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The Septin CDCrel-1 Is Dispensable for Normal Development and Neurotransmitter Release

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Septins are GTPases required for the completion of cytokinesis in a variety of organisms, yet their role in this process is not known. Septins may have additional functions since the mammalian septin CDCrel-1 is predominantly expressed in the nervous system, a largely postmitotic tissue. While relatively little is known about the function of this protein, we have previously shown that it is involved in regulated secretion. In addition, the gene encoding this protein maps to a locus often deleted in velo-cardiofacial and DiGeorge syndromes, and CDCrel-1 has recently been shown to be a direct target of the E3 ubiquitin ligase activity of Parkin, a causative agent in autosomal recessive forms of Parkinson's disease. Here we show that CDCrel-1 expression rises at the time of synaptic maturation and that CDCrel-1 is present in a complex that includes the septins Nedd5 and CDC10. To investigate its function in the nervous system, we generated homozygotic CDCrel-1 null mice and showed that these mice appear normal with respect to synaptic properties and hippocampal neuron growth in vitro. Moreover, we found that while the expression of a number of synaptic proteins is not affected in the CDCrel-1 mutant mice, the expression of other septins is altered. Together, these data suggest that CDCrel-1 is not essential for neuronal development or function, and that changes in expression of other septins may account for its functional redundancy.

Septins are a family of GTPase-domain proteins that were first identified in the budding yeast *Saccharomyces cerevisiae* during screens for temperature-sensitive mutations in the cell cycle (15). Four such mutants, CDC3, CDC10, CDC11, and CDC12, all exhibited similar phenotypes with elongated buds that failed to separate from the mother cell, resulting in multinucleated cells that eventually died. Loss of function of any of these four septins was later linked to the loss of bud-neck filaments (3, 4), suggesting that each of the septins comprised, or was important for the structure of, these filaments. Subsequent cloning studies revealed that CDC3p, CDC10p, CDC11p, and CDC12p were structurally related proteins. Near the amino terminus of each of these proteins are motifs found in all GTPases, and CDC3p, CDC11p, and CDC12p also contain helical regions near their carboxyl termini with the probability of forming coiled coils (12, 14, 21, 27). Immunoisolated septin complexes were found to contain the four proteins in near-stoichiometric ratios, indicating that the four proteins assemble in an ordered structure (13). Moreover, the isolated complexes had a width of about 7 nm and lengths in multiples of 32 nm, suggesting that filaments may be comprised of multiples of a unit complex (13). A fifth yeast septin, Sep7, has been shown to be expressed during vegetative growth and may be important in Gin4 kinase regulation. However, it is not required for viability in a wild-type background (6). Two additional septin-like proteins, Spr3p and Spr28p, are also found in *S. cerevisiae*, but they are only expressed in sporulating cells where they associate with the forming prospore wall (8, 32). Hence, some septins may have specialized functions distinct from cytokinesis. Moreover, neither of these proteins is essential for sporulation, suggesting that septin functions may be partially redundant during this process (27).

The conserved requirement for septins in cell division was revealed by the discovery that the *peanut* gene in *Drosophila melanogaster* encoded a septin homolog (30). *peanut* mutations result in the formation of multinucleated syncytia in imaginal discs during larval development due to failure to complete cytokinesis. Antibodies to the *peanut* protein (pnut) revealed that it accumulated at the invaginating cleavage furrow in cells undergoing mitosis (30). As in yeast, *Drosophila* septins appear to associate as part of a complex since immunoisolated pnut copurifies with two other septins, Sep1 and Sep2, in stoichiometric ratios. In addition, these septins can also be induced to polymerize in vitro (10).

In humans there are at least 11 different septin genes, many of which also appear to undergo alternative splicing to produce multiple protein products (11, 20, 27, 38). As in yeast and *Drosophila*, a primary function of at least some septins appears to be in the process of cytokinesis. For example, injection of antibodies against a ubiquitously expressed mammalian septin, Nedd5, led to the failure of cytokinesis and the appearance of multiple nuclei within injected cells (23). The perturbation of septin gene products has also been linked to several cancers (33, 36). However, it is unlikely that the only function for mammalian septins is in cytokinesis since many forms are abundantly expressed in tissues such as the brain and heart that are predominantly nonmitotic. As well, some septin genes, such as the CDCrel-1 gene, are almost exclusively expressed in the brain (2, 5, 41). Analysis of such specialized forms may

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shed light on the general function of septins and thereby clarify their role in cytokinesis.

CDCrel-1, also known as PNUTL1 due to its homology with *Drosophila* pnut, was originally identified as a novel 42-kDa protein recognized by monoclonal antibodies raised against proteins associated with immunoprecipitated human synaptophysin (16). The relationship between CDCrel-1 and the septins was discovered when it was subsequently cloned in two independent studies. In the first study, CDCrel-1 was found as the upstream part of a fusion transcript containing the β-subunit of the glycoprotein 1b platelet receptor (43) due to its nonconsensus polyadenylation sequence. As well, CDCrel-1 was identified as a gene within the segment of chromosome 22q11.2 that is commonly deleted in velo-cardiofacial and DiGeorge syndromes in humans (28). The latter is a complex congenital disorder including parathyroid and thymic hypoplasia as well as defects of the heart, which results, in part, in impaired migration of neural crest cells into the pharyngeal arches and pouches. The contribution of CDCrel-1 deficiency to the complexity of these phenotypes is not known.

CDCrel-1 shares a high level of homology (40 to 76% sequence identity) with other mammalian septins and also contains the highly conserved GTPase motifs. In the brain, CDCrel-1 is physically associated with synaptic vesicles as well as other membrane fractions (2) and is localized in presynaptic terminals (22). It has previously been shown, by immunoprecipitation experiments and by affinity binding studies using a glutathione *S*-transferase fusion protein, that CDCrel-1 interacts directly with the target membrane SNARE syntaxin 1A. Overexpression of wild-type CDCrel-1 in HIT-T15 cells inhibited evoked secretion, whereas a predicted dominant-negative form mutated in the GTPase domain enhanced secretion (2). Since septins appear filamentous, and their GTPase domain may contribute to this filamentous appearance, these observations suggest that septin filaments may regulate exocytosis by acting as a physical barrier to release.

Recently, CDCrel-1 was shown to be a direct substrate of the ring finger protein Parkin, an E3 ubiquitin ligase that is a causative agent in autosomal recessive forms of Parkinson's disease (7). Wild-type Parkin binds to and ubiquitinates CDCrel-1, leading to its degradation, whereas familial linked Parkin mutants are defective in the degradation of CDCrel-1 (42). Failure to degrade CDCrel-1 through the ubiquitin-mediated proteasome pathway could lead to reduced exocytosis of dopamine-containing synaptic vesicles and thus contribute to the development of Parkinson's disease.

To further investigate the function of CDCrel-1, we characterized its developmental expression in the brain and its association with other septins. In addition, we created targeted insertions in the gene to determine the loss-of-function phenotype. Despite our in vitro evidence that it had an important function in secretion, disruption of the CDCrel-1 locus did not cause any obvious developmental abnormalities, nor did the loss of CDCrel-1 perturb brain function significantly. The lack of an observable phenotype may be due to compensatory changes in the expression of other septins within the brain.

MATERIALS AND METHODS

Isolation of the CDCrel-1 gene and construction of the target vector. A 15-kb genomic sequence containing the CDCrel-1 gene was obtained from a P1 clone isolated from a murine 129/SvJ library and subcloned into pBluescript KS. The exon and intron boundaries of the CDCrel-1 gene were determined by restriction enzyme mapping and sequencing (see Fig. 3). Plasmid pPNT (39) containing the *pgk-neo* and *hsv-tk* (thymidine kinase) selection markers was used to construct the CDCrel-1 targeting vector. A 7-kb *Eco*RI-*Xho*I fragment containing intron 2 was used as the 5′ homologous region of the construct, while a 2.5-kb fragment containing exons 6 to 11 was used as the 3′ region of homology. The orientations of both inserted fragments in the targeting vector were confirmed by sequencing. The *neo* cassette from pPNT (*Xho*I-*Xba*I) was used as a positive marker and replaced a 1.5-kb fragment containing exons 4 and 5 and part of exon 6. Exons 4, 5, and 6 encode amino acids 19 to 92, which includes the P loop consensus sequence of the septin protein family. The negative selection marker (*hsv-tk*) was placed 5′ of the CDCrel-1 homology region.

The targeting vector was linearized with *Not*I and electroporated (Gene pulser; Bio-Rad, Hercules, Calif.) into R1 embryonic stem (ES) cells. Cells were selected by simultaneous treatment with G418 and ganciclovir. Positive clones were screened by Southern blotting following *Hin*dIII digestion of genomic DNA and probed with a radiolabeled 3′ probe (see Fig. 3A). Correct homologous recombinants were further confirmed by *Bgl*II digestion of genomic DNA and hybridized with a radiolabeled 5′ probe. The targeted ES cell lines (2 out of 600 doubly resistant clones) were expanded and subsequently aggregated with CD-1 morulae to generate chimeras. Chimeric males were crossed with CD-1 female mice and the offspring heterozygous for CDCrel-1 (CDCrel-$1^{+/-}$) were bred to homozygosity (CDCrel-$1^{-/-}$). The genotyping of offspring was carried out by Southern blotting of *Bgl*II-digested genomic DNA from the tail and hybridization with the radiolabeled 5′ probe. Experiments were performed using CDCrel-$1^{+/+}$ and CDCrel-$1^{-/-}$ littermates from heterozygous breeding pairs of mice of the hybrid 129/SvJ–CD-1 background. Similar results were obtained for experiments using either of the knockout lines.

Immunoblotting analysis. To analyze CDCrel-1 expression, brains were removed from mice and homogenized with a glass-Teflon homogenizer in 2 ml of ice-cold H buffer (10 mM HEPES-KOH [pH 7.5], 0.32 M sucrose, 1 mM $MgCl_2$, 0.1 mM EGTA) in the presence of protease inhibitors (0.3 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin/ml, 1 μg of pepstatin/ml). The concentrations of the protein samples were determined by bicinchoninic acid assay (Pierce, Rockford, Ill.). Proteins (5 to 40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on Immobilon-P membranes (Millipore, Bedford, Mass.) as described previously (2). The following primary antibodies were used in the immunoblotting experiments: mouse anti-CDCrel-1 (5), rabbit anti-CDCrel-1 (fetal), rabbit anti-Nedd5, rabbit anti-hCDC10, rabbit anti-H5, rabbit anti-VAMP2, mouse anti-α-subunit CaM kinase II (Affinity Bioreagents, Inc., Golden, Colo.), mouse anti-Sec8 (kindly provided by S. C. Hsu, Rutgers University), and mouse anti-PSD-95 (Chemicon, Temecula, Calif.).

Subcellular fractionation and immunoprecipitation. Brains were removed from five wild-type and five mutant mice and homogenized in 20 ml of H buffer as described above. The homogenate was then fractionated by differential centrifugation as previously described (18), and protein concentrations were determined for each fraction. Briefly, crude homogenates were spun at 800 × *g* to remove large unlysed cells and nuclei, and the pellet, P1, was discarded. The supernatant, S1, was then spun at 9,000 × *g*, and the synaptosome-enriched pellet was washed and repelleted (P2). The supernatant was collected (S2) and subjected to a high-speed spin at 100,000 × *g* to collect the high-speed pellet (P3) and the cytosolic fraction (S3). The P2 fraction was lysed by the addition of ice-cold water and sedimented first at 25,000 × *g* to collect the membrane-enriched pellet (LP1). The supernatant (LS1) was then sedimented at high speeds to produce the LP2 pellet that is enriched in synaptic vesicles. An equal amount of protein from each fraction was separated by SDS-PAGE and analyzed by Western blotting. For immunoprecipitations, the P2 fraction (synaptosome-enriched fraction) was first solubilized by the addition of *n*-octylglucoside to a concentration of 2% and incubated for 2 h at 4°C. Soluble material was then collected by centrifugation at 30,000 × *g* for 15 min, and the supernatant was mixed with protein A- or protein G-agarose beads coupled with septin antibodies for 2 h at 4°C. The agarose beads were washed 4 times with HKA buffer containing 0.1% Triton X-100 and 5% glycerol. Immunoprecipitates were eluted with nonreducing SDS sample buffer in the presence of 10 mM *N*-ethylmaleimide to prevent immunoglobulin dissociation and heavy chain contamination and analyzed by SDS-PAGE and Western blotting.

Hippocampal neuron culture. Postnatal hippocampal cell cultures were prepared from 1- to 2-day-old mice as described previously (19). Hippocampi were dissected and treated with papain (20 U/ml in neurobasal A medium) at 37°C for 30 min. Dissociated cells were plated at a low density on poly-D-lysine-coated glass coverslips in plating medium (neurobasal A plus B27 and supplemented

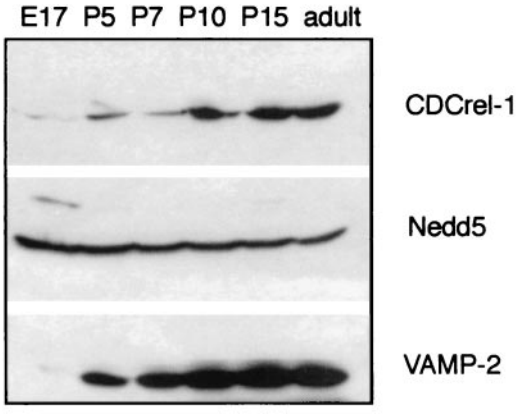

FIG. 1. Developmental expression of CDCrel-1 in the mouse brain. Brains were isolated from embryonic (embryonic day 17 [E17]) and postnatal (postnatal day 5 [P5], P7, P10, P15, and adult) mice. Total homogenates were prepared for each, and 40 μg of protein was electrophoresed and blotted with antibodies specific to the proteins indicated at the right.

with 0.5 mM L-glutamine and 25 μM L-glutamate [Life Technologies, Rockville, Md.]) for 2 h to allow the cell to adhere to the coverslips. Plating medium was removed and replaced with fresh feeding medium containing 25 μM glutamate and 5 ng of β-fibroblast growth factor/ml. The feeding medium was changed every 4 days. Cultured 11-day neurons were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline (PBS) for 15 min and then quenched by 25 mM glycine-PBS for 10 min. The coverslips were washed twice with PBS and then permeabilized with PBS containing 0.5% Triton X-100. After blocking with PBS containing 1% bovine serum albumin and 0.1% nonfat powdered milk, the coverslips were incubated with primary antibody overnight at 4°C. Secondary antibodies were then added after 3 washes with PBS (5 min/wash) and incubated for 1 h at room temperature. Coverslips were washed three times with PBS and then mounted in antifade mounting medium (Molecular Probes, Eugene, Oreg.), and the images were processed by confocal microscopy.

Hippocampal slice recording. The procedure for electrophysiological recordings was described previously (19). Briefly, hippocampal slices (400 μm thick) were prepared from 2- to 3-month-old mice and allowed to recover for at least 1 h in a holding chamber filled with artificial cerebrospinal fluid (ACSF) saturated with 95% O_2–5% CO_2. A single slice was then transferred to a recording chamber (28 ± 0.5°C) where it was submerged and superfused with ACSF (2 ml/min). ACSF contained the following: 125 mM NaCl, 2.5 mM KCl, 1.6 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 2.0 mM $CaCl_2$, and 10 mM D-glucose. Field excitatory postsynaptic potentials (fEPSPs) were evoked at a frequency of 0.03 Hz by bipolar tungsten electrodes and recorded with micropipettes filled with ACSF. The responses were amplified with an Axoclamp 2B and analyzed using pCLAMP 7 software (Axon Instruments, Union City, Calif.). The field potentials were measured by taking the slopes of the rising phase between 10 and 90% of the peak response and were verified by the addition of 5 μM 6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione at the end of each experiment. The stimulation intensity was adjusted to obtain approximately 25 to 30% of the maximum response.

RESULTS

CDCrel-1 expression increases in the brain during postnatal development. It has been previously shown that, among adult tissues, CDCrel-1 is mainly expressed in the nervous system (2, 5). To investigate its developmental expression profile, we prepared total homogenates of brains collected at different stages of embryonic and postnatal development. As a control protein, we examined the expression of the synaptic vesicle protein, VAMP-2, that is known to increase during postnatal synaptogenesis (35). As shown in Fig. 1, VAMP-2 expression is extremely low in the embryonic brain and dramatically increases during postnatal life to a plateau at approximately postnatal day 10, consistent with observations in rats (35). This is concurrent with the onset and peak of synaptogenesis that occurs in the cortex (24). Interestingly, CDCrel-1 expression (Fig. 1) follows a similar time course with maximal expression reached by postnatal day 10. As a control, the expression of the ubiquitous septin Nedd5 was examined. Nedd5 was originally identified by Northern blotting as a protein that was abundantly expressed in neuronal precursor cells and down regulated during the differentiation of adult brain (25). As seen in Fig. 1, Nedd5 expression decreases slightly during this time course but remains relatively stable throughout. The observation that CDCrel-1 expression coincides with that of VAMP-2 is consistent with previous observations that CDCrel-1 contributed to the regulation of exocytosis. Two alternate splice forms of CDCrel-1 in rats have been described, one which is most abundant in early postnatal life and a second that is prevalent in adults (37). The antibody shown in Fig. 1 was a polyclonal antibody specific to the amino terminus of the fetal form. However, similar results were obtained using a monoclonal antibody (not shown) that recognizes an undefined epitope in the carboxyl terminal half of the protein and recognizes both forms.

CDCrel-1 is associated in a multimolecular complex with other septins. Yeast and *Drosophila* septins are found in multimolecular complexes comprised of several septins in stoichiometric ratios (10). To determine if CDCrel-1 is also present in such a complex, we immunoprecipitated the septins from mouse brains and probed the precipitate for the presence of other septins. As a starting material for these precipitations, we purified the P2 fraction from adult mouse brains as described by Huttner et al. (18). The P2 fraction is obtained by differential sedimentation of brain homogenates and is enriched in intact synaptosomes. This fraction was then solubilized with the detergent *n*-octylglucoside and sedimented at 30,000 × *g*. As shown in Fig. 2, like two other septins, Nedd5 and CDC10, the majority of CDCrel-1 was found in the detergent supernatant.

This fraction was then used as the starting material for immunoprecipitations using antibodies against CDCrel-1 or three other septins as described in Materials and Methods. As shown in Fig. 2, antibodies specific to CDCrel-1 were capable of immunoprecipitating CDCrel-1, and they also immunoprecipitated Nedd5 and CDC10. Similarly, antibodies to Nedd5 coprecipitate CDCrel-1 and CDC10, and antibodies to CDC10 bring down CDCrel-1 and Nedd5. Only antibodies to the septin H5 failed to precipitate Nedd5 and CDCrel-1 efficiently despite their ability to precipitate H5 (not shown). Together, these results indicate that in the P2 synaptosomal fraction of mouse brains, the septins Nedd5, CDC10, and CDCrel-1 are part of a multimolecular complex that can coimmunoprecipitate.

Generation of CDCrel-1-deficient mice. To directly examine the function of CDCrel-1 in vivo, we generated mice with a targeted mutation in the CDCrel-1 gene. Genomic clones of the murine CDCrel-1 gene were obtained from a 129/SvJ mouse ES library and were used for the construction of a targeting vector. This vector was designed to permit the replacement of exons 4, 5, and 6 with a *neo* cassette (Fig. 3A). These three exons encode amino acids 19 to 92 of CDCrel-1,

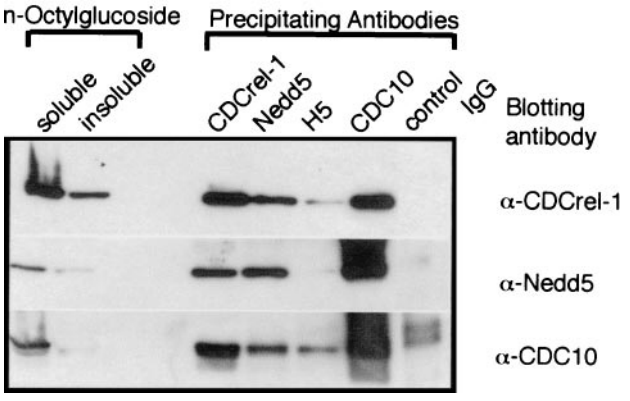

FIG. 2. CDCrel-1 coimmunoprecipitates with other brain septins. The P2 fraction from a mouse brain homogenate (18) was prepared and solubilized with *n*-octylglucoside, and then it was centrifuged to separate the soluble and insoluble proteins (left side of blot). The soluble fraction was then used as the starting material for immunoprecipitations with the antibodies indicated above the right portion of the blots. Precipitates were electrophoresed under nonreducing conditions and probed with the antibodies indicated at the right. Control immunoglobulin G (IgG) was nonspecific rabbit serum. Reactivity to the free heavy chain was observed in the anti-CDC10 immunoprecipitation lanes (bottom two blots) and in the IgG lane (bottom blot) because these were crude sera, but these were located above the septin bands. No reactivity was seen on the top blot since the blotting antibody was from a mouse. Of the *n*-octylglucoside soluble material used for the immunoprecipitations, 2% was loaded in the first lane.

including the P loop consensus sequence found in all GTPases. Of 600 neomycin-resistant ES cell lines analyzed, two demonstrated the desired replacement mutation as determined by Southern blotting. These two clones were both used to generate chimeric founder mice and heterozygous mice in the F_1 generation, as identified by Southern blotting of genomic DNA derived from the tail (not shown). Heterozygotes were then crossed to produce homozygous CDCrel-1-deficient mice. A typical Southern blot of wild-type, heterozygous, and homozygous deficient mice obtained from one of the ES lines is shown in Fig. 3B.

To confirm the lack of CDCrel-1 protein in the knockout mice, we probed brain tissue for the expression of CDCrel-1 using two different antibodies. The first was the monoclonal antibody that recognizes an epitope within the carboxyl terminal half of the protein. As shown in Fig. 3C, no protein could be detected in the homozygous deficient mice and levels appear reduced in the heterozygotes. We also probed blots with a polyclonal antibody raised to the first 15 amino acids of the fetal form of CDCrel-1 and detected no product in the mice (data not shown).

CDCrel-1-deficient mice have no gross developmental abnormalities. CDCrel-1-deficient mice were obtained with the expected Mendelian frequencies in both sexes. Deficient mice developed at the same rate as their littermates and displayed identical adult weights (not shown). Deficient mice were also fertile with litters of the normal size. Since CDCrel-1 is predominantly expressed in the brain, we examined the morphology of brain sections by hematoxylin and eosin staining. No significant differences were seen in the thickness of cortical layers, the appearance or location of nuclei, or in any other aspect of the gross morphology of the brain in any of the regions analyzed, including the hippocampus (not shown). These results indicate that CDCrel-1 is not required for normal development of the central nervous system or metabolic processes in mice. Several other tissues were also examined using hematoxylin- and eosin-stained paraffin-embedded sections, and these tissues included the pancreas, heart, lung, testis, and bone marrow. However, no abnormal morphology was observed.

Primary cultures of hippocampal neurons from CDCrel-1-deficient mice appear normal. The expression of CDCrel-1 at the time of synaptogenesis raised the possibility that it may participate in some aspect of neurite outgrowth such as the development of neuronal polarity or synapse formation. We therefore established primary cultures of hippocampal neurons from CDCrel-1-deficient mice and their wild-type littermates. At all stages of in vitro culture, the staining of CDCrel-1 was absent in all cells positive for the glial cell marker and present in cells that were positive for MAP2 and VAMP-2, suggesting that CDCrel-1 is only expressed in neurons. The 11-day in vitro cultures were stained with antibodies to CDCrel-1 and with antibodies to the synaptic vesicle protein VAMP-2. As shown in Fig. 4, wild-type hippocampal neurons produce extensive dendritic branches that are decorated with synapses, as judged by immunostaining for the presynaptic terminal marker VAMP-2. CDCrel-1 can be seen both throughout the dendrites and cell body but also concentrated in the puncta. As seen in the overlay image, both punctate patterns colocalize. CDCrel-1 staining is clearly within close proximity to that of VAMP-2, but it is difficult to determine if it is within presynaptic or postsynaptic structures. However, it had previously been shown that CDCrel-1 associated biochemically with synaptic vesicles, and more recent ultrastructural analysis localized CDCrel-1 among synaptic vesicles in the presynaptic nerve terminal (22) using immunoelectron microscopy, supporting the idea that these two proteins colocalize. In cultures established from the CDCrel-1-deficient mice, there appeared to be normal numbers of synaptic structures detected by VAMP-2 staining, and these appeared to be properly distributed over the surface of the dendrites (Fig. 4). CDCrel-1 staining confirmed the lack of expression of this septin because only background fluorescence could be detected in the images. Hence, CDCrel-1 appears to be dispensable for the formation of dendrites and synapses on hippocampal neurons in vitro.

CDCrel-1 is dispensable for normal synaptic function. We had previously shown that CDCrel-1 overexpression inhibited secretion while dominant-negative forms of the protein potentiated secretion (2). In addition, CDCrel-1 is enriched at synaptic sites. It therefore seemed plausible that deficient mice may have altered synaptic transmission. To examine this possibility, we measured fEPSPs from the CA1 region of the hippocampus, a region in the brain in which CDCrel-1 is expressed (5). In response to stimulation of Schaeffer collateral fibers in slice preparations, comparable sizes of fEPSPs were obtained in the knockout mice, indicating that synaptic transmission was normal. We then measured two forms of short-term plasticity in the CA1 region of the hippocampus, paired-pulse facilitation and posttetanic potentiation. In the paired-pulse paradigm, two sequential stimuli are delivered with varied interpulse intervals, and the facilitation caused by

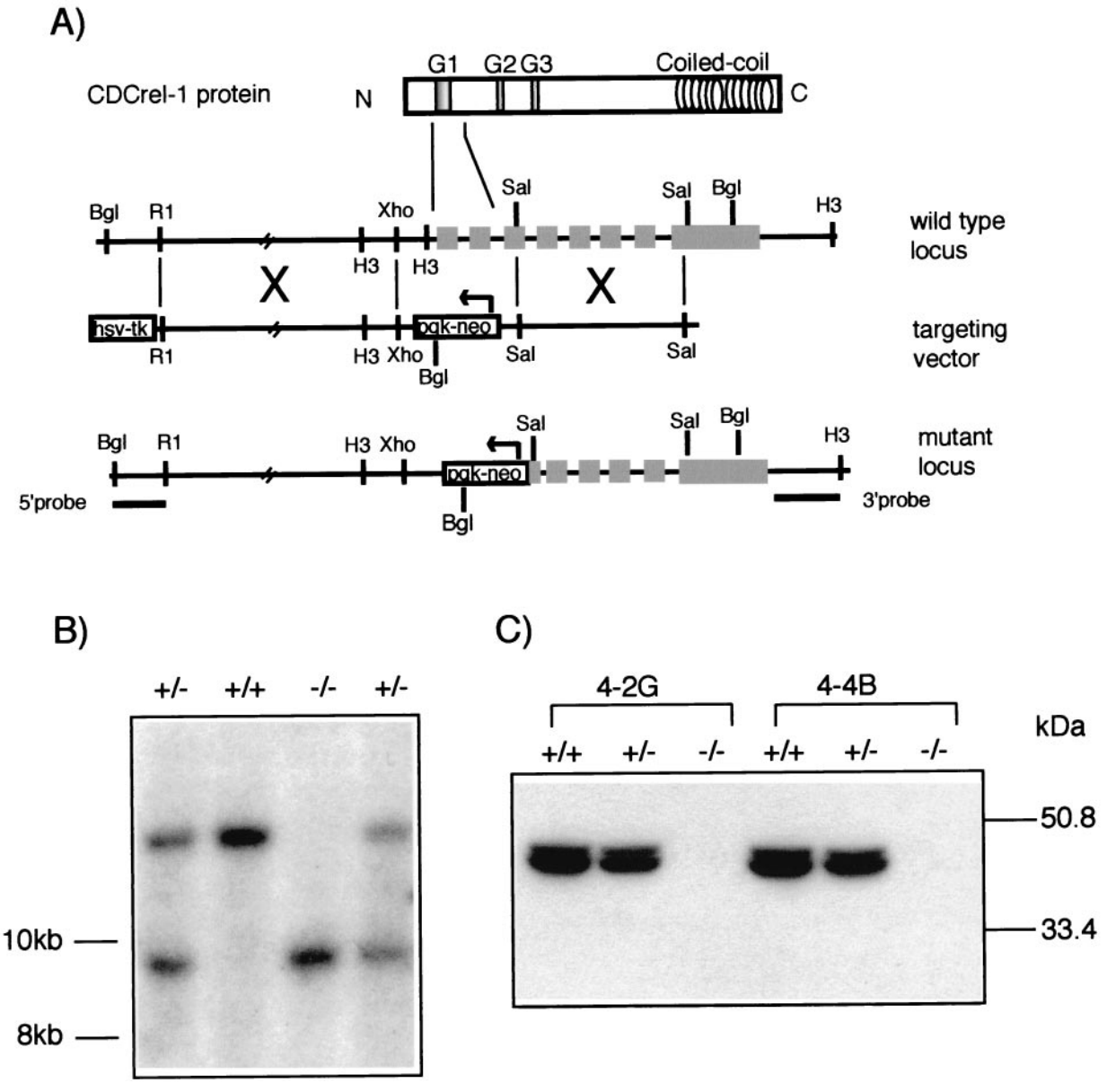

FIG. 3. Generation of CDCrel-1-deficient mice by homologous recombination. (A) Construction of targeting vector. Schematic representation of the CDCrel-1 protein (top) and the wild-type genomic locus, showing the targeting vector and targeted genomic locus. The location of the probes used for Southern blotting to identify targeting events are shown below the protein. Exons are indicated by grey boxes, and exons 1 to 3 are not shown. The restriction enzymes indicated are *Bgl*II (Bgl), *Eco*RI (R1), *Hin*dIII (H3), *Xho*I (Xho), and *Sal*I (Sal). (B) Southern blots of DNA extracted from mouse tails. Genomic DNA digested with *Bgl*II and probed with the 5′ probe reveals the presence of the *neo* cassette replacing exons 4 to 6. The *Bgl*II fragment on the wild-type CDCrel-1 locus is approximately 16.5 kb while the 5′ *Bgl*II fragment of the targeted CDCrel-1 locus is approximately 9.5 kb. (C) Mutant mice lack the CDCrel-1 protein. Western blots show ~10 μg of protein extracted from total brain electrophoresed and probed with anti-CDCrel-1 monoclonal antibody. Chimeras were generated from two independent ES clones (4-2G and 4-4B), and the progeny of both were tested. A reduction in CDCrel-1 levels is apparent in heterozygotes, and the signal is absent from homozygous mutant mice even after prolonged exposure.

this pairing is measured. As shown in Fig. 5A, there was no difference seen in this type of facilitation between the deficient mice and their wild-type littermates at any interpulse interval. High-frequency stimulation trains can lead to posttetanic potentiation of release lasting for several minutes, and that likely reflects changes in the release probability of synaptic vesicles. However, as shown in Fig. 5B, following a 100-Hz stimulation (1 s) train there was no significant difference in the magnitude of potentiation of the fEPSPs obtained for CDCrel-1-deficient mice and their wild-type littermates. These results indicate that CDCrel-1 is not required for these two forms of short-term synaptic plasticity.

Since septins have been shown to be filamentous proteins and we have shown that they can affect secretion, they may do so by acting as a physical barrier to release. In the neuron this could affect synaptic transmission either by controlling the

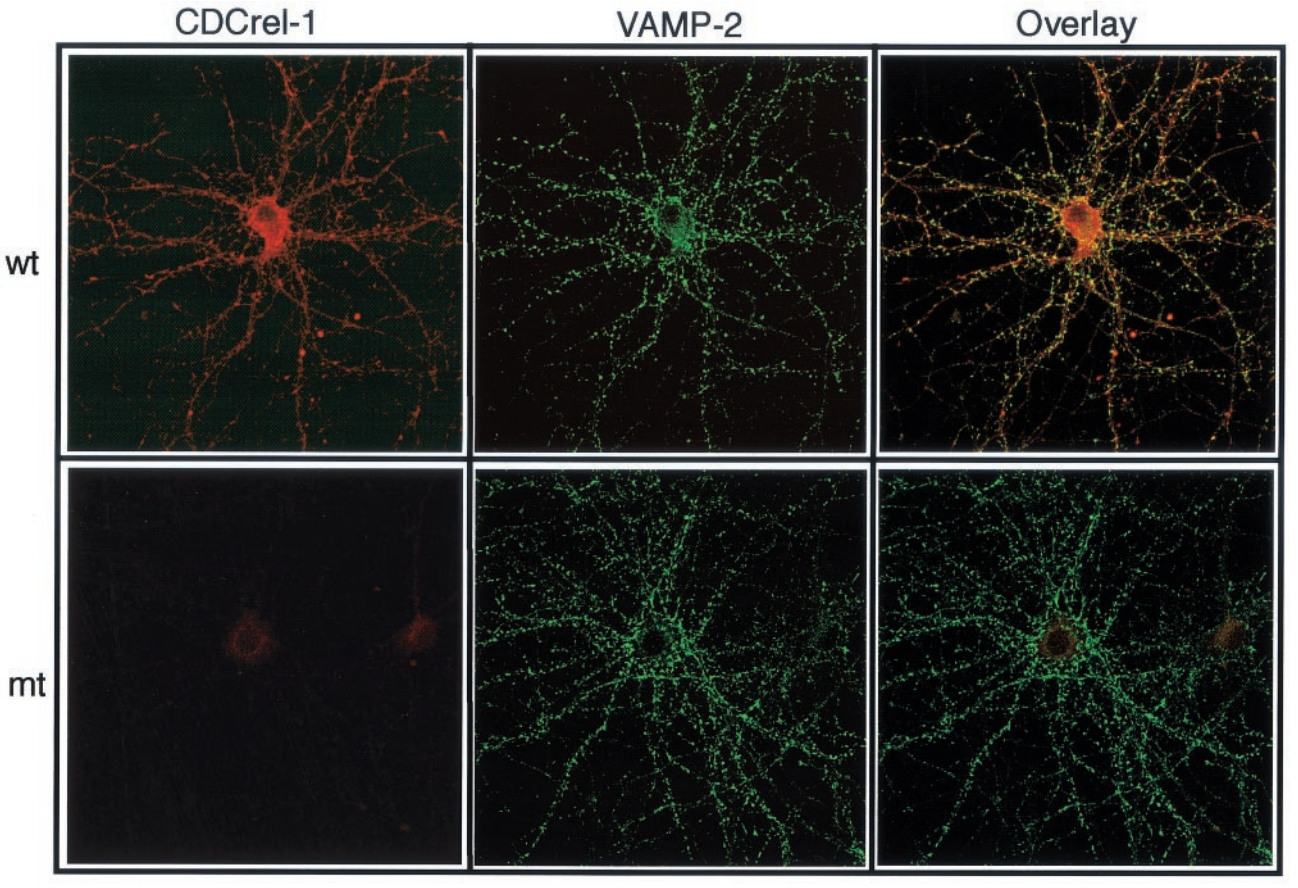

FIG. 4. Hippocampal neurons from CDCrel-1-deficient mice appear normal in vitro. Primary cultures were prepared from the hippocampi of CDCrel-1-deficient mice (mt) and their wild-type littermates (wt). After 11 days in vitro, the cells were fixed and stained with antibodies against CDCrel-1 (red) and VAMP-2 (green). Confocal images were collected and shown. CDCrel-1-deficient neurons had only background labeling due to the secondary antibody. The merged images are shown in the overlay column.

number of synaptic vesicles in the readily releasable pool or by affecting their rate of replenishment. To measure the size of the releasable pool, we used conditions of high release probability to deplete the synapses by repetitive stimulation at a frequency of 5 Hz for 120 s and monitored the slope of fEPSPs. As shown in Fig. 5C, we found no major difference in the rate or degree of synaptic depression using this paradigm, although the mutants appeared to fatigue slightly more slowly at the earliest time points.

Finally, we examined the capability of CDCrel-1-deficient mice to acquire the long-lasting enhancement of synaptic activity often associated with increased efficacy of postsynaptic receptors. Hippocampal long-term potentiation (LTP) is a widely studied form of synaptic plasticity that requires activation of the *N*-methyl-D-aspartic acid subtype of postsynaptic receptors. LTP was generated by delivering two trains of 100-Hz stimuli of 1-s duration separated by a 10-s interval. As shown in Fig. 5D, both CDCrel-1-deficient and wild-type littermates exhibited a very similar 40% increase in fEPSPs that lasted well beyond the 1-h course of the experiment. Taken together with the other physiological studies done, these results indicate that the absence of CDCrel-1 does not cause a significant change in the physiological properties of the synapse.

Changes in septin expression correlate with CDCrel-1 deficiency. In order to explain the absence of a physiological correlate for the lack of CDCrel-1 protein, we examined the expression of a number of synaptic proteins. If septins form filaments in the presynaptic terminal that function as tethers for synaptic vesicles, the absence of septin complexes might lead to subtle changes of synaptic vesicle distribution. Such changes may be detected by examining the synaptic proteins in different membrane fractions. Brains from CDCrel-1-deficient mice and their wild-type littermates were fractionated according to the procedure of Huttner et al. (18) to prepare crude membrane, synaptosomal, cytosolic, and synaptic vesicle fractions. All lanes were confirmed to contain equivalent amounts of protein by Ponceau S staining and by parallel blots for tubulin (not shown). The septin CDC10 had previously been identified within the postsynaptic density in proteomic studies of this protein complex (40). To determine if the lack of CDCrel-1 altered this structure, we examined the postsynaptic density proteins PSD-95 and CaMKII. The protein Sec8, a component of the mammalian exocyst complex, was monitored because previous studies had demonstrated an association between this protein and the septins (17). Finally, we examined the presence and fractionation of the synaptic vesicle protein VAMP-2 as a marker for synaptic vesicles. As shown in Fig.

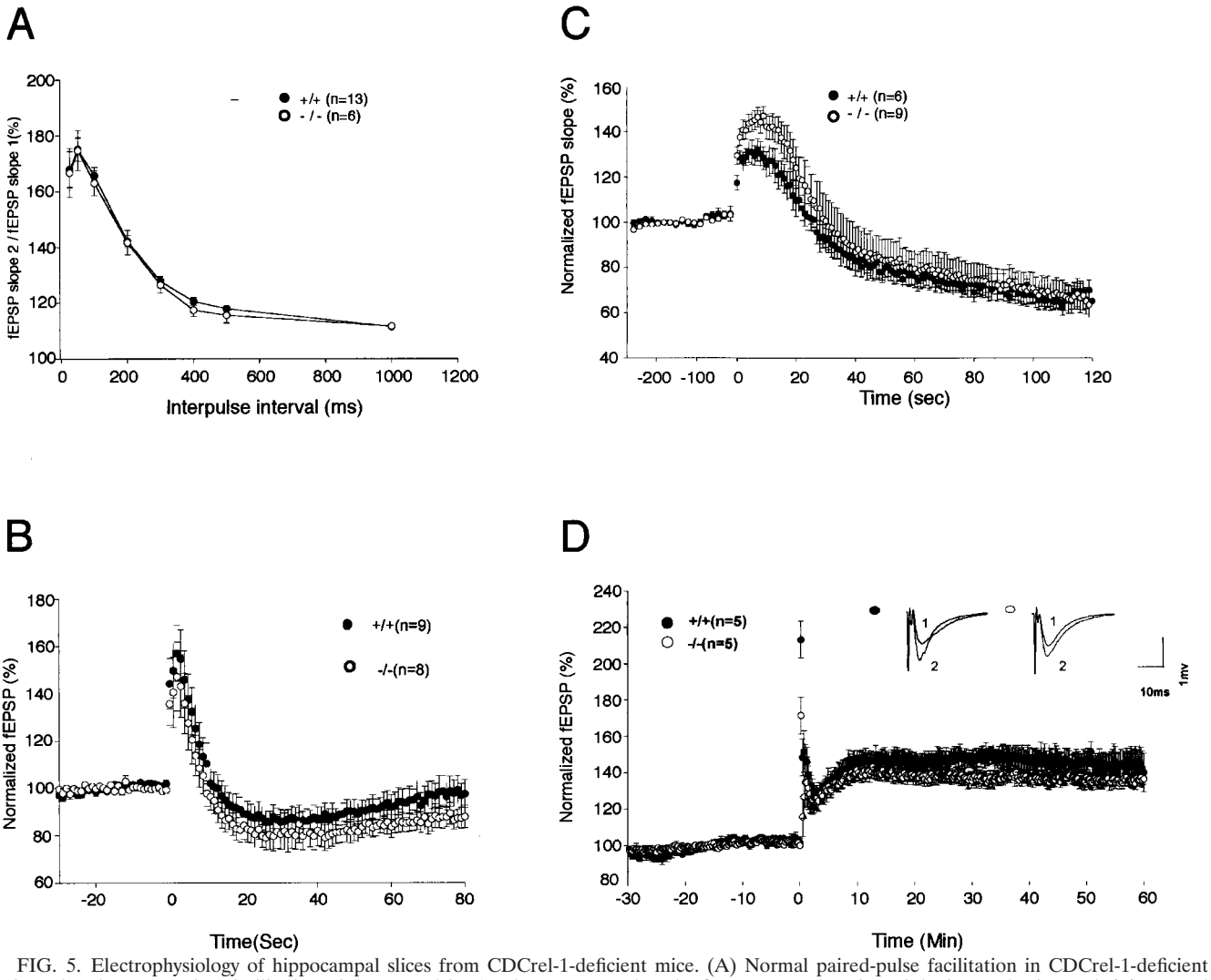

FIG. 5. Electrophysiology of hippocampal slices from CDCrel-1-deficient mice. (A) Normal paired-pulse facilitation in CDCrel-1-deficient mice. The plot summarizes facilitation of the second fEPSP slope compared to the first one as a function of the interpulse interval. (B) Normal posttetanic potentiation in CDCrel-1-deficient mice. Brief (1 s) 100-Hz stimulation was given at the zero time point in the presence of 50 μM (−)-2-amino-5-phosphonopentanoic acid, and the fEPSPs were recorded immediately after the tetanus. (C) Synaptic depression in response to sustained synaptic activities in the knockout mice. Repetitive stimuli (5 Hz, lasting 120 s) were applied at the zero time point, and the fEPSP to each stimulus was recorded. Each data point represents the averaged slope of 20 responses (2 s). (D) Hippocampal CA1 LTP. LTP was induced by tetanic stimulation consisting of 2 trains of 100 Hz lasting 1 s, at an intertrain interval of 10 s, delivered at the zero time point. Above the plot are the representative traces (average of 4 sweeps) of fEPSP obtained immediately before (trace 1) and 50 min after (trace 2) the tetanic stimulation.

6A, neither the levels of nor fractionation of any of these proteins was affected by the absence of CDCrel-1. Other presynaptic proteins were also examined, including syntaxin, SNAP-25 and nsec1, but no changes were noted between wild-type and deficient mice in their distribution either (data not shown).

Given the large number of septin genes in mammals, we considered the possibility that functional redundancy might account for the lack of a phenotype seen in the CDCrel-1-deficient mice. However, since dominant-negative forms of the protein resulted in altered secretion in transfection experiments, simple redundancy seemed less likely and raised the possibility that chronic CDCrel-1 deficiency may have led to compensatory changes in the mice. To examine this possibility, we probed blots for the presence of two mammalian septins, Nedd5 and CDC10. As shown in Fig. 6B, CDCrel-1 is present in all fractions except the cytosolic S3 fraction in the wild-type mice, but it is absent from all fractions in deficient mice. Interestingly, there are clear increases in the levels of Nedd5 and decreases in the levels of CDC10 in every fraction in the CDCrel-1-deficient mice. The signal obtained with the CDC10 antibody reveals a doublet, and it appears that the bottom band of the doublet is specifically decreased in the CDCrel-1-deficient mice. Although the relationship between the two bands seen with anti-CDC10 are not known, many of the septins undergo alternative splicing and it is possible that a specific form of this protein is decreased. These results indicate that alterations in the levels of specific septins have occurred in the

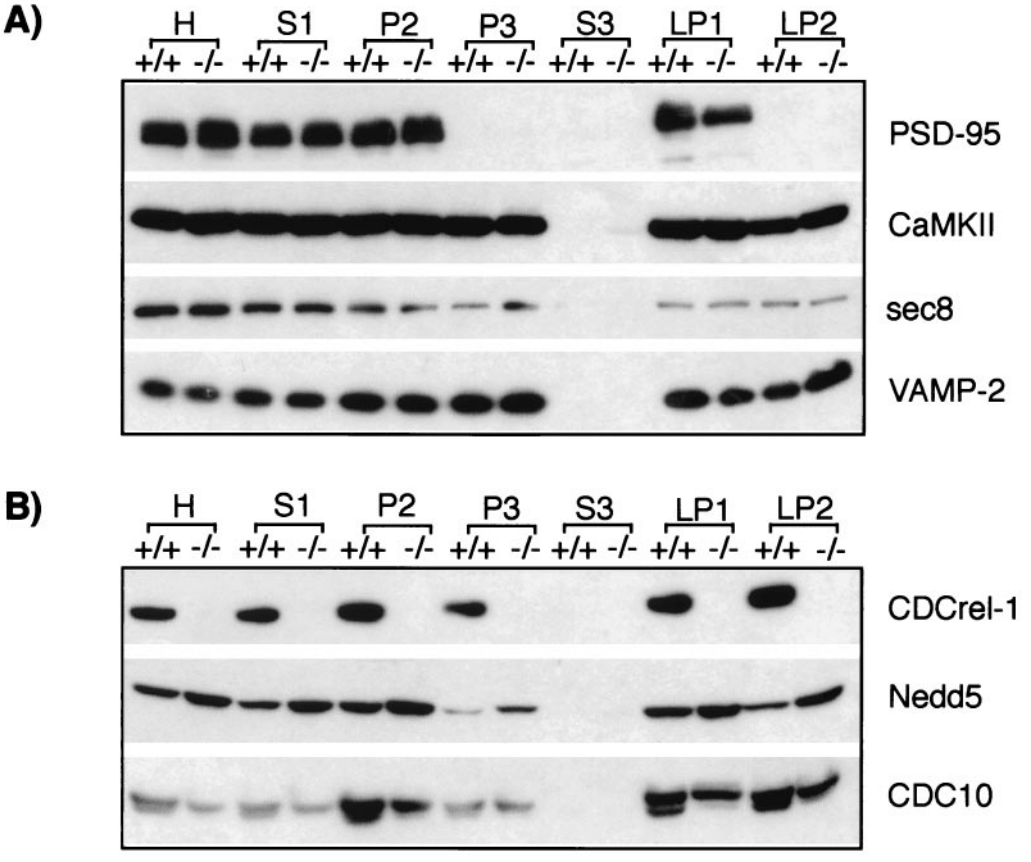

FIG. 6. Altered expression of septins, but not other synaptic proteins, in the brains of CDCrel-1-deficient mice. Wild-type and CDCrel-1-deficient brains were fractionated according to the procedure of Huttner et al. (18), and 20 μg of protein from each fraction was loaded onto each lane. Lanes: H, total homogenate; S1, low-speed supernatant; P2, crude synaptosomal fraction; P3, high-speed pellet of synaptosome-depleted sample; S3, cytosolic high-speed supernatant; LP1, low-speed pellet of lysed synaptosome containing membrane fractions; and LP2, high-speed pellet of lysed synaptosomes containing synaptic vesicles. Blots were probed with the antibodies indicated at the right.

CDCrel-1-deficient mice and these may permit functional compensation for the lack of CDCrel-1.

DISCUSSION

The septin CDCrel-1 is a member of a large and growing family of GTPases that have only recently gained much attention. Septins were first identified in yeast when specific mutations revealed the requisite role for Cdc3p, Cdc10p, Cdc11p, and Cdc12p in budding yeast cytokinesis (15). In addition, a septin was identified in *Drosophila* that was also required for cytokinesis (30), suggesting that this may be the main function of these GTPases (34). However, in yeast there are two other septin-encoding genes that are only expressed during sporulation and that are not essential for viability or cytokinesis (8, 9). One of these, Spr3p, was found to colocalize with Cdc3p and Cdc11p near the leading edges of the membrane sacs that form near the spindle-pole bodies and that engulf the nuclear lobes to form the spores (9). Two hybrid studies using the other sporulation-specific septin, Spr28p, indicated that it could bind to Spr3p, Cdc3p, and Cdc11p (8) but not to Cdc10p or Cdc12p. Together these observations raise the possibility that Spr3p, Spr28p, Cdc3p, and Cdc11p may interact in a unique septin complex specifically involved in sporulation and distinct from that formed during cytokinesis. Interestingly, the function of neither Spr3 nor Spr28 is required for sporulation, suggesting that functional redundancy with other septin proteins may exist.

Specialized septin complexes may also exist in more complex organisms as well. In mammalian cells, at least 11 different septin genes have been identified, several of which have multiple splice forms. Some of these appear to have broad expression patterns while others have unique, tissue-specific patterns. CDCrel-1 and G-septin are primarily expressed in the brain (2, 5, 41) while most of the other forms are broadly expressed (unpublished observations). These complex expression patterns may result in the assembly of distinct septin complexes in each cell type that could possess the combined properties contributed by each of its members. We have shown here that CDCrel-1 can be immunoprecipitated from the brain in a complex that includes Nedd5 and CDC10 but not H5.

The targeted disruption of CDCrel-1 in mice has no overt developmental or neurophysiological consequences. This is surprising given the number of linkages of CDCrel-1 to disease and neuronal function. First, CDCrel-1 had been identified within the locus that is commonly deleted in patients with DiGeorge syndrome (28). In these individuals, haploinsuffi-

ciency of an approximately 3-Mb region is often seen, and it had been assumed that a single gene within this locus might have caused the complex phenotypes associated with this syndrome. More recent studies have implicated the T-box transcription factor Tbx1 as a major cause of this syndrome, although the loss of other genes in the locus may also contribute to the phenotype (26, 29). However, our data indicate that despite its position within the deleted locus, the loss of one or both copies of the CDCrel-1 gene is unlikely to contribute significantly to the development of this disorder.

Recent evidence has also linked CDCrel-1 to the neurodegenerative disorder Parkinson's disease on the basis of its binding and ubiquitination by the E3 ubiquitin ligase Parkin (42). Parkin is the gene product linked to autosomal recessive forms of Parkinson's disease due to point mutations or deletions that result in the loss of function of this protein. The discovery that CDCrel-1 may be a target of Parkin led to suggestions that one of the contributing factors in Parkinson's disease may be the failure to degrade CDCrel-1 (42). Because it had previously been shown that CDCrel-1 overexpression inhibits secretion, the possibility was raised that failure to eliminate CDCrel-1 by ubiquitin-mediated degradation may contribute to Parkinson's disease by limiting the release of dopamine from neurons of the substantia nigra. We have seen no motor or neurological disorders in these mice, but based on this hypothesis we might expect that the CDCrel-1-deficient mice would be less prone to Parkinsonian disorders. It will be of interest to examine the relative sensitivity of the CDCrel-1-deficient mice to conditions that mimic or induce loss of substantia nigra cells.

It had previously been shown that CDCrel-1 was colocalized with the syntaxin and SNAP-25 tSNARE proteins in PC12 cells (2), and we have shown here that it is present in synaptic sites overlapping with the presence of synaptic vesicles. This distribution is consistent with recently published immunoelectron microscopic data that demonstrated the presence of CDCrel-1 among vesicles within presynaptic nerve terminals, particularly in inhibitory neurons (22). Our previous observations that dominant-negative forms of CDCrel-1 caused enhanced secretion led to our hypothesis that CDCrel-1-deficient mice would exhibit enhanced neurotransmitter release or reveal evidence of synaptic depression due to depleted synaptic vesicle stores. At least two possibilities could account for the lack of such results—different phenotypes resulting from the dominant-negative and null mutation approaches or compensatory changes that may not be seen in acute transfection experiments but that could occur during mouse development.

The previous dominant-negative approach was based on the observations of Kinoshita et al. (23) and our own unpublished results showing that GTP binding and/or hydrolysis may be necessary for the formation of septin polymers. Mutations that inhibit GTP binding prevented filament assembly and thereby appeared to act in a dominant-negative manner, possibly by competing with endogenous CDCrel-1. However, we cannot rule out the possibility that the dominant-negative approach affects the function of all endogenous septins while the null mutation only eliminates the function of just one of them. In this case, redundancy could account for the lack of phenotype. Evidence for functional redundancy among the septins has been observed in *Drosophila*. In this case, both female germ cell division and early stages of embryo cellularization occur normally in the absence of the septin *peanut*, suggesting either that it is not required for these steps or that other septins may compensate for its loss (1). In the case of *Caenorhabditis elegans*, mutations in either of the two known septins, *unc-59* or *unc-61*, resulted in the same phenotype. Mutations in either gene disrupted the localization of the other septin to the cleavage furrow and prevented postembryonic but not embryonic cytokinesis (31). Whether this indicates a lack of a role for these septins in embryonic cytokinesis or the presence of unknown proteins with redundant septin functions remains to be seen.

Aside from direct functional redundancy, an alternative form of redundancy could be achieved if alterations in the expression of other septins could compensate for the loss of a particular one. Analysis of the mice deficient in CDCrel-1 clearly shows that they have altered levels of other septin proteins in the brain. At the protein level, decreased amounts of CDC10 could reflect increased rates of degradation if the lack of CDCrel-1 decreased its stability. Alternatively, if CDCrel-1 is degraded due to its interaction with Parkin, associated proteins may suffer the same fate. Hence, in the absence of CDCrel-1, elevated Nedd5 levels could result from decreased degradation. However, as Nedd5 and CDC10 appear to be present within the same coimmunoprecipitating complex, it is unclear why their relative levels would have changed in opposite directions. Another explanation is that a change in the pattern of septin transcription that has occurred in the knockout mice permits the assembly of alternative combinations of septins with compensatory functions. In either case, our data demonstrate that CDCrel-1 is not required for neuronal development or regulated neurotransmission.

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