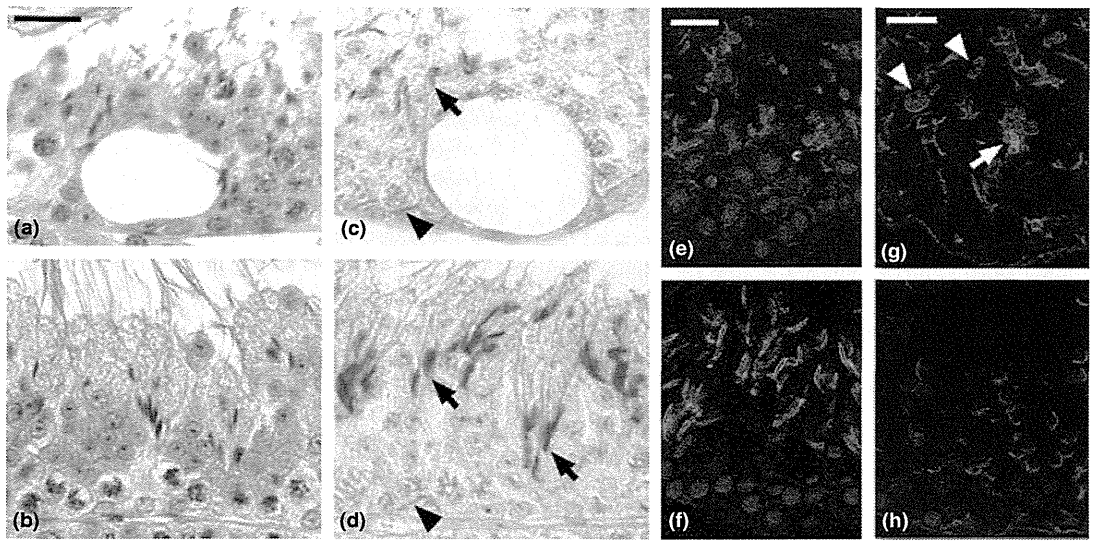

Fig. 5 Immunohistochemical localisation of basigin (a, b), nectin-2 (c, d) and nectin-3 (e, f), and distribution of actin filaments (g, h) in the testis. Testes from *RA175*$^{-/-}$ (a, c, e, g) and wild-type mice (b, d, f, h) were stained with anti-basigin antibody (a, b), anti-nectin-2 antibody (c, d), anti-nectin-3 antibody (e, f), or FITC-labelled phalloidin (g, h), and counterstained with haematoxylin (a–d) or propidium iodide (red; e–h). Arrowheads in (c), (d); basal ectoplasmic specialisations. Arrows in (c), (d); apical ectoplasmic specialisations. (g, h) In the *RA175*$^{-/-}$ testes, actin filaments were not observed around the malformed head of the elongate spermatids (arrowheads in g), and were observed to accumulate in the seminiferous tubules (arrow in g). Bar = 20 μm.

RA175$^{-/-}$ testis and is one of the candidates for such factors.

An ultrastructural investigation of elongate spermatids in the *RA175*$^{-/-}$ testis demonstrated abnormal morphology of the manchette, a microtubular structure around the nucleus of the elongate spermatid. The ectopic manchette of the spermatid has been reported in other gene-deficient mice showing male infertility, such as *GOPC*$^{-/-}$ (Ito *et al.*, 2004) and *Hrb*$^{-/-}$ (Kierszenbaum *et al.*, 2004) mice. The manchette is involved in the nuclear elongation and condensation of the spermatid, and spermatozoa from *GOPC*$^{-/-}$ and *Hrb*$^{-/-}$ mice have round heads without acrosomes, which are different from the *RA175*$^{-/-}$ phenotype. Another structure termed the acroplaxome, an F-actin/keratin 5-containing cytoskeletal plate anchored to the spermatid nucleus (Kierszenbaum *et al.*, 2003), is also important in determining the shape of the spermatid head. The acroplaxomes appeared to be normal in *RA175*$^{-/-}$ mice, and the abnormal shapes of the heads and acrosomes of the elongate spermatids in the *RA175*$^{-/-}$ testis were not triggered by the acroplaxome abnormality, and could be a secondary effect of *RA175* deficiency.

An unusual accumulation of actin filaments near the lumen of the seminiferous tubules was observed in the *RA175*$^{-/-}$ testis. These actin filaments were components of the apical ectoplasmic specialisations between Sertoli cells and spermatids, and were formed from the discarded ectoplasmic specialisations of exfoliated spermatids. Such structures are frequently found in seminiferous tubules lacking spermatids (Hosoi *et al.*, 2002).

Nectin-2 and nectin-3 are associated with the actin cytoskeleton through afadin, and nectin-2 on Sertoli cells and nectin-3 on spermatids interact at the apical ectoplasmic specialisations (Ozaki-Kuroda *et al.*, 2002). We examined the immunolocalisation of nectin-2 and nectin-3 in the *RA175*$^{-/-}$ testis, and their signals were decreased or lost around the malformed heads of the elongate spermatids, indicating the detachment of spermatids from the Sertoli cells in the seminiferous epithelium. However, a small number of elongate spermatids that survived and appeared to have normal morphology showed positive immunoreactions for nectins. Although nectin-3 is reported to interact with RA175 (necl-2) (Takai *et al.*, 2003), their expression sites were differed in the testis, and the roles of RA175 and nectins are different in spermatogenesis as discussed in the previous paper (Fujita *et al.*, 2006).

In this report, we revealed the characteristic pattern of RA175 expression in the testis, and demonstrated that basigin, nectin-2 and nectin-3, the other members of IgSF, were also expressed in the *RA175*$^{-/-}$ testis. Many IgSF members are necessary to achieve normal spermatogenesis in the testis, and RA175 plays an important role as an adherent molecule between the germ cells and Sertoli cells in the testis. Further studies will provide

important clues regarding the regulation of the mechanism of the cell adhesion system in the testis and in understanding human infertility.

References

Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, Kavalali ET, Sudhof TC (2002) SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297:1525–1531.

Bouchard MJ, Dong Y, McDermott BM Jr, Lam DH, Brown KR, Shelanski M, Bellve AR, Racaniello VR (2000) Defects in nuclear and cytoskeletal morphology and mitochondrial localization in spermatozoa of mice lacking nectin-2, a component of cell-cell adherens junctions. *Mol Cell Biol* 20:2865–2873.

Fujita E, Soyama A, Momoi T (2003) RA175, which is the mouse ortholog of TSLC1, a tumor suppressor gene in human lung cancer, is a cell adhesion molecule. *Exp Cell Res* 287:57–66.

Fujita E, Kouroku Y, Ozeki S, Tanabe Y, Toyama Y, Maekawa M, Kojima N, Senoo H, Toshimori K, Momoi T (2006) Oligo-astheno-teratozoospermia in mice lacking RA175/TSLC1/SynCAM/IGSF4A, a cell adhesion molecule in the immunoglobulin superfamily. *Mol Cell Biol* 26:718–726.

Fujita E, Tanabe Y, Hirose T, Aurrand-Lions M, Kasahara T, Imhof BA, Ohno S, Momoi T (2007) Loss of partitioning-defective-3/isotype-specific interacting protein (par-3/ASIP) in the elongating spermatid of RA175 (IGSF4A/SynCAM)-deficient mice. *Am J Pathol* 171:1800–1810.

Gliki G, Ebnet K, Aurrand-Lions M, Imhof BA, Adams RH (2004) Spermatid differentiation requires the assembly of a cell polarity complex downstream of junctional adhesion molecule-C. *Nature* 431:320–324.

Gomyo H, Arai Y, Tanigami A, Murakami Y, Hattori M, Hosoda F, Arai K, Aikawa Y, Tsuda H, Hirohashi S, Asakawa S, Shimizu N, Soeda E, Sakaki Y, Ohki M (1999) A 2-Mb sequence-ready contig map and a novel immunoglobulin superfamily gene IGSF4 in the LOH region of chromosome 11q23.2. *Genomics* 62:139–146.

Holness CL, Simmons DL (1994) Structural motifs for recognition and adhesion in members of the immunoglobulin superfamily. *J Cell Sci* 107(Pt 8):2065–2070.

Hosoi I, Toyama Y, Maekawa M, Ito H, Yuasa S (2002) Development of the blood-testis barrier in the mouse is delayed by neonatally administered diethylstilbestrol but not by beta-estradiol 3-benzoate. *Andrologia* 34:255–262.

Hynes RO (1999) Cell adhesion: old and new questions. *Trends Cell Biol* 9:M33–M37.

Inagaki M, Irie K, Ishizaki H, Tanaka-Okamoto M, Miyoshi J, Takai Y (2006) Role of cell adhesion molecule nectin-3 in spermatid development. *Genes Cells* 11:1125–1132.

Ito C, Suzuki-Toyota F, Maekawa M, Toyama Y, Yao R, Noda T, Toshimori K (2004) Failure to assemble the peri-nuclear structures in GOPC deficient spermatids as found in round-headed spermatozoa. *Arch Histol Cytol* 67:349–360.

Kierszenbaum AL, Rivkin E, Tres LL (2003) Acroplaxome, an F-actin-keratin-containing plate, anchors the acrosome to the nucleus during shaping of the spermatid head. *Mol Biol Cell* 14:4628–4640.

Kierszenbaum AL, Tres LL, Rivkin E, Kang-Decker N, van Deursen JM (2004) The acroplaxome is the docking site of Golgi-derived myosin Va/Rab27a/b- containing proacrosomal vesicles in wild-type and Hrb mutant mouse spermatids. *Biol Reprod* 70:1400–1410.

Kuramochi M, Fukuhara H, Nobukuni T, Kanbe T, Maruyama T, Ghosh HP, Pletcher M, Isomura M, Onizuka M, Kitamura T, Sekiya T, Reeves RH, Murakami Y (2001) TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. *Nat Genet* 27:427–430.

Maekawa M, Suzuki-Toyota F, Toyama Y, Kadomatsu K, Hagihara M, Kuno N, Muramatsu T, Dohmae K, Yuasa S (1998) Stage-specific localization of basigin, a member of the immunoglobulin superfamily, during mouse spermatogenesis. *Arch Histol Cytol* 61:405–415.

Masuda M, Yageta M, Fukuhara H, Kuramochi M, Maruyama T, Nomoto A, Murakami Y (2002) The tumor suppressor protein TSLC1 is involved in cell-cell adhesion. *J Biol Chem* 277:31014–31019.

Ozaki-Kuroda K, Nakanishi H, Ohta H, Tanaka H, Kurihara H, Mueller S, Irie K, Ikeda W, Sakai T, Wimmer E, Nishimune Y, Takai Y (2002) Nectin couples cell-cell adhesion and the actin scaffold at heterotypic testicular junctions. *Curr Biol* 12:1145–1150.

de Rooij DG, Grootegoed JA (1998) Spermatogonial stem cells. *Curr Opin Cell Biol* 10:694–701.

Shingai T, Ikeda W, Kakunaga S, Morimoto K, Takekuni K, Itoh S, Satoh K, Takeuchi M, Imai T, Monden M, Takai Y (2003) Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell-cell adhesion and transmembrane protein localization in epithelial cells. *J Biol Chem* 278:35421–35427.

Surace EI, Strickland A, Hess RA, Gutmann DH, Naughton CK (2006) Tslc1 (nectin-like molecule-2) is essential for spermatozoa motility and male fertility. *J Androl* 27: 816–825.

Takai Y, Irie K, Shimizu K, Sakisaka T, Ikeda W (2003) Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization. *Cancer Sci* 94:655–667.

Tanaka H, Pereira LA, Nozaki M, Tsuchida J, Sawada K, Mori H, Nishimune Y (1997) A germ cell-specific nuclear antigen recognized by a monoclonal antibody raised against mouse testicular germ cells. *Int J Androl* 20:361–366.

Toshimori K, Maekawa M, Ito C, Toyama Y, Suzuki-Toyota F, Saxena D (2006) The involvement of immunoglobulin superfamily proteins in spermatogenesis and sperm-egg interaction. *Reprod Med Biol* 5:87–93.

Toyama Y, Maekawa M, Kadomatsu K, Miyauchi T, Muramatsu T, Yuasa S (1999) Histological characterization of defective spermatogenesis in mice lacking the basigin gene. *Anat Histol Embryol* 28:205–213.

Urase K, Soyama A, Fujita E, Momoi T (2001) Expression of RA175 mRNA, a new member of the immunoglobulin superfamily, in developing mouse brain. *Neuroreport* 12:3217–3221.

Wakayama T, Iseki S (2009) Role of the spermatogenic-Sertoli cell interaction through cell adhesion molecule-1 (CADM1) in spermatogenesis. *Anat Sci Int* 84:112–121.

Wakayama T, Ohashi K, Mizuno K, Iseki S (2001) Cloning and characterization of a novel mouse immunoglobulin superfamily gene expressed in early spermatogenic cells. *Mol Reprod Dev* 60:158–164.

Wakayama T, Koami H, Ariga H, Kobayashi D, Sai Y, Tsuji A, Yamamoto M, Iseki S (2003) Expression and functional characterization of the adhesion molecule spermatogenic immunoglobulin superfamily in the mouse testis. *Biol Reprod* 68:1755–1763.

Wakayama T, Sai Y, Ito A, Kato Y, Kurobo M, Murakami Y, Nakashima E, Tsuji A, Kitamura Y, Iseki S (2007) Heterophilic binding of the adhesion molecules poliovirus receptor and immunoglobulin superfamily 4A in the interaction between mouse spermatogenic and Sertoli cells. *Biol Reprod* 76:1081–1090.

van der Weyden L, Arends MJ, Chausiaux OE, Ellis PJ, Lange UC, Surani MA, Affara N, Murakami Y, Adams DJ, Bradley A (2006) Loss of TSLC1 causes male infertility due to a defect at the spermatid stage of spermatogenesis. *Mol Cell Biol* 26:3595–3609.

Yamada D, Yoshida M, Williams YN, Fukami T, Kikuchi S, Masuda M, Maruyama T, Ohta T, Nakae D, Maekawa A, Kitamura T, Murakami Y (2006) Disruption of spermatogenic cell adhesion and male infertility in mice lacking TSLC1/IGSF4, an immunoglobulin superfamily cell adhesion molecule. *Mol Cell Biol* 26:3610–3624.

Biochemical and Biophysical Research Communications 396 (2010) 703–708

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Impairment of social and emotional behaviors in *Cadm1*-knockout mice

Yuki Takayanagi [a], Eriko Fujita [b,c,1], Zhiling Yu [c,2], Takanori Yamagata [c], Mariko Y. Momoi [c], Takashi Momoi [b,1], Tatsushi Onaka [a,*]

[a] Department of Physiology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi-ken 329-0498, Japan
[b] Division of Development and Differentiation, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi-machi, Kodaira, Tokyo 187-8502, Japan
[c] Department of Pediatrics, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi-ken 329-0498, Japan

ARTICLE INFO

Article history:
Received 23 April 2010
Available online 5 May 2010

Keywords:
Cell adhesion molecule 1 (CADM1)
Social behavior
Anxiety-related behavior
Motor function
Autism

ABSTRACT

Cell adhesion molecule 1 (CADM1), a member of the immunoglobulin superfamily, mediates synaptic cell adhesion. Missense mutations in the *CADM1* gene have been identified in autism spectrum disorder (ASD) patients. In the present study, we examined emotional behaviors, social behaviors and motor performances in *Cadm1*-knockout (KO) mice. *Cadm1*-KO mice showed increased anxiety-related behavior in open-field and light–dark transition tests. Social behaviors of *Cadm1*-KO mice were impaired in social interaction, resident-intruder and social memory/recognition tests. Furthermore, motor coordination and gait of *Cadm1*-KO mice were impaired in rotarod and footprint tests. Our study demonstrates that CADM1 plays roles in regulating emotional behaviors, social behaviors and motor performances, and that CADM1 has important implications for psychiatric disorders with disruptions in social behavior, such as autism.

1. Introduction

CADM1 (previously known as TSLC1, IGSF4, SgIGSF, SynCAM1, Necl2, RA175), a member of the immunoglobulin (Ig) superfamily, is a synaptic cell adhesion molecule [1–4]. CADM1 contains three extracellular Ig-like domains followed by a single transmembrane region and a short cytosolic sequence that interacts with PDZ (postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1) domains of synaptic scaffolding molecules [1]. CADM1 is required for synapse formation and maturation of presynaptic terminals [1–4].

Recently, we identified two missense mutations, C739A (H246N) and A755C (Y251S), in the *CADM1* gene of male Caucasian autism spectrum disorder (ASD) patients and their family members [5]. Both mutations are located in the third Ig domain of CADM1, which is essential for cell adhesive *trans*-interaction [1]. The mutated CADM1 exhibited defective trafficking to the cell surface and more susceptibility to the cleavage and/or degradation. From these results, we suppose that phenotypes in ASD patients might partly correlate with impaired synaptogenesis induced by these mutations.

In the present study, to assess the roles of endogenous CADM1 in behavioral functions, we examined emotional behaviors, social behaviors and motor performances in *Cadm1*-KO mice. We used *Cadm1*-KO mice on a C57BL/6J background.

2. Materials and methods

2.1. Animals

Cadm1-KO mice [6], heterozygous mice and wild-type mice were obtained from heterozygous intercrosses on the C57BL/6J background for 10 generations. All behavioral studies were performed between 13:00 and 17:00 using *Cadm1*-KO, heterozygous and their littermate wild-type male mice. Animals had free access to food and water under the condition of 12-h light–dark cycle (22 ± 2 °C, 40–70% humidity, light on between 7:30 and 19:30). Experimental protocols used throughout the study were approved by an institutional committee and were in accord with Japanese legislation concerning animal experiments.

2.2. Behavioral studies

2.2.1. Open-field test

To assess spontaneous locomotor activity and anxiety-related behavior, 10–15-week-old mice were placed in a corner of an

Abbreviations: CADM1, cell adhesion molecule 1; ASD, autism spectrum disorder; KO, knockout; Ig, immunoglobulin; ANOVA, analysis of variance; ER stress, endoplasmic reticulum stress.

* Corresponding author. Address: Division of Brain and Neurophysiology, Department of Physiology, Jichi Medical University, Shimotsuke-shi, Tochigi-ken 329-0498, Japan. Fax: +81 285 44 8147.
E-mail address: tonaka@jichi.ac.jp (T. Onaka).
[1] Present address: Center for Medical Science, International University of Health and Welfare, 2600-1 Kitakanemaru, Otawara, Tochigi 324-8501, Japan.
[2] Present address: Department of Pediatrics, Shenjing Hospital of China Medical University, Heping District, Shenyang, Liaoning Province 110004, China.

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.04.165

open-field apparatus (60 × 60 × 40 cm; O'Hara and Co., Tokyo, Japan) with an illuminated (60 lux) chamber. The distance traveled and time spent in the center area were recorded over a 10-min period.

2.2.2. Light–dark transition test

Anxiety-related behavior was tested using the light–dark transition test. The apparatus consisted of a cage (40 × 40 × 30 cm) divided into two equal chambers by a black partition containing a small opening (O'Hara and Co., Tokyo, Japan). One chamber was made of white plastic and was brightly illuminated (100 lux), whereas the other chamber was black and dark. Ten to fifteen-week-old mice were placed in the dark chamber and allowed to move freely between the two chambers for 5 min. The time spent in each chamber and the total distance traveled was recorded.

2.2.3. Elevated plus-maze test

The apparatus consisted of two open (25 × 5 cm) and two enclosed arms of the same size with 15-cm-high transparent walls (60 lux, O'Hara and Co., Tokyo, Japan). The frequency of entry into open arms, the time spent in open arms and locomotion activity were measured, as described previously [7]. Eleven to sixteen-week-old mice were used.

2.2.4. Social interaction test

Social behavior between two mice in a familiar environment was tested with a system that automatically analyzes behavior in home cages [7]. The system consisted of a cage (29 × 18 × 12 cm) and a filtered cage top containing an infrared video camera and infrared light emitting diodes. Two genetically identical mice (20–25 weeks old) that had been housed separately were placed together in a home cage and their behavior was video-monitored over 2 days. Images from each cage were captured at a rate of one frame per second. Social interaction was measured by counting the number of particles in each frame; two particles indicated that the mice were not in contact with each other and one particle indicated contact between the two mice.

2.2.5. Hand-scored social behaviors in social interaction test

Behaviors were recorded on digital video and social behaviors of two mice (23–31 weeks old) were observed during the first 5 min following placement of the two mice into the test cage. Behaviors were categorized into the following nine indices; non-social exploratory behaviors, approaching, following, anogenital sniffing, nose-to-nose sniffing, crawling over and under, grooming each other, sleeping together, and fighting.

2.2.6. Resident-intruder test

Resident males (51–56 weeks old) were individually housed for 4 weeks before testing. Male C57BL/6J mice at the age of 4–5-week-old housed in groups were used as intruders. Two tests of 5-min duration were performed at a 5-min interval. New intruder mice were used in each test. Latency to attack the intruder, total duration of attack, and total frequencies of attack were measured.

2.2.7. Social memory/recognition test

Social investigating behavior was measured by social memory/recognition test. Eleven to seventeen-week-old male mice were transferred to an individual cage. Stimulus mice were ovariectomized more than one week prior under Avertin anesthesia [8,9]. A transparent acrylate cylinder (7 cm diameter) with several small holes in its lower portion was placed in the center of the cage. Two hours after transferring the test mice, the ovariectomized stimulus mouse was introduced into the cylinder for a 5-min confrontation. At the end of the 5-min trial, the stimulus animal was removed. This was repeated with the same stimulus mouse for three trials with 15-min intervals between them. In a fourth trial, a different ovariectomized stimulus mouse was introduced to the resident male mouse. The time spent investigating the intruder was measured in each trial. Ratios of the time spent by each subject on olfactory investigation during the fourth trial (exposure to a novel mouse) as compared with the time spent during the third trial (exposure to the familiar mouse) was calculated as an index of social memory/recognition [10].

2.2.8. Rotarod test

Motor coordination and motor learning were tested using the rotarod test. Mice (13–19 weeks old) were placed on a rotating drum (3 cm diameter) (O'Hara and Co., Tokyo, Japan). The speed of the rotarod accelerated from 4 to 40 rotations over a 5-min period. How long it took for the mice to fall off the rotarod was recorded. Three trials were performed per day for two days.

2.2.9. Footprint test

Mice (14–22 weeks old) were trained three times to walk along a 50-cm-long, 10-cm-wide corridor (with 30-cm-high walls) toward a dark compartment. Before the fourth trial, their hind paws were dipped in India ink and were then allowed to walk on paper strips. Stride length, interstep and hindpaw base width were measured from three steps.

2.2.10. Buried food pellet test

The food was buried under 5 cm of bedding in one corner of the cage. The mouse, which had been fasted for 18 h, was placed in the center of the cage, and the time taken to find the food was recorded for wild-type and *Cadm1*-KO mice (21–28 weeks old).

2.2.11. Grip strength test

Grip strength was measured by letting forehands of 14–20-week-old mice grip mesh connected to a spring balance and pulling the bodies until the mice released the mesh (O'Hara and Co., Tokyo).

2.2.12. Tail immersion and hot plate tests

Those tests were used to examine pain sensitivity. At 14–19 weeks of age, the tail of each mouse was immersed 2 cm into water at 48 °C and the latency time of a rapid tail flick was measured five separate times on each animal over a 15-min period, allowing a 3-min recovery period between each trial. For the hot plate test, mice were placed on a 54 °C hot plate and latency of the first paw response (a foot shake or a paw lick) was recorded.

2.2.13. Tail suspension test

The tail suspension test was used to assess depression-related behavior. Mice (14–19 weeks old) were suspended by the tail for 6 min and cumulative immobility time was recorded.

2.3. Image analysis and statistics

The applications used for the behavioral studies were Image OP, Image LD, Image EP, Image FZ (O'Hara and Co.), which were produced on the basis of the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/), and Image J program (http://rsb.info.nih.gov/ij/). Data, presented as means ± SEM, were analyzed using one-way analysis of variance (ANOVA) or repeated measures ANOVA followed by a Dunnett's *t*-test for multiple comparisons. Single comparisons were made using a Mann–Whitney U test or Wilcoxon signed-rank test.

3. Results

We tested the mice for anxiety-related behaviors using the open field, light–dark transition, and elevated plus-maze tests. The *Cadm1*-KO mice showed reduced center time in the open-field test (Fig. 1A) and less time in the light box of the light–dark test (Fig. 1B). Because the total distance in the open field or light–dark test did not show a significant genotype-dependent difference, the lessened time in the center or in the light box was not attributable to locomotor impairment of *Cadm1*-KO mice. These two test results suggested enhanced anxiety-like behavior in *Cadm1*-KO mice. However, the *Cadm1*-KO mice did not show significant differences in stay time in or entries into open arms of the elevated plus-maze test (Fig. 1C). The increased anxiety-like behaviors in *Cadm1*-KO mice may only be manifested under certain situations.

We also evaluated social behaviors of the mice. First, direct contact between pairs of genotypically identical *Cadm1*-KO or wild-type mice was analyzed for 24 h. In the light period, wild-type mice spent majority of time staying still in contact with each other, while the *Cadm1*-KO mice showed higher locomotion and spent less time in contact with each other (Fig. 2A). Second, we also hand scored the actual social interaction behaviors during the first 5-min period after placement of two mice into a new cage. The *Cadm1*-KO mice spent less time exploring the cage and more time following the other mice as compared to wild-type mice (Fig. 2B). The time spent fighting was also longer for the *Cadm1*-KO mice. The social interaction behaviors of heterozygous mice were not significantly different as compared to wild-type mice (Fig. 2B). In a resident-intruder test, the *Cadm1*-KO mice showed shorter attack latency, longer attack duration, and higher attack frequency (Fig. 2C), suggesting higher aggressive behavior in these mice.

Furthermore, we measured social investigating behavior in a social memory/recognition test, in which the same ovariectomized female mouse was repeatedly used for exposure. The wild-type mice exhibited a characteristic decrease in the time spent investigating the female upon repeat exposures (85.6 ± 6.2 s for the first exposure, 61.8 ± 7.2 s for the third exposures. $P < 0.05$), with a full recovery following the introduction of a new female (91.0 ± 10.2 s). The *Cadm1*-KO mice exhibited no decrease in investigation time of the same female mouse upon repeat exposures (89.8 ± 8.1 s for the first exposure, 85.1 ± 11.1 s for the third exposures. $P > 0.05$). When we presented the *Cadm1*-KO males with a new female, they spent a similar time investigating the new female (79.9 ± 12.1 s) as compared to that investigating the familiar animal. The ratio of time spent investigating a novel mouse (fourth trial) compared to a familiar animal (third trial) was significantly less in the *Cadm1*-KO mice than for wild-type mice (Fig. 2D). Thus, the *Cadm1*-KO mice showed impairment in forming familiarity, a core symptom of ASD patients.

The *Cadm1*-KO mice also had deficits in motor learning using the rotarod test (Fig. 3A). The time to falling off the rotating cylinder was longer after repeated training for wild-type mice, but not for the *Cadm1*-KO mice. This result may be related to the clumsiness in gross motor function that people with ASD often exhibit.

We also examined gait pattern in a foot print test. Neither stride length nor interstep were significantly different between wild-type

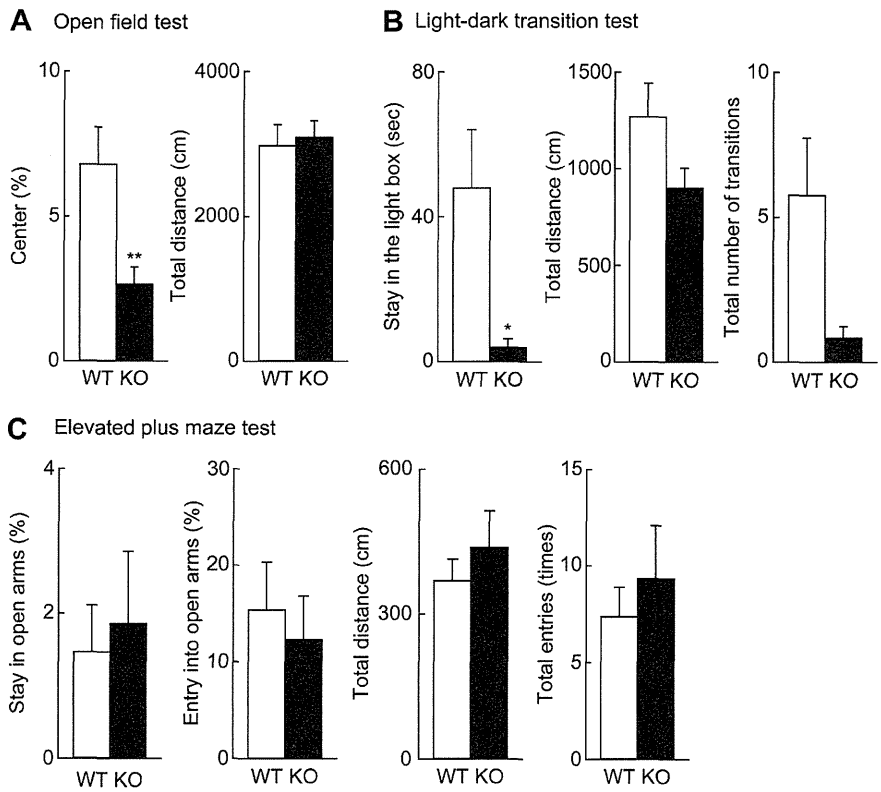

Fig. 1. Anxiety-related behaviors of *Cadm1*-KO mice. (A) The percentage of time spent in the center area of an open field cage and total distance traveled during the open-field test. *Cadm1*-KO mice ($n = 11$) spent less time in the center of the open field cage than wild-type mice ($n = 12$), but locomotor activity is similar between KO and wild-type mice. (B) The time spent in the lighted side of a cage during the light–dark transition test and total distance traveled. *Cadm1*-KO mice ($n = 11$) spent less time in the lighted side as compared to wild-type mice ($n = 12$). (C) The percentage of time spent in the open arms, that of the number of entries into the open arms, total distance of moving, and the total number of entries during the elevated plus-maze test. There were no significant differences in these parameters between wild-type ($n = 12$) and *Cadm1*-KO ($n = 10$) mice. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to wild-type mice.

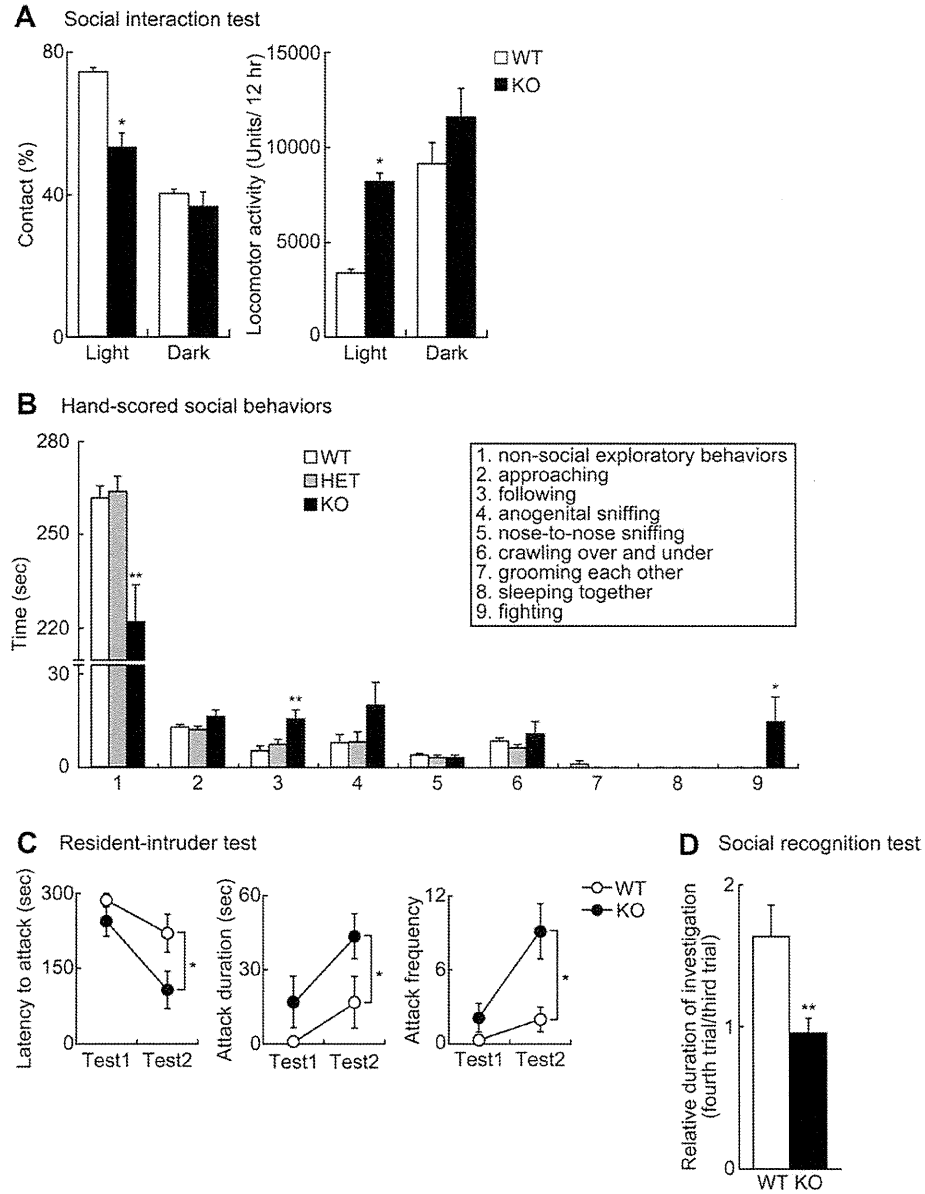

Fig. 2. Social behaviors of *Cadm1*-KO mice. (A, B) The percentage of time spent in contact and locomotor activity (A), and social behaviors (B) in the social interaction test. *Cadm1*-KO mice spent less time in contact with each other in the light period. In the light period, exploratory activity was higher in *Cadm1*-KO mice ($n = 4$ pairs) than in wild-type mice ($n = 4$ pairs). *Cadm1*-KO mice ($n = 7$ pairs) spent less time for exploring the cage and more for following the other mice and for fighting as compared to wild-type mice (n = 8 pairs) or heterozygous mice ($n = 8$ pairs). The one-way ANOVA F and P values were as follows. 9.00 and 0.0016 for non-social exploratory behaviors, 2.28 and 0.13 for approaching, 6.62 and 0.0062 for following, 2.39 and 0.12 for anogenital sniffing, 0.50 and 0.61 for nose-to-nose sniffing, 0.86 and 0.44 for crawling over and under, 0.93 and 0.41 for grooming each other, and 3.91 and 0.037 for fighting. (C) Latency to attack the intruder, total duration of attack, and total frequencies of attack in the resident-intruder test. *Cadm1*-KO mice ($n = 7$) showed shorter attack latency, longer attack duration and higher attack frequency than wild-type mice ($n = 9$). The ANOVA F and P values were as follows. Latency to attack: genotypes: $F = 5.01$, $P = 0.042$, trials: $F = 13.2$, $P = 0.0027$, interaction between genotypes and trials: $F = 1.71$, $P = 0.21$. Attack duration; genotypes: $F = 5.01$, $P = 0.042$, trials: $F = 8.13$, $P = 0.013$, interaction between genotypes and trials: $F = 0.55$, $P = 0.47$. Attack frequency; genotypes: $F = 8.60$, $P = 0.011$, trials: $F = 20.13$, $P = 0.0005$, interaction between genotypes and trials: $F = 8.81$, $P = 0.010$. (D) Relative duration of investigation in the social memory/recognition test. Ratio of time spent for investigating the familiar animal (the third trial) as compared to that for a novel animal (fourth trial) were less in *Cadm1*-KO mice ($n = 9$) than in wild-type mice ($n = 11$), indicating that wild-type but not *Cadm1*-KO mice discriminated between familiar and novel mice. $^*P < 0.05$, $^{**}P < 0.01$ as compared to wild-type mice.

and *Cadm1*-KO mice (Fig. 3B). Only hindpaw base width was significantly increased in the *Cadm1*-KO mice compared to wild-type mice (Fig. 3B), suggesting a wide-based gait, a sign of cerebellar dysfunction.

On the other hand, we identified no significant changes in sensory functions between wild-type and *Cadm1*-KO mice. We found no significant differences between wild-type and KO mice for olfactory function (buried food pellet test) (Fig. 4A), visual function (visual placing test) (data not shown), auditory function (startle response test) (data not shown).

We also found no significant differences in muscle strength between *Cadm1*-KO and wild-type mice as measured by grip strength (Fig. 4B), or in pain sensitivity as measured by tail immersion and hot plate tests (Fig. 4C and D). There was also no significant difference in immobility time in a tail suspension test, suggesting no differences in depression-like behavior in *Cadm1*-KO mice (Fig. 4E).

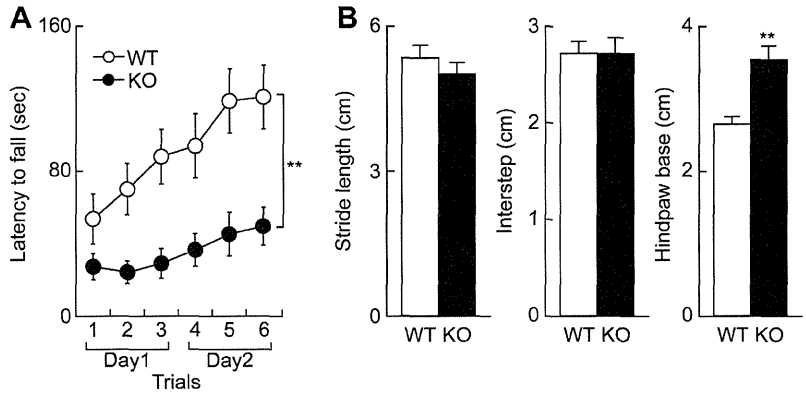

Fig. 3. Rotarod test and footprint test of *Cadm1*-KO mice. (A) The latency at which mice fell off a rotarod during multiple trials. *Cadm1*-KO mice ($n = 10$) showed no improvement in motor coordination, while wild-type mice ($n = 12$) showed improvement after repeated training. The ANOVA F and P values were as follows. genotypes: $F = 10.92$, $P = 0.0035$, trials: $F = 11.97$, $P < 0.0001$, interaction between genotypes and trials: $F = 2.57$, $P = 0.031$. (B) *Cadm1*-KO mice ($n = 11$) showed significantly longer hindpaw bases as compared to wild-type mice ($n = 16$). $^{**}P < 0.01$ as compared to wild-type mice.

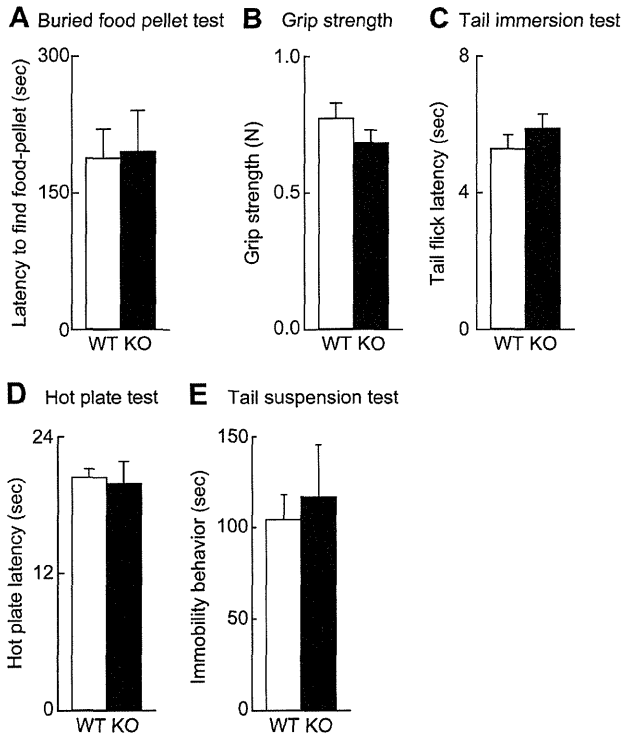

Fig. 4. Olfactory function test (A), grip strength test (B), pain sensitivity test (C and D) and depression-like behavior test (E) of *Cadm1*-KO mice. (A) There was no significant change in latency to find a buried food pellet between wild-type ($n = 16$) and *Cadm1*-KO ($n = 15$) mice. (B) Grip strength was not significantly different between *Cadm1*-KO ($n = 10$) and wild-type ($n = 12$) mice. (C, D) Latencies of tail flick in a tail immersion test (C) and of paw responses in a hot plate test (D) were not significantly different between *Cadm1*-KO ($n = 9$ or 10) and wild-type ($n = 12$) mice. (E) Cumulative time spent for immobility, depression-related behavior, in a tail suspension test was not significantly different between *Cadm1*-KO ($n = 9$) and wild-type ($n = 11$) mice.

4. Discussion

In the present study, *Cadm1*-KO mice exhibited increased anxiety-related behaviors, impaired social behaviors and impaired motor function, while they showed no significant changes in sensory functions. Similar psychiatric or psychosocial symptoms are often observed in people with ASD. Missense mutations in the *CADM1* gene have been identified in ASD patients [5]. These results are consistent with an idea that deficiency in CADM1 functions is involved in pathogenesis of ASD.

The open-field and light–dark transition tests indicated increased anxiety-related behaviors, but the elevated plus-maze test did not show any genotype-dependent difference; it may be that increased anxiety-like behaviors in *Cadm1*-KO mice manifest only under certain conditions. The social interaction and social memory/recognition tests indicated impaired social behaviors in *Cadm1*-KO mice. Furthermore, aggressive behaviors were observed in the *Cadm1*-KO mice. Results with the resident-intruder test suggested that *Cadm1*-KO mice have qualitative impairment in social interaction. Impaired social interaction is one of the core symptoms of ASD. Additional psychiatric or psychosocial symptoms that are often observed in people with ASD are anxiety and aggression [11]: anxiety has been reported to be related to social deficits and loneliness in people with ASD, and aggression is identified in the ASD-BPA (Autism Spectrum Disorders-Behavior Problems for Adults) as an associated symptom of ASD, although the precise nature of the relationship remains unclear [12].

The *Cadm1*-KO mice showed deficits in motor learning, based on findings using the rotarod test, and also showed the increased hindpaw base width, suggesting possible cerebellar dysfunction in *Cadm1*-KO mice. These phenotypes may be an indication of what underlies the problems with gross motor function that people with ASD often exhibit [13]. CADM1 is expressed in the Purkinje cells in the developing brain [14]. It is of interest that impaired development of Purkinje cells has been observed at autopsy in the brains of people with ASD [15]. Cerebellar impairment may be one of the major loci of ASD associated with communication impairment, an interesting question to address in future studies. On the other hand, we found no significant difference in immobility time in a tail suspension test (i.e., no depression-like behavior) and pain reactions in tail immersion or hot plate tests in *Cadm1*-KO mice. While depression and changes in pain reactions can be associated with patients with ASD, association of these comorbid symptoms depends on clinical situations of patients [16,17].

CADM1 is required for synapse formation and maturation of presynaptic terminals [1–4]. Thus the abnormal behavioral and motor phenotypes observed in *Cadm1*-KO mice might be caused by abnormal synaptogenesis due to loss-of function of CADM1. On the other hand, cells expressing the mutated CADM1, which was identified in some people with ASD, show defective trafficking of the mutated protein to the cell surface and its increased intracel-

lular accumulation [5]. Intracellular accumulation of the mutated molecules induces endoplasmic reticulum (ER) stress, which is a possible pathogenesis of ASD [18,19]. Thus both a loss-of function and a gain of function due to mutation of CADM1 may be involved in the pathogenesis of ASD.

Our study provides direct evidence that CADM1 is involved in the development of psychiatric disorders with disruptions in social behavior. *Cadm1*-KO mice could be used as a tool for a therapeutic model for psychiatric disorders with disruptions in social behavior such as ASD. Further study on the assembly of CADM1 with scaffold proteins and receptors on the postsynaptic membrane and its impairment in the *Cadm1* mutation knock-in mice will provide further insights into the pathogenesis of ASD.

Acknowledgment

This work was supported by KAKENHI (20590237, 20020023, 20790194, 22120512, 22659050) from MEXT and JSPS.

References

[1] T. Biederer, Y. Sara, M. Mozhayeva, D. Atasoy, X. Liu, E.T. Kavalali, T.C. Sudhof, SynCAM, a synaptic adhesion molecule that drives synapse assembly, Science 297 (2002) 1525–1531.
[2] E. Fujita, A. Soyama, T. Momoi, RA175, which is the mouse ortholog of TSLC1, a tumor suppressor gene in human lung cancer, is a cell adhesion molecule, Exp. Cell Res. 287 (2003) 57–66.
[3] A.I. Fogel, M.R. Akins, A.J. Krupp, M. Stagi, V. Stein, T. Biederer, SynCAMs organize synapses through heterophilic adhesion, J. Neurosci. 27 (2007) 12516–12530.
[4] Y. Sara, T. Biederer, D. Atasoy, A. Chubykin, M.G. Mozhayeva, T.C. Sudhof, E.T. Kavalali, Selective capability of SynCAM and neuroligin for functional synapse assembly, J. Neurosci. 25 (2005) 260–270.
[5] Y. Zhiling, E. Fujita, Y. Tanabe, T. Yamagata, T. Momoi, M.Y. Momoi, Mutations in the gene encoding CADM1 are associated with autism spectrum disorder, Biochem. Biophys. Res. Commun. 377 (2008) 926–929.
[6] E. Fujita, Y. Kouroku, S. Ozeki, Y. Tanabe, Y. Toyama, M. Maekawa, N. Kojima, H. Senoo, K. Toshimori, T. Momoi, Oligo-astheno-teratozoospermia in mice lacking RA175/TSLC1/SynCAM/IGSF4A, a cell adhesion molecule in the immunoglobulin superfamily, Mol. Cell Biol. 26 (2006) 718–726.
[7] Y. Sato, Y. Takayanagi, T. Onaka, E. Kobayashi, Impact of cyclosporine upon emotional and social behavior in mice, Transplantation 83 (2007) 1365–1370.
[8] J.N. Ferguson, L.J. Young, E.F. Hearn, M.M. Matzuk, T.R. Insel, J.T. Winslow, Social amnesia in mice lacking the oxytocin gene, Nat. Genet. 25 (2000) 284–288.
[9] Y. Takayanagi, M. Yoshida, I.F. Bielsky, H.E. Ross, M. Kawamata, T. Onaka, T. Yanagisawa, T. Kimura, M.M. Matzuk, L.J. Young, K. Nishimori, Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice, Proc. Natl. Acad. Sci. USA 102 (2005) 16096–16101.
[10] J.N. Ferguson, J.M. Aldag, T.R. Insel, L.J. Young, Oxytocin in the medial amygdala is essential for social recognition in the mouse, J. Neurosci. 21 (2001) 8278–8285.
[11] S.W. White, R. Roberson-Nay, Anxiety, social deficits, and loneliness in youth with autism spectrum disorders, J. Autism Dev. Disord. 39 (2009) 1006–1013.
[12] M.S. Parikh, A. Kolevzon, E. Hollander, Psychopharmacology of aggression in children and adolescents with autism: a critical review of efficacy and tolerability, J. Child Adolesc. Psychopharmacol. 18 (2008) 157–178.
[13] J. Manjiviona, M. Prior, Comparison of Asperger syndrome and high-functioning autistic children on a test of motor impairment, J. Autism Dev. Disord. 25 (1995) 23–39.
[14] K. Urase, A. Soyama, E. Fujita, T. Momoi, Expression of RA175 mRNA, a new member of the immunoglobulin superfamily, in developing mouse brain, Neuroreport 12 (2001) 3217–3221.
[15] S.H. Fatemi, A.R. Halt, G. Realmuto, J. Earle, D.A. Kist, P. Thuras, A. Merz, Purkinje cell size is reduced in cerebellum of patients with autism, Cell. Mol. Neurobiol. 22 (2002) 171–175.
[16] L. Sterling, G. Dawson, A. Estes, J. Greenson, Characteristics associated with presence of depressive symptoms in adults with autism spectrum disorder, J. Autism Dev. Disord. 38 (2008) 1011–1018.
[17] S. Tordjman, G.M. Anderson, M. Botbol, S. Brailly-Tabard, F. Perez-Diaz, R. Graignic, M. Carlier, G. Schmit, A.C. Rolland, O. Bonnot, S. Trabado, P. Roubertoux, G. Bronsard, Pain reactivity and plasma beta-endorphin in children and adolescents with autistic disorder, PLoS One 4 (2009) e5289.
[18] T. Momoi, E. Fujita, H. Senoo, M. Momoi, Genetic factors and epigenetic factors for autism: endoplasmic reticulum stress and impaired synaptic function, Cell Biol. Int. 34 (2009) 13–19.
[19] E. Fujita, H. Dai, Y. Tanabe, Y. Zhiling, T. Yamagata, T. Miyakawa, M. Tanokura, M. Momoi, T. Momoi, Autism spectrum disorder is related to endoplasmic reticulum stress induced by mutations in the synaptic cell adhesion molecule, CADM1, Cell Death Dis., in press.

Citation: Cell Death and Disease (2010) 1, e47; doi:10.1038/cddis.2010.23

www.nature.com/cddis

Autism spectrum disorder is related to endoplasmic reticulum stress induced by mutations in the synaptic cell adhesion molecule, CADM1

E Fujita[1,2], H Dai[1,3], Y Tanabe[1,3], Y Zhiling[2], T Yamagata[2], T Miyakawa[4], M Tanokura[4], MY Momoi[2] and T Momoi*[,1,3]

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with an unknown molecular pathogenesis. A recent molecular focus has been the mutated neuroligin 3, neuroligin 3(R451C), in gain-of-function studies and for its role in induced impairment of synaptic function, but endoplasmic reticulum (ER) stress induced by mutated molecules also deserves investigation. We previously found two missense mutations, H246N and Y251S, in the gene-encoding synaptic cell adhesion molecule-1 (CADM1) in ASD patients, including cleavage of the mutated CADM1 and its intracellular accumulation. In this study, we found that the mutated CADM1 showed slightly reduced homophilic interactions *in vitro* but that most of its interactions persist. The mutated CADM1 also showed morphological abnormalities, including shorter dendrites, and impaired synaptogenesis in neurons. Wild-type CADM1 was partly localized to the ER of C2C5 cells, whereas mutated CADM1 mainly accumulated in the ER despite different sensitivities toward 4-phenyl butyric acid with chemical chaperone activity and rapamycin with promotion activity for degradation of the aggregated protein. Modeling analysis suggested a direct relationship between the mutations and the conformation alteration. Both mutated CADM1 and neuroligin 3(R451C) induced upregulation of C/EBP-homologous protein (CHOP), an ER stress marker, suggesting that in addition to the trafficking impairment, this CHOP upregulation may also be involved in ASD pathogenesis.

Cell Death and Disease (2010) **1**, e47; doi:10.1038/cddis.2010.23; published online 3 June 2010
Subject Category: Neuroscience

Autism spectrum disorder (ASD) is the most common heritable neurodevelopmental disorder, characterized by impaired social interactions, communication impairments, and restricted and repetitive behaviors; over 15 susceptibility loci are currently estimated,[1] but the major mode of inheritance remains unknown. Genetic testing in individuals with ASD has identified mutations in the genes encoding several synaptic cell adhesion molecules, including neuroligin (NLGN) 3 and 4, cell adhesion molecule-1 (CADM1), and contactin-associated protein-like 2.[2–4] NLGNs are postsynaptic cell adhesion proteins that interact with neurexins on the presynaptic membrane,[5] and they are required for synapse maturation.[6] Neurexin–NLGN interactions induce differentiation of γ-aminobutyric acid (GABA) and glutamate postsynaptic specializations.[7] The ASD-related mutant, NLGN3(R451C), is retained intracellularly, which limits its pre–post synaptic interactions.[8] Some of the mutated NLGN4 also fails to transport to the cell surface and is instead retained in the endoplasmic reticulum (ER).[9]

CADM1 (also known as RA175/SynCAM1) is a membrane glycoprotein belonging to the immunoglobulin (Ig) superfamily and is localized to both sides of the synaptic cleft. Its extracellular domain displays calcium-independent homophilic *trans*-cell adhesion activity,[10,11] and its intracellular domain associates with calmodulin-associated serine/threonine kinase via individual PDZ-binding domains (PDZ: post synaptic density protein/Drosophila disc large tumor suppressor/zonula occludens-1 protein).[10] Recently, we identified two missense mutations, C739A (amino acid: H246N) and A755C(Y251S), in the *CADM1* gene of male Caucasian patients with ASD and their family members.[3] Both mutations are located in the third Ig (Ig3) domain of CADM1, which is essential for *trans*-active interactions. These ASD-related mutations stimulate the cleavage of CADM1 and induce defective trafficking to the cell surface.[3] These results suggest an association between impaired synaptogenesis and the pathogenesis of ASD.

[1]Division of Differentiation and Development, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi-machi, Kodaira, Tokyo 187-8502, Japan; [2]Department of Pediatrics, Jichi Medical University, 3311-1 Yakushiji, Shimotsukeshi, Tochigi 329-0498, Japan; [3]Center for Medical Science, International University of Health and Welfare, Kitakanemaru 2600-1, Ohtawara, Tochigi, 324-8501, Japan and [4]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
*Corresponding author: T Momoi, Division of Differentiation and Development, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi-machi, Kodaira, Tokyo 187-8502, Japan or Center for Medical Science, International University of Health and Welfare, Kitakanemaru 2600-1, Ohtawara, Tochigi, 324-8501, Japan.
Tel: +81 42 341 2711 (ext. 5273); Tel/Fax +81-287-24-3162; E-mail: momoi@ncnp.go.jp or momoi@iuhw.ac.jp
Keywords: autism; Cadm1; ER stress
Abbreviations: ASD, autism spectrum disorder; CADM1, cell adhesion molecule-1; Ig, immunoglobulin; NLGN, neuroligin; CHOP, upregulation of C/EBP-homologous protein; PDZ, post synaptic density protein/Drosophila disc large tumor suppressor/zonula occludens-1 protein; eIF2α, eukaryotic initiation factor-2 α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; UPR, unfolded protein response; TSC, tuberous sclerosis complex; GABA, aminobutyric acid; 4-PBA, phenyl butyric acid; Thap, thapsigargin
Received 08.2.10; revised 29.3.10; accepted 31.3.10; Edited by V De Laurenzi

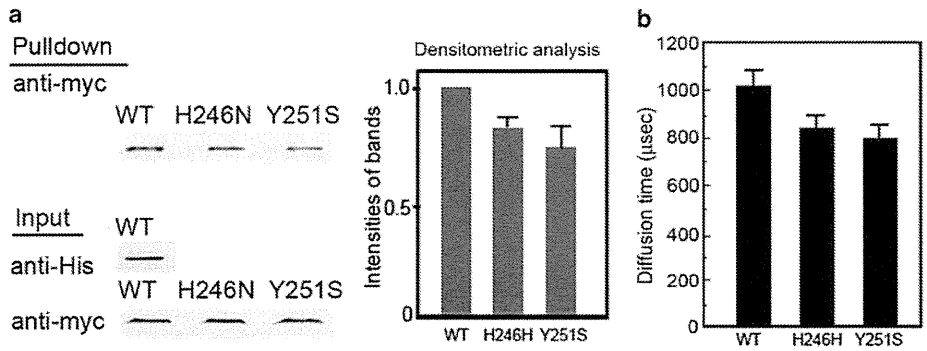

Figure 1 Effects of the mutations on the interaction activity. (**a**) Interaction between His-tagged wild-type (WT) CADM1 and myc-tagged WT or mutated CADM1. (left panel) Immunoblot analysis. Myc-WT and mutated (H246N- or Y251S-) CADM1 interacting with His-WT CADM1 were detected by western blot analysis using anti-myc antibody. Compared with the WT, the mutated CADM1 was not associated with His tagged-CADM1. (right panel) Densitometric analysis. Data from three experiments were scanned. Band intensities for the bound CADM1(H246N) and (Y251S) were normalized to the bound wild-type CADM1 and presented as the mean ± s.d. All experiments were performed three times, and typical data are shown. $P<0.05$ compared with wild-type. (**b**) FCS analysis of the protein–protein interaction between recombinant proteins of WT CADM1 or those of mutated CADM1. The interaction was detected by FCS using TAMRA-labeled molecules as probe. We examined the interaction between TAMRA-labeled and non-labeled WT-CADM1, TAMRA-labeled and non-labeled CADM1(H246N), and TAMRA-labeled and non-labeled CADM1(Y251S). All experiments were performed three times. Results are the mean ± s.d. of three different determinations. $P<0.05$ compared with wild-type

However, *nlgn3*-deficient mice do not show the core symptoms observed in patients with ASD.[6] In contrast, knock-in mice that express NLGN3(R451C), a mutation implicated in ASD,[12] do show behaviors analogous to the core symptoms of ASD, including impaired social behavior.[12,13] This finding suggests that both a loss-of-function and a gain-of-function mutation are involved in the pathogenesis of ASD. ER stress is a gain-of-function associated with the mutated protein.

The ER quality control system recognizes unfolded or misfolded proteins and activates stress-signaling pathways termed the unfolded protein response (UPR), via ER stress sensors.[14–16] The PKR-like ER kinase, one of the stress sensors in the ER, specifically upregulates the translation of the CAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP/Gadd153)[17] via phosphorylation of eukaryotic initiation factor-2 α (eIF2α). It is of interest that CHOP regulates synaptic function by regulating membrane trafficking[18] and that an eIF2α kinase, GCN2, controls synaptic plasticity, learning, and memory.[19,20] However, little is known regarding the association between ASD-related mutated molecules and ER stress.

In this study, we show that the ASD-related CADM1 mutations, H246N and Y251S, and the NLGN3 mutation, R451C, cause an UPR response with upregulation of CHOP as a gain of function.

Results

At first, we examined the *trans*-interaction between wild-type and mutated CADM1 molecules *in vitro* by pull-down and western blot analysis (Figure 1a). We compared the *trans*-interactions between bead-conjugated recombinant CADM1-His-tag proteins that lacked the transmembrane domain and either wild-type CADM1 or mutated CADM1 proteins (Figure 1a). Compared with the interaction between wild-type and wild-type, interactions between the mutant and wild-type proteins were slightly reduced and most of their interaction, 83.5% of CADM1(H246N) and 74.6% of CADM1(Y251S), remained. To evaluate this homophilic interaction quantitatively, we prepared recombinant extracellular domains of the wild-type and mutated CADM1 proteins and examined their interactions by fluorescence correlation spectroscopy (Figure 1b). Mutated CADM1 homophilic interaction activity was about 20 ± 5% weaker than that of wild-type CADM1.

We also modeled the chemical structure of CADM1 (Figure 2a) with a model in which the H246 and Y251 residues are located in the region that corresponds to the NH2-terminal strand of the Ig3 domain (amino acid sequence 160–324). The model showed that H246N and Y251S mutations represented reductions in the size of the side-chains of the NH2-terminal strand. Mutations also induced a conformational change (Figure 2b), and compared with H246N, the Y251S mutation induced a larger conformational change in the region.

To examine the relationship between a mutated CADM1 and the pathogenesis of ASD, we transfected myc-tagged wild-type CADM1 and H246N- or Y251S-mutated CADM1 sequences into neurons and examined their colocalization with synaptophysin, a marker of synapse. We excluded influences of the endogenous Cadm1 by using neurons isolated from Cadm1-deficient mouse embryos at embryonic day (E)16. Dendrites of the transfected neurons mainly showed three types of morphology (Figure 3, Table 1): most neurons that expressed wild-type CADM1 had long dendrites ($>100\,\mu$m) with synaptophysin-positive spines; neurons that expressed mutated CADM1 either had short dendrites ($<100\,\mu$m) without synaptophysin or no dendrites at all. Thus, neurons that expressed mutated CADM1 showed abnormal dendrites and impaired synaptogenesis.

Compared with the wild-type molecules, the mutated CADM1 more frequently showed intracellular accumulation. We examined the intracellular localization of wild-type CADM1 and CADM1(Y251S) proteins in transfected C2C5

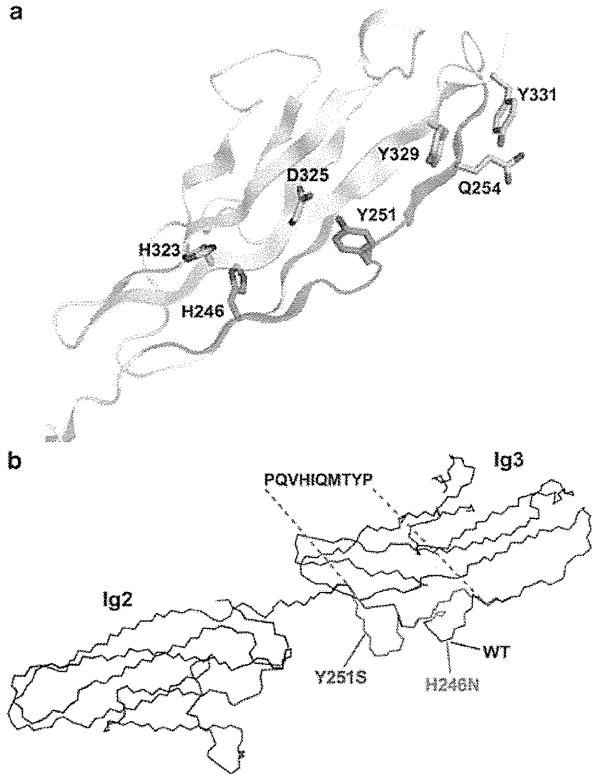

Figure 2 The modeled structure of mutated CADM1. (**a**) The Ig3 domain of CADM1, which was built using the Ig3 domain of NCAM as a template and registered in MODBASE.[39] The NH_2- and COOH-terminal strands of the model are red and yellow, respectively. The residues shown as sticks are possibly involved in the *trans*-interaction. The mutant residues, H246 and Y251, are in cyan. (**b**) The superimposed models of wild-type (black), H246N mutant (green), and Y251S mutant (blue) CADM1 using MOLMOL.[40] The amino acid sequence, PQVHIQMTYP, shows the altered region in the Y251S mutant compared with the wild-type

cells and neurons (Figure 4a). Wild-type CADM1 was partly colocalized with anti-KDEL, a marker for ER, whereas CADM1(Y251S) was predominantly accumulated in the ER. Wild-type CADM1 was also partly colocalized with beclin, which is localized on the various organelle including *trans*-Golgi, mitochondria, and ER,[21] whereas the accumulated CADM1(Y251S) was colocalized with ER-localized beclin (Figure 4b). The beclin was mainly localized on the ER under ER stress (Figure 4c), suggesting that the accumulation of CADM1(Y251S) in the ER caused the ER stress.

The cells expressing CADM1(Y251S) frequently showed an abnormal round cell morphology (Figures 4 and 5a). 4-phenyl butyric acid (4-PBA), a potential chemical chaperone in the ER[22] reduced the intracellular accumulation and the abnormal morphology of the cells expressing the mutated CADM1(Y251S) (Figure 5a and b). ER-localized beclin has a role in regulating autophagy.[21] Rapamycin, which stimulates the degradation of the accumulated protein via activation of the autophagosome,[23] inhibited the intracellular accumulation of CADM1(Y251S) (Figure 5a and b).

We also examined the mutated CADM1-induced CHOP upregulation downstream of the ER stress.[24,25] Both CADM1(H246N) and CADM1(Y251S) induced CHOP upregulation (Figure 6a), and CHOP-positive cells were more frequently detected in cells that expressed CADM1(H246) and (Y251S) (Figure 6b). In the cells expressing NLGN3(R451C), CHOP-positive cells are also more frequently detected compared with the cells expressing wild-type NLGN3 (Figure 6c). Thus, the mutated CADM1(H246) and (Y251S) proteins as well as the NLGN3(R451C) protein most likely stimulated ER stress, causing CHOP upregulation.

Discussion

ER stress and the mutated CADM1. The mutated CADM1 showed the most *trans*-interaction activity *in vitro* but accumulated in the ER and showed impaired trafficking, suggesting that the impaired synaptic function caused by defective trafficking of the mutated CADM1 could be related to the pathogenesis of ASD. However, *cadm1*-deficient mice[26] as well as *nlgn3*-deficient mice[6] did not show all of the core symptoms of ASD (Takayanagi *et al.*, submitted elsewhere); in addition to infertility,[26] *cadm1*-deficient mice manifested abnormal anxieties and impaired ultrasonic vocalization but did not show the impaired social interaction and restricted stereotyped behaviors. In contrast with cadm1 in the testis, the loss of synaptic function of cadm1 in *cadm1*-deficient mice could be partly compensated by other members of the Cadm family. This possibility suggests that the loss of function of CADM1 or NLGN3 is one of the factors but not a sufficient factor alone to cause ASD. The gain of function of their mutated molecules could be another feature related to the pathogenesis of ASD.[27]

The ER stress is a gain of function associated with the mutated proteins.[25] The misfolded and unfolded proteins are checked under the surveillance of the ER quality control system in the ER, followed by processing by the ER-associated degradation (ERAD) system, but if degradation is not sufficient, they are accumulated and cause ER stress with UPR.[25] ER stress is the signal elicited by the quality control system in the initial stages of the UPR. Rapamycin inhibits ER stress by stimulating the activation of autophagosome formation,[25,28] which also contributes to the degradation of the accumulated mutated proteins on the ER membrane in cooperation with ubiquitin/proteasome-mediated ERAD.[23,29] The sensitivity of CADM1(Y251S) to the rapamycin suggests that ubiquitin/proteasome-mediated ERAD is not sufficient for its degradation. This inference is also supported by the altered conformation and the sensitivity to 4-PBA. Furthermore, the mutated CADM1 as well as NLGN3(R451C) caused CHOP upregulation, suggesting that the mutated CADM1 as well as NLGN3(R451C) were retained in the ER, causing ER stress with CHOP upregulation as the UPR. This will be one of the important issues in the future.

Relationship between ER stress and the pathogenesis of ASD. Chronic and excess ER stress leads to neuronal cell death and may be related to the pathogenesis of

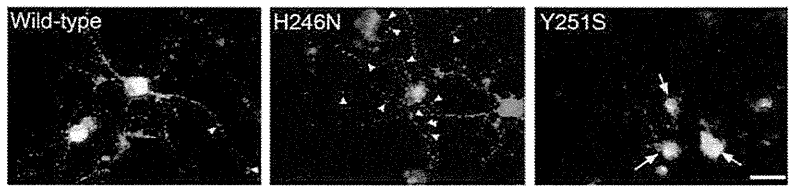

Figure 3 Localization of wild-type and mutated CADM1 in the neurons. Isolated neurons from *cadm1*-deficient mice embryos (E16) were transfected with wild-type and mutated CADM1 (H246N or Y251S). Their localization at the synapse was examined by immunostaining with anti-SynCAM (Cadm1; red), anti-synaptophysin (green), and Hoechst (blue). Closed arrowheads: CADM1 with no synaptophysin; arrows, neurons with short or no dendrites. Scale bars: 25 μm

Table 1 Percentages of the neurons expressing three types of dendrites in the neurons transfected with wild-type or mutated CADM1

Types[a] (8 DIV[b])	Wild-type	H246N	Y251S
Neurons with long dendrites, $>1000\,\mu$m	63.6 ± 6.0%	30.0 ± 2.9%	9.1 ± 1.8%
Neurons with short dendrites, $<1000\,\mu$m	27.3 ± 2.6%	40.0 ± 3.8%	36.4 ± 3.5%
Neuron with no dendrites, $<100\,\mu$m	9.1 ± 0.8%	30.0 ± 2.2%	54.5 ± 4.3%

The schematic illustration and evaluation of the length of the dendrites are described in Supplementary Figure S1. [a]Total dendritic length/cell. [b]DIV = days *in vitro*

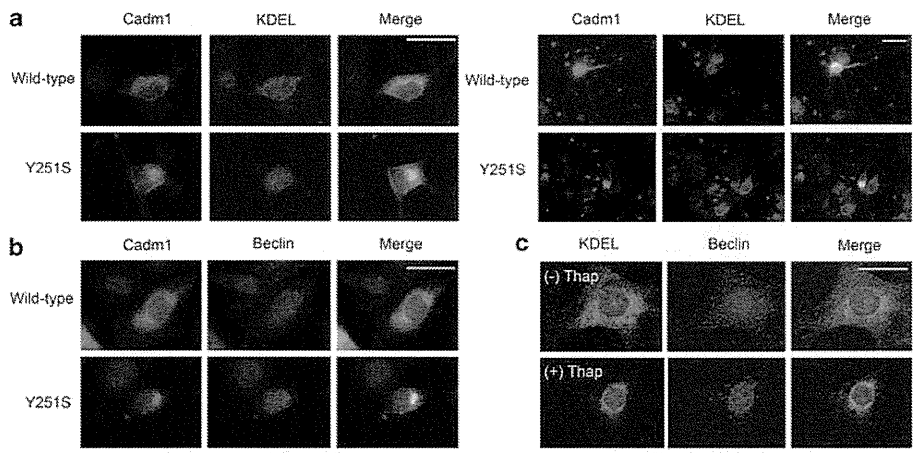

Figure 4 The localization of wild-type and mutated CADM1. (**a**) C2C5 cells and neurons were transfected with wild-type and mutated CADM1 (Y251S) for 26 h. After fixation, cells were immunostained with anti-SynCAM (Cadm1, green), anti-KDEL (red). (**b**) Transfected C2C5 cells were immunostained with anti-SynCAM (green), anti-beclin (red), and Hoechst (blue). (**c**) C2C5 cells were also treated with or without thapsigargin (Thap, 1 μM) for 24 h and were detected by anti-KDEL (green), anti-beclin (red), and Hoechst (blue). Scale bars: 20 μm

neurodegenerative diseases.[30] However, little is known about the relationship between ER stress and the pathogenesis of ASD. Other studies have shown that tuberous sclerosis complex (TSC), a neurogenetic disorder caused by a loss-of-function mutation in either the *TSC-1* or *TSC-2* genes, may also be related to ER stress. TSC frequently results in prominent central nervous system manifestations, including epilepsy, mental retardation, and ASD.[31,32] TSC-deficient cells have shown constitutive activation of mammalian target of rapamycin and proved to be highly susceptible to ER stress.[33] Thus, a wide variety of mutations that cause ER stress may be linked to the pathogenesis of ASD.

ASD may be the result of abnormal membrane trafficking of the synaptic functional molecules induced by ER stress. CHOP interacts with the heterodimeric receptors $GABA_{B1a}R$/$GABA_{B2}R$ and inhibits the formation of heterodimeric complexes; this results in intracellular accumulation and reduced cell surface expression of receptors.[18] In ASD patients, the $GABA_{B1}R$ level is significantly decreased in Brodmann area 9 and Brodmann area 40 of the cerebrum and cerebellum, whereas the $GABA_{B2}R$ level is significantly reduced in the cerebellum.[34] Therefore, it is possible that relatively low levels of ER stress may alter the intracellular transport of $GABA_BR$ to the cell surface by upregulation of CHOP without affecting the cell death of the neurons in the brain.

Abnormal morphology of neurons expressing mutated molecules may be due to the ER stress and ER stress-associated the abnormal membrane trafficking. At present, however, it is not clear whether the mutated molecules-mediated ER stress is linked with ER stress-mediated autophagosome activation in the pathogenesis of ASD.

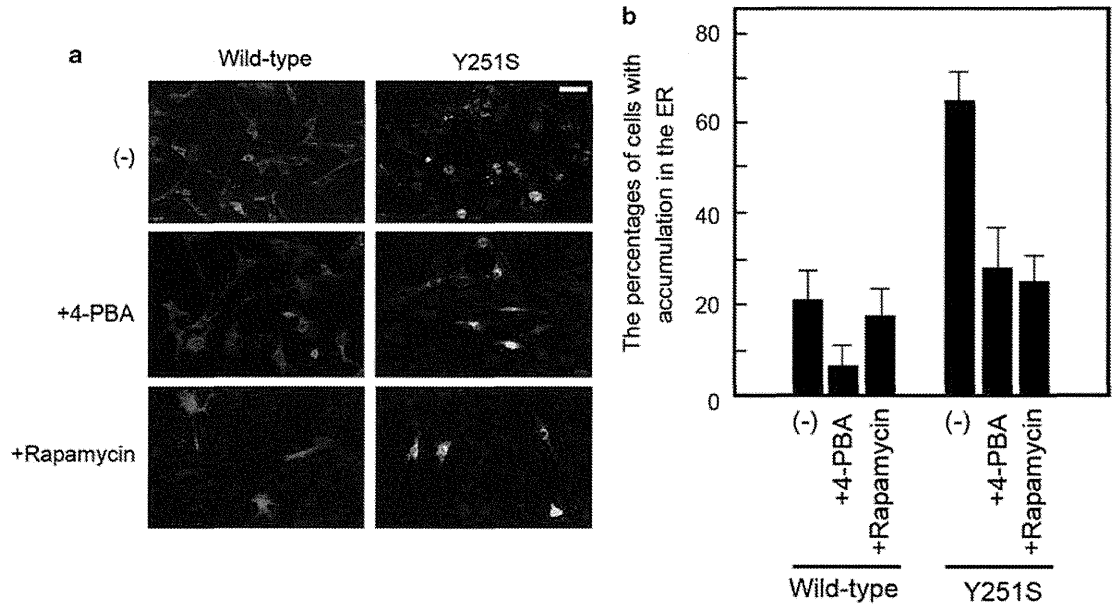

Figure 5 The effects of 4-phenyl butyric acid (4-PBA) or rapamycin on the intracellular accumulation of the mutated CADM1 in C2C5 cells. (**a**) The accumulation of the mutated CADM1 (Y251S) in the presence or absence of 4-PBA (7.5 mM) or rapamycin (10 μg/ml). C2C5 cells were transfected with wild-type and CADM1(Y251S) for 28 h and then fixed with paraformaldehyde and immunostained with anti-SynCAM (Cadm1). Scale bars: 50 μm. (**b**) The cell population showing accumulation of wild-type and mutated CADM1 (Y251S) in the presence or absence of 4-PBA or rapamycin. Three experiments were done. Bars indicate s.d.

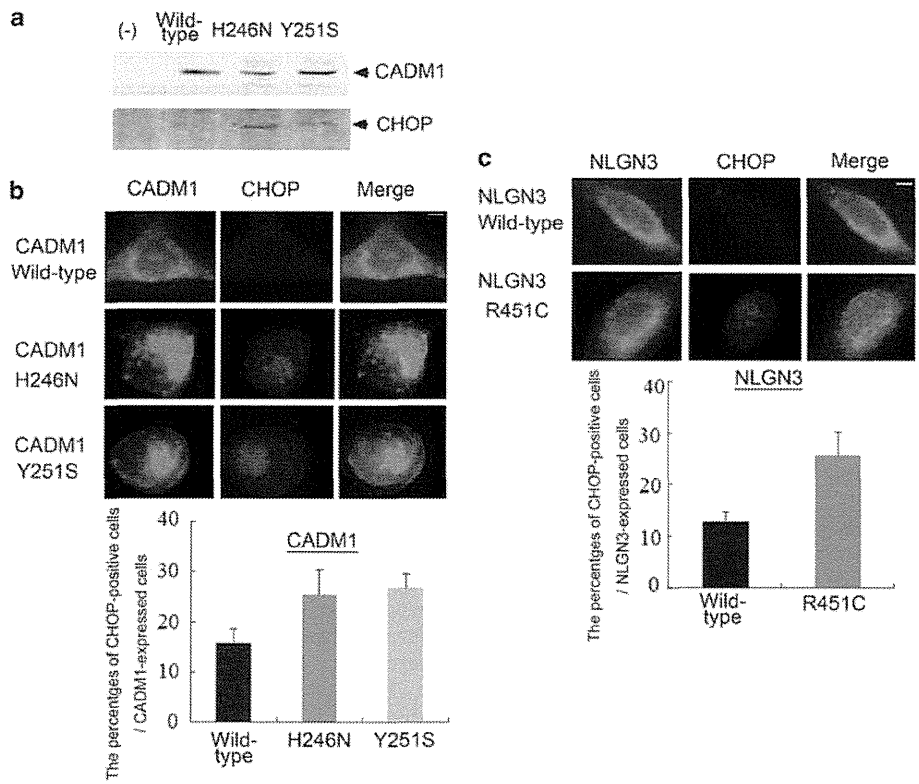

Figure 6 The expression of CHOP in the cells expressing the mutated CADM1 and the mutated neuroligin 3. (**a**) The mutated CADM1-induced upregulation of CHOP. C2C5 cells were transfected with wild-type, CADM1 (H246N), or (Y251S) cDNA and incubated for 24 h. The upregulation of CHOP was analyzed by immunoblot analysis using anti-CHOP. (**b**) The percentage of the CHOP-positive cells in the cells expressing wild-type or mutated CADM1s. (**c**) The percentage of the CHOP-positive cells in the cells expressing wild-type or R451C-mutated neuroligin (NLGN) 3. Scale bars: 5 μm. Bars indicate s.d.

Regulation of the mutated molecule-mediated ER stress will be another important issue in the future. Knock-in mice that express the mutated cadm1 related to the human CADM1 (H246N) or (Y251S) will provide more insight into the relationship between the ER stress and the pathogenesis of ASD.

Materials and Methods

Protein–protein interaction assay. His-tagged recombinant protein wild-type-CADM1 (48–334 a.a. including three Ig domains) lacking the transmembrane domain were prepared using silkworm cells (Katakura Industries Co., Tokyo, Japan).[35] His-tagged CADM1 (48–334 a.a.) was purified by Ni-column according to the manufacturer's protocol (Qiagen Science, Germantown, MD, USA).

Wild-type or mutated CADM1 in a pcDNA vector was transfected into COS cells using Lipofectamine 2000 (Invitrogen). After incubation for 28 h, the cells were lysed with PBS containing 1% Triton X-100, and then centrifuged at 12 000 r.p.m. for 20 min, COS-cell extracts. His-tagged recombinant Cadm1 (48–334) protein (2 μg protein) was incubated with the extracts (500 μg protein) from COS cells expressing wild-type, H246N-, or Y251S-mutated Cadm1 (48–334) -myc at 4°C overnight. The complexes were isolated from the incubation mixture by binding with the Ni-column and detected by immunoblot analysis using anti-myc (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and evaluated by densitometric analysis. Data from three experiments were scanned and analyzed for quantification with Image J software (National Institutes of Health).

Fluorescence correlation spectroscopy (FCS). TAMRA-labeled recombinant protein (mutated or wild-type Cadm1 (48–334 a.a. including three Ig domains)) lacking the transmembrane domain was prepared using the *in vitro* Pinpoint Fluorescence Labeling Kit 543).[36] TAMRA-labeled and non-labeled recombinant protein were purified using the RTS 100, *E. coli* HY Kit (Roche, Basel, Switzerland). FCS measurements were performed using an MF20 single molecule fluorescence detection system (Olympus, Tokyo, Japan). A helium–neon laser (543 nm) was used for the detection of TAMRA-labeled recombinant protein. TAMRA-labeled recombinant mutated or wild-type Cadm1 (4 nM) was mixed with non-labeled recombinant mutated or wild-type Cadm1 (0–40 nM) and added to the mixture in PBS with 0.05% Tween 20. After the mixtures were incubated at 37°C for 1 h, an aliquot (50 μl) of each sample was transferred to a microplate (24 × 16 wells, Olympus). A standard solution (MF-D543PX, Olympus) was used to derive the optical parameters necessary for a proper measurement. All measurements were carried out in more than duplicate and with 10 scans, each lasting 10 s at room temperature. The obtained data were fitted according to an autocorrelation function embodied in the accompanying software.

Modeling the structure of mutated CADM1. The structure of the Ig2 and Ig3 domains of wild-type and mutant CADM1 were built by SWISS-MODEL[37] using the crystal structure of MuSK (PDB entry: 2IEP) as a template and the amino acid sequence from 163–327 of CADM1. For the modeling of the H246N and Y251S mutants of CADM1, the input sequences were modified corresponding to their mutations.

Transfection of the wild-type and mutated CADM1-myc into neurons. Neurons were isolated from the brains of *cadm1*-deficient mice at embryonic day 16 as described.[38] Neurons were cultured using Neurobasal medium with 2% B27 supplement (Invitrogen, Carlsbad, CA, USA) and L-glutamine (0.5 mM). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 6 days *in vitro* (DIV), neurons were transfected with wild-type or mutated (H246N) or (Y251S) myc-tagged CADM1 using the calcium phosphate method and incubated for 2 DIV.

Immunostaining. For the immunostaining assay for intracellular localization of CADM1 and synaptophysin in the neurons and C2C5 cells, cells were transfected with wild-type and H246N- or Y251S-mutated pcDNA–CADM1 in the presence or absence of 4-PBA (7.5 mM) or rapamycin (10 μg/ml), and fixed in 4% paraformaldehyde, washed with PBS, and then incubated with mouse anti-synaptophysin (Sigma, St Louis, MO, USA), mouse anti-KDEL (Stressgen Biotechnologies Corp., Victoria, BC, Canada), rabbit anti-beclin (Cell Signaling Technology, Beverly, MA, USA), mouse anti-CHOP (Santa Cruz), or chicken anti-SynCAM1 (Cadm1) (MBL, Nagoya, Japan) overnight at 4°C. Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies against chicken and mouse or rabbit IgG were purchased from Molecular Probes (Eugene, OR, USA). Nuclei were detected by Hoechst 33342 staining (Molecular Probes). The reactivity was viewed using a confocal laser-scanning microscope (CSU-10, Yokogawa, Yokokawa Electric Co., Tokyo, Japan).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. We express our deepest sympathy at the untimely passing of an excellent neuroscientist, Dr. Alaa El-Husseini. We gratefully acknowledge the resources provided by the Autism Genetic Resource Exchange (AGRE) Consortium and the participating AGRE families. AGRE is a program of Autism Speaks and is supported, in part, by Grant 1U24MH081810 from the National Institute of Mental Health to Clara M Lajonchere (PI). This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology (21200011, 21500334, 21700377), for research into nervous and mental disorders from the Ministry of Health, Labor and Welfare (20B-13), for research on Publicly Essential Drugs and Medical Devices from the Japan Health Science Foundation.

1. Pickett J, London E. The neuropathology of autism. *J Neuropathol Exp Neurol* 2005; **64**: 925–935.
2. Jamain S, Quach H, Betancur C, Råstam M, Colineaux C, Gillberg IC *et al.* Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* 2003; **34**: 27–29.
3. Zhiling Y, Fujita E, Tanabe Y, Yamagata T, Momoi T, Momoi MY. Mutations in the gene encoding CADM1 are associated with autism spectrum disorder. *Biochem Biophys Res Commun* 2008; **377**: 926–929.
4. Bakkaloglu B, O'Roak BJ, Louvi A, Gupta AR, Abelson JF, Morgan TM *et al.* Molecular cytogenetic analysis and resequencing of contactin associated protein-like 2 in autism spectrum disorders. *Am J Hum Genet* 2008; **82**: 165–173.
5. Scheiffele P, Fan J, Choih J, Fetter R, Serafini T. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 2000; **101**: 657–669.
6. Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K *et al.* Neuroligins determine synapse maturation and function. *Neuron* 2006; **51**: 741–754.
7. Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM. Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 2004; **119**: 1013–1026.
8. Comoletti D, De Jaco A, Jennings LL, Flynn RE, Gaietta G, Tsigelny I *et al.* The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing. *J Neurosci* 2004; **24**: 4889–4893.
9. Zhang C, Milunsky JM, Newton S, Ko J, Zhao G, Maher TA *et al.* A neuroligin-4 missense mutation associated with autism impairs neuroligin-4 folding and endoplasmic reticulum export. *J Neurosci* 2009; **29**: 10843–10854.
10. Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, Kavalali ET *et al.* SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 2002; **297**: 1525–1531.
11. Fujita E, Soyama A, Momoi T. RA175, which is the mouse ortholog of TSLC1, a tumor suppressor gene in human lung cancer, is a cell adhesion molecule. *Exp Cell Res* 2003; **287**: 57–66.
12. Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM *et al.* A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 2007; **318**: 71–76.
13. Jamain S, Radyushkin K, Hammerschmidt K, Granon S, Boretius S, Varoqueaux F *et al.* Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. *Proc Natl Acad Sci USA* 2008; **105**: 1710–1715.
14. Kopito RR. ER quality control: the cytoplasmic connection. *Cell* 1997; **88**: 427–430.
15. Ron D. Translational control in the endoplasmic reticulum stress response. *J Clin Invest* 2002; **110**: 1383–1388.
16. Sitia R, Braakman I. Quality control in the endoplasmic reticulum protein factory. *Nature* 2003; **426**: 891–894.
17. Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ *et al.* Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol Cell Biol* 1996; **16**: 4273–4280.
18. Sauter K, Grampp T, Fritschy J-M, Kaupmann K, Bettler B, Mohler H *et al.* Subtype-selective interaction with the transcription factor CCAAT/Enhancer-binding protein (C/EBP) homologous protein (CHOP) regulates cell surface expression of $GABA_B$ receptors. *J Biol Chem* 2005; **280**: 33566–33572.

19. Chen A, Muzzio IA, Malleret G, Bartsch D, Verbitsky M, Pavlidis P *et al.* Inducible enhancement of memory storage and synaptic plasticity in transgenic mice expressing an inhibitor of ATF4 (CREB-2) and C/EBP proteins. *Neuron* 2003; **39**: 655–669.
20. Costa-Mattioli M, Gobert D, Stern E, Gamache K, Colina R, Cuello C *et al.* eIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory. *Cell* 2007; **129**: 195–206.
21. Cao Y, Klionsky DJ. Physiological functions of Atg6/Beclin: a unique autophagy-related protein. *Cell Res* 2007; **17**: 839–849.
22. Burrows JA, Willis LK, Perlmutter DH. Chemical chaperones mediate increased secretion of mutant alpha 1-antitrypsin (alpha 1-AT) Z: A potential pharmacological strategy for prevention of liver injury and emphysema in alpha 1-AT deficiency. *Proc Natl Acad Sci USA* 2000; **97**: 1796–1801.
23. Fujita E, Kouroku Y, Isoai A, Kumagai H, Misutani A, Matsuda C *et al.* Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). *Hum Mol Genet* 2007; **16**: 618–629.
24. Wang XZ, Kuroda M, Sok J, Batchvarova N, Kimmel R, Chung P *et al.* Identification of novel stress-induced genes downstream of chop. *EMBO J* 1998; **17**: 3619–3630.
25. Momoi T. Conformational diseases and ER stress-mediated cell death: apoptotic cell death and autophagic cell death. *Curr Mol Med* 2006; **6**: 111–118.
26. Fujita E, Kouroku Y, Ozeki S, Tanabe Y, Toyama Y, Maekawa M *et al.* Oligo-astheno-teratozoospermia in mice lacking RA175/TSLC1/SynCAM/IGSF4A, a cell adhesion molecule in the immunoglobulin superfamily. *Mol Cell Biol* 2006; **26**: 718–726.
27. Momoi T, Fujita E. Heritable neurodevelopmental disorders and endoplasmic reticulum stress. In: Hetz C (ed). *Protein Misfolding Disorders: A Trip into the ER*. Bentham science publishers Ltd.: United Arab Emirates (U.A.E.), 2009, pp 88–93.
28. Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H *et al.* ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ* 2007; **14**: 230–239.
29. Fujita E, Kanki T, Kouroku Y, Kundu M, Momoi T, Ney PA *et al.* Mechanisms of selective organellar clearance in cells. *Autophagy* 2010 (in press).
30. Muhle R, Trentacoste SV, Rapin I. The genetics of autism. *Pediatrics* 2004; **113**: e472–e486.
31. Curatolo P, Verdecchia M, Bombardieri R. Tuberous sclerosis complex: a review of neurological aspects. *Eur J Paediatr Neurol* 2002; **6**: 15–23.
32. Asato MR, Hardan AY. Neuropsychiatric problems in tuberous sclerosis complex. *J Child Neurol* 2004; **19**: 241–249.
33. Nardo A, Kramvis I, Cho N, Sadowski A, Meikle L, Kwiatkowski DJ *et al.* Tuberous sclerosis complex activity is required to control neuronal stress responses in an mTOR-dependent manner. *J Neurosci* 2009; **29**: 5926–5937.
34. Fatemi SH, Folsom TD, Reutiman TJ, Thuras PD. Expression of GABA(B) receptors is altered in brains of subjects with autism. *Cerebellum* 2009; **8**: 64–69.
35. Suzuki T, Kaki H, Naya S, Murayama S, Tatsui A, Nagai A *et al.* Recombinant human chymase produced by silkworm-baculovirus expression system: its application for a chymase detection kit. *Jpn J Pharmacol* 2002; **90**: 210–213.
36. Kajihara D, Abe R, Iijima I, Komiyama C, Sisido M, Hohsaka T. FRET analysis of protein conformational change through position-specific incorporation of fluorescent amino acids. *Nat Method* 2006; **3**: 923–929.
37. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res* 2005; **31**: 3381–3385.
38. Goslin K, Banker G. Rat hippocampal neurons in low density culture. In: Banker G, Goslin K (eds). *Culturing Nerve Cells*. MIT: Cambridge, MA, 1998, pp 251–282.
39. Pieper U, Eswar N, Davis FP, Braberg H, Madhusudhan MS, Rossi A *et al.* MODBASE: a database of annotated comparative protein structure model and associated resources. *Nucleic Acids Res* 2006; **34**: D291–D295.
40. Koradi R, Billeter M, Wüthrich K. MOLMOL: a program for display and analysis of macromolecular structures. *J Mol Graph* 1996; **14**: 51–55.

Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)