

Deletion of CASK in mice is lethal and impairs synaptic function

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CASK is an evolutionarily conserved multidomain protein composed of an N-terminal Ca²⁺/calmodulin-kinase domain, central PDZ and SH3 domains, and a C-terminal guanylate kinase domain. Many potential activities for CASK have been suggested, including functions in scaffolding the synapse, in organizing ion channels, and in regulating neuronal gene transcription. To better define the physiological importance of CASK, we have now analyzed CASK “knockdown” mice in which CASK expression was suppressed by ≈70%, and CASK knockout (KO) mice, in which CASK expression was abolished. CASK knockdown mice are viable but smaller than WT mice, whereas CASK KO mice die at first day after birth. CASK KO mice exhibit no major developmental abnormalities apart from a partially penetrant cleft palate syndrome. In CASK-deficient neurons, the levels of the CASK-interacting proteins Mints, Veli/Mals, and neuexins are decreased, whereas the level of neuroligin 1 (which binds to neuexins that in turn bind to CASK) is increased. Neurons lacking CASK display overall normal electrical properties and form ultrastructurally normal synapses. However, glutamatergic spontaneous synaptic release events are increased, and GABAergic synaptic release events are decreased in CASK-deficient neurons. In contrast to spontaneous neurotransmitter release, evoked release exhibited no major changes. Our data suggest that CASK, the only member of the membrane-associated guanylate kinase protein family that contains a Ca²⁺/calmodulin-dependent kinase domain, is required for mouse survival and performs a selectively essential function without being in itself required for core activities of neurons, such as membrane excitability, Ca²⁺-triggered presynaptic release, or postsynaptic receptor functions.

CaM kinase | MAGUK | neuexin | neurotransmitter release | synapse

Neurons in the brain communicate with each other mainly at synapses, specialized intercellular junctions. Like other intercellular junctions, synapses are thought to be organized by cytoplasmic scaffolding proteins that anchor cell-adhesion molecules and receptors to the submembranous compartments. Membrane-associated guanylate kinase proteins (MAGUKs) form the most prominent family of scaffolding molecules associated with intercellular junctions. MAGUKs are characterized by three canonical domains: N-terminal PDZ domains, a central SH3 domain, and a C-terminal guanylate kinase domain (1, 2). A large number of MAGUKs with these domains were described, but CASK is the only MAGUK that contains an additional large N-terminal domain with homology to calcium/calmodulin-dependent protein kinase II α (3). CASK was independently discovered in vertebrates because it binds to neuexins, cell-adhesion molecules with a possible function in synapse formation (3), in *Drosophila* (where it is called CamGUK) because its mutation causes a behavioral phenotype (4), and in *Caenorhabditis elegans* (where it is called lin-2) because its mutation induces abnormal vulva development (5).

Despite a large effort, the function of CASK remains unclear. Biochemical studies in vertebrates showed that CASK forms a stoichiometric complex with Mint 1 (also called X11 or Lin-10)

and Veli (also called MALS or Lin-7) that may be involved in organizing synapses (6, 7). Consistent with this notion, CASK binds to neuexins and to SynCAMs, which are putative synaptic cell-adhesion molecules (3, 8). In addition, CASK may traffic Ca²⁺ channels to the synapse (9), target potassium channels (10), and/or the Ca²⁺ pump 4b/Cl (11) to the plasma membrane, interact with liprins (12) or kinesin (13), and/or regulate transcription by interacting with transcription factors in the nucleus (14). Moreover, analysis of CASK mutations in *Drosophila melanogaster* and *C. elegans* suggested several other functions. In *Drosophila*, CASK mutations produce a discrete neurological phenotype that includes aberrant regulation of activities mediated by calcium/calmodulin-dependent kinase II (15, 16), and CASK may function by modulating Ca²⁺-calmodulin dependent protein kinase (17). In contrast, in *C. elegans* the CASK homolog Lin-2 is selectively required for vulval differentiation and proper localization of the EGF receptor LET-23 (5).

In the present study, we generated and analyzed knockout (KO) mice for CASK to study its function. CASK KO mice die within the first few hours after birth and exhibit a partially penetrant cleft palate syndrome and increased apoptosis in the thalamus, but display no other major developmental changes. Although CASK-deficient neurons exhibit no detectable change in electrical properties, the rate of spontaneous release events is changed, despite an apparently normal evoked release. Our data suggest that CASK performs an essential brain function but is not required for the fundamental development or activities of neurons.

Results

Generation of CASK Mutant Mice. Using homologous recombination experiments with the targeting vector described in Fig. 1A, we generated mutant mice in which the first coding exon of the CASK gene is flanked by loxP sites (i.e., is floxed) and a neomycin resistance gene cassette is inserted into the intron adjacent to the floxed exon. Immunoblotting demonstrated that, in floxed mutant mice, CASK expression is significantly sup-

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The authors declare no conflict of interest.

Abbreviation: KO, knockout.

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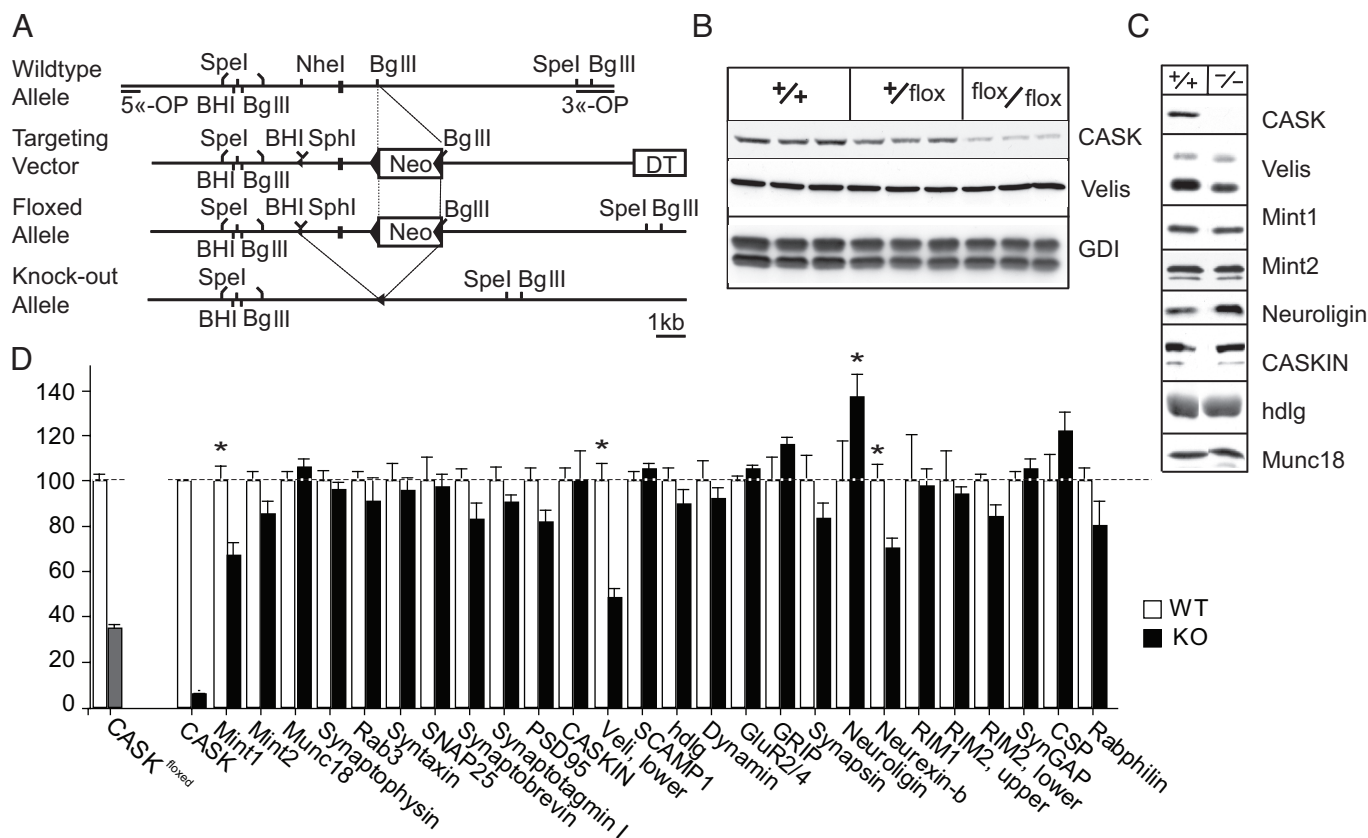


Fig. 1. Gene targeting strategy for CASK: protein levels in knockin and KO mice. (A) Targeting strategy. Genomic clones containing the 5' end of the murine CASK gene (top diagram) were used to construct a targeting vector in which the first coding exon is flanked by loxP sites (triangles) and a neomycin resistance gene cassette (Neo) is inserted into the intron following the exon. In addition, a diphtheria toxin gene (DT) is attached to the long arm of the vector. Homologous recombination of the targeting vector with the endogenous CASK gene replaces the endogenous with the modified genomic sequence and eliminates the diphtheria toxin gene. The resulting "floxed allele" retains the exon in the knockin mice but includes a neomycin resistance gene cassette in the intron, both of which are excised by cre recombinase in the KO. The scale bar on the lower right applies to all panels. (B) Immunoblotting analysis of CASK, Velis, and GDI (GDP dissociation inhibitor) in WT (+/+), heterozygous floxed mutant (+/flox), and homozygous floxed mutant (flox/flox) mice. (C) Immunoblotting analysis of CASK and selected proteins in WT and homozygous KO mice. (D) Quantitation of CASK levels in knockin and KO mice and of various indicated proteins in KO mice. Protein levels were measured by using quantitative immunoblotting with ¹²⁵I-labeled secondary antibodies and phosphorimager detection. Samples were derived from littermate offspring of heterozygous matings.

pressed (Fig. 1B), with quantitations showing that CASK levels were decreased $\approx 66\%$ in homozygous knockin mice (Fig. 1D). Thus, the changes in the CASK gene introduced by the homologous recombination event (i.e., insertion of loxP sites and/or the neomycin resistance gene cassette) partly inhibit CASK expression. Crossing of the floxed mutants with mice expressing cre recombinase in the male germ line (18) caused excision of the floxed exon and the neomycin resistance gene cassette, thus generating KO mice in which CASK expression was decreased $\approx 33\%$ in heterozygous KO mice and abolished in homozygous KO mice (Fig. 1C and D and data not shown).

Deletion of CASK Is Lethal. Homozygous CASK knockin mice are viable and fertile, whereas CASK KO mice die within a few hours after birth, with no surviving mouse observed in >100 crossings [supporting information (SI) Fig. 6A]. Knockin mice, however, are significantly smaller than littermate control mice and exhibit a slightly increased mortality (SI Fig. 6A and B). The floxed knockin mice may be useful for future studies because they have two potentially attractive properties: they represent hypomorphs in which CASK expression is decreased by two-thirds in the homozygous state, and they are conditional KOs in which CASK expression can be deleted in a particular cell type by tissue-specific cre recombinase expression. In the present study, however, we aimed to investigate the essential roles of CASK, and we

will focus on the KO mice. Because CASK KO mice are not viable, an efficient breeding strategy is required to generate sufficient numbers of KO mice for study. For this purpose, we produced hypomorphic mutant male mice that contain a single copy of the floxed CASK gene on their X chromosome and additionally carry a transgene expressing cre recombinase in the germ line. We then crossed these male mice with heterozygous CASK KO female mutant mice, resulting in offspring in which 50% are homozygous KO mice and 25% are WT or heterozygous KO mice (SI Fig. 6C).

Development of CASK KO Mice. A previous transgenic insertion mutant in mice showed that CASK-deficient mice exhibit a cleft palate (19). This finding, in addition to the abundant evidence for a major developmental role of the *C. elegans* CASK homolog Lin-2 (5) and the suggested role for CASK as a transcription factor involved in the development of the cerebral cortex (14, 20), raised the possibility that CASK KO mice may suffer from major developmental abnormalities that could be the cause of the KO lethality. As an initial screen for such developmental abnormalities, we examined newborn CASK KO mice morphologically (Fig. 2A). However, we detected only one major developmental impairment in CASK-deficient mice: the cleft palate reported earlier for a mouse that contains a random transgene insertion in the CASK gene (19). The cleft palate syndrome was

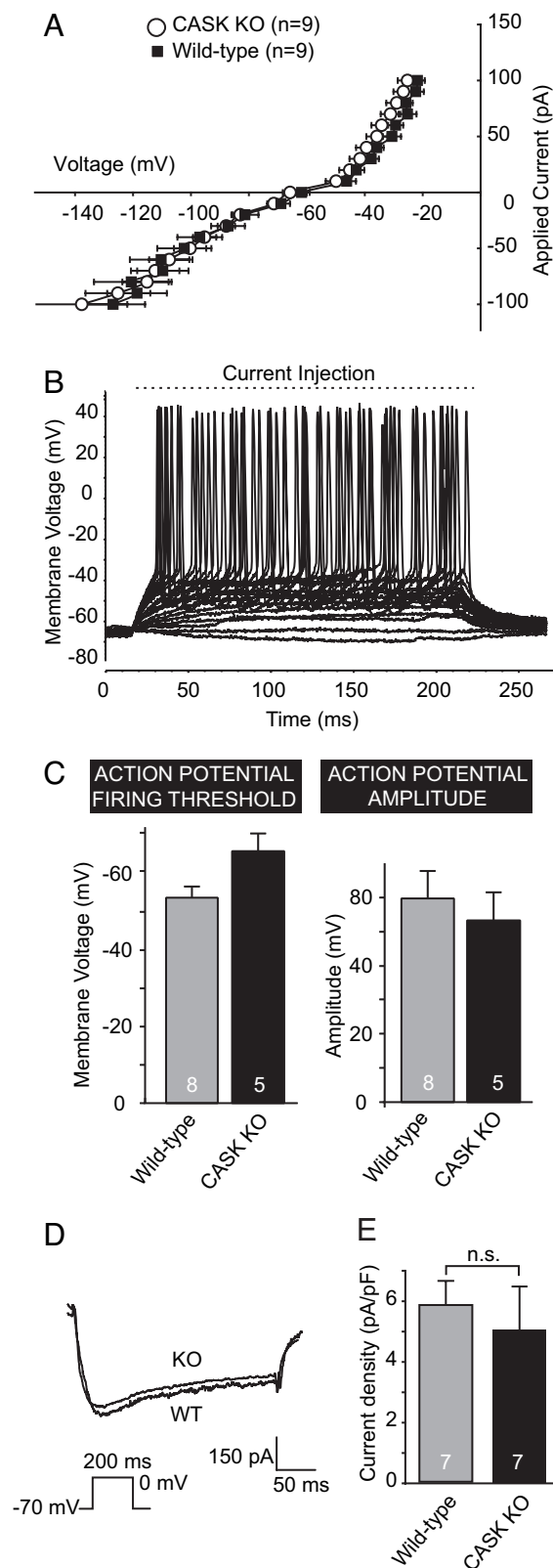


Fig. 3. Electrical properties of CASK KO neurons. (A) Comparative analysis of membrane conductance in neurons at 14 days *in vitro* from littermate WT and CASK-deficient mice. Neurons were examined in current-clamp mode in the presence of 1 μ M tetrodotoxin (mean input resistance: 357.08 ± 19.4 M Ω). The neuronal membrane potential was measured in response to 200-ms current injections, with an 800-ms interval between current injections. The graph plots the membrane potential as a function of injected current; in coincident values for WT and KO neurons, the symbol for the KO neuron is superposed on the

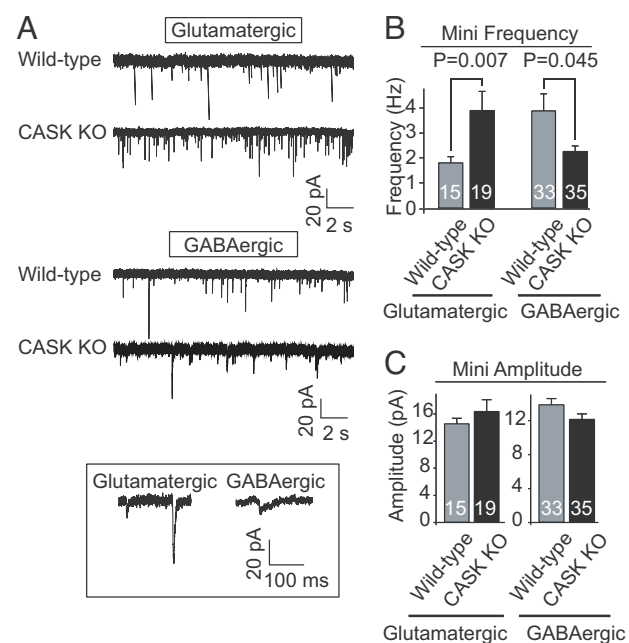


Fig. 4. Analysis of spontaneous release events in CASK KO mice. (A) Representative traces from recordings of the spontaneous miniature synaptic events (minis). Cultured cortical neurons at 14 days *in vitro* were analyzed in voltage-clamp mode in the presence of 1 μ M tetrodotoxin. Glutamatergic and GABAergic responses were examined separately upon addition of 50 μ M picrotoxin or 10 μ M CNQX and 50 μ M AP-5, respectively. The box at the bottom displays a representative single event at high resolution. (B and C) Comparison of the minifrequencies (B) and miniampplitudes (C) for glutamatergic and GABAergic minievents in WT and CASK-deficient neurons (means \pm SEMs; $n = 3$ independent cultures for glutamatergic, $n = 15$ neurons for WT, and $n = 19$ neurons for KO; $n = 4$ independent cultures GABAergic, $n = 33$ neurons for WT, and $n = 35$ neurons for KO). Statistical significance was assessed in pairwise comparisons by using Student's *t* test.

overall responsiveness of synaptic vesicle pools in CASK-deficient neurons.

Evoked Neurotransmitter Release in CASK KO Mice. In a final set of experiments, we analyzed excitatory and inhibitory postsynaptic currents induced by field stimulation. We analyzed the mean amplitudes of responses under four separate pharmacological conditions that either measured the total synaptic responses (i.e., both excitatory and inhibitory postsynaptic currents), separately examined glutamatergic responses using inhibition of GABAergic responses by picrotoxin (50 μ M) or of GABAergic responses using inhibition of glutamatergic responses by CNQX (10 μ M) and AP5 (50 μ M), or measure only NMDA-receptor-mediated glutamatergic responses. For all of these recordings, the holding potential in the postsynaptic patched cell was kept at -70 mV except for the measurements of NMDA-receptor responses where the holding potential was kept at $+40$ mV. The latter

symbol for the WT neuron ($n = 9$ mice used for cultures). (B and C) Analysis of action potential generation in WT and CASK-deficient neurons. Neurons held in current-clamp mode were injected with 200-ms pulses of current in the absence of tetrodotoxin, and the amplitude and firing threshold of the resulting action potentials were analyzed. B shows representative recordings from a KO neuron, and C shows summary graphs from WT and KO neurons ($n = 8$ and 5 independent cultures, respectively; data are not corrected for junction potential). (D) Representative traces of HVA Ca²⁺ currents evoked by a step depolarization from -70 mV to 0 mV in brainstem pre-Bötzing complex neurons of WT and CASK KO mice. (E) Summary graph depicting average HVA Ca²⁺ current densities.

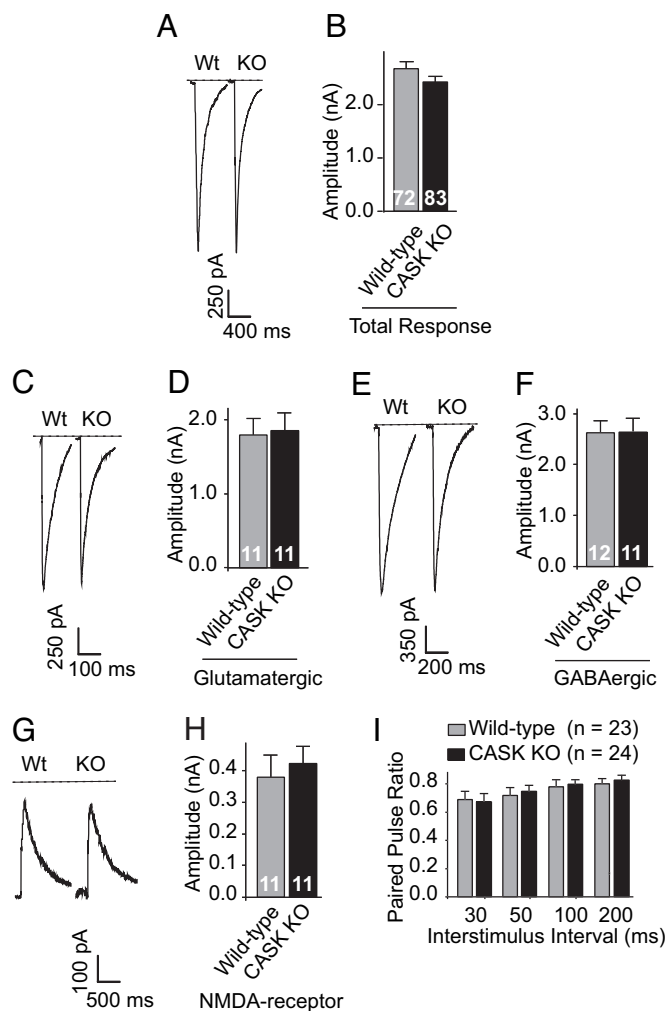


Fig. 5. Evoked synaptic responses in CASK KO mice. Whole-cell recordings in voltage-clamp mode were obtained from cultured cortical neurons; responses were triggered by field stimulation. (A–F) Amplitudes of evoked synaptic responses in WT and CASK-deficient neurons. Synaptic responses to isolated action potentials were measured in cultured cortical neurons (14 days *in vitro*) in voltage-clamp mode by whole-cell recordings. Responses were monitored at a holding potential of -70 mV in the absence of receptor blockers (A and B show total responses; $n = 83$ CASK KO and $n = 72$ WT neurons), in the presence of $10 \mu\text{M}$ CNQX and $50 \mu\text{M}$ AP-5 (C and D show glutamatergic responses; $n = 12$ CASK KO and $n = 11$ WT neurons), or in the presence of $50 \mu\text{M}$ picrotoxin (E and F show GABAergic responses; $n = 11$ CASK KO and WT neurons). (G and H) NMDA receptor-dependent responses were recorded from a holding potential of $+40$ mV in $50 \mu\text{M}$ picrotoxin (H; $n = 11$ CASK KO and WT neurons). (I) Paired-pulse facilitation. Shown is a summary graph [size of the second response divided by the size of the first response to two closely spaced stimuli (paired-pulse ratio); $n = 24$ KO and $n = 23$ WT neurons]. All data shown are means \pm SEMs.

experiments were performed because indirect data have implicated CASK in NMDA receptor trafficking (13).

In all of these experiments, we detected no significant difference between CASK-deficient and WT neurons obtained from littermate mice (Fig. 5 A–H), suggesting that CASK performs no essential function in synaptic transmission, either in presynaptic channel trafficking or in postsynaptic receptor trafficking. However, it is possible that a subtle modulatory effect of CASK would have been missed in these experiments. To address this possibility at least in part, we monitored the release probability in CASK-deficient neurons by measuring the size of synaptic responses upon application of two closely spaced stimuli (Fig.

5I). The relative size of the second to the first response in such measurements is very sensitive to alterations in release probability (25). However, we failed to detect any significant difference between CASK KO and WT synapse in paired-pulse ratio.

To complete our search for potential subtle abnormalities in synaptic vesicle function in CASK-deficient synapses, we investigated the synaptic properties in CASK KO mice during sustained stimulation. We measured synaptic responses in whole-cell voltage-clamped neurons during field stimulations with 300 pulses administered at 1, 5, 10, and 20 Hz. Plots of synaptic currents, normalized to the first response, revealed no significant difference between WT and CASK-deficient neurons (SI Fig. 12). Overall, these results establish that any essential synaptic function of CASK would have to be relatively subtle to be missed in the current experiments.

Discussion

In the present study we produced two mouse models to investigate the function of CASK, an unusual MAGUK protein with a unique N-terminal domain that is homologous to calcium/calmodulin-dependent protein kinase II α : CASK knockin mice that contain a floxed CASK gene and express only $\approx 35\%$ of normal CASK, and CASK KO mice that express no CASK. Using these mouse mutants, we demonstrate that CASK is essential for survival. Deletion of CASK leads to a partially penetrant cleft palate phenotype, as previously observed with mutant mice containing a transgenic insertion in the CASK gene (19). However, in the previous study it was unclear whether the insertion mutant represents a null mutant or whether a truncated protein with a dominant-negative activity is responsible. This question is clarified in the present study by showing that this phenotype is also caused by a null mutation. Cleft palate is a syndrome observed with many mouse mutants, including that of another MAGUK, *dlg* (26). The presence of a cleft palate in CASK KO mice agrees well with the ubiquitous distribution of the protein (3) but is not in itself indicative of a particular function for CASK. Interestingly, mutations in PVRL1, a cell-adhesion molecule that contains a C-terminal CASK binding sequence, also cause a cleft palate syndrome in humans, suggesting a possible pathway by which CASK deletion induces cleft palate (27). We did not observe any other developmental phenotype in the CASK KO mice besides the cleft palate; moreover, even the cleft palate phenotype was not uniformly penetrant in all CASK-deficient mice. Thus, although CASK performs a central developmental function in *C. elegans*, where it is encoded by the *Lin-2* gene (5), it is not absolutely required for normal development in mice.

In our analysis of the CASK KO mice, we focused on the brain because this organ expresses by far the highest levels of CASK (3). Quantitations of brain protein levels revealed discrete changes that confirm the importance of previously reported interactions of CASK with neuroligins: a decrease in β -neuroligins, supposed ligands for CASK, and an increase in neuroligins, supposed ligands for neuroligins (Fig. 1). In addition, we observed changes in Mint 1 and in Velis, which form a tripartite complex with CASK in the brain (7). As an important control, we did not observe massive changes in other synaptic or nonsynaptic proteins, a finding that confirms the selective nature of the CASK KO.

To search for functional deficits resulting from the CASK deletion, we studied the properties of CASK-deficient neurons electrophysiologically. These experiments tested the multiple hypotheses that were previously advanced about CASK functions, e.g., roles in trafficking Ca^{2+} channels to the synapses (9), in targeting potassium channels (10) and/or the Ca^{2+} pump 4b/Cl (11) to the plasma membrane, or in building active zones by interacting with liprins (12). Our results did not establish major deficits in any of these parameters. The basic electrical properties of the neurons were not significantly altered, and evoked

synaptic transmission was not affected by a major change. The only notable alteration we observed was a change in the frequency of spontaneous release events, consistent with the notion that CASK functions in the presynaptic terminal at the active zone. Although our data do not rule out the many functions suggested in previous studies (e.g., functions in trafficking Ca^{2+} or potassium channels, or as transcription factors), our data establish that such functions are not responsible for the lethality observed in the CASK KO mice.

Our study raises several important questions. First, clearly CASK is not functionally redundant in all of its activities with other MAGUKs, because deletion of CASK causes lethality, and does not perform a central role in neuronal ion channel function, synaptic transmission, or development. What then is the function of CASK that is responsible for the lethality of CASK KO mice? Second, in a related question raised by our data, why does CASK contain a domain that is homologous to calcium/calmodulin-dependent protein kinase II but (that are probably related) is missing from all other MAGUKs? Addressing these questions will now be possible with the availability of the constitutive and conditional KO mice reported here.

Materials and Methods

Generation of CASK Knockin and KO Mice. Using genomic clones containing the first coding exon of the CASK gene, we constructed a targeting vector for homologous recombination by standard procedures (28) and used this vector in homologous recombination experiments with embryonic R1 stem cells (29) to generate mutant mice (see *SI Materials and Methods* for a detailed description). All analyses reported here were performed on littermate mice derived from heterozygous breedings.

Immunoblotting Analyses. Protein quantifications were performed on brain tissue homogenized in PBS, 10 mM EDTA, 1 mM

PMSE, and proteinase inhibitors from three pairs of adult littermate mice per genotype, using quantitative immunoblotting with 40 μg of protein per lane. Blots were reacted with ^{125}I -labeled secondary antibodies followed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) detection and GDP dissociation inhibitor or vasolin-containing protein as internal controls as described (24).

Cell Culture. Mixed cultures from cortex of newborn CASK KO mice and WT littermates were prepared as described (30).

Optical Imaging. Optical imaging was performed as described in ref. 31 with cultured cortical neurons at 8 and 13 days *in vitro* loaded with styryl dyes (FM1–43 at 8 μM or FM2–10 at 400 μM ; both from Molecular Probes, Eugene, OR).

Electrophysiology. Synaptic responses were monitored in cultured cortical neurons or from pre-Bötzing complex neurons in acute brainstem slices by using whole-cell recordings (see *SI Materials and Methods* for details). Excitatory and inhibitory responses were obtained after pharmacological isolation with 50 μM picrotoxin or 10 μM CNQX and 50 μM AP-5, respectively. All minianalyses were performed in the presence of 1 μM TTX.

Microscopy. Light microscopy analyses were performed on sections from fixed newborn mouse brains, and electron microscopy was performed on cultured neurons at 13 days *in vitro* (see *SI Materials and Methods* for a detailed description).

Miscellaneous. All data shown are means + SEMs. Statistical significance was determined by Student's *t* test.

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