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

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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. 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.

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 (Špaček 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 Tóth, 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

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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 and the 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 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 phosphate buffered saline (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 Mg^{2+} (Thermo Fisher Scientific, Waltham, MA) containing 10 mM HEPES, 1 mM sodium pyruvate, and 4% sucrose. After dehydration with 30% sucrose in PBS, whole brains were embedded in OCT compounds (Sakura Finetek, Tokyo, Japan). Cryostat sections were incubated at 65 °C for 20 min in HistoVT One antigen retrieval solution (Nacalai Tesque) and then incubated with 1% bovine serum albumin, 10% normal goat serum, and 0.25% Triton X-100 in PBS at room temperature for 30 min. The sections were stained with the indicated antibodies (Abs), and then with appropriate fluorophore-conjugated secondary Abs (1:300). Confocal image acquisition was performed on a LSM510 META confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) under the same conditions for both the control and *afadin*-deficient brain sections.

2.3. Dissociated culture of hippocampal neurons

Dissociated hippocampal neurons were prepared as described (Toyoshima et al., 2014). In brief, the hippocampal neurons dissociated with trypsin were plated at a density of 5 to 7 × 10^3 cells/cm^2 on poly-