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Involvement of l-afadin, but not s-afadin, in the formation of puncta adherentia junctions of hippocampal synapse

Tomohiko Maruo[1, 2], Shotaro Sakakibara[1], Muneaki Miyata[1], Yu Itoh[1], Souichi Kurita[1], Kenji Mandai[1, 3], Takuya Sasaki[2], and Yoshimi Takai[1]

[1]Division of Pathogenetic Signaling, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, 1-5-6 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan.

[2]Department of Biochemistry, Tokushima University Graduate School of Medical Sciences, 3-18-15, Kuramoto-cho, Tokushima 770-8503, Japan

[3]Department of Biochemistry, Kitasato University School of Medicine, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0374, Japan

Corresponding author:

Yoshimi Takai MD, PhD

Division of Pathogenetic Signaling, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, 1-5-6 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

E-mail: ytakai@med.kobe-u.ac.jp

ABSTRACT

A hippocampal mossy fiber synapse has a complex structure in which presynaptic boutons attach to the dendritic trunk by puncta adherentia junctions (PAJs) and wrap multiply-branched spines, forming synaptic junctions. It was previously shown that afadin regulates the formation of the PAJs cooperatively with nectin-1, nectin-3, and N-cadherin. Afadin is a nectin-binding protein with two splice variants, l-afadin and s-afadin: l-afadin has an actin filament-binding domain, whereas s-afadin lacks it. It remains unknown which variant is involved in the formation of the PAJs or how afadin regulates it. We showed here that re-expression of l-afadin, but not s-afadin, in the *afadin*-deficient cultured hippocampal neurons in which the PAJ-like structure was disrupted, restored this structure as estimated by the accumulation of N-cadherin and αN-catenin. The l-afadin mutant, in which the actin filament-binding domain was deleted, or the l-afadin mutant, in which the αN-catenin-binding domain was deleted, did not restore the PAJ-like structure. Both l-afadin and s-afadin have the same αN-catenin-binding domain, but s-afadin less efficiently recruited αN-catenin to form the PAJ-like structure than l-afadin, although l-afadin showed this activity. These results indicate that l-afadin, but not s-afadin, regulates the formation of the hippocampal synapse PAJ-like structure through the binding to actin filaments and αN-catenin. We further found here that l-afadin bound αN-catenin, but not γ-catenin, whereas s-afadin bound γ-catenin, but hardly αN-catenin. These results suggest that the inability of s-afadin to form the hippocampal synapse PAJ-like structure is due to its inability to efficiently bind αN-catenin.

Keywords

24 synapse, puncta adherentia junction, nectin, afadin, catenin, actin filament

1. Introduction

In the nervous system, an axon of one neuron adheres to the dendrites of other neurons to form synapses, leading to the formation of neural circuits. At least two adhesion apparatuses have been identified at synapses: one is synaptic junctions that are present in all chemical synapses and the other is puncta adherentia junctions (PAJs) that are present in many, but not all, synapses (Spacek and Lieberman, 1974; Uchida et al., 1996). PAJs are particularly developed at subsets of large synapses including a hippocampal mossy fiber synapse, which is formed between an axon terminal of a dentate granule cell, called hippocampal mossy fiber bouton, and the proximal dendrites of CA3 pyramidal cells in the hippocampus, and has a large and complex structure (Rollenhagen et al., 2007; Wilke et al., 2013). Usually two to four boutons, sometimes one bouton, attach to a dendritic shaft by multiple PAJs and wrap around a highly branched dendritic spine, known as thorny excrescences, where multiple synaptic junctions, neurotransmitter release sites, are formed (Amaral and Dent, 1981). Postsynaptic densities (PSDs) are located at the heads of the spine branches and face toward active zones (AZs). A single mossy fiber bouton has about 20 AZs in mice. Many synaptic vesicles (SVs) are present in the mossy fiber bouton (Chamberland and Toth, 2016; Santos et al., 2009). Because of the enormous size of the mossy fiber synapse, which triggers large synaptic responses in the postsynapse at the proximal dendrite of the CA3 pyramidal cell (Bischofberger et al., 2006; Henze et al., 2000), each synaptic transmission via a single mossy fiber is thought to reliably evoke an action potential in a CA3 pyramidal cell, allowing information to flow efficiently. In addition, the mossy fiber synapse exhibits presynaptic long-term potentiation and long-term depression by high and low frequency stimulations, respectively (Yokoi et al., 1996;

Zalutsky and Nicoll, 1990), which are implicated in cellular bases for learning and memory (Kim and Linden, 2007; Malenka and Bear, 2004). The mossy fiber synapse alters its morphologies in response to electrical stimulation and learning (Bell et al., 2014; De Paola et al., 2003; Maruo et al., 2016; Zhao et al., 2012). Despite the functional importance of this synapse for information transfer in the hippocampus, the molecular mechanisms for the formation of such a large and complex structure of the hippocampal mossy fiber synapse and its structural and functional relationship remain to be elucidated.

Afadin (also known as an *Afdn* gene product) was originally purified from rat brain as a novel actin filament (F-actin)-binding protein and displayed a primary sequence similar to that of the human *ALL-1 fused gene from chromosome 6* (*AF-6*) gene product. (Mandai et al., 1997). It was purified as two variants and the larger and smaller ones with molecular masses of 205 kDa and 190 kDa, which were named l-afadin and s-afadin, respectively (Mandai et al., 1997). The *AF-6* gene was identified to be a fusion partner of the *lysine-specific methyltransferase 2A* (*Kmt2a*) gene (alias *ALL-1*) in pediatric acute myeloid leukemia with chromosome translocation (Prasad et al., 1993). The putative *AF-6* gene product lacked the C-terminal 61 amino acids compared with s-afadin. Instead, the *AF-6* gene contained an intron sequence in its C-terminus. Therefore, the *AF-6* gene product is now considered not to exist in nature. l-Afadin has multiple domains, including two Ras-association domains, a forkhead-associated domain, a dilute domain, a PSD-95/Dlg/ZO-1 domain, three proline-rich regions, and an F-actin-binding (FAB) domain in this order from the N-terminus, whereas s-afadin lacks the third proline-rich region and the FAB domain (Takai et al., 2008). Thus, afadin is largely classified into two groups: l-afadin with the FAB domain and s-afadin without the third proline-rich region and

this domain. l-Afadin is expressed widely in all tissues thus far examined whereas s-afadin is specifically expressed in the brain (Mandai et al., 1997).

Both l-afadin and s-afadin are bound to cell adhesion molecule nectin, which comprises a family consisting of four members (nectin-1, nectin-2, nectin-3, and nectin-4) (Takai et al., 2008). l-Afadin as well as N-cadherin and αN-catenin is symmetrically localized at the presynaptic and postsynaptic sides of the mossy fiber synapse PAJs whereas nectin-1 and nectin-3 are asymmetrically localized at the presynaptic and postsynaptic sides, respectively (Mizoguchi et al., 2002; Nishioka et al., 2000). Studies using the cultured hippocampal neurons from *afadin*$^{f/f}$;Nestin-Cre mice have demonstrated that the accumulation of the representatives of the PAJ proteins, nectin-1, nectin-3, and N-cadherin, is markedly reduced, whereas the accumulation of the AZ protein bassoon and the SV protein of excitatory synapses VGLUT1 is partly reduced (Toyoshima et al., 2014). These results suggest that afadin plays roles in the structural and functional differentiations of the hippocampal mossy fiber synapse. However, the detailed roles of afadin in the structural and functional differentiations of the hippocampal mossy fiber synapse *in vivo* remained elusive.

The *in vivo* roles of afadin in the structural and functional differentiations of the hippocampal mossy fiber synapse were recently investigated using the *afadin*$^{f/f}$;Emx1-Cre mice (Geng et al., 2017; Sai et al., 2017). Transmission electron microscopy analysis revealed that typical PAJs with prominent symmetrical plasma membrane darkening undercoated with the thick filamentous cytoskeleton are observed at the synapse of the control mice, whereas at the synapse of the *afadin*-deficient mice, atypical PAJs with the symmetrical plasma membrane darkening, which is much less in thickness and darkness

than those of the typical PAJs, are observed (Sai et al., 2017). Immunoelectron microscopy analysis revealed that nectin-1, nectin-3, and N-cadherin are localized at the typical PAJs, whereas nectin-1 and nectin-3 are localized at the atypical PAJs to extents lower than those at the typical PAJs and N-cadherin is localized at their non-junctional flanking regions (Sai et al., 2017). These results indicate that the atypical PAJs are formed by *trans*-interacting nectin-1 and nectin-3 independently of afadin and N-cadherin whereas the typical PAJs are formed by afadin and *trans*-interacting nectin-1, nectin-3, and N-cadherin, implying that afadin plays a role in the formation of the typical PAJs by recruiting N-cadherin to the atypical PAJs. The studies by serial block face-scanning electron microscopy further showed that the complexity of postsynaptic spines and mossy fiber boutons, the number of spine heads, the area of the PSDs, and the density of the SVs docked to the AZs are decreased in the *afadin*-deficient synapse (Sai et al., 2017). These results collectively indicate that afadin plays multiple roles in the complex ultrastructural morphogenesis of the hippocampal mossy fiber synapse. Consistent with these morphological results, the electrophysiological studies revealed that both the release probability of glutamate and the postsynaptic responsiveness to glutamate are markedly, but not completely, reduced in the *afadin*-deficient mossy fiber synapse, whereas neither long-term potentiation nor long-term depression is affected (Geng et al., 2017). These results collectively indicate that afadin plays multiple roles in the presynaptic and postsynaptic functions of the hippocampal mossy fiber synapse.

However, it remains unknown which splice variant of afadin, l-afadin or s-afadin, regulates the formation of the mossy fiber synapse typical PAJs or how the afadin variant(s) regulates it. In the present study, by using the *afadin*-deficient cultured hippocampal neurons in which the PAJ-like structure was not formed, we addressed these issues and

showed that l-afadin, but not s-afadin, is involved in the formation of the PAJ-like structure through binding to F-actin and αN-catenin and that the inability of s-afadin to form the PAJs-like structure is due to its inability to efficiently bind αN-catenin.

2. Materials and Methods

2.1. Mice

The *afadin*-floxed mice, Nestin-Cre mice, and Emx1-Cre mice were described previously (Gorski et al., 2002; Majima et al., 2009; Tronche et al., 1999). They were kept on a C57BL/6J background. The heterozygous mice carrying the *afadin* conditional allele are referred to as *afadin*$^{+/f}$. The mutant and control samples were prepared from the same litter. All animal experiments were performed in accordance with the guidelines of the institution and approved by the administrative panel on laboratory animal care of Kobe University. This study was approved by the president of Kobe University after being reviewed by the Kobe University Animal Care and Use Committee (Permit Numbers: P130205 and 2-24-03-02).

2.2. Immunofluorescence microscopy

Immunofluorescence microscopy of brain sections was performed as described previously (Toyoshima et al., 2014). In brief, mice were deeply anesthetized and transcardially perfused at room temperature with PBS containing heparin and (*p*-amidinophenyl)methanesulfonyl fluoride (Nacalai Tesque, Kyoto, Japan), followed by perfusion of 2% paraformaldehyde in 1 × Hanks' Balanced Salt Solution with Ca^{2+} and

142 Mg^{2+} (Thermo Fisher Scientific, Waltham, MA) containing 10 mM HEPES, 1 mM sodium
143 pyruvate, and 4% sucrose. After dehydration with 30% sucrose in phosphate buffered saline,
144 whole brains were embedded in OCT compounds (Sakura Finetek, Tokyo, Japan). Cryostat
145 sections were incubated at 65°C for 20 min in HistoVT One antigen retrieval solution
146 (Nacalai Tesque) and then incubated with 1% bovine serum albumin, 10% normal goat
147 serum, and 0.25% Triton X-100 in phosphate buffered saline at room temperature for 30
148 min. The sections were stained with the indicated antibodies (Abs), and then with
149 appropriate fluorophore-conjugated secondary Abs (1:300). Confocal image acquisition was
150 performed on a LSM510 META confocal laser-scanning microscope (Carl Zeiss, Jena,
151 Germany) under the same conditions for both the control and *afadin*-deficient brain
152 sections.
153
154 *2.3. Dissociated culture of hippocampal neurons*
155
156 Dissociated hippocampal neurons were prepared as described (Toyoshima et al., 2014).
157 In brief, the hippocampal neurons dissociated with trypsin were plated at a density of 5 to 7
158 $\times 10^3$ cells/cm^2 on poly-L-lysine-coated coverslips in Neurobasal Medium (Thermo Fisher
159 Scientific) containing B27 Supplement (Thermo Fisher Scientific) and GlutaMAX (Thermo
160 Fisher Scientific) and cultured in a 5% CO_2 incubator. For re-expression of EGFP-afadin in
161 the *afadin*-deficient hippocampal neurons obtained from *afadin*$^{f/f}$;Nestin-Cre mice, pEGFP
162 (Takara, Kusatsu, Japan), pEGFP-l-afadin, or pEGFP-s-afadin was introduced with an
163 Amaxa Nucleofector (Lonza, Cologne, Germany) at the time of seeding according to the
164 manufacturer's protocol. At 14 days *in vitro* (DIV), the cells were fixed and immunostained
165 with the indicated Abs. The fields, which contained EGFP-positive and EGFP-negative

neuronal dendrites (judged by the presence of MAP2 immunoreactivities) of the neurons showing typical pyramidal cell-like morphologies, were chosen and imaged by the confocal microscopy using identical image acquiring setting throughout the experiments without saturation of the signals. Cluster intensities of N-cadherin and αN-catenin were measured using the ImageJ software and averaged for both neurons and the ratio was calculated per each image.

2.4. Abs

Rabbit polyclonal anti-l-afadin, rabbit anti-l/s-afadin, rabbit polyclonal anti-nectin-1, and rabbit polyclonal anti-nectin-3 were prepared as described (Mandai et al., 1997; Satoh-Horikawa et al., 2000; Takahashi et al., 1999). Rat monoclonal anti-αN-catenin clones NCAT2 and NCAT5 are kind gifts from Masatoshi Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan). The Abs listed below were purchased from commercial sources: guinea pig polyclonal anti-VGLUT1 (Merck Millipore, Billerica, MA), mouse monoclonal anti-N-cadherin, clone 32 (BD Biosciences, San Jose, CA); rabbit polyclonal anti-GFP (Thermo Fisher Scientific); mouse monoclonal anti-FLAG M2 (Merck Millipore); rabbit polyclonal anti-FLAG (Merck Millipore); mouse monoclonal anti–γ-catenin (BD Biosciences); rabbit polyclonal anti-β-catenin (Merck Millipore); and chicken polyclonal anti-MAP2 (abcam, Cambridge, UK). Alexa Fluor-conjugated goat secondary Abs (Thermo Fisher Scientific) were used for immunocytochemistry.

2.5. Constructs

The expression plasmids for EGFP-tagged or FLAG-tagged rat l-afadin and rat s-afadin, were constructed as described previously (Kurita et al., 2013). The cDNAs for mouse αE-catenin and αN-catenin are kind gifts from Masatoshi Takeichi (RIKEN Center for Developmental Biology). The expression plasmid for γ-catenin is a kind gift from Akira Nagafuchi (Nara Medical University, Nara, Japan). The expression plasmid for an EGFP-tagged l-afadin mutant lacking the FAB domain, or an EGFP-tagged l-afadin mutant lacking the αE-catenin-binding domain, FLAG-tagged afadin fragments, a glutathione S-transferase (GST)-tagged αE-catenin fragment (385–651 amino acids (aa)), a GST-tagged αE-catenin fragment (507–631 aa), and a GST-tagged αN-catenin fragment (505–629 aa) were constructed using PCR-based standard molecular biology techniques.

2.6. Immunocytochemistry

For immunostaining of the cultured hippocampal neurons, the cells were fixed with the fixative containing 2% paraformaldehyde, 4% sucrose, 1 mM sodium pyruvate, Hanks' Balanced Salt Solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Thermo Fisher Scientific), and 10 mM HEPES (pH 7.3) at 37°C for 10 min. The fixed cells were permeabilized with 0.25% Triton-X in Tris-buffered saline (T-TBS) containing 1 mM $CaCl_2$ and 0.005% Tween-20 at room temperature for 5 min, and then blocked in T-TBS containing 10% goat serum at room temperature for 20 min. Then, the cells were incubated with primary Abs in T-TBS containing 10% goat serum at 4°C overnight. After three washes in T-TBS at room temperature for 5 min each, the cells were incubated with Alexa

Fluor-conjugated secondary Abs (Thermo Fisher Scientific) at room temperature for 45 min, followed by three washes in T-TBS at room temperature for 5 min each. The samples were then mounted in a FluorSave reagent (Merck Millipore). Maximum signal intensity projection images were created from around 10 confocal images collected at a 0.4-μm step along the z-axis with an LSM510 META (Carl Zeiss) confocal laser-scanning microscope under the same conditions for both the control and *afadin*-deficient neurons.

2.7. Immunoprecipitation assay

Procedures were conducted at 4°C or on ice. The brains of male ICR mice at postnatal day 21 were homogenized in 8-fold brain volumes of a buffer containing 20 mM HEPES (pH 7.4), 320 mM sucrose, 5 mM EDTA, 5 mM EGTA supplemented with protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupetin, 1 μg/ml pepstatin, and 500 μM benzamidine). The homogenate was subjected to centrifugation at 3,000 × *g* for 15 min. The supernatant was further subjected to centrifugation at 38,400 × *g* for 15 min. The membrane pellet was further washed with a buffer containing 20 mM HEPES (pH 7.4), 5 mM EDTA, and 5 mM EGTA supplemented with the protease inhibitors. The membrane pellet was re-suspended in a buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.25% dodecyl maltoside supplemented with the protease inhibitors. After a 3-h incubation on a rocking platform, insoluble materials were removed by a centrifugation at 40,000 × *g* for 30 min and the supernatant was used as the lysate of the light membrane fraction. This lysate was then incubated overnight on a rocking platform with the control rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), the anti-l-afadin polyclonal Ab (pAb), or the anti-l/s-afadin pAb each of which

was covalently linked to protein A magnetic beads (New England Biolabs, Ipswich, MA). After extensive washes, l-afadin and its binding proteins or l-afadin, s-afadin, and their binding proteins captured by the immune-affinity beads were eluted with a buffer containing 0.1 M glycine (pH 2.5) and 0.25% dodecyl maltoside. The samples were then neutralized with 100 mM Tris-HCl (pH 8.8), and subjected to SDS-PAGE followed by Western blotting.

2.8. Western blotting

The lysates of the light membrane fraction of mouse brain, and the immunoprecipitated samples were mixed with an SDS sample buffer and boiled for 5 min. The samples were then separated by SDS-PAGE and transferred to PVDF membranes (Merck Millipore). After being blocked with 5% skim milk in Tris-buffered saline plus 0.05% Tween 20, the membranes were incubated with the indicated Abs. After three washes in Tris-buffered saline plus 0.05% Tween 20, the membranes were incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG Ab. The signals for the proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore).

2.9. Statistical analysis

Statistical analysis of the difference between mean values was performed with the two-tailed Student's *t*-test or paired *t*-test. The criterion for statistical significance was set at $p < 0.05$. All values are reported as the mean ± s.e.m.

3. Results

3.1. Requirement of afadin for the localization of αN-catenin at the hippocampal mossy fiber synapse typical PAJs

It was previously shown that *trans*-interacting nectin-1, nectin-3, and N-cadherin and their respective binding proteins l-afadin and αN-catenin are localized at the hippocampal mossy fiber synapse typical PAJs of the wild-type mice (Mizoguchi et al., 2002), whereas *trans*-interacting nectin-1 and nectin-3, but not *trans*-interacting N-cadherin or l-afadin, are localized at the hippocampal mossy fiber synapse atypical PAJs of the *afadin*$^{f/f}$;Emx1-Cre mice (Sai et al., 2017). On the basis of these observations, it was previously described that afadin plays a role in the formation of the typical PAJs by recruiting N-cadherin to the atypical PAJs (Sai et al., 2017). However, the localization of αN-catenin at the hippocampal mossy fiber synapse atypical PAJs has not been studied in the *afadin*-deficient mice. We therefore first examined this issue by immunofluorescence microscopy. The immunofluorescence signal for αN-catenin was observed as dots in the stratum lucidum of the CA3 area of the hippocampus of the wild-type mice (**Fig. 1, A and B)**. This signal pattern for αN-catenin was similar to that for l-afadin. However, in the stratum lucidum of the CA3 area of the hippocampus of the *afadin*-deficient mice, the signal for αN-catenin was markedly decreased, compared with those in the control stratum lucidum (**Fig. 1, A and B**). Together with the previous findings (Sai et al., 2017), these results indicate that afadin is required for the localization of αN-catenin as well as nectin-1, nectin-3, and N-cadherin at the hippocampal mossy fiber synapse typical PAJs.

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284 *3.2. Involvement of l-afadin, but not s-afadin, in the formation of the PAJ-like structure in*
285 *the cultured hippocampal neurons*

286

287 It was previously shown that the immunofluorescence signals for nectin-1, nectin-3,
288 l-afadin, N-cadherin, and β-catenin are accumulated along the dendrites to form the
289 PAJ-like structure in the control cultured hippocampal neurons obtained from the wild-type
290 mice, whereas the signals for all of these components are decreased along the dendrites in
291 the *afadin*-deficient cultured hippocampal neurons (Toyoshima et al., 2014). We next
292 examined by using the control and *afadin*-deficient cultured hippocampal neurons obtained
293 from the wild-type and the *afadin*$^{f/f}$;Nestin-Cre mice, respectively, which splice variant,
294 l-afadin or s-afadin, is involved in the formation of the PAJ-like structure. In Western
295 blotting, l-afadin and s-afadin were expressed in the control cultured hippocampal neurons
296 at 14 DIV whereas both the proteins were undetectable in the *afadin*-deficient neurons (**Fig.**
297 **2A**). Re-expression of EGFP-tagged l-afadin in the *afadin*-deficient cultured hippocampal
298 neurons significantly increased the signals for N-cadherin and αN-catenin, compared with
299 those of the adjacent untransfected neurons (**Fig. 2, B-D**). In contrast, re-expression of
300 neither EGFP-tagged s-afadin nor EGFP affected the signal for N-cadherin or αN-catenin.
301 These results indicate that l-afadin more efficiently accumulates N-cadherin and
302 αN-catenin at the PAJ-like structure than s-afadin in the cultured hippocampal neurons.

303

304 *3.3. Requirement of the FAB domain of l-afadin for the formation of the PAJ-like structure*

305

306 To elucidate whether the inability of s-afadin to regulate the accumulation of
307 N-cadherin and αN-catenin at the PAJ-like structure in the cultured hippocampal neurons
308 was caused by its lack of the FAB domain including the third proline-rich region, we
309 examined the effect of re-expression of the l-afadin mutant, of which FAB domain was
310 deleted, in the *afadin*-deficient cultured hippocampal neurons on the re-accumulation of
311 N-cadherin and αN-catenin at the PAJ-like structure. Re-expression of this l-afadin mutant
312 did not restore the re-accumulation of the immunofluorescence signal for N-cadherin or
313 αN-catenin, compared with those of the adjacent untransfected neurons **(Fig. 3, A and B)**.
314 These results indicate that the FAB domain of l-afadin is required for the accumulation of
315 N-cadherin and αN-catenin at the PAJ-like structure in the cultured hippocampal neurons
316 and that the inability of s-afadin to regulate it is caused by the lack of the FAB domain.
317
318 *3.4. Determination of the αE-catenin- and αN-catenin-binding domain of l-afadin*
319
320 It was previously shown that αE-catenin is indirectly bound to E-cadherin via
321 β-catenin and is directly bound to l-afadin in epithelial cells (Tachibana et al., 2000). It was
322 also shown that the *αN-catenin*-deficient cultured hippocampal neurons have thinner and
323 shorter spines similar to those of the *afadin*-deficient cultured hippocampal neurons,
324 although the accumulation of the PAJ components was not previously studied in the
325 *αN-catenin*-deficient cultured hippocampal neurons (Abe et al., 2004; Togashi et al., 2002).
326 Therefore, we examined whether the binding of αN-catenin to l-afadin is required for the
327 formation of the PAJ-like structure in the cultured hippocampal neurons. For this purpose,
328 we first determined the αE-catenin-binding domain of l-afadin. The reasons why we used

αE-catenin instead of αN-catenin were because (1) it was shown that l-afadin binds αE-catenin; (2) the binding region of αE-catenin to l-afadin was determined; and (3) this binding region of αE-catenin was more than 95% (255 aa out of 268 aa) homologous to the corresponding region of αN-catenin. αE-Catenin has three vinculin homology (VH) domains (Maiden and Hardin, 2011). The VH1 domain (22–224 aa) includes the β-catenin-binding domain and the dimerization interface; the VH2 domain (377–585 aa) includes the formin-biding domain, the α-actinin-binding domain, and the afadin-binding domain; and the VH3 domain (698–848 aa) includes the FAB domain, the ZO-1-binding domain, and the EPLIN-binding domain (Abe and Takeichi, 2008; Maiden and Hardin, 2011). The l-afadin-binding domain of αE-catenin was determined to be 385–651 aa or 507–631 aa (Pokutta et al., 2002). We constructed the GST-tagged l-afadin-binding domain of αE-catenin and various truncated mutants of l-afadin with a FLAG tag as schematically shown in **Fig. 4A**. The FLAG-tagged l-afadin or each of the various deletion mutants were expressed in HEK293 cells and the lysates were subjected to the GST pull-down assay using the GST-tagged αE-catenin fragment (385–651 aa). The cell lysates and the pull-down samples were analyzed by Western blotting with an anti-FLAG mAb and by amido black staining. The mutants containing 1,400–1,460 aa, but not the mutants without this region, were pulled-down by the GST-tagged αE-catenin fragment (385–651 aa) **(Fig. 4, A–E)**. These results indicate that the region between the second and third proline-rich regions (1,400–1,460 aa) is the αE-catenin-binding domain of l-afadin. We furthermore confirmed that a shorter fragment of αE-catenin (507–631 aa) and its identical fragment of αN-catenin (505–629 aa) bound to l-afadin, but not to the l-afadin mutant lacking the region between the second and third proline-rich regions (1,400–1,460 aa) **(Fig. 4F)**. These

results indicate that the same region of l-afadin is both the αE-catenin- and αN-catenin-binding domain.

3.5. Requirement of the binding of αN-catenin to l-afadin for the formation of the PAJ-like structure in the cultured hippocampal neurons

We then examined whether re-expression of the l-afadin mutant, in which the αN-catenin-binding domain was deleted, restored the re-accumulation of the immunofluorescence signals for N-cadherin and αN-catenin in the *afadin*-deficient cultured hippocampal neurons. Re-expression of the l-afadin mutant, in which the αN-catenin-binding domain was deleted, did not restore the signal for N-cadherin or αN-catenin, compared with those of the adjacent untransfected neurons (**Fig. 5, A and B**). These results, together with the previous report which showed that αN-catenin is likely to be the dominant α-catenin in mouse hippocampal neurons (Abe et al., 2004; Togashi et al., 2002), indicate that l-afadin regulates the accumulation of N-cadherin and αN-catenin at the PAJ-like structure through binding of αN-catenin to l-afadin.

3.6. Binding of γ-catenin to s-afadin, but not to l-afadin, and binding of αN-catenin to l-afadin, but not to s-afadin

Although both l-afadin and s-afadin have the α-catenin-binding domain, re-expression of s-afadin was unable to accumulate αN-catenin as well as N-cadherin at the PAJ-like structure in the *afadin*-deficient cultured hippocampal neurons. We therefore next examined

why s-afadin was unable to show this activity. In the preceding paper, we prepared the lysate of the light membrane fraction of mouse brain and examined which proteins are co-immunoprecipitated from this lysate with the anti-l-afadin Ab, which recognizes l-afadin, but not s-afadin, and the anti-l/s-afadin Ab, which recognizes both l-afadin and s-afadin (Maruo et al., 2017). We presented the Western blotting data in that paper showing that nectin-1, nectin-3, β-catenin, and δ2-catenin were co-immunoprecipitated with both l-afadin and s-afadin, and that γ-catenin was co-immunoprecipitated with s-afadin, but faintly with l-afadin. However, we did not mention in the text that γ-catenin was co-immunoprecipitated with s-afadin, but faintly with l-afadin. We first confirmed these results by the same method. The lysate of the light membrane fraction of mouse brain was immunoprecipitated with the anti-l-afadin Ab and the anti-l/s-afadin Ab and each precipitate was subjected to SDS-PAGE, followed by Western blotting with the indicated Abs. Nectin-1 and nectin-3 were co-immunoprecipitated with both the anti-l-afadin Ab and the anti-l/s-afadin Ab **(Fig. 6A)**. γ-Catenin was co-immunoprecipitated with the anti-l/s-afadin Ab, although it was faintly co-immunoprecipitated with the anti-l-afadin Ab. β-Catenin was co-immunoprecipitated with the anti-l-afadin Ab and less with the anti-l/s-afadin Ab. These results were consistent with the previous observations (Maruo et al., 2017). αN-Catenin was co-immunoprecipitated with the anti-l-afadin Ab, although it was faintly co-immunoprecipitated with the anti-l/s-afadin Ab. These results indicate that l-afadin forms a complex with αN-catenin and β-catenin, but not with γ-catenin, whereas s-afadin forms a complex with γ-catenin, but presumably not with αN-catenin and β-catenin, although the possibility that s-afadin forms a complex with αN-catenin cannot be completely excluded. The specific binding of γ-catenin to s-afadin, but not to l-afadin, was

398 further confirmed by an *in vitro* co-immunoprecipitation assay. γ-Catenin was co-expressed
399 with FLAG-tagged GFP, FLAG-tagged l-afadin, or FLAG-tagged s-afadin. When
400 FLAG-tagged proteins were immunoprecipitated with the anti-FLAG mAb, γ-catenin was
401 co-immunoprecipitated with FLAG-tagged s-afadin, but not with FLAG-tagged EGFP or
402 FLAG-tagged l-afadin, **(Fig. 6B)**. These results indicate that s-afadin binds γ-catenin both
403 *in vivo* and *in vitro*.

4. Discussion

The electron microscopic features of the hippocampal mossy fiber synapse typical PAJs resemble those of epithelial adherens junctions (AJs) except that the epithelial AJs completely enclose the lateral membranes of the apposing cells along with F-actin lining (Farquhar and Palade, 1963) whereas the mossy fiber synapse typical PAJs are organized into tiny puncta and discontinuous (Uchida et al., 1996). A major function of the epithelial AJs is to maintain the physical association between cells, as disruption of the AJs causes loosening of cell–cell contacts, leading to disorganization of tissue architecture (Takeichi, 1991), but the role of the mossy fiber synapse typical PAJs still remains unknown. The components of the epithelial AJs have been intensively investigated and it was shown that nectin-1, nectin-2, nectin-3, l-afadin, E-cadherin, β-catenin, and αE-catenin, are localized at the epithelial AJs (Takai et al., 2008; Takeichi, 2014). α-Actinin, vinculin, EPLIN, PLEKHA7, ponsin, ADIP, and LMO-7 are also localized at the epithelial AJs (Abe and Takeichi, 2008; Meng et al., 2008; Takai et al., 2008). In contrast, it was shown that N-cadherin, β-catenin, αN-catenin, nectin-1, nectin-3, l-afadin, S-SCAM, and ZO-1 are localized at the mossy fiber synapse typical PAJs (Inagaki et al., 2003; Mizoguchi et al., 2002; Takai et al., 2008; Yamada et al., 2003). ZO-1 is localized at the epithelial tight junctions, but not at the epithelial AJs (Itoh et al., 1993). The epithelial AJs is associated with F-actin via the F-actin-binding proteins, such as αE-catenin, l-afadin, α-actinin, vinculin, EPLIN, and drebrin, and the reorganization of F-actin by these proteins is involved in the formation of the epithelial AJs (Rehm et al., 2013; Takai et al., 2008; Takeichi, 2014). This reorganization of the actin cytoskeleton is induced by the

427 *trans*-interaction of nectins and the *trans*-interaction of E-cadherin through the activation of
428 the Rap1, Cdc42, and Rac small G proteins (Takai et al., 2008).

429 In contrast, the presence of F-actin at the mossy fiber synapse typical PAJs has not
430 been well investigated and the involvement of the reorganization of F-actin in the formation
431 of the mossy fiber synapse typical PAJs has not been elucidated. We performed here a
432 series of experiments using the *afadin*-deficient cultured hippocampal neurons, in which the
433 PAJ-like structure is markedly disrupted, and showed that l-afadin, but not s-afadin, is
434 involved in the formation of the PAJ-like structure. The FAB domain of l-afadin is required
435 for the formation of this structure, indicating that the inability of s-afadin to form the
436 PAJ-like structure is caused by its lack of the FAB domain. These results suggest that
437 F-actin associated with l-afadin is involved in the formation of the hippocampal synapse
438 PAJ-like structure. Because both the *trans*-interaction of nectins and that of N-cadherin are
439 involved in the formation of the mossy fiber synapse typical PAJs as well as the epithelial
440 AJs (Honda et al., 2006; Sai et al., 2017; Toyoshima et al., 2014), it could be speculated
441 that the mechanisms similar to those for the formation of the epithelial AJs may be also
442 involved in the formation of the mossy fiber synapse typical PAJs.

443 The l-afadin-binding domain of αE-catenin was previously determined (Pokutta et al.,
444 2002), but the α-catenin-binding domain of l-afadin has not been determined. In addition, it
445 remained unknown whether the binding of αE-catenin or αN-catenin to l-afadin is required
446 for the formation of the epithelial AJs or the hippocampal mossy fiber synapse typical PAJs.
447 We determined here the αE-catenin- and αN-catenin-binding domain of l-afadin which
448 were the same region of l-afadin and showed the requirement of this domain for the
449 formation of the PAJ-like structure. We further confirmed here that afadin was required for

the localization of αN-catenin as well as nectin-1, nectin-3, and N-cadherin at the hippocampal mossy fiber synapse typical PAJs *in vivo*. A model for the formation of the hippocampal mossy fiber synapse typical PAJs was previously proposed as follows: (1) the *trans*-interaction of presynaptic nectin-1 and post-synaptic nectin-3 forms the atypical PAJs with small sizes independently of afadin and N-cadherin; (2) afadin then binds to the *trans*-interacting nectins and also to non-*trans*-interacting nectins, promotes the *trans*-interaction of the non-*trans*-interacting nectins, and enlarges the atypical PAJs with the small sizes; (3) afadin, which is bound to nectins, further binds to αN-catenin, and recruits non-*trans*-interacting N-cadherin, which binds to β-catenin, to the developing atypical PAJs; (4) the non-*trans*-interacting N-cadherin recruited there *trans*-interacts with each other, resulting in the formation of the typical PAJs; and (5) these sequential reactions enlarge the typical PAJs with the small sizes, resulting in the formation of the maturated typical PAJs (Sai et al., 2017). Our present results indicate that l-afadin, but not s-afadin, is involved in these processes for the formation of the hippocampal synapse PAJ-like structure, and presumably the hippocampal mossy fiber synapse typical PAJs, and have provided the evidence for the requirement of the binding of αN-catenin to l-afadin at the abovementioned process of (3).

Both l-afadin and s-afadin have the αN-catenin-binding domain and are postulated to have a potency to bind αN-catenin *in vitro*. However, we showed here by the immunoprecipitation assay using the lysate of the light membrane fraction of mouse brain that l-afadin binds αN-catenin, but not γ-catenin whereas s-afadin binds γ-catenin, but hardly αN-catenin, although the possibility that s-afadin does not bind αN-catenin at all cannot be completely excluded. These results suggest that the binding of γ-catenin to

s-afadin inhibits the binding of αN-catenin to s-afadin presumably in a competitive inhibition manner, thus making l-afadin bind αN-catenin more preferentially than s-afadin to regulate the formation of the mossy fiber synapse typical PAJs, and inversely making s-afadin not bind αN-catenin to regulate the formation of the mossy fiber synapse typical PAJs. γ-Catenin, also known as junction plakoglobin, is a protein homologous to β-catenin, but is localized at desmosomes in various types of cells including epithelial cells and associates desmosomal cadherins with the intermediate filament cytoskeleton through desmoplakin (Delva et al., 2009), but its role in synapses remains unknown. We have not investigated here the physiological functions of the binding of γ-catenin to s-afadin, but the present results raised the possibility that γ-catenin and its binding to s-afadin regulate the formation of the synaptic junctions, because it was previously shown that s-afadin is localized at the synaptic junctions (Maruo et al., 2017; Nishioka et al., 2000).

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628 as revealed by high-pressure freezing. J Comp Neurol 520, 2340-2351.
629

Figures and Figure legends

Figure. 1

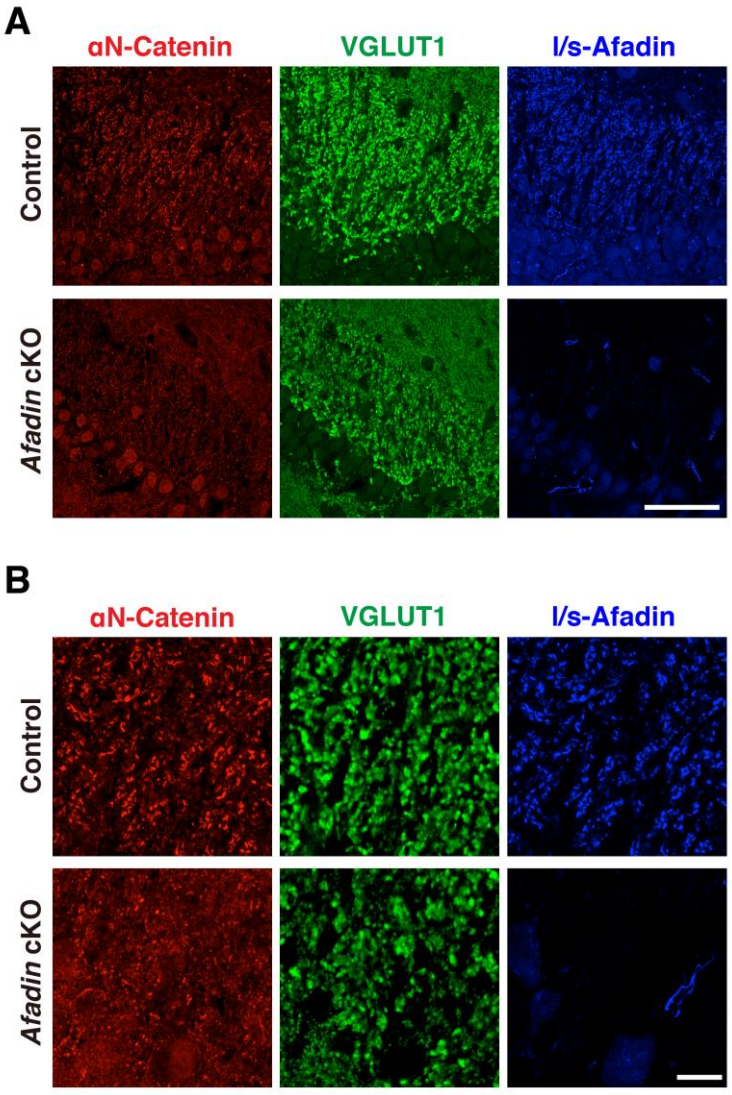


Fig. 1. Requirement of *afadin* for the localization of αN-catenin in the CA3 stratum lucidum of the hippocampus. **A and B,** Coronal hippocampal sections obtained from the brains of the control and *afadin*-deficient mice at P31. The sections were stained with the indicated Abs. **A,** Lower magnification images. **B,** Higher magnification images. Control, *afadin*$^{+/f}$;Emx1-Cre; and *Afadin* cKO, *afadin*$^{f/f}$;Emx1-Cre. Scale bars, 50 μm in **A** and 10 μm in **B**.

Figure. 2

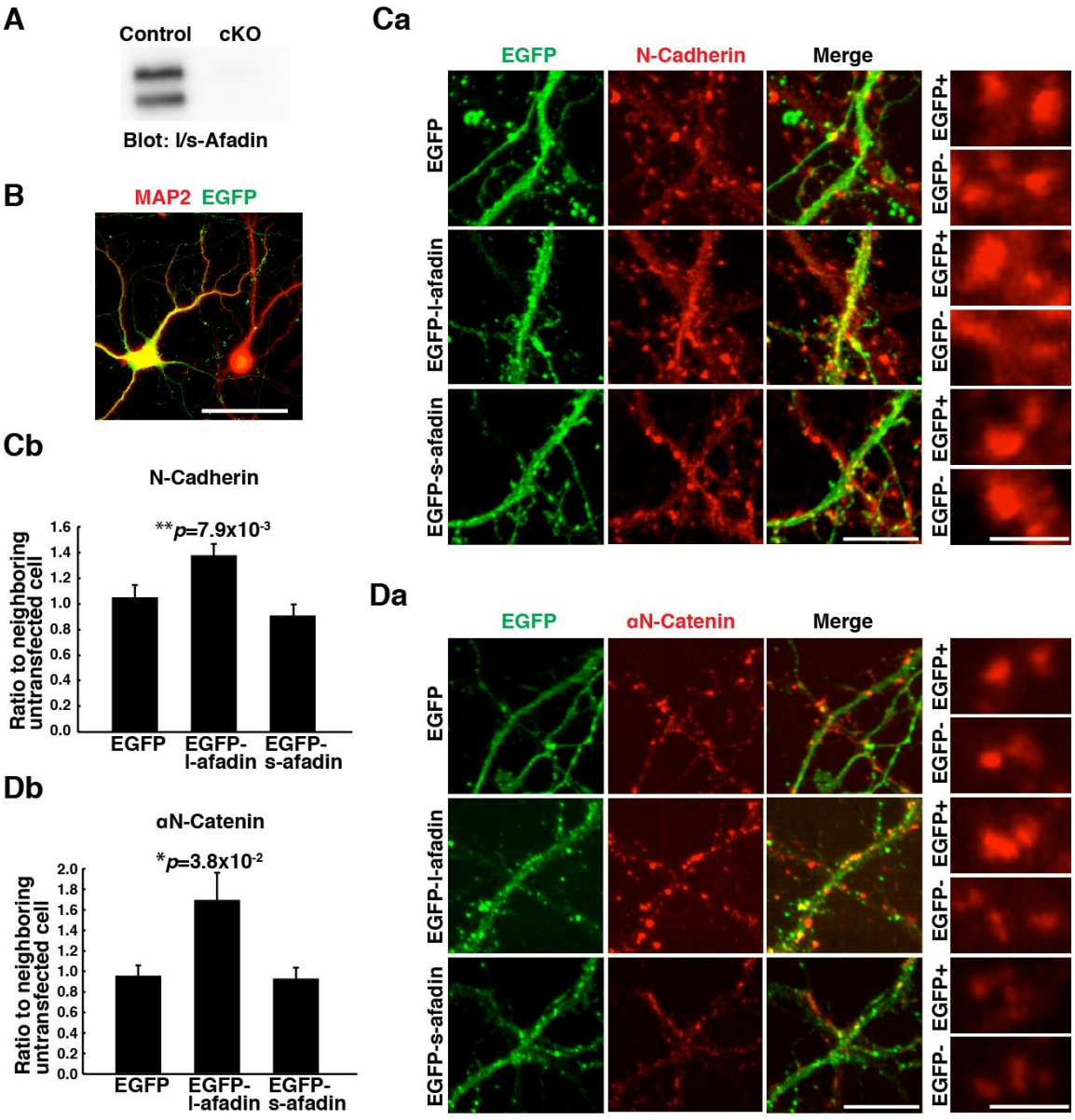


Fig. 2. Requirement of l-afadin, but not s-afadin, in the formation of the PAJ-like structure in the cultured hippocampal neurons. **A,** Western blotting of the hippocampal neurons obtained from the brains of the control and *afadin*-deficient mice (*afadin*$^{f/f}$;Nestin-Cre mice). The hippocampal neurons at 14 DIV were subjected to SDS-PAGE, followed by Western blotting with the Ab recognizing both l-afadin (upper band) and s-afadin (lower

band). cKO, *afadin*$^{f/f}$;Nestin-Cre mice. **B,** Representative image of a field containing EGFP-positive and EGFP-negative neurons showing typical pyramidal cell-like morphologies judged by MAP2 immunoreactivities. **C and D,** Restoration of the accumulation of the PAJ proteins, N-cadherin and αN-catenin, by expression of l-afadin, but not s-afadin. **C,** The signal for N-cadherin. **D,** The signal for αN-catenin. **Ca and Da,** The cultured hippocampal neurons stained with the indicated Abs at 14 DIV. The neurons were obtained from the *afadin*-deficient mice and were transfected with the indicated constructs. Enlarged images of the N-cadherin or αN-catenin clusters on the EGFP-positive and -negative dendrites were shown at the right sides. Scale bars, 50 μm in **B**, 10 μm in the left three columns in **Ca** and **Da**, and 2 μm in the right enlarged images in **Ca** and **Da**. **Cb and Db,** Quantification of the ratio of the intensity of the signals for N-cadherin and αN-catenin in the neurons transfected with the indicated constructs relative to that of the adjacent untransfected neurons in *afadin*-deficient neuron culture. * and ** indicate statistical significance (∗, $p < 0.05$; ∗∗, $p < 0.01$).

Figure. 3

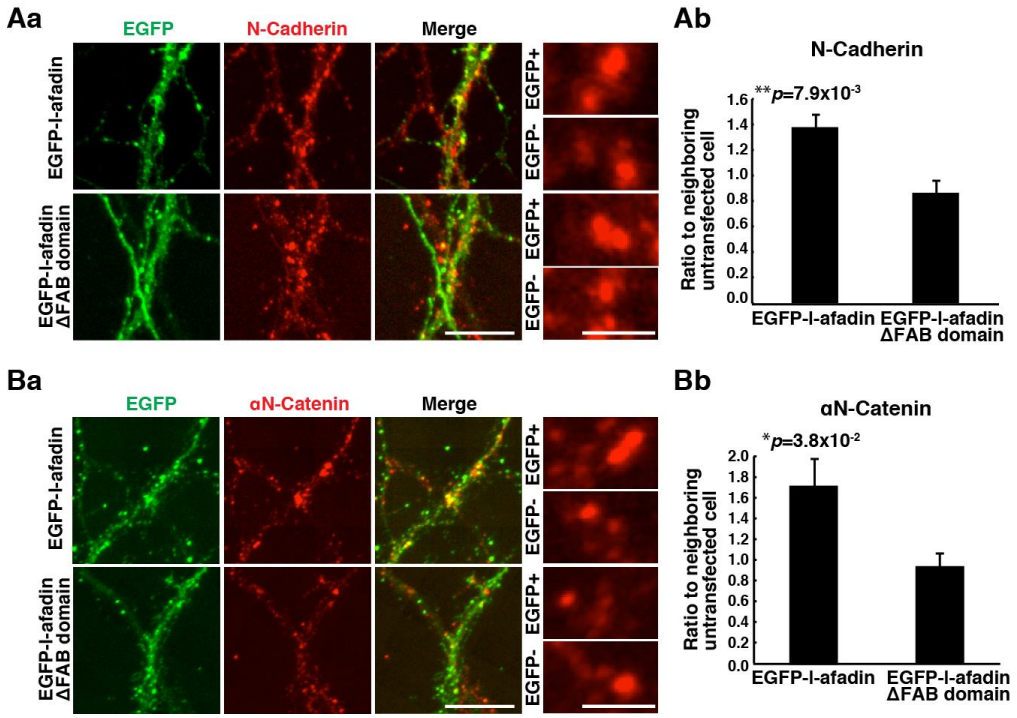
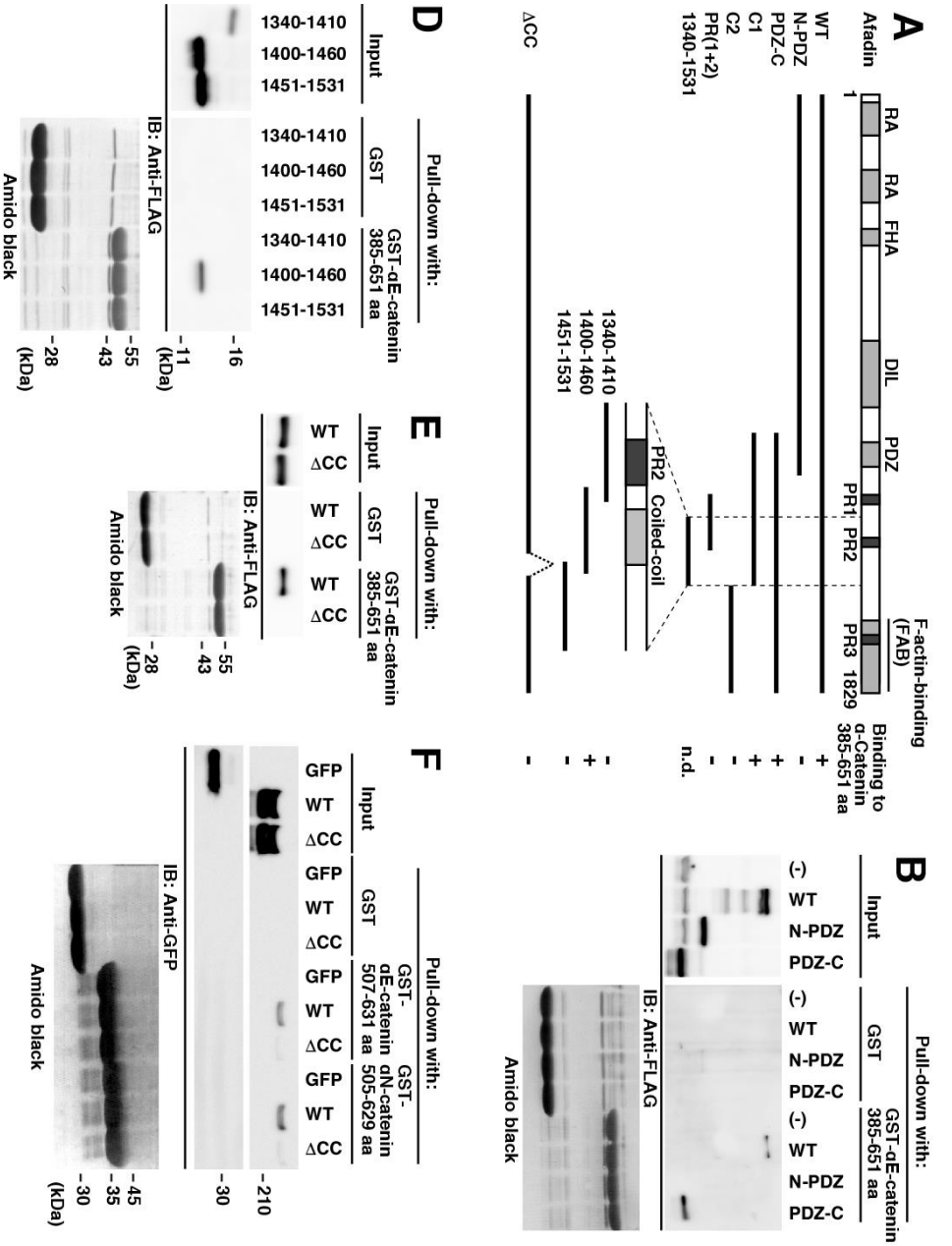


Fig. 3. Requirement of the FAB domain of l-afadin for the formation of the PAJ-like structure in the cultured hippocampal neurons. **A,** The signal for N-cadherin. **B,** The signal for αN-catenin. **Aa and Ba,** The cultured hippocampal neurons stained with the indicated Abs at 14 DIV. The neurons were obtained from the *afadin*-deficient mice and were transfected with the indicated constructs. Enlarged images of the N-cadherin or αN-catenin clusters on the EGFP-positive and -negative dendrites were shown at the right sides. Scale bars, 10 μm in left three columns and 2 μm in the right enlarged images in **Aa** and **Ba**. **Ab and Bb,** Quantification of the ratio of the intensity of the signals for N-cadherin and αN-catenin in the neurons transfected with the indicated constructs relative to that of the adjacent untransfected neurons in *afadin*-deficient neuron culture. The same data sets shown in **Figure 2D and 2E** were used for EGFP-l-afadin. * and ** indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$).

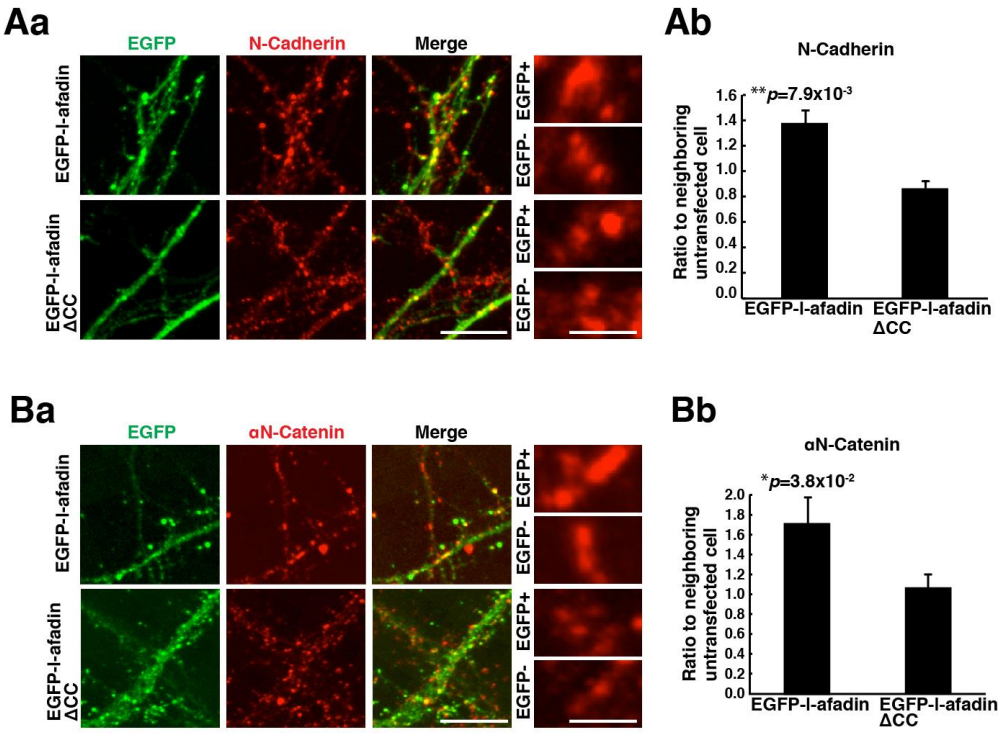
Figure. 4


A
Afadin
1
RA
RA
FHA
DIL
PDZ
F-actin-binding (FAB)
PR1
PR2
PR3
1829
Binding to α-Catenin 385-651 aa
WT + N-PDZ - PDZ-C + C1 + C2 - PR(1+2) - 1340-1531 n.d.
ΔCC -
PR2
Coiled-coil
1340-1410 -
1400-1460 +
1451-1531 -
B
Input
(-)
WT
N-PDZ
PDZ-C
Pull-down with:
GST
GST-αE-catenin 385-651 aa
IB: Anti-FLAG
Amido black
C
Input
PDZ-C
C1
C2
PR(1+2)
Pull-down with:
GST
GST-αE-catenin 385-651 aa
IB: Anti-FLAG
Amido black
114
85
47
31
62
47
31
26
(kDa)
D
Input
Pull-down with:
GST
GST-αE-catenin 385-651 aa
1340-1410
1400-1460
1451-1531
IB: Anti-FLAG
Amido black
16
11
55
43
28
(kDa)
E
Input
Pull-down with:
GST
GST-αE-catenin 385-651 aa
WT
ΔCC
IB: Anti-FLAG
Amido black
55
43
28
(kDa)
F
Input
Pull-down with:
GST
GST-αE-catenin 507-631 aa
GST-αN-catenin 505-629 aa
GFP
WT
ΔCC
IB: Anti-GFP
Amido black
210
30
45
35
30
(kDa)

671

Fig. 4. The binding region of l-afadin to αE-catenin and αN-catenin. The lysates from HEK293 cells expressing FLAG-tagged l-afadin or various deletion mutants with a FLAG tag were subjected to GST pull-down assay using GST-tagged αE-catenin fragment (385–651 aa). The cell lysates and the pull-down samples were analyzed by Western blotting with an anti-FLAG mAb and by amido black staining. **A,** Schematic drawings for the deletion mutants of l-afadin used in the experiments and their binding to the αE-catenin fragment (385–651 aa). RA, Ras-association domain; FHA, forkhead-associated domain; DIL, dilute domain; PDZ, PSD-95/Dlg/ZO-1 domain; PR, proline-rich region; and n.d., not determined. **B,** Binding of the C-terminal half of afadin (PDZ-C) to GST-tagged αE-catenin fragment (385–651 aa). **C,** Binding of the C1 fragment to GST-tagged αE-catenin fragment (385–651 aa). **D,** Binding of the afadin fragment (1,400–1,460 aa) containing a putative coiled-coil (CC) domain to GST-tagged αE-catenin fragment (385–651 aa). **E and F,** No binding of the afadin mutant lacking the putative coiled-coil domain (ΔCC) to GST-tagged αE-catenin fragment and GST-tagged αN-catenin fragment. **E,** GST-tagged αE-catenin fragment (385–651 aa). **F,** GST-tagged αE-catenin fragment (507–631 aa) and GST-tagged αN-catenin fragment (505–629 aa).

Figure. 5



688

689 **Fig. 5.** Requirement of the binding of αN-catenin to l-afadin for the formation of PAJ-like
690 structure in the cultured hippocampal neurons. **A,** The signal for N-cadherin. **B,** The signal
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694 clusters on the EGFP-positive and -negative dendrites were shown at the right sides. Scale
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697 αN-catenin in the neurons transfected with the indicated constructs relative to that of the
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699 shown in **Figure 2D and 2E** were used for EGFP-l-afadin. * and ** indicate statistical
700 significance (*, $p < 0.05$; **, $p < 0.01$).

Figure. 6

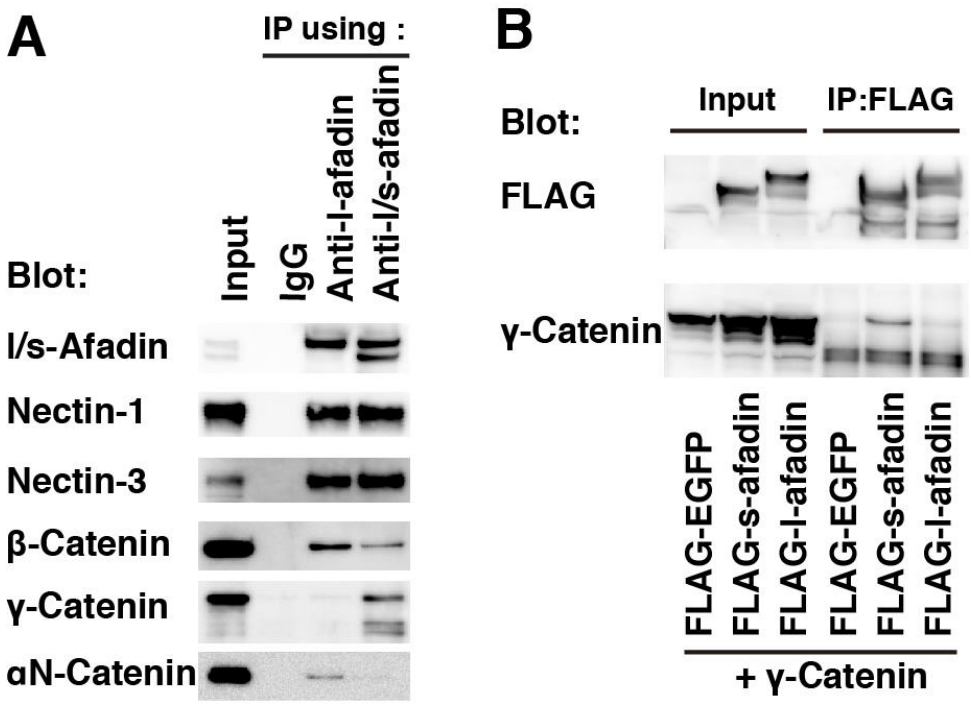

Fig. 6. Specific binding of γ-catenin to s-afadin, but not to l-afadin. **A**, *In vivo* immunoprecipitation assay for the l-afadin-binding or s-afadin-binding proteins. The lysate of the light membrane fraction of the mouse brain was immunoprecipitated by rabbit IgG, the anti-l-afadin pAb, or the anti-l/s afadin pAb. The lysates and the immunoprecipitated samples were subjected to SDS-PAGE, followed by Western blotting with the indicated Abs. **B**, *In vitro* immunoprecipitation assay for the l-afadin-binding or s-afadin-binding proteins. The lysates from HEK293 cells expressing FLAG-tagged EGFP, FLAG-tagged l-afadin, or FLAG-tagged s-afadin with γ-catenin were immunoprecipitated with the anti-FLAG M2 mAb. The cell lysates and the immunoprecipitated samples were subjected to SDS-PAGE, followed by Western blotting with the anti-FLAG pAb and the anti-γ-catenin mAb. IP, immunoprecipitation.