Fbxo45, a Novel Ubiquitin Ligase, Regulates Synaptic Activity*3

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Neurons communicate with each other through synapses. To establish the precise yet flexible connections that make up neural networks in the brain, continuous synaptic modulation is required. The ubiquitin-proteasome system of protein degradation is one of the critical mechanisms that underlie this process, playing crucial roles in the regulation of synaptic structure and function. We identified a novel ubiquitin ligase, Fbxo45, that functions at synapses. Fbxo45 is evolutionarily conserved and selectively expressed in the nervous system. We demonstrated that the knockdown of Fbxo45 in primary cultured hippocampal neurons resulted in a greater frequency of miniature excitatory postsynaptic currents. We also found that Fbxo45 induces the degradation of a synaptic vesicle-priming factor, Munc13-1. We propose that Fbxo45 plays an important role in the regulation of neurotransmission by modulating Munc13-1 at the synapse.

The nervous system stores and retrieves information via synapses, which are its primary means of communication. Synapses are specialized intercellular junctions dedicated to the transfer of information from a neuron to another neuron. Synaptic transmission is rapidly, dynamically, efficiently, and tightly regulated by several molecular mechanisms (1–4). Among these mechanisms, recent studies have shown that synaptic components are modified by protein activation (5) and degradation (6–9).

Protein degradation can be mediated by the ubiquitin-proteasome system (UPS)4 (10–12). The modification of proteins by the ligation of ubiquitin molecules is critical in regulating the degradation of specific proteins, thereby controlling protein turnover. This control mechanism is extremely effective because it allows the rapid elimination of particular regulatory proteins, ensuring that the biological process regulated by the proteins can be shut down immediately. Protein ubiquitination is catalyzed by a cascade of reactions involving three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase). E1 activates ubiquitin in an ATP-dependent reaction and transfers it to E2 with the formation of a thiol ester bond between the C terminus of ubiquitin and a cysteine residue of E2. Next, E2, either by itself or together with E3, transfers the ubiquitin moiety to a lysine residue of the substrate protein. Various E3s have been reported, and the action of each is substrate-specific. Finally, the polyubiquitinated protein is attacked by the 26S proteasome complex, which rapidly degrades it into small fragments (13).

The E3s are presently categorized into four major classes based on their specific structural motif: HECT, RING finger, U-box, and PHD-finger types (14). The RING finger-type E3s are thought to be the largest family and are further divided into subfamilies: one of these, the cullin-based E3 subfamily, is one of the largest single classes of E3. There are seven cullin-based E3s, including the Skp1-Cul1-F-box-protein (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C) (14). The SCF complex E3s are composed of the scaffold protein Cul1, the RING-domain protein Rbx1/Roc1, the adaptor pro-

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4 The abbreviations used are: UPS, ubiquitin-proteasome system; SCF, Skp1-Cul1-F-box-protein; PBS, phosphate-buffered saline; GFP, green fluorescent protein; DIV, days in vitro; siRNA, short interfering RNA; aa, amino acids; PSD, postsynaptic density; mEPSC, miniature excitatory postsynaptic current; EGFP, enhanced green fluorescent protein; HA, hemagglutinin.
tein Skp1, and one of many F-box proteins, which function to bind the substrate and constitute the largest known class of E3-specificity components. Rbx1 associates with Cul1 and E2, whereas Skp1 interacts with Cul1 and the F-box protein. The F-box protein interacts with Skp1 via its F-box motif and with substrates via its C-terminal protein-protein interaction domain (15, 16). Target molecules of several F-box proteins have been reported; for example, β-TrCP, an F-box protein that contains WD40 domain repeats, was identified as the intracellular receptor in SCF E3 for β-catenin and IκBα (17, 18); Skp2, which recognizes cyclin-dependent kinase inhibitors such as p27kip1, p21cip1, and p57kip2, is an F-box protein containing seven leucine-rich repeats (19).

The UPS in synaptic sites plays various roles throughout the life cycle of the synapse, i.e. in synaptic development, maintenance, and plasticity (6). Genetic studies in Drosophila and Caenorhabditis elegans have demonstrated the importance of E3s in synaptic development and function. For example, mutations of highwire, a putative E3 in Drosophila, or of its orthologue in C. elegans (rpm-1) lead to aberrant synaptic development (20–23). Moreover, recent studies have identified several additional mammalian E3s at the synaptic site, including SCRAPPER (9), Siah (24, 25), Staring (26), and Parkin (27). Although ample evidence supports the role of protein ubiquitination in synaptic development and plasticity in vertebrates (28), the specific molecular mechanisms underlying these effects remain to be elucidated.

Here we identified a novel synaptic E3, Fbxo45, by expression screening using the autoimmune antiserum from a patient with stomach cancer associated with psychiatric symptoms. To examine E3 functions at the synapse with potential relevance to neuronal disease, we recorded the miniature excitatory postsynaptic current (mEPSC) in hippocampal neuronal cultures and postnatal brain and from adult mouse tissues were incubated overnight at 55 °C in a moist chamber with 200 μg of the sense or antisense probe per 50 μl of a buffer containing 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate), 50 μg/ml Escherichia coli tRNA, 50 μg/ml heparin sodium, and 1% SDS. The specimens were washed in 50% formamide, 5 × SSC (30 min at 55 °C), 2 × SSC (3 times for 30 min at 55 °C), and TBST (twice for 20 min at room temperature). After being blocked in 0.5% bovine serum albumin in TBST (60 min), the slides were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) in the above buffer (overnight at 4 °C), then washed in TBST (3 times for 20 min) and NTM (100 mM NaCl; 50 mM MgCl₂, and 100 mM Tris-HCl, pH 9.5) for 10 min. Signals were detected in NTM containing 450 μg/ml 4-nitroblue tetrazolium chloride and 175 μg/ml 5-bromo-4-chloro-3-indolyolphosphate according to the digoxigenin RNA detection kit (Roche Applied Science).

**Northern Blot Analysis**—Total RNAs (20 μg) from embryonic and postnatal brain and from adult mouse tissues were prepared with TRizol reagent (Invitrogen) following the manufacturer’s protocol. Purified RNAs were loaded onto a 1% formaldehyde-agarose gel after being denatured at 70 °C for 10 min. The RNAs were transferred to a nylon membrane (Amersham Biosciences) and UV-cross-linked using a Stratalinker (Stratagene). The membrane was hybridized at 51 °C with a full-length mouse Fbxo45 or gyceraldehyde-3-phosphate dehydrogenase (G3PDH) CDNA probe that had been labeled with [α-32P]dCTP using a Priime-It labeling kit (Stratagene). The blots were washed at 65 °C in 2, 1, and then 0.1% standard sodium citrate containing 0.1% SDS and were exposed to MR film (Eastman Kodak Co.).

**Antibodies**—The following antibodies were used: Synapsin-I, Munc13-1 (Synaptic Systems), synaptophysin, β-actin, FLAG, and Myc (Sigma), GFP (Wako), PSD-95 and Myc (Upstate Biotechnology), VGlut1 (Chemicon), PSD-95 (Cell Signaling), pan-Munc13, synaptotagmin, SNAP25, synapticyn, and Skp1 (BD Biosciences), and GFP and hemagglutinin (HA) (MBL).

To produce an anti-Fbxo45 polyclonal antibody, glutathione S-transferase-tagged full-length Fbxo45 (for immunohistochemistry, immunocytochemistry, and immuno-electron microscopy) or C- (257–286) terminal Fbxo45 (for immuno-
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blot) bacterial fusion proteins were purified and dialyzed against PBS as described above. Rabbits were given injections of 2.5 mg of a mixture of the proteins at 3-week intervals. The antibodies were obtained by passing the serum through a column containing glutathione S-transferase fusion versions of the proteins.

Plasmid Construction—GFP-tagged Fbxo45 was inserted into a pEGFP-C1 vector (Clontech), and FLAG-tagged Fbxo45 and Fbxo45 deletion mutants (1–140, 54–140, and 101–286 aa) were inserted into a pFLAG-CMV-2 vector (Eastman Kodak Co.) or p3xFLAG-CMV-7.1 vector (Sigma). FLAG-Msi1, FLAG-HuC, Myc-Skp1, Myc-Munc13-1, Myc-RIM1, and HA-ubiquitin were prepared as described previously (9, 31–34, 55).

Immunoelectron Microscopy—For immunoelectron microscopic analysis, frozen sections were incubated with the anti-Fbxo45 primary antibody (1:500) followed by incubation with a nanogold-conjugated anti-rabbit secondary antibody (1:100; Invitrogen). After glutaraldehyde fixation, the signals were enhanced by a 10-min HQ-Silver kit reaction (Nanoprobes Inc.). The sections were postfixed with 0.5% osmium tetroxide, dehydrated through ethanol, and embedded in Epon. Ultrathin sections (70 μm) were cut and stained with uranyl acetate and lead citrate, observed under a transmission electron microscopy (JEOL model 1230), and photographed with a Digital Micrograph 3.3 (Gatan Inc.). For quantitative analysis, nanogold density was calculated in each neuronal compartment by counting the number of nanogold particles in every immunoelectron microscopic images using MetaMorph software (Molecular Devices, Toronto, Canada). The mean number of nanogold particles and the mean area of each compartment was determined, and the values were entered into Microsoft Excel.

Immunocytochemistry and Image Analysis—The cultured hippocampal neurons were prepared as described previously (9, 36). Cultured cells were fixed with 4% paraformaldehyde in PBS for 30 min at day in vitro (DIV) 17–18. The cells were permeabilized with 0.2% Triton X-100 for 10 min, washed with PBS, and then incubated in PBS containing TNB (PerkinElmer Life Sciences) for 1 h. The preparation was then incubated with Fbxo45 (1:100), VGlut1 (1:5000), anti-Synapsin-I (1:1000), and anti-PSD-95 (1:100) antibodies overnight at 4 °C, and after extensive washes with PBS, the preparation was overlaid with secondary antibody solution for 1 h at room temperature.

Image acquisition was performed using an LSM 510 (Carl Zeiss) and Fluoview 1000 (Olympus) confocal microscope. The clusters of Synapsin-I (red)/PSD-95 (white) colocalization were counted. The red and white images were merged, and the colocalized signals over the dendrites of GFP-expressing neurons were determined, and the values were entered into Microsoft Excel. The transfected neurons were chosen randomly from three independent experiments for each construct. All experiments on live animals were performed in accordance with Keio University guidelines and regulations.

Recording of the mEPSC—Cultured cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) 3 days before electrophysiological analysis. For the mEPSC recording, the culture medium was replaced with a saline solution containing 168 mM NaCl, 2.4 mM KCl, 2, 5, or 10 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 0.5 μM tetrodotoxin, pH 7.3. The electrodes (5–8 MΩ) were filled with whole-cell pipette solution containing 120 mM potassium acetate, 20 mM KCl, 0.1 mM CaCl₂, 5 mM MgCl₂, 0.2 mM EGTA, 5 mM ATP, and 10 mM HEPES, pH 7.3. The whole-cell recording of GFP-expressing neurons was configured using an EPC-7 amplifier (HEKA) and a Digidata 1200 acquisition board (Axon Instruments). The membrane potential was clamped at 60 mV, and the signals were filtered at 10 kHz with the gain set at 0.5 mV/pA for 40-s recording periods. In all instances cells were excluded from analysis if a leak current >300 pA was observed. Membrane resistance (Rm), series resistance (Rs), and membrane capacitance (Cm) were monitored. Only recordings with Rm > 100 MΩ and Rs < 20 MΩ were included for analysis. The mean Rm, Rs, and Cm were not different (p > 0.05) among or within groups of cells being compared. Measurements of the frequency and amplitude of mEPSC were acquired for a period of 40 s. The mEPSC was detected by setting the amplitude threshold to the background noise level ×3 (in all electrophysiological experiments, a similar amount of data from GFP-expressing neurons transfected with each construct was acquired on the same day). The data from each neuron were then averaged and tested for statistical significance. All electrophysiological experiments were performed from at least three different platings of neurons for each of three different transfections. See our previously published method for details (5).

RNA Interference—Short interfering RNA (siRNA) duplexes for Fbxo45 were: siRNA Fbxo45-1, sense (5′-GGACCAAAGAUUGGUUUACGtt-3′) and antisense (5′-UCGGAAACCAAUUUGGUCc-3′); siRNA Fbxo45-2, sense (5′-GGUUUUACAUAUCGGAGAtt-3′) and antisense (5′-UCCGAUGUAUG-UAAACCAt-3′). The recommended negative control were obtained from Ambion in annealed and purified forms. Transfection of the siRNA duplexes was carried out using Lipofectamine 2000 (Invitrogen). Three microliters of Lipofectamine 2000 reagent with 100 pmol of siRNA duplex and 0.5 μg of pEGFP were transfected into cultured hippocampal neurons at DIV14. Three days after transfection, the cells were subjected to mEPSC recording.

Transfection, Immunoprecipitation, and Ubiquitination Assay—293T cells or COS cells were transfected using the FuGENE transfection reagent (Roche Applied Science) and harvested 48 h later. For the ubiquitination assay, the cells were treated with 50 μg/ml MG132 (Peptide Institute) for 3 h before they were harvested. Immunoprecipitates prepared with anti-FLAG M2 affinity gel or anti-c-Myc-agarose conjugate (Sigma) were used for immunoblotting with the appropriate antibodies.

Miscellaneous Procedures—Immunoblotting analyses were performed as described (37). The subcellular fractionation analysis of mouse brain extract was performed as described (38, 39). The stability of Munc13-1 was determined by treatment with 50 mg/ml cycloheximide (Calbiochem) for the indicated times.
RESULTS

Identification of a Novel F-box Protein, Fbxo45—To identify the target molecule recognized by the autoantibody of a cancer patient with psychiatric symptoms, we performed expression screening of a mouse brain cDNA library using the patient’s serum. From $1 \times 10^6$ clones screened, four positive clones were isolated. Sequence analysis showed that all four clones encoded overlapping sequences of the same cDNA, Fbxo45.

The Fbxo45 gene was located on chromosome 16 in Mus musculus and encoded a 286-amino acid protein with an estimated molecular mass of 30 kDa. Data base (National Center for Biotechnology Information) searches with the deduced amino acid sequence of Fbxo45 revealed that it was a novel protein with striking similarity (99% identity) to a Homo sapiens F-box protein encoded by a gene located on chromosome 3. Recently, mammalian F-box proteins were systematically classified, and the protein corresponding to Fbxo45 was named FBXO45 (40, 41). The amino acid sequence of the Fbxo45 protein was highly conserved among species, including Drosophila DFsn/CG4643-PA; isoform A, CG4643-PA; and isoform B (GenBank™ accession no. NP_610849, NP_725266), and C. elegans FSN-1 (GenBank™ accession no. NP_498046) (Fig. 1).

The deduced Fbxo45 amino acid sequence displayed some notable primary structural characteristics: an F-box domain of $\sim 45$ amino acids, located in its N-terminal region, and a SPRY (SPla and the ryanodine receptor) domain, the function of which remains unknown (42). F-box motifs are found in proteins that function as the substrate recognition component of SCF E3 complexes. The patient’s autoantibodies in the serum and cerebrospinal fluid recognized the F-box domain in Fbxo45 (supplemental Fig. S1).

Tissue Distribution of Fbxo45—To elucidate the expression pattern of Fbxo45 mRNA, we performed Northern blots, immunoblotting analyses, and in situ hybridization on mouse tissue preparations. The Fbxo45 mRNA, which was $\sim 2.7$ kb, was detected preferentially in the brain at similar levels throughout development, from E12 to adulthood, and was undetectable in the tissues outside the nervous system (Fig. 2A). We next raised a Fbxo45-specific polyclonal antibody that recognized the 30-kDa endogenous Fbxo45 protein. Immunoblot analysis of mouse brain tissues revealed that Fbxo45 was expressed widely in the central nervous system, including the olfactory bulb, hippocampus, cortex, cerebellum, and brain stem (Fig. 3A, supplemental Fig. S3). In situ hybridization analysis showed that the
Fbxo45 mRNA was highly expressed in the granule cell layer of the lateral ventricle (supplemental Fig. S2), mRNA in embryonic brain and adult tissues from mice. Fbxo45 (LP1), synaptic plasma membrane fraction (SPM), and the synaptosomal membrane fraction (PSM), and the postsynaptic density (PSD) fraction (Fig. 3B).

Fbxo45 mRNA was highly expressed in the granule cell layer of the cerebellum, dentate gyrus of the hippocampus (Fig. 2, B–G), granule cell layer of the olfactory bulb, and subventricular zone of the lateral ventricle (supplemental Fig. S2, A and B). It was also expressed in some cells in the cortex (supplemental Fig. S2C).

Expression of Fbxo45 in the Nervous System—To determine the precise location of the Fbxo45 protein in the nervous system, we examined the subcellular distribution of Fbxo45 by subcellular fractionation. Fbxo45 was enriched in the synaptic fractions, which included the synaptosomal membrane fraction (LP1), synaptic plasma membrane fraction (SPM), and the postsynaptic density (PSD) fraction (Fig. 3B).

We also performed immunohistochemistry using the anti-Fbxo45 polyclonal antibody, whose immunoreactivity disappeared in Fbxo45 knock-out mice (supplemental Fig. S4). Endogenous Fbxo45 protein was detected as a spotty and fibrous pattern in the primary cultured rat hippocampal neuron at DIV 16 (Fig. 3C). The Fbxo45 immunoreactivity was colocalized in part with a presynaptic marker, VGlut1, and a postsynaptic marker, PSD-95. Analyses using immunoelectron microscopy revealed that the significantly higher expression of Fbxo45 in both pre- and postsynaptic structures of excitatory neuron was detected compared with that in myelin cytoplasm (Fig. 3, D and E, supplemental Fig. S6). Taken together, these results support the idea that Fbxo45 could have a physiological function at the synapse.

Involvement of Fbxo45 in Neurotransmission in Rat Hippocampal Neurons—Because FSN-1, the Fbxo45 homologue in C. elegans, is involved in aspects of synapse formation such as the clustering of periactive zones (43), we investigated Fbxo45 involvement in synaptic function. First, we immunostained primary cultured hippocampal neurons at DIV 14 transfected with an expression vector bearing FLAG-tagged full-length Fbxo45 (FLAG-Fbxo45), vector control (FLAG-Vec), Fbxo45 siRNAs (siRNA Fbxo45-1 and -2), which significantly decreased the level of endogenous Fbxo45 protein (supplemental Fig. S5), or siRNA control (siRNA Cont.) for Synapsin-1 and PSD-95 (Fig. 4A) as a marker for synapses and then evaluated whether the expression of Fbxo45 altered the number of the synapses, defined as sites in which Synapsin-1 and PSD-95 were colocalized. A quantitative analysis showed no significant change in the synaptic number (Fig. 4B), indicating that Fbxo45 did not influence synapse formation in mature neurons.

Next, we recorded the mEPSC under whole-cell voltage clamp after blocking the sodium channel-mediated action potential activity with tetrodotoxin. Primary cultured rat hippocampal neurons at DIV 14 were transfected with Fbxo45, Fbxo45 deletion mutants (54–140 or 101–286 aa), vector control, siRNA Fbxo45-1 and -2, or siRNA control along with an EGFP-bearing vector, and the mEPSC was recorded 3 days later. We found that the knockdown of Fbxo45 significantly increased the mEPSC frequency without changing its amplitude. In contrast, the overexpression of Fbxo45 decreased the mEPSC frequency. Furthermore, a dominant-negative deletion mutant Fbxo45 (101–286 aa) lacking its F-box domain increased the mEPSC as did the siRNAs (Fig. 4, C and D). The frequency of the mEPSC was increased or decreased by the change of Fbxo45 protein level with an extracellular Ca$^{2+}$ concentration of 2 mm; however, there was no significant difference in the mEPSC frequency compared with the control at a higher extracellular Ca$^{2+}$ concentration (10 mM) (Fig. 4E). These results show that Fbxo45 functions as a negative regulator of mEPSC frequency in an extracellular Ca$^{2+}$ concentration-dependent manner. Because Fbxo45 did not alter synapse formation in mature neurons, these findings strongly suggest that Fbxo45 regulates neurotransmission machinery.

Fbxo45 Interacts with Skp1 and Induces Protein Ubiquitination—It is known that the F-box proteins interact with an adapter protein Skp1 and constitute the larger class of E3 specificity components. To evaluate whether Fbxo45 interacts with Skp1, we performed co-immunoprecipitation analyses. FLAG-Fbxo45, FLAG-Musashi1 (FLAG-Msi1) (31, 44), or FLAG-HuC were overexpressed in 293T cells and immunoprecipitated with a monoclonal antibody against the FLAG epitope. In the result, endogenous Skp1 was co-immunoprecipitated with overexpressed FLAG-Fbxo45 (Fig. 5A). The structure of Fbxo45 to form SCF complex has been described elsewhere (45).

To clarify the relationship between Fbxo45 and the ubiquitin proteolytic system, we examined whether Fbxo45 traps target proteins for the ultimate ubiquitination. Immunoblots showed multiple ubiquitinated bands of high molecular mass in the anti-FLAG immunoprecipitates from 293T cells co-transfected with FLAG-Fbxo45 and HA-tagged ubiquitin after pretreatment with the proteasome inhibitor MG132. In contrast, no
ubiquitinated bands were detected in immunoprecipitates prepared from cells without FLAG-Fbxo45 (Fig. 5B). These results indicate that Fbxo45 indeed induced the ubiquitination.

Involvement of Fbxo45 in the Degradation of Munc13-1—The findings that Fbxo45 regulates mEPSC frequency and protein ubiquitination led us to speculate that Fbxo45 might control the degradation of a substrate in the nerve terminal to regulate neurotransmission. To find candidate substrates of Fbxo45, we first evaluated several synaptic proteins immunoprecipitated with Fbxo45. Munc13-1, the synaptic vesicle priming factor, was identified as one of Fbxo45-binding proteins. Moreover, we found that Munc13-1 interacted with the Fbxo45 deletion mutant (101–286 aa) that included the SPRY domain but not with deletion mutants lacking the SPRY domain (54–140 and 1–140 aa) (Fig. 6A).

To examine whether Fbxo45 affects the protein level of Munc13-1, we first co-expressed FLAG-Fbxo45 and Myc-Munc13-1 or Myc-RIM1 (rab3-interacting molecule 1), another vesicle priming factor in COS cells. With the expression of Fbxo45, the level of Munc13-1 decreased in a dose-dependent manner, but in sharp contrast the level of RIM1 did not change (Fig. 6B).

Next, to examine whether Fbxo45 affects the stability of Munc13-1, we co-transfected 293T cells with Munc13-1, Fbxo45, and Fbxo45 deletion mutants and then inhibited new protein synthesis by incubating the cells with cycloheximide for various times. The stability of Munc13-1 was assessed by immunoblotting analysis. The overexpression of Fbxo45 promoted a significantly faster degradation of Munc13-1 (half-life 4 h) compared with the control (Vec, Fbxo45 deletion mutants). In contrast, degradation of Munc13-1 was inhibited and rebuilt to control the level in Fbxo45-expressing cells treated with MG132 (Fig. 6C). The knockdown of Fbxo45 also showed delay of degradation of endogenous Munc13-1 (Fig. 6D). These results indicate that Fbxo45 interacts with Munc13-1 via SPRY domain and leads to degradation by UPS.

DISCUSSION

Information is passed from one neuron to the next by the release of neurotransmitters at the synapse. Neurotransmitter release is mediated by recycled neurotransmitter-containing synaptic vesicles at the presynaptic plasma membrane. A key event in neurotransmitter release is the priming step, which takes place before the fusion of a synaptic vesicle with the presynaptic active zone membrane. The priming step dramatically increases the propensity for vesicular release by lowering the energy barrier for fusion, thereby enabling the vesicles to use the action potential-induced transient Ca\(^{2+}\) increase efficiently for rapid exocytosis (46). Munc13-1 plays an essential role in

![FIGURE 3. Fbxo45 protein expression. A, immunoblot analysis of Fbxo45 in adult mice is shown. B, subcellular distribution of Fbxo45 in the mouse brain is shown. Immunoblot analysis of Fbxo45, Munc13, SNAP25, synaptophysin (Syph), synaptotagmin (Syt), synaptogyrin (Syngr), or PSD-95 in the indicated fractions showed that Fbxo45 was enriched at the synaptic region, including the synaptosomal membrane fraction (LP1) and postsynaptic density (PSD). H, homogenate; P1, nuclear fraction; S1, crude synaptosomal fraction; S2, cytosolic synaptosomal fraction; P2, crude synaptosomal pellet fraction; LP2, synaptosomal vesicle fraction; SPM, synaptic plasma membrane fraction. C, immunocytochemistry of primary cultured rat hippocampal neurons expressing Fbxo45 is shown. Shown are Fbxo45 (green), VGlut1 (red), and PSD-95 (purple) (upper panels). Magnifications of the boxed areas in the upper panels are shown in the lower panels. Arrowheads indicate synapses, at which VGlut1 and PSD-95 were colocalized. Scale bars: 20 μm in the upper panels, 5 μm in the lower panels. D, shown is immunoelectron microscopy of endogenous Fbxo45. The lower panel shows a magnified image of the boxed area in the upper panel. S, synapse; Pre, presynaptic terminal; Post, postsynaptic terminal. Scale bars: 0.5 μm in the upper panel, 0.2 μm in the lower panel. E, localization of nanogold particles was quantitatively evaluated on each indicated structures (count/μm²). Asterisk, unpaired t test, p < 0.01.](image-url)
the neurotransmitter release machinery. In Munc13-1-deficient neurons, both spontaneous and evoked transmitter secretion are dramatically reduced, while vesicle docking to the active zone is essentially normal (47–50). On the other hand, the overexpression of Munc13-1 enhances neurotransmitter release at neuromuscular junctions in *Xenopus* (51). To control the level of Munc13-1 protein at synaptic sites, a spatially regulated protein degradation system like UPS is required. In support of this concept, the *Drosophila* Munc13-1 homologue *DUNC13* is ubiquitinated, and it selectively accumulates in the neuron.

**FIGURE 4. Regulation of synaptic activity by Fbxo45.** A, shown is immunocytochemistry of cultured hippocampal neurons transfected with full-length of Fbxo45, vector control (Vec), siRNAs against Fbxo45 (siRNA Fbxo45-1, siRNA Fbxo45-2), or a control siRNA and EGFP. GFP (green), Synapsin-I (Syn; red), and PSD-95 (white) (scale bars: 5 μm). B, shown is quantification of the clusters that Synapsin-I and PSD-95 colocalized in the dendrites shown in A. Vec, n = 18; Fbxo45, n = 29; siRNA control, n = 24; siRNA Fbxo45-1, n = 20; siRNA Fbxo45-2, n = 11 dendrites. C, shown is a representative recording of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor-mediated mEPSC from neurons transfected with Fbxo45 or the control vector in 2, 5, or 10 mM extracellular Ca²⁺. (Vec, n = 11; Fbxo45, n = 9; Fbxo45(154–140aa), n = 9; Fbxo45(101–286aa), n = 9; siRNA control, n = 9; siRNA Fbxo45-1, n = 12; siRNA Fbxo45-2, n = 6 neurons; asterisk, unpaired t test, p < 0.05). D, shown is mEPSC frequency in neurons transfected with Fbxo45 or the control vector in 2, 5, or 10 mM extracellular Ca²⁺. (Vec, n = 11, 14, 14; Fbxo45, n = 12, 8, 13; siRNA control, n = 9, 6, 7; siRNA Fbxo45-1, n = 12, 9, 8; siRNA Fbxo45-2, n = 6, 6, 7 neurons (2, 5, 10 mM extracellular Ca²⁺); asterisk, unpaired t test, p < 0.05.)
FIGURE 6. Involvement of Fbxo45 in the degradation of Munc13-1. A, Fbxo45 interacts with Munc13-1 through the SPRY domain. FLAG-tagged full-length or deletion mutants of Fbxo45 were co-expressed with Myc-Munc13-1 in 293T cells and then immunoprecipitated (IP) with an anti-Myc antibody. Vec, vector. B, the level of Munc13-1 protein was decreased by Fbxo45 in a dose-dependent manner. In contrast, the RIM1 level was not affected by Fbxo45. C, shown is regulation of Munc13-1 stability by Fbxo45. Munc13-1 was co-expressed with Fbxo45 full-length, Fbxo45 deletion mutants, or Fbxo45 with MG132 and EGFP in 293T cells. D, shown is regulation of endogenous Munc13-1 protein stability by endogenous Fbxo45 in Neuro2A cells. siRNA control or siRNAs against Fbxo45 were transfected into Neuro2A cell. The protein stability was assessed by immunoblot analysis after incubation with cycloheximide for various times. Protein decay curves were determined from these studies. Asterisk, p < 0.05, Mann-Whitney U test.
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presynaptic terminal after proteasome inhibition (52), although the E3 for DUNC13 is still unknown.

In the present study we cloned and identified the Fbxo45 gene, which encodes an F-box ubiquitin ligase that is specifically expressed in the nervous system. Our newly generated anti-Fbxo45 antibody demonstrated the synaptic localization of Fbxo45. Subcellular distribution assay showed that Fbxo45 expression overlapped with presynaptic protein Munc13 in L1, synaptic plasma membrane fraction, and PSD. Fbxo45 functions as a ubiquitin ligase of vesicle-priming factor Munc13-1 and regulated the spontaneous mEPSC activity in mature neurons. Munc13-1-deficient mice have been shown to exhibit a decrease of mEPSC frequency (53), which was almost the same quantitative changes as overexpression of Fbxo45. Furthermore, we showed that the level of the Munc13-1 was regulated by Fbxo45, suggesting that Fbxo45 is critically involved in the regulation of synaptic activity and functions by controlling Munc13-1 protein via ubiquitin dependent proteolysis. It remains to be studied whether there are additional substrates of Fbxo45 involved in the regulation of synaptic activity at the postsynaptic site, because Fbxo45 expresses at not only the presynaptic but also the postsynaptic site.

Fbxo45 is highly conserved among species from C. elegans to mammals. The Fbxo45 homologues FSN-1 (C. elegans) and DFn (Drosophila) have been shown to be important for synapse formation in the presynaptic neurons by interacting with RING finger-type E3 RPM-1 (C. elegans) and Highwire (Drosophila), respectively (43, 54). Details on the interaction between Fbxo45 and PAM (protein associated with Mhc), RPM-1/Highwire mammalian homologue, will be described elsewhere (45). The fsn-1 mutant in C. elegans and the DFn mutant in Drosophila exhibit increased numbers of synapses and abnormal transmission at the neuromuscular junction, fsn-1-null animals also exhibit enhanced levels of a receptor-tyro- sinase kinase, anaplastic lymphoma kinase (ALK), and their phenotype is suppressed by mutations in ALK, suggesting that FSN-1 contributes to the stabilization of synapse formation through a protein degradation pathway (43). In Drosophila, DFn down-regulates the levels of a MAP kinase kinase kinase, Wallenda/dual leucine zipper kinase (DLK), and restrains the growth of synaptic terminals (54). These findings indicate that FSN-1 and DFn are required for synaptic growth in developing neurons. However, the role of DFn/FSN-1 in mature neurons is still unclear. Here we showed that Fbxo45 negatively regulates neurotransmission without affecting synapse formation in mature hippocampal neurons. This difference may be because of context-dependent functions of Fbxo45/DFsn/FSN-1 in addition to differences in the species and neuronal regions examined (i.e. the hippocampus versus the neuromuscular junction). That is, the roles of Fbxo45/DFsn/FSN-1 might switch from those regulating synapse formation to those regulating neurotransmission as the development of the nervous system progresses.

The UPS has recently emerged as an important mechanism for regulating synaptic function. Several ubiquitin ligases are known to regulate presynaptic protein turnover and synaptic efficacy. For example, the E3 ligase SCRAPPER ubiquitinates and mediates the proteasomal degradation of RIM1, a vesicle-priming protein located in the presynaptic active zone. In Scrapper knock-out mice, the RIM1 protein levels have an increased half-life, and the mice exhibit enhanced mEPSC frequency. These results indicate that SCRAPPER regulates presynaptic transmitter release by targeting RIM1 for degradation through the UPS (9).

Interestingly, the levels of other presynaptic proteins, including Munc13-1, are also elevated in Scrapper-knock-out mice and/or reduced upon SCRAPPER overexpression. However, there is no direct interaction between SCRAPPER and Munc13-1. Therefore, other factors, like the Fbxo45 pathway, appear to regulate the amount of Munc13-1 protein and Munc13-1-dependent neurotransmitter release. A bidirec- tional pathway between Fbxo45-Munc13-1 and SCRAPPER-RIM1 may cooperatively regulate the priming step of synaptic vesicle release by protein degradation to modulate synaptic transmission. Therefore, an impairment in this pathway may lead to dysfunctional neurotransmission and disruptions in neuronal communication.

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