Drebrin A regulates hippocampal LTP and hippocampus-dependent fear learning in adult mice

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Abstract

Structural plasticity of dendritic spines, which underlies higher brain functions including learning and memory, is dynamically regulated by the actin cytoskeleton and its associated proteins. Drebrin A is an F-actin-binding protein preferentially expressed in the brain and localized in the dendritic spines of mature neurons. Isoform conversion from drebrin E to drebrin A and accumulation of the latter in dendritic spines occurs during synapse maturation. We have previously demonstrated that drebrin A plays a pivotal role in spine morphogenesis and plasticity. However, it is unclear whether drebrin A plays a specific role in processes required for fear learning, and whether drebrin E can substitute in this role. To answer these questions, we analyzed mutant mice (named DAKO mice), in which isoform conversion from drebrin E to drebrin A is disrupted. In DAKO mouse brain, drebrin E continues to be expressed throughout life instead of drebrin A. Electrophysiological studies using hippocampal slices revealed that long-term potentiation of CA1 synapses was impaired in adult DAKO mice, but not in adolescents. In parallel with this age-dependent impairment, DAKO mice exhibited impaired hippocampus-dependent fear learning in an age-dependent manner; the impairment was evident in adult mice, but not in adolescents. In addition, histological investigation revealed that the spine length of the apical dendrite of CA1 pyramidal cells was significantly longer in adult DAKO mice than in wild-type mice. Our data indicate that the roles of drebrin E and drebrin A in brain function are different from each other, that the isoform conversion of drebrin is critical, and that drebrin A is indispensable for normal synaptic plasticity and hippocampus-dependent fear memory in the adult brain.

Keywords

drebrin A, drebrin E, isoform conversion, synaptic plasticity, fear learning, hippocampus

Abbreviations

DAKO mouse, drebrin A knockout mouse; LTP, long-term potentiation; NMDAR, N-methyl-D-aspartate receptor; WT, wild-type; fEPSP, field excitatory postsynaptic potential; D-APV, D-(-)-2-amino-5-phosphonovaleric acid; CS, conditioned stimulus; US, unconditioned stimulus; APP,

amyloid precursor protein; AMPA receptor, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

Introduction

Dendritic spines are tiny protrusions on the dendrites of neurons that serve as the postsynaptic sites of excitatory synapses. Dendritic spines contribute to the synaptic plasticity underlying higher brain functions such as learning and memory. Pathological changes associated with spine morphology and structural plasticity occur in a variety of neurological disorders accompanied by cognitive deficits, including Alzheimer's disease, fragile X syndrome, Down syndrome, alcohol abuse, epilepsy, and schizophrenia (Fiala et al., 2002). Regulation of the actin cytoskeleton, which is a major cytoskeletal component of dendritic spines (Landis and Reese, 1983), is important for spine morphology and structural plasticity (Matus, 2000; Carlisle and Kennedy, 2005; Tada and Sheng, 2006).

Using primary cultured neurons, we have demonstrated that the actin-binding protein drebrin plays a pivotal role in spine morphogenesis and plasticity (Sekino et al., 2007; Takahashi et al., 2009). Drebrin has two major isoforms in mammals: drebrin E (Embryonic isoform) and drebrin A (Adult isoform) (Shirao, 1989). These isoforms are derived from a single gene by alternative splicing (Kojima et al., 1993; Jin et al., 2002). The amino acid sequences are almost identical between the two isoforms, which include an actin-depolymerizing factor homology domain (Poukkula et al., 2011), an actin-remodeling domain (Hayashi et al., 1999; Grintsevich et al., 2010), a membrane-targeting domain (Xu and Stamnes, 2006), and Homer-binding sites (Shiraishi-Yamaguchi et al., 2009). One structural difference between the two isoforms is a 46-amino-acid sequence (named ins2) deleted or inserted just downstream of the actin-remodeling domain in drebrin E and drebrin A, respectively (Hayashi et al., 1999). The two drebrins exhibit differences in: (1) the time course of their expression during neuronal development (Shirao, 1989); (2) the type of cells or tissues expressing each isoform (Keon et al., 2000); and (3) their intracellular distribution (Aoki et al., 2005; Song et al., 2008). We have, therefore, hypothesized that drebrin E and drebrin A are functionally different in the brain.

To address this issue we have generated drebrin A-specific knockout (DAKO) mice, in which isoform conversion from drebrin E to drebrin A is disrupted (Kojima et al., 2010). We reported that drebrin A is involved in the homeostatic synaptic plasticity of the N-methyl-D-aspartate receptor (NMDAR) (Aoki et al., 2009) and context-dependent fear learning (Kojima et al., 2010). These results suggested a functional difference between drebrins E and A in NMDAR-dependent synaptic plasticity

in the hippocampus.

In the present study, we examined the electrophysiological and behavioral phenotypes of DAKO mice to elucidate the specific roles of drebrin A in synaptic plasticity. We found that hippocampal long-term potentiation (LTP) and hippocampus-dependent learning were impaired in adult DAKO mice.

Experimental procedures

Animals

Generation of DAKO mice has been described previously (Kojima et al., 2010). To maintain the strain of these mutant mice, heterozygous DAKO mice were mated with C57BL/6N mice purchased from Clea Japan, Inc. (Tokyo, Japan). Heterozygous offspring were then intercrossed to generate homozygous DAKO mice. Mice were housed in groups of three to five in a controlled environment with a 12-h light/dark cycle (lights on at 6:00 AM), an ambient humidity of $50 \pm 10\%$, and a room temperature of $23 \pm 1^\circ\text{C}$. Food and water were available *ad libitum*. After the mice were weaned, their genotypes were determined by PCR from tail DNA. At postnatal 8–52 weeks of age, male offspring of the DAKO mice were used in the experiments. Wild-type (WT) littermates were used as control animals. Experiments were designed so as to minimize the number of animals used and their suffering. All animal experiments were performed in accordance with regulations outlined by Japanese law and National Institutes of Health guidelines, and were approved by the Animal Care and Experimentation Committee of Gunma University.

Electrophysiological analyses

Hippocampal slices were prepared from adolescent (postnatal 8–9 weeks old), young adult (16–20 weeks old), and adult (28–42 weeks old) DAKO mice and their WT littermates using a standard procedure (Yasuda et al., 2003; Yasuda and Mukai, 2015). Slices were incubated in a submersion-type incubation chamber for at least two hours at a room temperature of $25 \pm 1^\circ\text{C}$, and then transferred to a recording chamber mounted on an upright microscope (BX51WI; Olympus, Tokyo, Japan) equipped with IR-DIC optics. Slices were perfused with a standard artificial cerebrospinal fluid at 32°C that was

bubbled continuously with 95% O₂ and 5% CO₂, and contained (in mM) 119 NaCl, 2.5 KCl, 4.0 CaCl₂, 4.0 MgSO₄, 1.0 NaH₂PO₄, 26.2 NaHCO₃, 11 glucose, and 0.1 picrotoxin. The Schaffer collateral/commissural pathway was stimulated using a glass electrode placed in the *stratum radiatum* and field excitatory postsynaptic potentials (fEPSPs) were recorded through a patch pipette that had a broken tip and was filled with normal artificial cerebrospinal fluid from the CA1 hippocampal region using a Multiclamp 700A (Molecular Devices, Sunnyvale, CA). Acquisition and analysis were performed using custom Igor Pro (WaveMetrics, Lake Oswego, OR) software routines. LTP was induced by two trains of 100 Hz 100 pulses at 0.05 Hz. Paired-pulse facilitation was induced by two pulses at intervals of 10, 20, 50, 100, 200, 500, and 1000 ms and paired-pulse ratios were calculated as the ratios of the slope of the second fEPSP to that of the first fEPSP. Post-tetanic potentiation was elicited by a train of 100 pulses at 100 Hz in the presence of 50 μ M D-(-)-2-amino-5-phosphonovaleric acid (D-APV). Results are represented as mean $\pm$ SEM.

Electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as described previously (Aoki et al., 2005; Ishizuka et al., 2014). Briefly, hippocampi were dissected from postnatal day 0 and 18, adolescent, young adult and adult mice. Then, the hippocampi were lysed and sonicated in 10-fold weight-to-volume ratio of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2% SDS, 20 mM NaF, 1 mM Na₃VO₄) supplemented with protease inhibitor cocktail, Complete Mini (Roche, Indianapolis, IN). After centrifugation (20,000x g), the supernatant was collected and protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories, Ind., Hercules, CA). Equal amounts of protein (15 μ g/lane) were subjected to SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA). The membranes were incubated with primary antibodies against pan-drebrin (clone M2F6, hybridoma supernatant) (Shirao and Obata, 1986), drebrin A (DAS2, Immuno-Biological Laboratories Co., Ltd. Gunma, Japan), and β -actin (clone AC-15; Sigma-Aldrich, St. Louis, MO). After incubation with horseradish peroxidase-conjugated secondary antibodies, peroxidase activity was detected using chemiluminescence reagents (Immobilon Western Chemiluminescent HRP Substrate, Merck

Millipore), and visualized with an image analyzer (LAS-3000, Fujifilm, Tokyo, Japan).

Golgi staining

Adult mice (26–36 weeks old) were deeply anesthetized with tribromoethanol (Avertin; 50 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Then brains were removed and processed by a rapid Golgi stain method with an FD Rapid GolgiStain Kit (FD NeuroTechnologies, Ellicott City, MD) according to the manufacturer's instructions. Briefly, whole brains were treated for silver impregnation for 2 weeks, cryoprotected for 1 week, and sectioned at 100 μ m on a cryostat. After sectioning, sections were developed, clarified, then coverslipped in resinous medium. During staining, image acquisition and analysis, observers were blind to the genotype of each animal. Using a microscope (Axioplan; Zeiss) with a 40 $\times$ 0.75 numerical aperture objective lens and a cooled CCD camera (CoolSnap fx; Photometrics, Tuscon, AZ), we obtained from each experimental animal 10 primary dendritic segments (100–250 μ m from the cell body) of CA1 pyramidal neurons in the hippocampus. For morphological analysis of dendritic spines, a z-series with 0.5 μ m intervals was created to visualize the entire extent of each dendritic process and also to capture spine details in all focal planes. We measured the number of dendritic spines per 100 μ m and spine length using Object-Image (N. Vischer; available at: <https://sils.fnwi.uva.nl/bcb/Object-Image/object-image.html>), which permits measurement of objects that extend across multiple focal planes.

Fear conditioning

Mice were divided into two age groups: adolescent (8–12 weeks old) and adult (26–52 weeks old). Two versions of fear conditioning, delayed fear conditioning and trace fear conditioning, were conducted.

Delayed fear conditioning was performed according to a previous procedure (Kojima et al., 2010). In brief, both initial assessment of electrical shock sensitivity and fear conditioning were performed in a clear acrylic cubic chamber of 1000 cm³ with a stainless steel grid floor (2-mm diameter rods, 5 mm apart) and a clear acrylic lid. The chamber was placed in a soundproof box with a

ceiling-mounted CCD camera connected to a video monitor and a computer. The grid floor was wired to a shock generator. An auditory signal was supplied from a loudspeaker located in the ceiling of the box. The fear conditioning experiment was controlled with TimeFZ1 software (O'Hara & Co., Tokyo, Japan). On the conditioning day, mice were placed individually in the chamber, and after a 60-s exploratory period, a tone (10 kHz, 70 dB) was delivered for 10 s; this served as the conditioned stimulus (CS). The CS co-terminated with the unconditioned stimulus (US), a scrambled electrical foot shock (0.3 mA, 1 s). The CS–US pairing was delivered twice at 20-s intervals, and 50 s later the animal was returned to its home cage. The chamber was wiped with ethanol after the conditioning of each animal. The mice were subjected to a context-dependent fear memory test 24 h after conditioning. Animals were placed in the original conditioning chamber for 30 s, and then freezing was measured for 60 s without delivery of either the tone or the footshock. In this test, the conditioning chamber served as the background CS for the animal. Behavior was recorded via CCD camera (two frames per second), and freezing was measured as an index of fear. Freezing was defined as complete immobility (excluding movement required for heartbeat and respiration) lasting longer than 1 s; freezing was calculated automatically by the TimeFZ1 software. Summed freezing time was scored as the percentage of freezing within a specified period (30 s).

Trace fear conditioning was performed essentially as described above except that we presented a CS and a US with a 30-s interval (Misane et al., 2005) and 50 s later the animal was returned to its home cage. Tone-dependent fear memory tests were conducted in a new chamber to separate tone-dependent fear memory from context-dependent fear memory. This chamber, although of the same size and located in the same soundproof box, differed from the conditioning chamber in that it was made of Plexiglas, had white screens on the side and back walls, and had a flat floor covered with wood chips. To further distinguish the experimental contexts, the tone-dependent tests were conducted under low illumination (30 lux during tone test vs. 120 lux during conditioning) and low background fan noise (50 dB during tone test vs. 55 dB during conditioning). The duration of the entire test was 180 s. Mice were placed individually in the chamber, and after 60 s, the tone was presented for 60 s without the foot shock. The mice were placed back in the home cage 60 s after the end of tone presentation. Freezing was measured as described above in the pre-tone, tone, and post-tone sessions.

Statistics

The statistical significance of differences between two groups was analyzed using the Student's *t* test or Welch's *t* test as applicable. For multiple comparisons, analysis of variance (ANOVA) with Tukey-Kramer test in EZR (Easy R) (Kanda, 2013) or Stat View software (SAS Institute Inc., Cary, NC) was used. For spine morphology analysis, the Kolmogorov-Smirnov test was also used. For all statistical analyses, a probability level of 0.01 or lower was considered statistically significant.

Results

Hippocampal LTP is attenuated in adult DAKO mice, but not in adolescent DAKO mice

We have previously reported that DAKO mice exhibit an impairment of context-dependent fear learning (Kojima et al., 2010). Many studies have suggested a close correlation between LTP and hippocampus-dependent learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Thus, we examined basal synaptic transmission and LTP in the CA1 region of the hippocampus. We compared general synaptic properties between adult WT and DAKO mice and there was no difference between the two groups in input-output relationship (Fig. 1A; WT, $n = 8$ from 2 mice; KO, $n = 7$ from 2 mice), paired pulse facilitation (Fig. 1B; WT, $n = 14$ from 3 mice; KO, $n = 18$ from 3 mice), or post-tetanic potentiation in the presence of APV (Fig. 1C; $n = 11$ from 3 mice; KO, $n = 11$ from 4 mice). These results suggest that the synaptic connections between Schaffer collaterals and CA1 pyramidal cells are functionally normal in DAKO mice.

In contrast, LTP induced by high-frequency stimulation (two 100-pulse trains at 100 Hz) was attenuated in adult DAKO mice (Fig. 2). The amplitude of LTP at 60 min after the stimulation was significantly reduced in adult DAKO mice (WT, $178.7 \pm 14.0\%$, $n = 18$ from 5 mice; KO, $133.2 \pm 8.7\%$, $n = 21$ from 4 mice; $p < 0.01$).

We have reported the time course of drebrin expression during neuronal development is different between the isoforms in the rat brains (Shirao, 1989), and we ensured that the drebrin isoforms express in a similar time course during neuronal development in the hippocampus of WT mice (Fig. 3). With drebrin-A-specific antibody drebrin A was not detected on postnatal day 0, but was

detected on postnatal day 18. Since then similar levels of drebrin A was detected (upper panel in Fig. 3). On the other hand, drebrin A was not detected in the hippocampus of DAKO mice. With a pan-drebrin antibody, drebrin E was strongly detected on postnatal day 0, and decreased on postnatal 18. After that drebrin E was hardly detected (middle panel in Fig. 3). In DAKO mice drebrin E was detected as previously reported (Fig. 3; Kojima et al., 2010). We also examined LTP in younger animals. LTP in young adults was not different between WT and DAKO mice (Fig. 4A; WT, $156.2 \pm 10.8\%$, $n = 13$ from 3 mice; KO, $144.6 \pm 16.8\%$, $n = 7$ from 3 mice). In adolescent animals, unexpectedly, LTP tended to be greater in DAKO mice than WT mice, although the difference was not significant (Fig. 4B; WT, $150.3 \pm 5.7\%$, $n = 17$ from 6 mice; KO, $184.0 \pm 14.6\%$, $n = 14$ from 4 mice; $p = 0.046$, Student's t test).

Spine length of the apical dendrite of CA1 pyramidal cells is longer in adult DAKO mice than in WT mice

Because abnormal spine shapes are often associated with impaired LTP (Bourne and Harris, 2008; Bosch and Hayashi, 2012; Bellot et al., 2014), we next analyzed spine morphology in the CA1 regions of the hippocampus of adult DAKO mice by Golgi staining (Fig. 5A). The spine length, width, and density of the apical dendrite of the CA1 pyramidal neurons of adult WT and DAKO mice were measured and compared (Fig. 5B–F). Spine density was comparable between WT and DAKO mice (Fig. 5B; WT, 102.1 ± 4.2 per 100 μm , $n = 30$ from 3 mice; KO, 99.0 ± 3.9 per 100 μm , $n = 30$ from 3 mice; Mann-Whitney's U test, $p = 0.584$). In contrast, spine length was significantly different between the two groups of mice (Fig. 5C; WT, 0.857 ± 0.017 μm , $n = 30$ neurons from 3 mice; KO, 0.978 ± 0.027 μm , $n = 30$ neurons from 3 mice; Student's t test, $p < 0.001$), although spine width was not different (Fig. 5D; WT, 0.539 ± 0.010 μm , $n = 30$ neurons from 3 mice; KO, 0.562 ± 0.011 μm , $n = 30$ neurons from 3 mice; Welch's t test, $p = 0.130$). A cumulative frequency plot of spine length confirmed a significant difference between WT and DAKO mice (Fig. 5E; WT, $n = 1476$ spines from 3 mice; KO, $n = 1421$ spines from 3 mice; Kolmogorov-Smirnov test, $p < 0.0001$). Interestingly, the cumulative frequency of spine width also showed a significant difference between groups (Fig. 5F; WT, $n = 1476$ spines from 3 mice; KO, $n = 1421$ spines from 3 mice; Kolmogorov-Smirnov test, $p <$

0.0001), showing that DAKO mice have spines whose width is more variable than those of WT mice. Thus, a lack of drebrin A seems to affect the morphology of dendritic spines in the adult brain, in spite of the presence of drebrin E.

Impairment of hippocampus-dependent fear in DAKO mice is age-dependent

The age-dependent phenotype of hippocampal synaptic plasticity described above suggests that the behavioral phenotype of DAKO mice might also show age-dependence. To address this possibility we divided DAKO mice into adult and adolescent groups, and examined fear conditioning.

In both groups, DAKO mice exhibited freezing comparable with that of WT mice during the last 30 s of the training session immediately after tone-footshock pairing. This indicates that DAKO mice possess a normal sensitivity to an aversive footshock. Adult DAKO mice exhibited a lower freezing rate in the conditioning chamber 1 day after conditioning (Fig. 6A) (WT, $n = 15$; KO, $n = 15$; Student's t test, $p < 0.01$), while adolescent groups of WT and DAKO mice exhibited equivalent levels of freezing (Fig. 6B) (WT, $n = 14$; KO, $n = 15$; Student's t test; $p = 0.20$). Thus, the impairment of context-dependent fear learning in DAKO mice was age-dependent, and consistent with the age-dependent impairment of hippocampal LTP.

To confirm this, we examined another hippocampus-dependent version of fear conditioning: trace fear conditioning (Misane et al., 2005). One day after the conditioning, the degrees of freezing in adult DAKO mice tended to be less during the tone session and the post-tone session than those in WT mice, although the difference was not significant (Fig. 6C) (two-way ANOVA (genotype and session), $F(1, 25) = 4.95$, $p = 0.035$). On the other hand, adolescent DAKO and WT mice exhibited similar degrees of freezing during the tone and post-tone sessions (Fig. 6D) (two-way ANOVA, $F(1, 18) = 1.76$, $p = 0.20$). We did however frequently observe exaggerated responses (tail wagging and jumping) during the tone and post-tone sessions in adolescent DAKO mice. These results indicate that the disruption of drebrin isoform conversion affects hippocampus-dependent fear learning in adulthood.

Discussion

We have previously demonstrated that DAKO mice exhibit an impairment in hippocampus-dependent

fear memory (Kojima et al., 2010). In this study, we demonstrated that DAKO mice exhibit an impairment of hippocampal LTP and that the impairment in both the LTP and the fear learning is age-dependent. Thus, our results indicate that the impairment of LTP causes impairment in adult hippocampus-dependent fear memory, and suggest that the isoform conversion from drebrin E to drebrin A plays an indispensable role in normal hippocampal function.

The conversion from drebrin E to drebrin A is regulated in a cell-type-specific and developmental stage-specific manner (Shirao, 1989; Keon et al., 2000). Drebrin A expression is restricted to neurons and is preferentially localized within the dendritic spines of mature neurons. Conversely, drebrin E is expressed in neuronal and non-neuronal cells. In embryonic neurons, drebrin E is distributed in cell somata and processes, but not in dendritic spines. However, we have previously shown that drebrin E is capable of locating to dendritic spines in mature neurons in the absence of drebrin A (Kojima et al., 2010).

We found that LTP is impaired in adult brain of DAKO mice, although drebrin E is abundantly expressed instead of drebrin A. LTP is induced by NMDARs and is associated with long-lasting incorporation of AMPA receptors in postsynaptic membrane and spine enlargement (for review, see (Bourne and Harris, 2008; Bosch and Hayashi, 2012; Bellot et al., 2014). Alteration in spine morphology is tightly coupled with dynamic changes in the actin-based cytoskeleton (Fifkova and Delay, 1982; Matus, 2000; Matsuzaki et al., 2004). Drebrin A forms a stable actin cytoskeleton in dendritic spines (Mikati et al., 2013; Shirao and Gonzalez-Billault, 2013). On the other hand, drebrin E does not bind to actin filament strongly as drebrin A in vivo (Aoki et al., 2005; Kojima et al., 2010), although in vitro study shows the equivalent F-actin-binding ability between drebrins E and A (Ishikawa et al., 1994). Therefore, the stability of drebrin A-actin complex may be critical for hippocampal function (Mizui et al., 2014), while the stability of drebrin E-actin complex might not be sufficiently strong to play the same role.

NMDAR activation induces a transient exodus of drebrin A from spines and a subsequent robust re-entry of drebrin A into dendritic spines in vitro (Sekino et al., 2006; Bosch and Hayashi, 2012; Mizui et al., 2014). Similarly in vivo, LTP is accompanied by a long-lasting increase in spine drebrin content (Fukazawa et al., 2003). On the other hand, blockade of NMDAR activity for 30 min increases

the proportion of drebrin-immunopositive spines (Fujisawa et al., 2006). Thus, the amount of drebrin A within spines is regulated by NMDAR activity. Therefore, another possible mechanism of impaired LTP in adult DAKO mice may be due to the difference in the regulation by NMDAR activity between drebrins A and E.

We show impaired LTP and spine elongation without affecting spine density in the hippocampus of adult DAKO mice. Although we do not know the causal relationship between impaired LTP and spine elongation in DAKO mice, the phenotypes caused by drebrin A-knockout have some similarity to those of myosin IIB-knockdown. Similar to the DAKO mice, the knockdown of myosin IIB results in elongation of dendritic spines (Ryu et al., 2006) and also shows LTP impairment without changes in basal synaptic transmission (Rex et al., 2010). Myosin II is known to interact with drebrin (Hayashi et al., 1996). Furthermore, we have previously reported that the LTP requires NMDAR-induced ATPase activity of myosin IIB via exodus of drebrin A-actin from spines during the early phase of chemical LTP (Mizui et al., 2014). Thus, it may be possible that both elongation of dendritic spines and LTP impairment are caused at least in part by the disruption of myosin IIB-drebrin interaction in DAKO mice. No change in basal transmission might be due to no change in spine density and spine width in DAKO mice.

Drebrin is capable of controlling NMDAR content at synapses. Antisense down-regulation of drebrin A blocks the homeostatic synaptic accumulation of NMDARs (Takahashi et al., 2006). The rapid increase in NR2A subunit-positive spines after a brief blockade of NMDAR activity does not occur in the cerebral cortex of DAKO mice (Aoki et al., 2009). Therefore, the impairment of activity-dependent NMDAR accumulation in synapses might cause LTP impairment in DAKO mice.

During aging, memory retention and the maintenance of LTP are progressively impaired (Rosenzweig and Barnes, 2003; Lynch et al., 2006), and aging-associated impairment of synaptic protein synthesis has been reported (Ryan et al., 2015). Indeed, drebrin A content has been reported to be decreased in aged human brain (Hatanpaa et al., 1999). Interestingly, in aged Tg2576 mice, which overexpress a mutant form of amyloid precursor protein, APP KM670/671NL, and develop progressive cognitive deficits, dietary depletion of n-3 polyunsaturated fatty acids leads to the degradation of actin (an increase in fractin) and a decrease in postsynaptic proteins including drebrin

(Calon et al., 2004). Thus, a decrease in drebrin A and the resulting impairment in synaptic plasticity may critically induce memory dysfunction in the aged brain.

At present we cannot interpret the molecular basis underlying the age-dependency of LTP impairment in DAKO mice. However, age-related modulation of LTP by antioxidative enzymes has been reported (Watson et al., 2006), suggesting that age-related changes in vulnerability to oxidative stress might be involved in the age-dependent impairment of LTP in DAKO mice. In fact, oxidative stress can increase multimerization of the drebrin-binding protein Homer, which accelerates Homer-drebrin binding (Nepliouev et al., 2011). It will be important to determine whether oxidative stress affects Homer-drebrin binding in a drebrin isoform-specific manner.

Age-dependent LTP impairment also occurs in calcium/calmodulin-dependent kinase II (CaMKII) knockout (KO) mice (Yasuda et al., 2003) and GluA1 KO mice (Jensen et al., 2003). It has been shown that protein kinase A (PKA) mediates LTP in the early postnatal hippocampus and CaMKII starts to replace PKA at around the second postnatal week, resulting in the delayed impairment of LTP in CaMKII KO mice (Yasuda et al., 2003). There might be unidentified mechanisms other than drebrin A-dependent mechanism for LTP in adolescents.

In conclusion, we demonstrate that isoform conversion of drebrin is critical, and that drebrin A is indispensable for adult hippocampal synaptic plasticity and hippocampus-dependent fear learning. The phenotype of DAKO mice suggests functional differences between drebrin A and drebrin E. Determining the protein dynamics of each drebrin isoform in dendritic spines under normal and LTP conditions might help us to understand the molecular basis of the functional differences between the two isoforms. In addition, the role of drebrins in early synaptic development before and just after drebrin E-A conversion occurs should also be evaluated in the future study.

Author contributions

K.N., H.Y., and T.S. designed this research; K.N., H.Y., K.H., and Y.I. collected data; and all authors wrote the paper.

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Figure legends

Fig. 1. General electrophysiological properties in the CA1 region of hippocampal slices. Synaptic transmission was examined in adult DAKO mice compared with age-matched WT mice. The fEPSP was evoked by Schaffer collateral stimulation and recorded from the CA1 region. (A) Relationship between the amplitude of fiber volley and the EPSP slope (input-output; WT, $n = 8$ from 2 mice; DAKO, $n = 7$ from 2 mice); (B) paired pulse ratio observed in the various inter-stimulus intervals (WT, $n = 14$ from 3 mice; DAKO, $n = 18$ from 3 mice); (C) post-tetanic potentiation evoked in the presence of APV (WT, $n = 11$ from 3 mice; DAKO, $n = 11$ from 4 mice). No measure was significantly different between WT and DAKO mice.

Fig. 2. LTP is impaired in adult DAKO mice. (A) Examples of LTP in the CA1 region induced by two 100-pulse stimulations at 100 Hz (arrows) of the Schaffer collateral/commissural fibers in adult WT and DAKO mice. (B) Summary of LTP in adult WT and DAKO mice. The amplitude of LTP 60 min after induction was significantly attenuated in adult DAKO mice compared with in adult WT mice (WT, $n = 18$ from 5 mice; DAKO, $n = 21$ from 4 mice; *: $p < 0.01$, Student's t test).

Fig. 3. Developmental changes in drebrin expression in WT and DAKO mice. Upper and middle panel shows western blot for drebrin A and drebrin E and A. Open and closed arrowheads indicate drebrin E and drebrin A. Lower panel shows b-actin as an internal control. 1: postnatal day 0, 2: postnatal day 18, 3: adolescent, 4: young adult, 5: adult, 6: young adult.

Fig. 4. LTP is not significantly altered in young adult and adolescent DAKO mice. Summary of LTP in young adult (A) and adolescent (B) mice. LTP in young adult DAKO mice was comparable to that in young adult WT mice (A; $n = 13$ from 3 mice; KO, $n = 7$ from 3 mice). LTP tended to be larger in adolescent DAKO mice than in WT mice, however the difference was not significant (B; WT, $n = 17$ from 6 mice; KO, $n = 14$ from 4 mice; $p = 0.046$, Student's t test).

Fig.5. Spine morphology of the pyramidal neurons in the CA1 region of the hippocampus. Spine morphology was examined in adult DAKO mice compared with age-matched wild-type mice. (A) Golgi-stained CA1 pyramidal neurons of adult WT and DAKO mice. Boxed regions in the upper panels are shown below at higher magnification in the middle panels. Drawings of the dendritic spines are shown in the bottom panels. Spine length and width were measured as shown in white bars in the bottom panels. Scale bars: 2 μm for upper panels and 1 μm for middle and bottom panels. (B) Quantitative analyses of the spine density (number of spines per 100 μm) (WT, $n = 30$ neurons from 3 mice; DAKO, $n = 30$ neurons from 3 mice; $p = 0.583$, Student's t test). (C) Quantitative analyses of spine length (WT, $n = 30$ neurons from 3 mice; DAKO, $n = 30$ neurons from 3 mice; $p < 0.001$, Student's t test). (D) Quantitative analyses of spine width (WT, $n = 30$ neurons from 3 mice; DAKO, $n = 30$ neurons from 3 mice; $p = 0.130$, Welch's t test). (E) Cumulative frequency plot of the distribution of spine length in adult WT and DAKO mice (WT, $n = 1476$ spines from 3 mice; DAKO, $n = 1421$ spines from 3 mice; $p < 0.0001$, Kolmogorov-Smirnov test). (F) Cumulative frequency plot of the distribution of spine width in adult WT and DAKO mice (WT, $n = 1476$ spines from 3 mice; KO, $n = 1421$ spines from 3 mice; $p < 0.0001$, Kolmogorov-Smirnov test).

Fig.6. Fear conditioning in DAKO mice. (A, B) Context-dependent freezing evoked in the shock chamber one day after delay conditioning was compared between DAKO and age-matched WT mice. The extent of freezing was significantly attenuated in adult DAKO mice (A, WT mice, $n = 15$; DAKO mice, $n = 15$, *: $p < 0.01$, Student's t test), but was not different between animal groups in adolescent mice (B, WT mice, $n = 14$; DAKO mice, $n = 15$). (C, D) Tone-dependent freezing in the test chamber one day after trace conditioning was compared between DAKO and age-matched WT mice. The extent of freezing during the tone and post-tone sessions tended to be attenuated in adult DAKO mice,

although the difference was not significant (C, WT mice, $n = 15$; DAKO mice, $n = 15$; $p = 0.035$, Tukey-Kramer test), while it was not different between groups of adolescent mice (D, WT mice, $n = 10$; DAKO mice, $n = 10$).

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A **B** **C**

A

B adult

fig 2

fig 3

A young adult

B adreescence

A WT DAKO

B

C

D

E

F