Reduced axonal localization of a Caps2 splice variant impairs axonal release of BDNF and causes autistic-like behavior in mice

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Ca2+-dependent activator protein for secretion 2 (CAPS2 or CADPS2) potently promotes the release of brain-derived neurotrophic factor (BDNF). A rare splicing form of CAPS2 with deletion of exon 3 (dex3) was identified to be overrepresented in some patients with autism. Here, we generated Caps2-dex3 mice and verified a severe impairment in axonal Caps2-dex3 localization, contributing to a reduction in BDNF release from axons. In addition, circuit connectivity, measured by spine and interneuron density, was diminished globally. The collective effect of reduced axonal BDNF release during development was a striking and selective repertoire of deficits in social and anxiety-related behaviors. Together, these findings represent a unique mouse model of a molecular mechanism linking BDNF-mediated coordination of brain development to autism-related behaviors and patient genotype.

Ca2+-dependent activator protein for secretion 2 (CAPS2 or CADPS2) is a member of the CAPS protein family that regulates the trafficking of dense-core vesicles by binding both phosphoinositides and dense-core vesicles (1–5). We initially identified mouse Caps2 as a potent factor promoting the release of brain-derived neurotrophic factor (BDNF) during cerebellar development (6, 7). Our subsequent knockout mouse study showed that Caps2 not only plays a role in neuronal development of the cerebrum and hippocampus as well as the cerebellum, but that it is also associated with social interaction, anxiety, and maternal and circadian behaviors in mice (7, 8). We also showed that the expression of an exon 3-skipped (or -spliced out) form of CAPS2 (designated CAPS2-dex3) (8), which is now known to be a rare alternative splicing variant (9, 10), is increased in a subgroup of patients with autism and is not properly localized in axons (8). Thus, neurons overexpressing dex3 may fail to coordinate local BDNF release from axons properly (8, 9), resulting in improper brain development and function. The human Caps2 gene locus (7q31.32) is intriguingly located within the autism susceptibility locus 1 (AUTS1) (11) on chromosome 7q31–q33, one of several susceptibility loci for autism (12). Moreover, an association of Caps2 with autism has been suggested recently, not only by the presence of copy number variations in the CAPS2 gene in autistic patients (13–15), but also by decreased transcription of CAPS2 in the brains of people with autism (16). Thus, clarifying the biological significance of dex3 expression is an important step in elucidating the association of CAPS2 with brain circuit development and behaviors related to autism.

The potential molecular risk factors for autism susceptibility have been increasingly reported (17–26) but are poorly characterized in animal models. In this report, we generated a mouse model expressing dex3 and analyzed the cellular and autistic-like behavioral phenotypes of dex3 mice. Our results support the involvement of the rare dex3 form of Caps2 in defective axonal BDNF secretion, affecting proper brain circuit development and/or function, and thereby contributing to an increased susceptibility to autism. Our results suggest that disturbance of the alternative splicing patterns of Caps2 can directly affect normal brain development and function and could contribute to a genetic background of autism susceptibility.

Results

Generation of a Mouse Line Expressing Exon 3-Skipped Caps2 (dex3). To clarify the in vivo effect of Caps2 exon 3 skipping, we used a Cre/loxP system to generate a mouse line carrying a deletion of exon 3 (which encodes 111 amino acids) of the Caps2 gene, which expresses the exon 3-skipped Caps2 (Caps2-dex3) protein. A conditional Caps2-dex3 allele was generated by gene targeting in embryonic stem cells (Fig. S1A). The resultant homozygotes, called Caps2 Δex3/Δex3 mice, produced a Caps2-immunoreactive protein with a slightly lower molecular weight than that of wild types (Fig. S1B). The Caps2 Δex3/Δex3 mice in standard breeding cages exhibited no differences in life expectancy from control mice, and both male and female Caps2 Δex3/Δex3 mice had normal reproductive ability.

Dex3 Mice Show a Severe Reduction in Caps2 Immunoreactivity in the Projection Areas of Caps2-Expressing Neurons. We first examined the intracellular localization of dex3 protein in vivo using an anti-Caps2 antibody. In the cerebellum, wild-type Caps2 protein was mostly localized in the granule cell axons (parallel fibers) extending into the molecular layer (Fig. 1 A and C), whereas dex3 protein was not localized in the axons and instead accumulated in the cell somas of the internal granule cell layer (Fig. 1 B and D). In the cerebellum of wild-type mice, diffuse signal was widely distributed over the cortical layers, and intense signals were observed in the soma of a subset of interneurons scattered throughout layers II/III and V (Fig. 1 E). Interestingly, in Caps2 Δex3/Δex3 mice, the diffuse signal was almost absent and mostly localized to cell somas in layer V, in addition to some intense signaling seen in interneurons in layers II/III and V (Fig. 1 F). This supported our hypothesis that full-length Caps2 protein [exon3-plus; the dominant form in mouse (27) and human (8) brains] can be transported into axons, whereas dex3 protein (exon3-minus) cannot (8).

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In the wild-type mouse hippocampus, Caps2 protein was expressed in dentate gyrus (DG) granule cells and cornu ammonis region 1 (CA1) pyramidal neurons in addition to a subset of interneurons scattered in the DG and CA1 regions (28), as shown in Fig. S2A. The latter immunosignal was strong in the cell soma, whereas Caps2 expressed in DG granule cells was localized in axons of the stratum lucidum. In Caps2Δex3/Δex3 hippocampus, diffuse signal of the stratum lucidum was almost diminished, as shown in Fig. S2 B, D, and E.

**Decreased Axonal Localization of BDNF in dex3 Neurons.** We analyzed the effect of dex3 expression on the axonal localization of BDNF immunoreactivity using primary-cultured hippocampal calbindin-positive granule cells from wild-type and Caps2Δex3/Δex3 mice. In wild-type neurons (Fig. 2 A–C), Caps2-immunopositive puncta were localized in the axon, as shown by colocalization with the axonal marker Tau. However, in Caps2Δex3/Δex3 neurons, Caps2-positive puncta were decreased in the axons (Fig. 2 D–G). Similarly, as shown in Fig. 2 H–J, BDNF-immunopositive puncta were localized in the axon of wild-type neurons, whereas BDNF-positive puncta were decreased in the axon of Caps2Δex3/Δex3 neurons (Fig. 2 K–N). These data suggest that the axonal localization of BDNF is affected in dex3-expressing neurons.

To exclude the possibility of a defect in cell polarization, we examined the immunoreactivity of MAP2 (a dendritic marker) and Tau (an axonal marker). Both were localized normally in Caps2Δex3/Δex3 hippocampal cells (Fig. S3).

**Decreased Axonal Secretion of BDNF in dex3 Neurons.** To analyze the axonal release of BDNF in Caps2Δex3/Δex3 neurons, a BDNF–green fluorescent protein (GFP) fusion construct was transfected into hippocampal dentate granule cell cultures prepared from either wild-type or Caps2Δex3/Δex3 mice. KCl-induced (i.e., depolarization-induced) BDNF–GFP release from transfected neurons was evaluated by immunostaining with an anti-GFP antibody before cell permeabilization and by counting immunopositive puncta on axons (Fig. 2O), as previously reported (29–31). The number of cell-surface (i.e., released) BDNF–GFP puncta in KCl-stimulated cells was normalized to that in unstimulated control cells. A graded increase in KCl concentration revealed an activity (depolarization)-dependent release of BDNF–GFP from the axons of wild-type but not Caps2Δex3/Δex3 cells (Fig. 2P). These results showed that regulated release of BDNF was impaired in the axons of Caps2Δex3/Δex3 neurons.

**Deficits in Hippocampal and Cortical Interneurons and Dendritic Spines in dex3 Mice.** There is a report showing disrupted architecture in the gamma-aminobutyric acid (GABA)ergic interneuron circuit of the neocortex in autism (32). It was also shown that differentiation of a subset of neocortical parvalbumin-positive GABAergic neurons is regulated by BDNF (33). Thus, we analyzed parvalbumin- and calbindin-positive interneurons in Caps2Δex3/Δex3 mice. In the hippocampus at postnatal days 17 (P17) and P21, there was no significant difference in the number of parvalbumin-positive interneurons among wild-type, Caps2Δex3/Δex3, and Caps2Δex3/Δex3 mice (Figs. S4 A and B and S5 A–D). On the other hand, in the neocortex, there were significantly fewer parvalbumin-positive interneurons in Caps2Δex3/Δex3 mice than in their wild-type littermates (Figs. S4 C and D and E–H), which showed a phenotype similar to that of Bdnf−/− mice (33) and Caps2-null mutants (8).

Fewer calbindin-positive interneurons were observed at P7 in the hippocampus of both Caps2Δex3/Δex3 and Caps2Δex3/Δex3 mice compared with wild types (Figs. S4E and S5 F and J). In contrast, there was no significant difference in the P21 hippocampus among wild-type, Caps2Δex3/Δex3, and Caps2Δex3/Δex3 mice (Figs. S4F...
Caps2 was decreased in those secrete BDNF. The total number of dendritic protrusions (J) in one corner. When the stranger mouse was introduced, both Caps2+/Δex3 and Caps2Δex3/+ mice showed a significant reduction, compared with wild-type mice, in the time spent approaching or interacting with the stranger mouse cage (Fig. 3C).

We also performed a three-chamber social interaction test (Fig. 3D and E). The mouse being tested was placed in the central chamber and could move freely among the three chambers. A stranger mouse was placed in one of the side chambers in a small cage, and an empty cage was placed in the opposite chamber. Wild-type mice tended to contact the stranger mouse, and the time spent in the quadrant containing the stranger mouse was significantly higher than the time spent in the other quadrants.
Therefore, we measured the olfactory ability of this result might be because of an impairment in olfaction. However, when placed in an open field containing a novel object in the central area (the black-and-white vertical object shown in the Inset of Fig. 4A), Caps2Δex3 mice became less active (Fig. 4A) without changing their speed of movement (Fig. 4B) and tended to contact the novel object less frequently than wild-type mice did (Fig. 4C). Representative traces from wild-type, Caps2Δex3, and Caps2Δex3 mice are shown in Fig. 4D.

In the elevated plus maze test, there was no difference in horizontal distance moved, suggesting that locomotor activity in this test was not affected by exon 3 skipping (Fig. 4E). However, the Caps2Δex3 mice spent less time in the open arms than did the wild types (Fig. 4F). In addition, the number of open-arm entries was decreased (Fig. 4G). Representative traces from wild-type, Caps2+/Δex3, and Caps2Δex3 mice are shown in Fig. 4H. However, Caps2Δex3 mice showed no significant anxiety-like behavior in the light/dark transition test (Fig. S11 A–C), indicating that their tendency to display increased anxiety-like behavior might depend on the particular behavioral test used; for example, they may be more sensitive to novelty or unfamiliarity than to a light/dark preference.

In the home cage, locomotor activity was similar between Caps2Δex3 mice and their wild-type littersmates when measured during a 3-d period (12-h light/dark cycle) after habituation to a fresh home cage for 24 h (Fig. S12). However, in the dark period of the 24-h cycle, even on the third day, both Caps2Δex3 and Caps2Δex3 mice exhibited reduced locomotion activity compared with wild-type mice (Fig. 4I), suggesting that Caps2Δex3 and Caps2Δex3 mice do not adapt well to a new environment.

Decreased dam–pup interaction and communication. To evaluate whether the maternal behavior of Caps2Δex3 mice was affected, we monitored the nurturing of newborns by their mothers, to assess maternal care as an important social interaction between dams and pups. To exclude the effect of pup genotype, Caps2Δex3 pups were used for this experiment. In many cages, the newborns of a Caps2Δex3 dam and a wild-type sire rarely survived beyond P1 (Fig. 4J). In contrast, the newborns of a Caps2Δex3 sire and a wild-type dam normally survived beyond P1 (Fig. 4J). These results indicated that Caps2Δex3 mothers display defective maternal behavior.

To investigate communication ability from pups to their dam, we studied the ultrasound vocalization (USV) of neonates using Caps2Δex3 mice. To exclude the effect of sire genotype, wild-type or Caps2Δex3 males were mated with wild-type females. The result wild-type or Caps2Δex3 pups were used to monitor USV. We found that the duration of USVs decreased from P5 to P10 in wild-type pups, but that Caps2Δex3 pups emitted very few USVs throughout this period (Fig. S13). This suggests that the skipping of exon 3 affects the dam–pup interaction by USV calls.

Impaired circadian rhythm. Autism is frequently accompanied by an abnormal sleep–wake rhythm (39, 40). Regarding the circadian rhythm of locomotor activity under a 12-h light/dark cycle, we detected no differences in the sleep–wake rhythm among wild-type, Caps2Δex3, and Caps2Δex3 mice. Under constant dark conditions, the sleep–wake rhythm of the Caps2Δex3 mice showed a shorter period than that of wild-type mice. However, there was no statistically significant difference among the three genotypes (Fig. S14). Because some Caps2Δex3 mice (4/14) showed impaired circadian rhythmicity (Fig. S14C) compared with wild types and this resulted in a large variance (Fig. S14B).
In this report, we developed a mouse line expressing exon 3-deleted Caps2 (dex3), the same as a rare alternatively spliced variant of human CAPS2 that was identified in some individuals with autism (8). Caps2-dex3 mice displayed deficits in axonal localization in cerebral, hippocampal, and cerebellar neurons, resulting in decreased local secretion of BDNF from axons. This local loss of BDNF would predict the abnormal splicing of Caps2, contributing to the abnormal circuit connectivity observed in cerebral, hippocampal, and cerebellar neurons, resulting in disturbance in the proper levels of local BDNF release, which we hypothesized might be attributable to the approximately equal balance of dex3-minus vs. dex3-plus isoform activity and any nucleotide sequence differences in the three CAPS2 sites and the branch point) of the mRNA. Therefore, we considered the possibility that there is a polymorphism(s) in an acceptor′ gene in autistic patients. We must also approach and avoid behaviors (43). Approach behavior or anxiety-related behavior is thought to reflect an animal’s fear of novelty. In this report, Caps2Δex3 mice showed increased anxiety or reduced environmental exploration compared with wild-type mice when placed in an open field containing a novel object. Moreover, Caps2Δex3 mice tended not to contact a novel object. Overall, Caps2Δex3 mice tended to show augmented anxiety or reduced environmental exploration in a novel environment. Caps2Δex3 mice showed impairments in both social interaction and social novelty preference. Caps2Δex3 mice also exhibited a significant reduction in interaction activity, suggesting that a social behavioral deficit might be attributable to the approximately equal balance of dex3-minus vs. dex3-plus isoform expression (Fig. S1B) that was found in some autistic patients (8). The link between social behavior in rodents and humans is difficult to establish. Our model may provide a powerful tool to explore its mechanisms.

Recently, it was suggested that splicing dysregulation is an underlying mechanism of neuronal dysfunction in autism (16). However, it remains unclear how the increased removal of exons 2 and 3 of CAPS2 is specifically caused in certain autistic patients. We did not find any nucleotide sequence differences in the three common splicing cis-elements (the 5′ donor and 3′ acceptor sites and the branch point) of the CAPS2 gene in autistic patients displaying exon 3 skipping (8). We also examined the sequence of introns 2 and 3 in the autistic subjects, and we found some variants specifically identified in patients displaying increased exon 3 skipping. We are currently investigating the association of these variants with exon 3 skipping. We must also consider the possibility that there is a polymorphism(s) in an unknown gene(s), which alters the splicing of CAPS2 mRNA (10). This scenario would predict the abnormal splicing of multiple genes besides CAPS2. However, the Caps2Δex3 Is a powerful tool to explore its mechanisms.
mice, in which only Caps2 was mutated, showed autistic-like phenotypes, arguing against the involvement of other abnormally spliced genes. This causality is reminiscent of that between mutation of MeCP2 (the cause) and altered expression of BDNF (the effect) in Rett syndrome. Bdnf is one of the affected genes in MeCP2-knockout mice; however, BDNF is strongly associated with the symptoms of Rett syndrome (44, 45). Clearly, the genetic involvement for autism is complex, but our approach of model validity—reverse engineering a mouse model for autistic patients with overexpression of a dex3 isoform of CAPS2, which shows relevant phenotypes—is certainly promising.

Materials and Methods

Animals. All experimental protocols were approved by the RIKEN Institutional Animal Care and Use Committee. Mice were housed on a 12-h light/dark cycle, with the dark cycle from 20:00–08:00.

Generation of Caps2 Exon 3-Skipped Targeting Vector. An 11-kb genomic fragment containing exon 3 of the mouse Caps2 gene was obtained from the genomic DNA of C57BL/6 mice and was used to construct the targeting vectors (Fig. S1A). One loxP sequence was inserted at the BstI site, and the phosphoglycerate kinase–neomycin resistance (neo) cassette, flanked by another loxP sequence and a pair of frt sequences, was inserted into the AatII site upstream of exon 3, to generate a loxP–frt-neo–frt-exon3–loxP cassette. For negative selection, the diphtheria toxin A-fragment gene cassette was inserted to the 3′ end of the targeting vector.

Generation of Caps2 exon 3-skipped mice, primary culture, immunohistochemistry, immunocytochemistry, and behavioral tests are described in detail in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Antibodies. The following primary antibodies were used for Western blotting: guinea pig polyclonal anti-Ca2+-dependent activator protein for secretion 2 (Caps2) (1:10,000 dilution) (1) and mouse monoclonal antiactin (1:500 dilution; A4700; Sigma-Aldrich). The following primary antibodies were used for immunohistochemistry and immunocytochemistry: guinea pig polyclonal anti-Ca2+-Caps2 (1:5,000 dilution) (1), rabbit polyclonal anti-brain-derived neurotrophic factor (BDNF) (1:100 dilution) (2), rabbit polyclonal anti-green fluorescent protein (GFP) (1:400 dilution; A1122; Life Technologies), anti-MAP2(a+b) (1:1,000 dilution; M1406; Sigma-Aldrich), mouse monoclonal antiparvalbumin (1:4,000 dilution; P3088; Sigma-Aldrich), mouse monoclonal anti-Tau (1:300 dilution; 610672; BD Biosciences), rabbit polyclonal antisyaptophysin (1:100 dilution; RB-1461-P0; Thermo Scientific), and rabbit polyclonal anticalbindin (1:1,000 dilution; AB1778; Millipore). A polyclonal antibody was raised against recombinant human BDNF (whole BDNF) in male New Zealand white rabbits (Japan SLC) (2). Purified anti-BDNF antibody was tested for specific reactivity with the antigen (recombinant human BDNF) but not with mouse nerve growth factor (NGF) or recombinant human neurotrophin-3 (NT-3) (2). It was also confirmed that the purified anti-BDNF antibody could immunoreact with specific areas of the hippocampus in wild-type mice but not in BDNF-knockout mice (3).

Generation of Caps2 Exon 3-Skipped Mice. After transfecting MS12 embryonic stem (ES) cells (a C57BL/6 mouse ES cell line) (4) by electroporation, targeted clones were screened for G418 resistance with the targeting vector and analyzed by Southern blotting. Chimeric mice were generated by injection of targeted MS12 ES cells into mouse BALB/c blastocysts; the chimeras were mated with wild-type C57BL/6 mice to obtain heterozygous mutant mice. All of the engineered animals studied were backcrossed with C57BL/6 mice for more than five generations.

Immunohistochemistry. C57BL/6J male mice were killed by anesthesia with diethyl ether. Mice were transcardially perfused, initially with PBS and then with Zamboni fixative [2% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.2% picric acid]. Tissues were dissected, postfixed in Zamboni’s fixative at 4 °C for 5 h, and cryoprotected by immersion in 15% (wt/vol) sucrose in PBS overnight at 4 °C. After embedding in Tissue-Tek OCT compound (Sakura Finetechnical), tissues were frozen in dry ice powder, and sectioned at a thickness of 14 μm using a cryostat (CM1850; Leica Microsystems) at −18 °C. The sections were air dried for 1 h and rinsed three times in PBS. After blocking with 5% (vol/vol) normal donkey serum/PBS at RT for 60 min, cells were incubated with primary antibodies at RT for 2 h, rinsed in PBS, permeabilized in PBS containing 0.02% Triton X-100 at RT for 5 min. After blocking with Image-iT FX signal enhancer (Invitrogen) at RT for 60 min, cells were incubated with primary antibodies at 4 °C overnight, rinsed in PBS, then incubated with Alexa-conjugated secondary antibodies (1:1,000 dilution; Invitrogen) at RT for 1 h, and again rinsed in PBS. Immunoreacted cells were mounted with Vectashield mounting medium (Vector Laboratories). Images were acquired with a microscope (BX51; Olympus) equipped with a CCD camera (VB-7000; Keyence). Digital images were processed using Adobe Photoshop 6.0 software (Adobe Systems).

Primary Cultures of Hippocampal Granule Cells. Primary-cultured hippocampal granule cells from mice were prepared using previously described methods (5), with slight modifications. Briefly, three-day-old C57BL/6J mice (Nippon SLC) were deeply anesthetized by ether, and the hippocampal formation was immediately dissected and placed in ice-cold Gey’s balanced salt solution bubbled with a gas mixture of 95% O2 and 5% CO2. After removal of the subicular complex along the sulcus hippocampi, the remaining part was divided into the dentate gyrus and Ammon’s horn. Both tissues were cut into pieces and treated with 0.25% trypsin (Difco Laboratories) and 0.01% DNase I (Sigma-Aldrich) at 37 °C for 30 min. The incubation was terminated by the addition of heat-inactivated horse serum. The tissue fragments were centrifuged at 250 × g for 5 min, the supernatant was removed, and the pellet was resuspended in a mixture of 50% Neurobasal/B-27 and 50% astrocyte-conditioned medium. The cells were plated at a density of 5.0 × 103 cells/cm² onto poly-l-lysine–coated cell culture plates. To prevent proliferation of glial cells, the culture medium was changed to conditioned medium-free Neurobasal/B-27 medium supplemented with 2 μM cytosine-β-arabinofuranoside (Sigma-Aldrich) 24 h after plating. Half of the medium was exchanged every 3 d.

On days in vitro (DIV) 6, neurons were transfected with BDNF–GFP using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cultured neurons were stimulated with medium containing various concentrations of KCl for 10 min, fixed with 4% (wt/vol) paraformaldehyde (PFA)/PBS, and then washed with PBS. After blocking with 5% (vol/vol) normal donkey serum/PBS (Vector Laboratories), cells were incubated with anti-GFP antibody at RT for 2 h, rinsed in PBS, permeabilized, incubated with anti-MAP2(a+b) antibody at RT for 2 h, rinsed in PBS, incubated with Alexa Fluor-conjugated secondary antibody at 4 °C overnight, and then rinsed in PBS. Immunoreacted cells were mounted with Vectashield mounting medium (Vector Laboratories). Images were acquired with a microscope (BX51; Olympus) equipped with a CCD camera (VB-7000; Keyence). Digital images were processed using Adobe Photoshop 6.0 software (Adobe Systems).

Behavioral Tests. Two-month-old male mice were used unless otherwise described. All mice used were the littermate progeny of intercrossed heterozygotes. The experimenter was blind to the genotype.

Rotarod testing. Rotarod testing was carried out as described previously (6) using a Rota-Rod Treadmill for Mice 7600 (Ugo Basile). All mice used in the experiments below were littermates from mated heterozygotes. Briefly, a male mouse was required to run backward to maintain its position on top of a rod revolving at 24 rpm. Each time it fell, the mouse was put back on the rod until it had run for a total of 3 min on the rod. The time was not
counted while the mouse was off the rod. The latency to first fall and the total number of falls within 3 min were recorded.

**Grip traction test.** Muscle strength was tested by a mouse’s ability to hang on by its forepaws to a horizontal wire placed above the table. The time during which the mouse remained hanging was recorded (7).

**Open-field test and novel object recognition test.** The test was performed as previously described (8, 9) with minor modifications. Locomotor activity was measured in an open field (60 × 60 cm) at 50 lx (at the surface level of the area). Each mouse was placed in the center of the open field, and its horizontal movements were monitored for 15 min with a charge-coupled device camera. The images were processed with National Institutes of Health (NIH) Image Open Field (O.F.) software (O’Hara & Co.).

**Social interaction test.** The social interaction test was performed as previously described (8) with minor modifications. A vacant small cage was placed in the home cage with the subject for 24 h. Social interaction was measured in the open field apparatus described above. A small cage containing a stranger C57BL/6J mouse was placed in one corner. Each mouse was placed into the open field for 15 min.

**Y-maze test.** Spontaneous alternation to explore new environments was assessed to test spatial recognition memory as described previously (10). The acryl symmetrical Y maze consists of three arms (40 × 3 cm) each separated by 120°, with 13-cm-high transparent walls (O’Hara & Co.). Each mouse (11–13 wk of age) was placed in the center of the Y maze and allowed to explore freely for 5 min. The sequence and total number of arms entered were recorded. The percentage of alternation is the maximum possible number of alternations (total number of arm entries = 2) × 100.

**Olfactory test.** The hidden-cookie test was performed as previously described (11). Male mice (n = 9 of each genotype) were food deprived overnight. A piece of butter cookie (Morinaga, <0.7 g per piece) was buried beneath about 3 cm of clean bedding in a random location. The mouse was placed in the cage, again in a random location, during the light phase. The latency time to locate the cookie was recorded. We defined finding the cookie as when the mouse held it in both paws.

**Elevated plus maze.** The elevated plus maze was set at a height of 65 cm and consisted of four gray plexiglass arms, each 8-cm-wide × 25-cm-long with 15-cm-high walls. Two arms were open, and two were enclosed. Individual mice were placed in the center of the maze, and the total distance and time spent in each arm were measured, and analyzed with NIH Image Elevated Plus (E.P.) software (O’Hara & Co.).

**Light/dark transition test.** The light/dark transition test was performed as previously described (12). The apparatus consisted of a cage (40 × 20 × 20 cm) bisected by a black partition containing a small opening (O’Hara & Co.). One chamber was open and brightly illuminated, whereas the other chamber was closed and dark. Mice were placed into the dark side and allowed to move freely between the two chambers for 10 min. The time spent in the light side was recorded, and analyzed with NIH Image Light/Dark (L.D.) software (O’Hara & Co.).

**Home-cage activity.** Home-cage activity was measured as previously described (13). Spontaneous locomotor activity in the home cage (18 cm wide × 14 cm high × 32 cm deep) was determined by counting photobeam interruptions using SCANET (6 channel SV-20 system; Melquest) for 6 d, after 24 h habituation to a fresh cage. The distance between the sensors was 0.5 cm. Mice were housed individually under the 12-h light/dark cycle described above. Food and water were available ad libitum. The experiment was begun at 08:00, and lasted 3 d.

**Neonate survival test.** The mice used were 2-mo-old primiparous females. Wild-type females were mated with Caps2Δex3/Δex3 males, and vice versa. Pregnant wild-type or Caps2Δex3/Δex3 females were housed separately and were not touched for at least 5 d before giving birth.

**Ultrasound vocalization test.** Animals were assessed for ultrasound vocalization (USV) between P5 and P10. Wild-type females were mated with wild-type or Caps2Δex3/Δex3 males. The pups were separated from their dam immediately before recording and were positioned in a sound-attenuating chamber below a condenser ultrasound microphone (40011; Avisoft Bioacoustics) to detect USVs at 50–100 kHz. The duration of calls was recorded and quantified with an Avisoft recorder (Avisoft Bioacoustics) during a 10-min test.

**Recording of circadian rhythm.** Circadian rhythm was recorded as previously described (14). Wheel running was measured with a wheel meter (WW-3302; O’Hara & Co.), which comprises a set of 20 rooms (14.3 cm wide × 14.8 cm high × 29.3 cm deep) containing a wheel cage (50 cm diameter × 5 cm wide). The mice were housed one per room, were able to move freely, and were given free access to food and water. Every 1/3 revolution of the wheel cage was recorded as one count. The circadian period was calculated using a χ² periodogram.

Fig. S1. Generation of Caps2Δex3/Δex3 mice. (A) Maps of the mouse Caps2 gene, the targeting vector, and the resultant targeted allele. The filled box denotes exon 3. Restriction enzyme sites: A, AatII; B, BamHI; Bt, BtrI; Sm, SmaI; and Sp, SpeI. DTA, diphtheria toxin fragment A. White triangles represent loxP sites, gray triangles represent FRT sites. (B and C) Immunoblot analysis of the cerebellum of P8 wild-type, Caps2+/Δex3, and Caps2Δex3/Δex3 mice. Protein lysates from the cerebellum were Western blotted (WB) with an anti-Caps2 (B) and an antiactin antibody (C).
Fig. S2. Distribution of Caps2 protein in the Caps2Δex3/Δex3 mouse hippocampus. (A and B) Sagittal sections of P21 wild-type (A) and Caps2Δex3/Δex3 (B) hippocampus were immunolabeled with an anti-Caps2 antibody. (Scale bars, 100 μm.) (C and D) Sagittal sections of P21 wild-type (C) and Caps2Δex3/Δex3 (D) hippocampal CA3 region were immunolabeled with an anti-Caps2 antibody. Dotted line indicates stratum lucidum. (Scale bars, 50 μm.) (E) Immunosignal intensities at stratum lucidum of wild-type (white bar) and Caps2Δex3/Δex3 mice (black bar) are shown. Error bars indicate the SD. **P < 0.01, by Student t test.

Fig. S3. Distribution of MAP2(a+b) and Tau in primary-cultured hippocampal cells. (A–F) Subcellular localization of MAP2(a+b) (A and D) and Tau (B and E) protein in wild-type (A–C) and Caps2Δex3/Δex3 (D–F) hippocampal primary cultures immunostained for MAP2(a+b) (green) and Tau (red) at 14 d in vitro. Arrows show the position of the axon. Merged image is shown in C and F. (Scale bars, 30 μm.)
Fig. S4. Reduction in parvalbumin- and calbindin-positive cells in the hippocampus and neocortex of Caps2<sub>Δex3/Δex3</sub> mice during postnatal development. (A–H) Cell densities of parvalbumin-positive neurons (A–D) and calbindin-positive neurons (E–H) for wild-type (white), Caps2<sup>+/Δex3</sup> (gray), and Caps2<sub>Δex3/Δex3</sub> (black) mice. (A) P17 hippocampus (n = 16, 19, and 14, respectively). (B) P21 hippocampus (n = 12, 16, and 16, respectively). (C) P17 neocortex (layers I–VI) (n = 14 for each) (P < 0.01, one-factor ANOVA). (D) P21 neocortex (layers I–VI) (n = 12, 16, and 16, respectively) (P < 0.01, one-factor ANOVA). (E) P7 hippocampus (n = 9, 18, and 12, respectively) (P < 0.01, one-factor ANOVA). (F) P21 hippocampus (n = 6, 6, and 8, respectively). (G) P7 neocortex (layers II–V) (n = 9, 8, and 12, respectively). (H) P21 neocortex (layers IV–V) (n = 8, 9, and 11, respectively). Error bars indicate SEM. *P < 0.05; **P < 0.01, by post hoc t test.

Fig. S5. Differences in the parvalbumin- and calbindin-immunostaining patterns in the hippocampus and neocortex between wild-type and Caps2<sub>Δex3/Δex3</sub> mice. (A–H) Sagittal sections of hippocampus (A–D) and neocortex (E–H) were immunolabeled with antiparvalbumin antibody. (A and E) Wild type at P17; (B and F) Caps2<sub>Δex3/Δex3</sub> at P17; (C and G) wild-type at P21; and (D and H) Caps2<sub>Δex3/Δex3</sub> at P21. (Scale bars, 200 μm.) (I–P) Sagittal sections of hippocampus (I–L) and neocortex (M–P) were immunolabeled with anticalbindin antibody. (I and M) Wild type at P7; (J and N) Caps2<sub>Δex3/Δex3</sub> at P7; (K and O) wild-type at P21; and (L and P) Caps2<sub>Δex3/Δex3</sub> at P21. (Scale bars, 200 μm.)
Fig. S6. Unchanged NeuN-positive neuronal density in the neocortex of Caps2Δex3/Δex3 mice compared with wild-type mice. Cell densities of NeuN-positive neurons for wild-type (white), Caps2+/-Δex3 (gray), and Caps2Δex3/Δex3 (black) mice in the P21 neocortex (layers II–VI) (n = 8, 8, and 8, respectively). Error bars indicate SEM.

Fig. S7. Decreased dendritic spine density of granule cells in the hippocampal dentate gyrus of Caps2Δex3/Δex3 mice. (A and B) Representative Golgi staining of a granule cell dendrite of the dentate gyrus from wild-type (A) and Caps2Δex3/Δex3 (B) mice at P21. (Scale bars, 5 μm.) (C) Protrusion density of primary and secondary dendrites in dentate gyrus granule cells at P21 in wild-type (white; n = 26 from 12 mice) and Caps2Δex3/Δex3 (black; n = 26 from 13 mice) littermates. Error bars indicate SEM. *P < 0.05, by Student t test.

Fig. S8. Grip traction test. Time during which the mice remained hanging from a horizontal wire is presented for wild-type (white; n = 9), Caps2+/-Δex3 (gray; n = 9), and Caps2Δex3/Δex3 (black; n = 9) mice. Error bars indicate SEM.
Fig. S9. Y-maze test and olfactory test. (A) Percentage correct alternation response in the Y-maze test is shown for wild-type (white; n = 9), Caps2<sup>+/Δex3</sup> (gray; n = 9), and Caps2<sup>Δex3/Δex3</sup> (black; n = 9) mice. (B) Latency to locate a piece of cookie was recorded. We defined finding the cookie as when the mouse held it in both paws. No significant differences were observed among wild-type, Caps2<sup>+/Δex3</sup>, and Caps2<sup>Δex3/Δex3</sup> mice in these tests.

Fig. S10. Open-field test. (A–C) Horizontal movement distance (A) and speed (B) of an open field (15 min) are shown for wild-type (white; n = 11), Caps2<sup>+/Δex3</sup> (gray; n = 13), and Caps2<sup>Δex3/Δex3</sup> (black; n = 12) mice. Representative movement traces are shown in C. Error bars indicate SEM.

Fig. S11. Light/dark box test. (A–C) Horizontal movement distance in the light box (A) and time spent in the light box (B) of a light/dark box (10 min) is shown for wild-type (white; n = 11), Caps2<sup>+/Δex3</sup> (gray; n = 13), and Caps2<sup>Δex3/Δex3</sup> (black; n = 12) mice. Representative movement traces are shown in C. Error bars indicate SEM.
Fig. 512. *Caps2Δex3/Δex3* mice display impaired habituation to a fresh cage. After habituation to a fresh cage for 24 h, the locomotor activity of wild-type (blue; *n* = 11), *Caps2Δex3* (orange; *n* = 13), and *Caps2Δex3/Δex3* (red; *n* = 12) mice was measured for 3 d (12-h light/dark period). Graph shows mean number of photobeam interruptions per 2 h. Error bars indicate SEM.

Fig. 513. Average duration of USVs per pup was reduced in *Caps2Δex3* mice compared with wild-type mice. Wild-type females were mated with wild-type or *Caps2Δex3/Δex3* males. The developmental change in USVs is shown for wild-type (open circles; *n* = 7 litters) and *Caps2Δex3* (gray circles; *n* = 8 litters) pups. Error bars indicate SEM. *P* < 0.01, repeated measures ANOVA. *P* < 0.05; **P* < 0.01, by post hoc *u* test.
Fig. S14. Circadian rhythm of Caps2Δex3/Δex3 mice. (A) Sleep–wake rhythms of locomotor activity under free wheel-running conditions in a constant dark cycle. Graph shows the circadian period (h) calculated by a χ² periodogram for wild-type (white; n = 20), Caps2+/−/Δex3 (gray; n = 25), and Caps2Δex3/Δex3 (black; n = 14) mice. Error bars indicate SEM. (B and C) Light–dark rhythms of locomotor activity under free wheel-running conditions under a 12-h light/dark cycle (LD) and a constant dark cycle (DD). Activity traces for wild-type (B) and Caps2Δex3/Δex3 (C) mice for 4 d of light/dark cycle and 4 d of constant darkness are represented as relative deflections from the horizontal line. Actograms are double-plotted over a 48-h period.