

**Figure 11** Functional analyses of *CADPS2* exon 3. (A) BDNF release induced by 50 mM KCl in PC12 cells transfected with *CADPS2*(WT) or *CADPS2*( $\Delta$ exon3) together with a BDNF expression plasmid. Average values obtained from 4 independent experiments are shown. (B) BDNF release activity in *Cadps2*<sup>-/-</sup> neocortical cultures transfected with control vector, *CADPS2*(WT), or *CADPS2*( $\Delta$ exon3) using the calcium phosphate method at 4 DIV was evaluated at 21 DIV by measuring the amounts of BDNF spontaneously secreted into the culture medium over the course of the previous 17 days. Activity is indicated in BDNF concentration (pg/ $\mu$ l) normalized to cell density (/mm<sup>2</sup>). Average values obtained from 4 independent experiments are shown. The error bars indicate SD. \*\**P* < 0.01, Student's *t* test.

Finally, we examined nonsynonymous SNPs over the entire *CADPS2*-coding sequence. A previous study showed that no patient-specific nonsynonymous (missense) SNPs were identified in the *CADPS2* gene from 90 autistic individuals, using the heteroduplex formation-detection method (9). However, our sequence analyses of 252 white autistic patients revealed 7 nonsynonymous SNPs in the *CADPS2* gene that we believe to be novel (Supplemental Table 2 and Supplemental Figure 11). By contrast, these SNPs were not observed in 218 biologically unrelated white subjects from cohorts recruited as bipolar disorder pedigrees (34, 35), as shown in Supplemental Table 2. In addition, they have not been reported in healthy human subjects. These results suggest the possibility that the 7 SNPs identified within the *CADPS2*-coding sequence are associated with some autistic patients, although whether these SNPs have any relation to the specific phenotypes of autism remains elusive.

**Discussion**

Our knockout mouse study shows that the loss of *CADPS2* function in mice causes impairments in behavioral phenotypes (social interaction, home cage activity, response to novel environment, circadian rhythm, and maternal behavior) that are reminiscent of the impairments that characterize autistic patients. Analyses of the cellular phenotypes of *Cadps2*-knockout mice revealed that *CADPS2* is critical for BDNF secretion from neocortical and cerebellar neurons, the differentiation of neocortical and hippocampal interneurons (deficits in which can be rescued by BDNF injection), and the survival of cerebellar Purkinje cells. Moreover, we found that *CADPS2* mRNA from the blood of some autistic patients is aberrantly spliced, resulting in a loss of exon 3, which has been shown to encode the domain that binds to p150<sup>Glucd</sup>. We also showed that exon 3-skipped *CADPS2* protein has almost normal BDNF releasing activity but is not properly transported into the axons of neocortical and cerebellar neurons. These results suggest

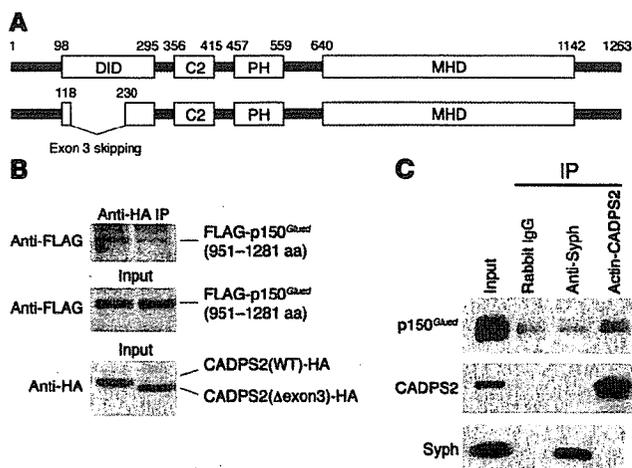
that, while BDNF localization at the axon terminal is not altered by the exon 3 skipping of *CADPS2*, the loss or relative decrease of BDNF release from the axon terminal is caused by the impaired translocation of *CADPS2* to the axon terminal (Supplemental Figure 10).

*CADPS2 plays an important role in the BDNF secretion.* *CADPS* was previously implicated in the regulation of dense-core vesicle secretion from endocrine and neuroendocrine cells through the activation of the phosphatidylinositol 4,5-bisphosphate-dependent and Ca<sup>2+</sup>-dependent priming step (36–39). On the other hand, a recent study of *Cadps1*-knockout mice, which die shortly after birth, suggested a role for *CADPS1* in the loading of catecholamines into secretory vesicles (40). Therefore, there is a possibility that *CAPS/CADPS* family proteins are involved in multiple steps in the secretory pathway, although their underlying molecular mechanisms remain elusive. Our previous study suggested the involvement of *CADPS2* in the release of BDNF and NT-3 from cerebellar granule cells (10). The present study confirms the indispensable role of *CADPS2* in BDNF secretion by showing that *CADPS2* deficiency causes impairment of BDNF release from neocortical and cerebellar primary cell cultures. It was of interest that the deficit of BDNF release was observed in both regulated and constitutive processes in cerebellar cultures. These

data suggest that *CADPS2* is a component involved in a few steps of the secretion pathway, including loading and exocytosis steps, and that *CADPS2* is likely involved in a common mechanism of the regulatory and constitutive secretion processes. On the other hand, an immunolocalization study indicated that the distribution patterns of *CADPS2* and BDNF completely overlap in many brain areas but overlap incompletely in other areas (11). Thus, although *CADPS2* is required for BDNF release from many brain areas, BDNF release from other areas, including the hippocampal CA1 region, might be attributed to the function of another *CAPS/CADPS* family protein, *CADPS1*. The differential immunolocalization of *CADPS2* and BDNF in some brain areas (11) suggests that *CADPS2* is involved in the release of secretory substances other than BDNF in these areas.

*Abnormal behavioral phenotypes characteristic of Cadps2-knockout mice.* *Cadps2*-knockout mice not only have a normal locomotion ability but also show normal basic sensory functions such as vision, olfaction, and audition. In a probe test of Morris water maze learning, however, *Cadps2*-knockout mice showed impaired spatial memory. It was shown that the probe test, for example, a hidden platform test, reflects spatial learning better than measures of escape latencies during training (41). Taking these results together, some part of cognitive function (that is required for Morris water maze learning) appears to be impaired in *Cadps2*-knockout mice.

Novel stimuli, such as unfamiliar environments or objects, are theorized to create conflict in rodents by concurrently evoking both approach and avoidance behaviors (22). Approach behavior, or “exploration”, reflects an animal’s tendency to explore novel stimuli or environments, whereas avoidance behavior, or “anxiety-related behavior,” is thought to reflect an animal’s fear of novelty. *Cadps2*<sup>-/-</sup> mice showed decreased locomotor activity compared with WT mice (Figure 2D) when placed in an open field containing a novel object, as shown in dopamine D4 receptor-knockout mice (42). Moreover, *Cadps2*<sup>-/-</sup> mice made significantly fewer contacts



**Figure 12**

Interacting protein of CADPS2 exon 3. (A) Schematic depiction of human CADPS2 protein (GenBank accession number NP\_060424). The C2- and pleckstrin homology (PH)-like domain, Munc13-1-homologous domain (MHD), and p150<sup>Glued</sup> (also known as dynactin 1)-interacting domain (DID) used as bait are shown. Exon 3 skipping leads to a deletion of 111 aa residues (from 119 to 229). (B) Coimmunoprecipitation experiments using lysates of COS-7 cells coexpressing p150<sup>Glued</sup> 951–1281 residues and CADPS2 constructs tagged with N-terminal FLAG and C-terminal HA epitopes, respectively. Coimmunoprecipitates with anti-HA antibody to CADPS2 constructs were blotted with anti-FLAG antibody (upper panel). Input lysates were blotted with anti-FLAG antibody (middle panel) and anti-HA antibody (lower panel). (C) The endogenous p150<sup>Glued</sup> was coimmunoprecipitated with the endogenous CADPS2 in P8 mouse neocortex extracts but not with endogenous synaptophysin (Syph). The blots were immunostained for p150<sup>Glued</sup> (upper panel), CADPS2 (middle panel), and synaptophysin (lower panel).

with the novel object. These results indicate that *Cadps2*<sup>-/-</sup> mice tend to show augmented anxiety or reduced environmental exploration in a novel environment, as was also the case in dopamine D4 receptor-knockout mice (42). Interestingly, in an 8-arm radial maze test, *Cadps2*<sup>+/-</sup> mice showed decreased locomotor activity and lower arm entries relative to WT mice (Supplemental Figure 5). Correspondingly, the selective dopamine D2 receptor agonist, LY-171555, has been found to cause decreased locomotion in an 8-arm radial maze test (43) along with hyperactivity in the home cages (44). As the CAPS/CADPS family proteins interact with a dopamine D2 receptor (45), the phenotypes observed in the present study might be associated with the dopamine pathway. Similar behaviors in unfamiliar environments are observed in patients with autism (1, 2, 19).

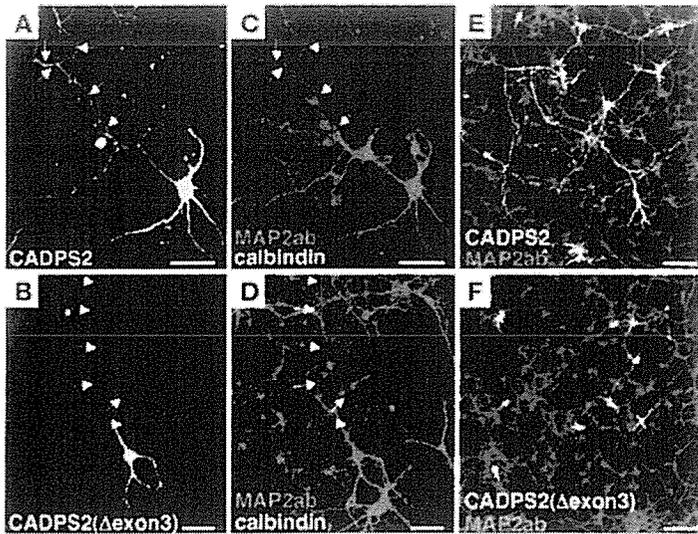
**BDNF release activity of exon 3-skipped CADPS2.** We found that some autistic patients express an aberrantly spliced, exon 3-skipped variant of *CADPS2* mRNA in their blood. It is a notable fact that this variant form was detected in both autistic monozygotic twins. Exon 3-skipped CADPS2 proteins exogenously expressed in PC12 cells and primary cultured cerebellar and neocortical neurons had a BDNF-releasing activity similar to that of WT CADPS2. From these in vitro expression data, we suggest that the levels of BDNF in the blood of autistic patients expressing exon 3-skipped CADPS2 might be unchanged in comparison with those from healthy control subjects or patients expressing only the WT CADPS2. On the other hand, there have been several reports showing different pathological results regarding BDNF levels in

the sera of autistic patients, including increased levels (46–48), no difference (49), and decreased levels (50). As for this point, the origin of blood BDNF is still an open question; that is, it remains unknown from which cell types and by which releasing mechanisms BDNF is released (constitutive versus regulated, CADPS1 mediated versus CADPS2 mediated, etc.). Therefore, at present, we cannot state anything conclusive about the association of BDNF levels in the sera with autism.

**Disturbance in subcellular localization of exon 3-skipped CADPS2.** We have shown here that the aberrant exon 3 skipping in *CADPS2* deletes the dynactin-binding domain of the protein product. Our previous study indicated that in the cerebellum, CADPS2 protein accumulates primarily in the presynaptic terminals of granule cells connecting Purkinje cell spines (10). CADPS2-mediated BDNF release seems to trans-synaptically act on Purkinje cells and promote their survival and differentiation. Exogenously expressed exon 3-skipped CADPS2, however, was not transported to the axons of primary cultured neocortical and cerebellar neurons. This improper subcellular targeting of exon 3-skipped CADPS2 seems to cause a disturbance of localized neurotrophin release in neurons (Supplemental Figure 10). The current data suggest that disturbance in the proper levels of localized BDNF release, which is assumed to be caused by aberrant subcellular targeting of CADPS2, results in the abnormal development of circuit connectivity observed in autism (51).

It remains to be resolved whether expression of exon 3-skipped *CADPS2* mRNA is paralleled by production of exon 3-skipped CADPS2 protein in autistic patient cells. We tried to analyze CADPS2 protein by Western blotting in the limited volume of blood samples from patients, but unfortunately we could not detect it even in samples from healthy persons. This is probably due to the very low levels of CADPS2 protein in blood cells being expressed only in basophilic leukocytes, which represent only about 0.5% of total leukocytes. Recombinant exon 3-skipped CADPS2 protein, however, could be stably expressed in PC12 cells and cultured neocortical and cerebellar neurons, as could WT CADPS2 protein. To correlate the present findings to the phenotypes of autistic patients, it must also be determined whether either mRNA and/or protein of exon 3-skipped CADPS2 is expressed in autistic brain tissues.

**Plausible mechanisms underlying the aberrant splicing of CADPS2 mRNA in autistic patients.** It is still unclear how the splicing out of exon 3 is specifically caused in some autistic patients. We could not find any nucleotide sequence differences in 3 common splicing *cis*-elements (the 5' donor and 3' acceptor sites and the branch point) of the *CADPS2* gene from autistic patients displaying exon 3 skipping. In addition to these authentic *cis*-elements, some splicing events are regulated by additional *cis*-elements known as exonic and intronic splicing enhancers/silencers. To further pursue the possibilities pointing to a “*cis*-hypothesis,” we examined exon 3 sequences and could find no change in the patients tested. We also tested whether there were microdeletion(s) in the intron 2 interval (73,385 bp) by quantitative genomic PCR, targeting 6 discrete portions within intron 2 sequences in DNA from the autistic subjects represented in Figure 10 (data not shown). As a result, we could not detect any genomic variants/abnormalities in the autistic DNA. Nonetheless the possibility of a “*cis*-hypothesis” cannot be ruled out because 1 or more mutations might still be present somewhere in the unexplored genomic region within the *CADPS2* genes of these autistic patients.



**Figure 13** Aberrant distribution patterns of exon 3–skipped CADPS2 protein. (A–D) Subcellular localization of C-terminal HA-tagged CADPS2(WT) (A) and CADPS2(Δexon3) protein (B) exogenously expressed in neocortical primary cultures immunostained for HA (green), MAP2ab (red), and calbindin (blue) at 14 DIV. Arrows show the position of MAP2ab-negative and calbindin-positive axons as shown in the same frame (C and D). (E and F) Subcellular localization of C-terminal HA-tagged CADPS2(WT) (E) and CADPS2(Δexon3) (F) protein exogenously expressed in cerebellar primary cultures immunostained for HA (green) and MAP2ab (red) at 7 DIV. Scale bars: 50 μm.

Alternatively, we must also consider the possibility of a “*trans*-hypothesis” as an underlying causality — that is, the possibility that there are 1 or more polymorphisms in unknown genes that alters the splicing of *CADPS2* mRNA. This scenario would also predict abnormal splicing of multiple genes in addition to the exon 3 skipping of *CADPS2*. However, as we do not have any evidence for aberrant splicing in other genes, we need to be cautious in any interpretation of the results of *CADPS2* exon 3 skipping based on a *trans*-hypothesis. In this regard, it seems to be a suggestive example that a mutation in methyl CpG binding protein 2 (*MECP2*) has been shown to be causative for Rett syndrome, a particular autism spectrum disorder, by affecting the expression level of the *BDNF* gene (16, 52, 53); however, *MECP2* likely has multiple target genes other than the *BDNF* gene.

Other potential mechanisms for pathological alternative splicing of *CADPS2* in autistic patients would include aberrant epigenetic regulation and de novo mutation. It should be noted, however, that none of these inferred mechanisms explain satisfactorily why the affected individuals in pedigree A show 2 bands, authentic and exon 3–skipped *CADPS2* bands (Figure 10), whereas the affected individual in pedigree B shows only an exon 3–skipped band. These results imply that the causal variants in pedigrees A and B should be different. We can at least exclude a dominant inheritance mechanism of causal variants in both pedigrees, because the parents are unaffected in both pedigrees. Future extensive studies are warranted to expose the precise pathological mechanisms.

Our study also identified 7 nonsynonymous SNPs by analyzing the *CADPS2* coding sequence of 252 white autistic patients and 1 case in which *CADPS2* mRNA expression was not detected in the blood specimen of that particular autistic patient (see Methods); however, any correlation of these findings with autistic phenotypes

remains elusive. We suggest that *CADPS2* disturbance due to genetic and/or allelic heterogeneities (e.g., disturbed function, subcellular localization, or expression caused by rare, nonsynonymous mutations, exon 3 skipping, aberrant expressional control, etc.) predisposes individuals to a higher risk for autism, at least in some cases. These phenomena may point to the scenario of “common disease — rare variants” rather than “common disease — common variants,” both of which are proposed to contribute to common disease mechanisms. It would be important that future autism studies include an intensive scrutiny of the genetic mechanisms that impact directly and indirectly on the function and structure of *CADPS2*, as one of the implicated susceptibility genes for autism.

### Methods

**Human subjects.** The study protocol was approved by the ethics committees of RIKEN Brain Science Institute, Hamamatsu University School of Medicine, and Tokyo Metropolitan Umegaoaka Hospital (Tokyo, Japan). RNA samples were obtained from 16 Japanese autistic patients, who were diagnosed according to the DSM-IV (2). Their ages ranged from 13 to 27 years. Informed consent was obtained from all the participants. Control samples were obtained from age-matched healthy Japanese volunteers. DNA samples from 252 white autistic patients were obtained from the Autism Genetic Resource Exchange (AGRE). The diagnostic assessment in the AGRE sample was based on the Autism Diagnostic Interview–Revised (ADI-R) diagnostic test (54). DNA samples from 218 biologically unrelated white subjects from cohorts recruited as bipolar disorder pedigrees (34, 35) were also used.

**RT-PCR.** RNA was extracted from fresh blood with TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions (1 ml TRIzol Reagent was added to 100 μl of blood). RT-PCR reactions were carried out in a 20-μl volume in the GeneAmp PCR System 9700 (PerkinElmer). The QIAGEN OneStep RT-PCR kit (QIAGEN) was used according to the manufacturer’s instructions. A 50-ng sample of total RNA template was used per reaction. The reverse transcription cycling conditions were 30 minutes at 50 °C followed by 15 minutes at 95 °C. PCR cycling conditions consisted of 48 cycles of 94 °C for 30 seconds, 67 °C for 30 seconds, and 72 °C for 30 seconds, followed by a final hold at 72 °C for 5 minutes. The forward primer was 5-GGCAGCAGAAGCTTAACAACAACAGTTGCAGT-TAC-3, and the reverse primer was 5-GGACCACCTTTCGAACTG-GAAGACTTTC-3 (normal, 661-bp product; exon 3 skipped, 328-bp product). PCR was carried out at least 4 separate times for each sample. PCR products were electrophoresed on 2% agarose gels with a 100-bp DNA Ladder (catalog no. 15628-050; Invitrogen). All 328-bp products were cloned into the pCR4-TOPO TA cloning vector (Invitrogen), and the exon 3 skipping was confirmed by sequencing. PCR reactions were run at the same time for patients and controls. The RT-PCR product band of *CADPS2* mRNA was not detected from 1 autistic patient, although the *GAPDH* band was detected (data not shown).

**Production of the mouse line.** A 12-kb genomic fragment containing exon 1 of mouse *CADPS2* from C57BL/6 mice was used to construct the targeting vectors. For positive selection, the *SmaI-SmaI* fragment containing the full length of exon 1 was replaced by the P<sub>gk</sub>-neo gene cassette flanked by the *loxP* sites (Figure 1A). For negative selection, the diphtheria toxin A fragment gene cassette was added to the 5’ end of the targeting vector. After transfection of MS12 ES cells (C57BL/6 mouse ES cell line [ref. 55]) by electroporation, targeted clones were screened for G418 resistance and analyzed by Southern blot analysis. Chimeric mice



were generated by injection of the targeted MS12 ES cells into Balb/c blastocysts and mated with WT C57BL/6J mice to obtain heterozygous mutant mice. All the engineered animals studied were backcrossed onto C57BL/6J for more than 5 generations.

**BDNF injection.** The icv injection of BDNF in neonatal mouse brains was carried out essentially as described in previous studies (56, 57). P5 C57BL/6J mice were anesthetized by diethyl ether and then given an icv injection of a 2-ml solution containing either vehicle (PBS, pH 7.4) or BDNF (5 mg in vehicle) into the left hemisphere. The location of each injection was 2.5 mm anterior to  $\lambda$ , 0.9 mm lateral to the midline, and 1.9 mm below the skull surface. Injections were performed using a Hamilton syringe with a 30-gauge beveled needle. The needle was withdrawn 2 minutes later and the scalp sutured. Mice were placed in a warm cage until they had fully recovered and then were returned to their home cages with a foster mother mouse. The brains of 8 mice (4 PBS-injected mice and 4 BDNF-injected mice) were fixed at P17 as described below, and 2 sagittal sections of left hemisphere from each mouse (a total of 16 sections) were immunostained.

**Immunohistochemistry of brain sections.** P8, P17, and P21 C57BL/6J mice anesthetized with diethyl ether were transcardially perfused with PBS and then with 4% paraformaldehyde in PBS. The brains were dissected, postfixed in 4% paraformaldehyde at 4°C for 5 hours, and cryoprotected by immersion in 15% sucrose in PBS overnight at 4°C. After embedding in Tissue-Tek OCT compound (Sakura Finetechnical Co.), the brains were frozen in dry ice powder and cut into 14- $\mu$ m sagittal sections with a cryostat (Leica CM1850; Leica Microsystems) at -18°C. The sections were then air dried for 1 hour and rinsed in PBS 3 times. After blocking with 5% normal donkey serum (Vector Laboratories) in PBS, the sections were reacted with the primary antibody at 4°C overnight, rinsed in PBS, reacted with the secondary antibody at room temperature for 1 hour, and again rinsed in PBS. The immunoreacted sections were mounted with VECTASHIELD Mounting Medium (Vector Laboratories) and examined with an epifluorescence microscope (Eclipse E800; Nikon) equipped with a cooled charge-coupled device camera (SPOT model 1.3.0; Diagnostic Instruments Inc.) or a confocal laser microscope (LSM 510 META; Zeiss).

**Immunocytochemistry of fractionated leukocytes.** Leukocytes were fractionated as described previously (58). Freshly separated leukocytes that had been smeared on 3-aminopropyltriethoxysilane (APS)-coated glass slides and fixed with acetone for 10 minutes at 4°C were used for immunocytochemistry.

**PC12 secretion assay.** Mouse BDNF cDNA was subcloned into the pEF4/MyC-His plasmid vector containing the EF-1a promoter (Invitrogen). Mouse CADPS2 cDNA (GenBank accession number AK038568) was subcloned into the pcDNA3 plasmid vector containing the CMV promoter (Invitrogen) to create pcDNA3-CADPS2(WT). pcDNA3-CADPS2( $\Delta$ exon3) had an internal deletion of 155–265 aa, which corresponded to exon 3 of human CADPS2. Twenty-four hours after transfection with the expression plasmids as described previously (10) using Lipofectamine 2000 Transfection Reagent (Invitrogen), PC12 cells were incubated in fresh assay medium (DMEM containing 0.2% BSA) for 10 minutes. Two different DMEMs were used: standard DMEM (catalog no. 11965; Invitrogen) and high-KCl DMEM (50 mM KCl/65 mM NaCl based on catalog no. 11965; Invitrogen). Both DMEMs were fully equilibrated in a 5% CO<sub>2</sub> atmosphere at 37°C before application. The control and high-KCl stimulation assay media were collected, and their neurotrophin contents were measured using the BDNF E<sub>max</sub> ImmunoAssay System (Promega) according to the manufacturer's instructions.

**Yeast 2-hybrid analysis.** The yeast 2-hybrid system (Matchmaker Two-Hybrid System 3; Clontech) was used to identify proteins that bind to the exon 3-containing region of mouse CADPS2 (134–331 aa of mouse CADPS2 [GenBank accession number BAD05017]). The aa sequence

is almost identical to the 98–295 aa sequence (Figure 12A) of human CADPS2 (GenBank accession number NP\_060424). The partial mouse CADPS2 cDNA (134–331 aa) was cloned in vector pGBKT7 (pGBKT7-CADPS2 [134–331 aa]) and used as the bait in yeast 2-hybrid screening. The bait plasmid pGBKT7-CADPS2 (134–331 aa) was transformed into yeast strain AH109 and plated on an SD-Trp plate. An overnight culture (concentrated culture) of positive pGBKT7-CADPS2 (134–331 aa) bait strain in SD/-Trp medium was used to mate with 1 ml of pretransformed adult mouse whole-brain cDNA library according to the manufacturer's instructions. The mating mixture was plated on 50 Quadruple Dropout Medium (QDO; SD/-Ade/-His/-Leu/-Trp/X- $\alpha$ -Gal) plates (BD Biosciences) (150 mm<sup>2</sup>, 200  $\mu$ l per plate). Plates were incubated at 30°C for up to 14 days. Approximately 18  $\times$  10<sup>6</sup> colonies were screened. Ten positive colonies were picked and processed for plasmid preparation according to the Clontech yeast plasmid protocol. The resulting yeast plasmids were transformed into *E. coli*, purified, and sequenced.

**Immunoprecipitation and immunoblotting.** Mouse CADPS2 cDNA (GenBank accession number AK038568) was subcloned into the pEF-BOS plasmid vector containing the EF-1a promoter (59) with the C-terminal HA primer to create pEF-BOS-CADPS2(WT)-HA. The pEF-BOS-CADPS2( $\Delta$ exon3)-HA had an internal deletion of 155–265 aa, which corresponds to the human CADPS2 exon 3 coding sequence. The cDNA fragment coding for aas 951–1281 of mouse p150<sup>Glial</sup> (GenBank accession number NP\_031861) was cloned into pEF-BOS with N-terminal FLAG primer to create pEF-BOS-FLAG-p150<sup>Glial</sup> (951–1281 aa). COS-7 cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO<sub>2</sub>, and 5  $\times$  10<sup>5</sup> cells in 6-well plates were transiently transfected with 1.25 mg pEF-BOS-CADPS2-HA and 3.75 mg pEF-BOS-FLAG-p150<sup>Glial</sup> (951–1281 aa) plasmids by using Lipofectamine 2000 Transfection Reagent (Invitrogen). At 24 hours after transfection, COS-7 cells were harvested and lysed in 1.3 ml of Nonidet P40 lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P40) supplemented with Complete protease inhibitor cocktail (Roche). After preabsorption with protein G-sepharose, the supernatants were incubated with 0.5 mg anti-HA antibody or anti-FLAG antibody, and the immunocomplexes were then associated with protein G-sepharose. The resins were washed 5 times with lysis buffer, and the bound proteins were separated on SDS-PAGE gel and transferred to a nitrocellulose membrane for analysis by Western blotting with anti-HA or anti-FLAG antibody.

**Cerebellar primary culture.** Cerebellar primary cultures were prepared basically as described in a previous study (10). In brief, after rapid decapitation, the P0 cerebella of C57BL/6 mice were dissected, digested with 0.1% Trypsin (Sigma-Aldrich) and 0.05% DNase I (Roche) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS-CaMg(-) (Sigma-Aldrich) for 13 minutes at 37°C, washed with HBSS-CaMg(-), triturated by repeated passage through a 1-ml plastic micropipette tip in HBSS-CaMg(-) containing 0.05% DNase I and 12 mM MgSO<sub>4</sub>, and washed with the following medium: serum-free Eagle's minimal essential medium-based chemical-conditioned medium supplemented with 0.25% (wt/vol) glucose (Nacalai Tesque), 10  $\mu$ g/ml insulin (Sigma-Aldrich), 0.1 nM L-thyroxine (Sigma-Aldrich), 0.1 mg/ml apotransferrin (Sigma-Aldrich), 1 mg/ml BSA (Sigma-Aldrich), 2 mM L-glutamine (Nacalai Tesque), 1  $\mu$ g/ml aprotinin (Sigma-Aldrich), 30 nM sodium selenite (Merck), 100 U/ml penicillin (Banyu Pharmaceutical Co.), and 135  $\mu$ g/ml streptomycin (Meiji Seika KK). The dissociated cells were plated at 5  $\times$  10<sup>5</sup> cells per glass coverslip (12 mm in diameter; Matsunami) coated with poly-L-lysine (Sigma-Aldrich). They were then cultured in the medium under a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Either CADPS2(WT)-HA or CADPS2( $\Delta$ exon3)-HA tagged with a C-terminal HA epitope was exogenously expressed by the calcium phosphate method at 2 DIV (10).



**Preparation and infection of recombinant adenoviruses.** A replication deficient adenovirus Ad-BDNF-GFP was generated by the cosmid-terminal protein complex (COS-TPC) method (60). Briefly, the full-length mouse BDNF cDNA C-terminal tagged with GFP was inserted into the CAG promoter expression unit of pAxCawt cosmid cassette (Takara Bio Inc.). Recombinant viruses were generated by homologous recombination between EcoT22I-digested Ad5-dlx DNA-terminal protein complex and recombinant cosmid vectors in HEK293 cells as described elsewhere (61). The generated recombinant adenoviruses were propagated in HEK293 cells, then concentrated and purified by double CsCl step gradient centrifugation. The virus titers were measured on HEK293 cells. The resulting Ad-BDNF-GFP was used for infection of primary cerebellar cultures at an MOI of 30 for 1 hour at 37°C. As a control, Ad-GFP (26), which expresses GFP, was used.

**Secretion assay in cerebellar primary cultures.** Cerebellar cultures at 48 hours after infection were incubated for 30 minutes in the chemical-conditioned medium described above. After the incubation, 3 different stimulation assay media were applied for 15 minutes: the standard chemical-conditioned medium described above, high-KCl chemical-conditioned medium (50 mM KCl), and chemical-conditioned medium with 1 mM TTX and 10 mM NBQX. All media were fully equilibrated in a 5% CO<sub>2</sub> atmosphere at 37°C before application. The stimulation assay media were collected, and their BDNF content was measured using the BDNF E<sub>max</sub> ImmunoAssay System (Promega) according to the manufacturer's instructions. BDNF content in cell lysate was also measured using lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P40, and 10% glycerol) supplemented with Complete protease inhibitor cocktail (Roche).

**Neocortical primary culture.** Pregnant C57BL/6 mice were anesthetized, and E16 embryos were collected. After rapid decapitation, the neocortices were dissected from the E16 brains and digested with 45 U papain (Worthington), 0.01% DNase I, 0.02% DL-cysteine, 0.02% BSA, and 0.5% glucose in PBS(-) for 10 minutes at 37°C. The culture medium was then supplemented with FBS to a final concentration of 20% and triturated by repeated passage through a 1-ml plastic micropipette tip. The dispersed cells were plated at a density of 5 × 10<sup>4</sup> cells/cm<sup>2</sup> onto a poly-L-lysine-coated glass coverslip in a neurobasal medium (Invitrogen) containing 2% B27 supplement (Invitrogen), 500 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin and cultured under a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

**Secretion assay in neocortical primary cultures.** Transfection of neocortical neurons was carried out by the calcium phosphate method using a CellPfect Transfection Kit (Amersham Biosciences) according to the manufacturer's instructions with minor modifications. Full-length CADPS2 [CADPS(WT)] and the exon 3 deletion variant [CADPS2(Δexon3)] were HA tagged at the C-terminal end and were used for transfection. The

reaction mixture, containing 1 μg of DNA for each cDNA construct, was added to neocortical primary dissociation cell cultures (5 × 10<sup>4</sup> cells in 1.9 cm<sup>2</sup> culture dish) at 4 DIV in a serum-free neurobasal medium. Cells were then incubated for 30 minutes at 1% CO<sub>2</sub> and 37°C followed by rinsing twice with prewarmed neurobasal medium. The transfection efficiency was approximately 30%–50%. The BDNF content of culture media collected at 21 DIV was measured using the BDNF E<sub>max</sub> ImmunoAssay System (Promega) according to the manufacturer's instructions. The amounts of BDNF, NGF, and NT-3 in the 21 DIV media of untransfected cultures were also measured using the BDNF E<sub>max</sub> ImmunoAssay System (Promega), NGF E<sub>max</sub> ImmunoAssay System (Promega), and a highly sensitive 1-site enzyme immunoassay (10, 62), respectively.

**Statistics.** Statistical analysis was performed using Statcel software (OMS). Data were compared using the 2-tailed Student's *t* test or the Mann-Whitney *U* test, according to unequal or equal variance. Fisher's exact test was used to compare the prevalence of exon skipping in patients and control subjects. A *P* value of less than 0.05 was considered statistically significant.

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- World Health Organization. 1992. The ICD-10 classification of mental and behavioural disorders: clinical descriptions and diagnostic guidelines. World Health Organization. Geneva, Switzerland. [http://www.who.int/substance\\_abuse/terminology/icd\\_10/en/index.html](http://www.who.int/substance_abuse/terminology/icd_10/en/index.html).
- American Psychiatric Association. 1994. Diagnostic and statistical manual of mental disorders: DSM-IV. American Psychiatric Publishing Inc. Washington, DC, USA. 886 pp.
- Muhle, R., Trentacoste, S.V., and Rapin, I. 2004. The genetics of autism. *Pediatrics*. **113**:e472–e486.
- Folstein, S.E., and Rosen-Sheidley, B. 2001. Genetics of autism: complex aetiology for a heterogeneous disorder. *Nat. Rev. Genet.* **2**:943–955.
- Jamain, S., et al. 2003. Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat. Genet.* **34**:27–29.
- Kwon, C.H., et al. 2006. Pten regulates neuronal arborization and social interaction in mice. *Neuron*. **50**:377–388.
- International Molecular Genetic Study of Autism Consortium (IMGSAC). 2001. Further characterization of the autism susceptibility locus AUTS1 on chromosome 7q. *Hum. Mol. Genet.* **10**:973–982.
- Grishanin, R.N., et al. 2004. CAPS acts at a pre-fusion step in dense-core vesicle exocytosis as a PIP<sub>2</sub> binding protein. *Neuron*. **43**:551–562.
- Cisternas, F.A., Vincent, J.B., Scherer, S.W., and Ray, P.N. 2003. Cloning and characterization of human CADPS and CADPS2, new members of the Ca<sup>2+</sup>-dependent activator for secretion protein family. *Genomics*. **81**:279–291.
- Sadakata, T., et al. 2004. The secretory granule-associated protein CAPS2 regulates neurotrophin release and cell survival. *J. Neurosci.* **24**:43–52.
- Sadakata, T., et al. 2006. Differential distributions of the Ca<sup>2+</sup>-dependent activator protein for secretion family proteins (CAPS2 and CAPS1) in the mouse brain. *J. Comp. Neurol.* **495**:735–753.
- Bibel, M., and Barde, Y.A. 2000. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* **14**:2919–2937.
- Lu, B. 2003. BDNF and activity-dependent synaptic modulation. *Learn. Mem.* **10**:86–98.
- Lessmann, V., Gottmann, K., and Malsangio, M. 2003. Neurotrophin secretion: current facts and future prospects. *Prog. Neurobiol.* **69**:341–374.
- Amir, R.E., et al. 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat. Genet.* **23**:185–188.
- Chang, Q., Khare, G., Dani, V., Nelson, S., and Jaenisch, R. 2006. The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. *Neuron*. **49**:341–348.
- Aman, M.G., and Langworthy, K.S. 2000. Pharmacotherapy for hyperactivity in children with autism and other pervasive developmental disorders. *J. Autism Dev. Disord.* **30**:451–459.
- Aman, M.G. 2004. Management of hyperactivity and other acting-out problems in patients with autism spectrum disorder. *Semin. Pediatr. Neurol.* **11**:225–228.



19. Pierce, K., and Courchesne, E. 2001. Evidence for a cerebellar role in reduced exploration and stereotyped behavior in autism. *Biol. Psychiatry*. **49**:655-664.
20. Filipek, P.A., et al. 2000. Practice parameter: screening and diagnosis of autism: report of the Quality Standards Subcommittee of the American Academy of Neurology and the Child Neurology Society. *Neurology*. **55**:468-479.
21. Richdale, A.L., and Prior, M.R. 1995. The sleep/wake rhythm in children with autism. *Eur. Child Adolesc. Psychiatry*. **4**:175-186.
22. Montgomery, K.C. 1955. The relation between fear induced by novel stimulation and exploratory behavior. *J. Comp. Physiol. Psychol.* **48**:254-260.
23. Casanova, M.F., Buxhoeveden, D., and Gomez, J. 2003. Disruption in the inhibitory architecture of the cell minicolumn: implications for autism. *Neuroscientist*. **9**:496-507.
24. Jones, K.R., Farinas, L., Backus, C., and Reichardt, L.F. 1994. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell*. **76**:989-999.
25. Bauman, M., and Kemper, T.L. 1985. Histoanatomic observations of the brain in early infantile autism. *Neurology*. **35**:866-874.
26. Sato, Y., Shiraiishi, Y., and Furuichi, T. 2004. Cell specificity and efficiency of the Semliki forest virus vector- and adenovirus vector-mediated gene expression in mouse cerebellum. *J. Neurosci. Methods*. **137**:111-121.
27. Ogawa, H., et al. 2003. A SWI2/SNF2-type ATPase/helicase protein, mDomino, interacts with myeloid zinc finger protein 2A (MZF-2A) to regulate its transcriptional activity. *Genes Cells*. **8**:325-339.
28. Shibata, N., et al. 2004. Mouse RanBPM is a partner gene to a germline specific RNA helicase, mouse vasa homolog protein. *Mol. Reprod. Dev.* **67**:1-7.
29. Schroer, T.A. 2004. Dynactin. *Annu. Rev. Cell Dev. Biol.* **20**:759-779.
30. Speidel, D., et al. 2003. A family of Ca<sup>2+</sup>-dependent activator proteins for secretion: comparative analysis of structure, expression, localization, and function. *J. Biol. Chem.* **278**:52802-52809.
31. Waterman-Storer, C.M., et al. 1997. The interaction between cytoplasmic dynein and dynactin is required for fast axonal transport. *Proc. Natl. Acad. Sci. U. S. A.* **94**:12180-12185.
32. Deacon, S.W., et al. 2003. Dynactin is required for bidirectional organelle transport. *J. Cell Biol.* **160**:297-301.
33. Burack, M.A., Silverman, M.A., and Banker, G. 2000. The role of selective transport in neuronal protein sorting. *Neuron*. **26**:465-472.
34. Detera-Wadleigh, S.D., et al. 1999. A high-density genome scan detects evidence for a bipolar-disorder susceptibility locus on 13q32 and other potential loci on 1q32 and 18p11.2. *Proc. Natl. Acad. Sci. U. S. A.* **96**:5604-5609.
35. Detera-Wadleigh, S.D., et al. 1997. Initial genome scan of the NIMH genetics initiative bipolar pedigrees: chromosomes 4, 7, 9, 18, 19, 20, and 21q. *Am. J. Med. Genet.* **74**:254-262.
36. Walent, J.H., Porter, B.W., and Martin, T.F. 1992. A novel 145 kd brain cytosolic protein reconstitutes Ca<sup>2+</sup>-regulated secretion in permeable neuroendocrine cells. *Cell*. **70**:765-775.
37. Berwin, B., Floor, E., and Martin, T.F. 1998. CAPS (mammalian UNC-31) protein localizes to membranes involved in dense-core vesicle exocytosis. *Neuron*. **21**:137-145.
38. Tandon, A., et al. 1998. Differential regulation of exocytosis by calcium and CAPS in semi-intact synaptosomes. *Neuron*. **21**:147-154.
39. Renden, R., et al. 2001. Drosophila CAPS is an essential gene that regulates dense-core vesicle release and synaptic vesicle fusion. *Neuron*. **31**:421-437.
40. Speidel, D., et al. 2005. CAPS1 regulates catecholamine loading of large dense-core vesicles. *Neuron*. **46**:75-88.
41. Brandeis, R., Brandys, Y., and Yehuda, S. 1989. The use of the Morris Water Maze in the study of memory and learning. *Int. J. Neurosci.* **48**:29-69.
42. Dulawa, S.C., Grandy, D.K., Low, M.J., Paulus, M.P., and Geyer, M.A. 1999. Dopamine D4 receptor-knock-out mice exhibit reduced exploration of novel stimuli. *J. Neurosci.* **19**:9550-9556.
43. Levin, E.D., and Bowman, R.E. 1986. Effects of the dopamine D-2 receptor agonist, LY 171555, on radial arm maze performance in rats. *Pharmacol. Biochem. Behav.* **25**:1117-1119.
44. Breese, G.R., and Mueller, R.A. 1985. SCH-23390 antagonism of a D-2 dopamine agonist depends upon catecholaminergic neurons. *Eur. J. Pharmacol.* **113**:109-114.
45. Binda, A.V., Kabbani, N., and Levenson, R. 2005. Regulation of dense core vesicle release from PC12 cells by interaction between the D2 dopamine receptor and calcium-dependent activator protein for secretion (CAPS). *Biochem. Pharmacol.* **69**:1451-1461.
46. Miyazaki, K., et al. 2004. Serum neurotrophin concentrations in autism and mental retardation: a pilot study. *Brain Dev.* **26**:292-295.
47. Nelson, K.B., et al. 2001. Neuropeptides and neurotrophins in neonatal blood of children with autism or mental retardation. *Ann. Neurol.* **49**:597-606.
48. Connolly, A.M., et al. 2006. Brain-derived neurotrophic factor and autoantibodies to neural antigens in sera of children with autistic spectrum disorders, Landau-Kleffner syndrome, and epilepsy. *Biol. Psychiatry*. **59**:354-363.
49. Nelson, P.G., et al. 2006. Selected neurotrophins, neuropeptides, and cytokines: developmental trajectory and concentrations in neonatal blood of children with autism or Down syndrome. *Int. J. Dev. Neurosci.* **24**:73-80.
50. Hashimoto, K., et al. 2006. Reduced serum levels of brain-derived neurotrophic factor in adult male patients with autism. *Prog. Neuropsychopharmacol. Biol. Psychiatry*. **30**:1529-1531.
51. Courchesne, E., and Pierce, K. 2005. Why the frontal cortex in autism might be talking only to itself: local over-connectivity but long-distance disconnection. *Curr. Opin. Neurobiol.* **15**:225-230.
52. Chen, W.G., et al. 2003. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science*. **302**:885-889.
53. Martinowich, K., et al. 2003. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science*. **302**:890-893.
54. Lord, C., Rutter, M., and Le Couteur, A. 1994. Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *J. Autism Dev. Disord.* **24**:659-685.
55. Kawase, E., et al. 1994. Strain difference in establishment of mouse embryonic stem (ES) cell lines. *Int. J. Dev. Biol.* **38**:385-390.
56. Han, B.H., and Holtzman, D.M. 2000. BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. *J. Neurosci.* **20**:5775-5781.
57. Nawa, H., Pellemounter, M.A., and Carnahan, J. 1994. Intraventricular administration of BDNF increases neuropeptide expression in newborn rat brain. *J. Neurosci.* **14**:3751-3765.
58. Fukuda, D., Sata, M., Tanaka, K., and Nagai, R. 2005. Potent inhibitory effect of sirolimus on circulating vascular progenitor cells. *Circulation*. **111**:926-931.
59. Mizushima, S., and Nagata, S. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**:5322.
60. Miyake, S., et al. 1996. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc. Natl. Acad. Sci. U. S. A.* **93**:1320-1324.
61. Kanegae, Y., Makimura, M., and Saito, I. 1994. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn. J. Med. Sci. Biol.* **47**:157-166.
62. Katoh-Semba, R., Takeuchi, I.K., Semba, R., and Kato, K. 2000. Neurotrophin-3 controls proliferation of granular precursors as well as survival of mature granule neurons in the developing rat cerebellum. *J. Neurochem.* **74**:1923-1930.