the molecular basis that determines their individual and redundant functions. However, our study clearly demonstrates a severe defect in synaptic transmission that can at least partially explain the dramatic lethal phenotype that we observe in the absence of  $\alpha$ -RIMs.

## Materials and methods

### Generation and characterization of RIM2 $\alpha$ KO mice

The targeting vector (Figure 2A) was constructed and employed to generate mutant embryonic R1 stem cells (Nagy *et al*, 1993) using standard procedures (Rosahl *et al*, 1995) (see Supplementary data for a detailed description). Mice were generated by blastocyst injections, and the floxed fifth exon was removed by cre-mediated recombination in the germ line (O'Gorman *et al*, 1997). Mice were bred using standard mouse husbandry, and genotyped by PCR. RIM1 $\alpha$  mutant alleles were genotyped as described (Schoch *et al*, 2002).

*In situ* hybridizations were performed on cryostat sections from the brains of male Wistar rats (4-week-old; see Supplementary data for detailed procedures) with  $^{35}$ S-labeled oligonucleotides. Anti-sense oligonucleotides used corresponded to bp 952–907 and 1381–1337 of RIM1 $\alpha$  (GenBank acc. no. NM\_052829.1); bp 536–491 and 745–701 of RIM2 $\alpha$  (GenBank acc. no. NM\_053945.1); bp 111–67 and 194–150 of RIM2 $\beta$  (GenBank acc. no. AF548738.1); bp 311–267 and 337–293 of RIM2 $\gamma$  (GenBank acc. no. NM\_145881.1). Oligonucleotide sequences (45-mers, 50–60% GC, low hairpin and dimer formation probability) were selected by using Oligo 6.7 (Cascade, CO, USA). In control experiments, hybridizations were performed with a 1000-fold excess of the respective unlabeled oligonucleotide. The hybridization signal was visualized either directly on Kodak BioMax film (Eastman Kodak, New Haven, CT, USA) or after dipping in the photographic emulsion (LM1; Amersham Biosciences) and development in Kodak D-19 developer (Eastman Kodak). For analysis with bright-field optics, sections were counterstained with H&E.

Protein quantifications were performed on brain homogenates from four 2-month-old RIM2 $\alpha$  littermate KO and wild-type mice, and four E18.5 RIM1/2 $\alpha$  DKO mice using littermate controls. Quantifications were performed by immunoblotting using  $^{125}$ I-labeled secondary antibodies and Phospholmager (Fuji) detection with annexin, VCP, and  $\alpha$ -tubulin as internal standards (Rosahl *et al*, 1995; Schoch *et al*, 2002). Phospholmager data were then quantified with the image data analyzer software AIDA 3.20.116 (raytest GmbH, Germany).

### Microscopy

Whole-body staining of eviscerated E18.5 embryos was performed after immersion fixation in absolute ethanol for 4 days and acetone for 3 days (see Supplementary data). For light and electron microscopy, brain sections from 2-month-old and E18.5 mice were examined using standard procedures (Rosahl *et al*, 1995) (see Supplementary data).

### Hippocampal electrophysiology

Vibratome-cut transverse hippocampal slices (400- $\mu$ m thick) from 3- to 5-week-old mice were used for field recordings using standard procedures (Castillo *et al*, 2002; Schoch *et al*, 2002).

### NMJ electrophysiology

NMJ recordings were carried out at room temperature using intracellular sharp glass microelectrodes filled with 3 M KCl on acutely isolated phrenic nerve/diaphragm preparations at E18.5. Muscles were dissected in oxygenated normal mouse Ringer's solution (136.8 mM NaCl, 5 mM KCl, 12 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 11 mM glucose; pH 7.4) (Liley, 1956), pinned to Sylgard coated dishes, and continuously perfused with oxygenated Ringer's solution. Evoked EPPs were elicited by suprathreshold stimulation (5 V, 1 ms) of the phrenic nerve via a suction electrode. Data were collected with an AxonClamp 2B amplifier, digitized, and analyzed using PClamp (v. 9.0) (Axon Instruments Inc.) and MiniAnalysis (Synaptosoft Inc.).

### Glucose tolerance test and body fat measurement

Mice at 10–12 weeks of age were fasted overnight with free access to water. Glucose was injected into the peritoneal cavity at 2 mg/g of body weight. Blood samples were collected from the tail vein immediately before and 15, 30, 60, 90, and 120 min after glucose injection. Plasma was prepared by low-speed centrifugation (5000 g for 10 min) and used for glucose measurement using Sigma Diagnostics glucose (Trinder) reagent. Body fat content in mice was measured with a Bruker Minispec mq7.5 NMR analyzer (Bruker Optics, TX, USA).

### Miscellaneous

All chemicals were of the highest available purity and were purchased from standard sources. SDS-PAGE and immunoblotting were performed following standard procedures (Laemmli, 1970; Towbin *et al*, 1979) using the antibodies described (Schoch *et al*, 2002).

### Statistical analysis

Data are presented as means  $\pm$  s.e.m. Statistical analysis of quantitative Western blots was performed using one sample

*t*-test (GraphPad Prism) to determine the significance of the difference from mean to control level (normalized to 100% for each blot). Differences were considered significant at  $P < 0.05$ . EM: Statistical analysis was performed with Student's *t*-tests (two-sided, unpaired data with unequal variances); data represent means and s.d.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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