Auxin-mediated rapid degradation of target proteins in hippocampal neurons

Risako Nakano[a], Naoki Ihara[a], Shota Morikawa[a], Ai Nakashima[a], Masato T. Kanemaki[c,d], Yuji Ikegaya[a,e] and Haruki Takeuchi[a,b]

Genetic manipulation of protein levels is a promising approach to identify the function of a specific protein in living organisms. Previous studies demonstrated that the auxin-inducible degron strategy provides rapid and reversible degradation of various proteins in fungi and mammalian mitotic cells. In this study, we employed this technology to postmitotic neurons to address whether the auxin-inducible degron system could be applied to the nervous system. Using adeno-associated viruses, we simultaneously introduced enhanced green fluorescent protein (EGFP) fused with an auxin-inducible degron tag and an F-box family protein, TIR1 from *Oryza sativa* (OsTIR1), into hippocampal neurons from mice. In dissociated hippocampal neurons, EGFP enhanced green fluorescent protein fluorescence signals rapidly decreased when adding a plant hormone, auxin. Furthermore, auxin-induced enhanced green fluorescent protein degradation was also observed in hippocampal acute slices. Taken together, these results open the door for neuroscientists to manipulate protein expression levels by the auxin-inducible degron system in a temporally controlled manner. *NeuroReport* 30: 908–913 Copyright © 2019 Wolters Kluwer Health, Inc. All rights reserved.

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[a]Laboratory of Chemical Pharmacology, [b]Social Cooperation Program of evolutional chemical safety assessment system, LECSAS, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan [c]Molecular Cell Engineering Laboratory, National Institute of Genetics, Research Organization of Information and Systems (ROIS), [d]Department of Genetics, The Graduate University for Advanced Studies (SOKENDAI), Mishima, Shizuoka, Japan and [e]Center for Information and Neural Networks, National Institute of Information and Communications Technology, Suita, Osaka, Japan

Correspondence to Haruki Takeuchi, PhD, Social Cooperation Program of evolutional chemical safety assessment system, LECSAS, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan
Tel: +81 3 5841 4784; fax: +81 3 5841 4786;
e-mail: haruki-t@mol.f.u-tokyo.ac.jp

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Introduction

Conditional manipulation of the protein expression level is indispensable for understanding not only the function of specific proteins but also complex biological systems. Various methods have been developed to regulate the expression levels of specific proteins at the level of transcription or translation [1–3]. General approaches to control the protein level are the disruption of DNA sequence coding for a specific protein by gene editing and suppression of mRNA level by RNA interference [4–7]. However, as these methods deplete proteins in an indirect way, their temporal specificities heavily rely on the stability of the target proteins.

In the field of neuroscience, there is no doubt that conventional and Cre recombinase-mediated conditional knockout approaches contributed heavily to the understanding of brain function. However, due to the lack of temporal specificity, researchers often could not address whether observed phenotypes are attributed to developmental defects or defects in adult brain function. In a shorter time scale, it is impossible to address protein function in a specific time frame such as sleep period. Therefore, conditional and reversible control of specific proteins is desirable to elucidate brain functions.

To achieve precise temporal control of protein expression, a variety of systems have been invented that target posttranslational protein degradation using cell-permeable small molecules [8–13]. The auxin-inducible degron (AID) system has been developed by modifying a plant-specific ubiquitin-proteasome pathway [14]. Skp1-Cullin-F-Box protein (SCF) complex catalyzes polyubiquitination of proteins destined for degradation by the 26S proteasome. The SCF complex is composed of the core CUL1-RING complex and an F-box substrate-recognition subunit. Although the SCF complex is highly conserved across species, a type of F-Box protein determines the specificity of substrate recruited to the SCF complex. TIR1 is an F-box protein that is only preserved within plant species and recognizes a degron sequence (hereafter called the AID tag) conserved in the auxin/indole-3-acetic acid (AUX/IAA) family proteins only in the presence of phytohormone auxin for degradation via the ubiquitin-proteasome pathway [15–17]. The AID system has been developed by introducing two components, the plant-specific TIR1 and the AID tag, the latter of which is fused with a protein of interest to promote the degradation of AID-tagged proteins through the binding of TIR1. To date, the AID system has been employed for conditional ablation of specific proteins in a variety of

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cells [14,18–20]. Previous studies demonstrated that the AID system achieves not only conditional but also rapid degradation of target proteins in approximately 90 minutes [14,18,21]. In this study, we examined whether the AID system can be used for manipulating specific protein levels in the nervous system.

Materials and methods

Animals

All procedures were performed with the approval of the animal experiment ethics committee of The University of Tokyo (approval number: P29-15) and in accordance with the guidelines for the care and use of laboratory animals of The University of Tokyo. C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan).

DNA construction and adeno-associated virus vector production

Adeno-associated virus (AAV) vector was generated as described previously [22]. pAAV-hSyn-OsTIR1-P2A-mAID-EGFP construct was synthesized by replacing the coding region of the pAAV-hSyn-EGFP (Addgene plasmid #50465) with the OsTIR1-P2A-mAID-EGFP-NES sequence. Nuclear export signal (NES) sequence was attached to the C-terminus region of enhanced green fluorescent protein (EGFP). A single 2A sequence from porcine teschovirus-1 (P2A) was inserted into the middle of OsTIR1 and mAID-EGFP. OsTIR1-P2A-mAID-EGFP-NES sequence was amplified by PCR from pAY8 using following primers; 5′ GGATCCGCCACC ATGAC ATACTTTCCTGAAGA GGTCGTC 3′ and 5′ GCT TTGTACGG AATTGGGA GGTGTGGGAGGAGGTTTT 3′. The PCR product was subcloned into the NcoI and EcoRI sites of the pAAV-hSyn-EGFP. 293AAV cells (Cell Biolabs, San Diego, California, USA) were transfected with the pAAV-hSyn-OsTIR1-P2A-mAID-EGFP and two AAV helper plasmids encoding serotype DJ (Cell Biolabs, San Diego, California, USA) using polyethylenimine (Polysciences, Warrington, Pennsylvania, USA). Three days after transfection, AAVs were collected from the 293AAV cells and purified using AAVpro Purification Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The AAV titer was determined to be 4.8×10^{13} vg/ml by real-time PCR using ITR2 primers [23].

Primary culture of hippocampal neuronal cells

Dissociated hippocampal neurons were prepared from postnatal day 0 C57BL/6J mice as previously described [24]. Mice were anesthetized by hypothermia and quickly decapitated. Hippocampal tissue was dissected and minced in prewarmed Hank's Balanced Salt Solution (HBSS) and treated with 0.25% Trypsin/EDTA at 37°C. After 15 minutes of incubation, the tissue was treated with DNaseI (Sigma-Aldrich, St Louis, Missouri, USA) at room temperature (RT) for 5 minutes and washed with HBSS three times. HBSS was replaced with Neurobasal plating medium [Neurobasal Medium containing B27 Supplement (1:50), 0.5 mM Glutamine Solution, 25 µM Glutamate, Penicillin/Streptomycin (1:200), 1 mM HEPES, 10% horse serum (heat-inactivated and filter-sterilized, Gibco, Inc., Grand Island, New York, USA)]. Tissue was triturated using fire-polished Pasteur pipettes and filtered through a 40-µm-pore cell strainer (Corning, New York City, New York, USA). Hippocampal cells were plated on poly-D-Lysine coated 35 mm glass bottom dishes (12 mm diameter × 0.15 mm thickness glass) (IWAKI) at a density of 8.0×10^4 cells/well, and placed in a 37°C, 5% CO_2 incubator. At 1 day *in vitro* (DIV), Neurobasal plating medium was replaced with serum-free Neurobasal feeding medium (Neurobasal medium containing B27 supplement (1:50), 0.5 mM Glutamate Solution, Penicillin/Streptomycin (1:200), 1 mM HEPES). At 2 DIV, the medium was replaced with fresh feeding medium containing a final concentration of 5 µM cytosine β-D-arabinofuranoside (AraC; Sigma-Aldrich) for 24 hours to inhibit the growth of non-neuronal cells. The medium was replaced with fresh feeding medium 24 hours after adding AraC. After 3 DIV, half of the Neurobasal medium was replaced with fresh Neurobasal feeding medium every 4 days. One microliter of the diluted AAV (4.8×10^{12} vg/ml) was dropped into the culture at 7 DIV and the degradation assay was performed at 13 or 14 DIV.

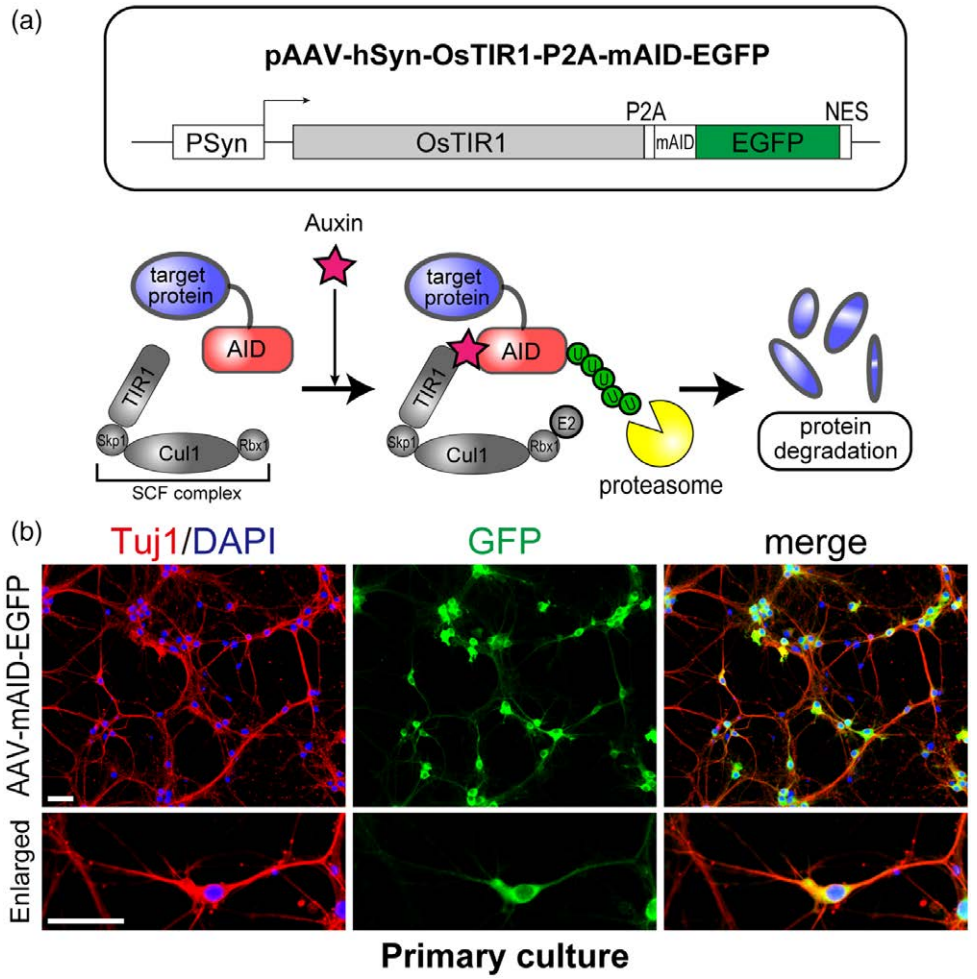
Hippocampal acute slice preparation

Adult C57BL mice were anesthetized by isoflurane and fixed in a stereotaxic frame. The skull was exposed and a glass micropipette containing the AAV was inserted to dentate gyrus (anterior-posterior = –2 mm; medial-lateral = +1.3 mm; dorsal-ventral = –2.05 mm). Five hundred nanoliters of AAV was injected at 50 nl/minute using a syringe pump (KD Scientific, Tokyo, Japan). Three weeks after the AAV injection, a posterior brain block was cut into 300-µm-thick coronal slices using a Vibratome VT1200S (Leica Microsystems, Wetzlar, Germany) in ice-cold oxygenated artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 24 mM $NaHCO_3$, 5 mM HEPES, 13 mM glucose, 2 mM $CaCl_2$). Slices were briefly transferred to an interface chamber containing oxygenated aCSF at RT. Slices were placed onto 35 mm glass base dish filled with Neurobasal feeding medium during imaging.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at RT. After fixation, cells were incubated in PBS containing 0.1% Triton X-100 for 15 minutes at RT. After washing twice with PBS, cells were blocked in a solution of PBS containing 5% normal donkey serum for 30 minutes at RT. Primary antibodies used are as follows: mouse anti-Tuj1 antibodies (Covance, 1:1000); chicken anti-GFP antibodies (Abcam, 1:1000). Secondary antibodies used are as follows: donkey antichicken IgY (IgG) (H+L) (Jackson ImmunoResearch, 1:400); donkey antimouse IgG (H+L) (Invitrogen, 1:400). Fluorescent

Fig. 1



An auxin-inducible degron system for rapid protein depletion in neurons. (a) Schematic drawings of AAV vector for a protein degradation assay using the AID system (top). Virally expressed TIR1 proteins are incorporated into SCF complex (bottom left). In the presence of auxin, TIR1 interacts with AID-fused target proteins and promotes polyubiquitination by the E2 ligase (bottom middle). Ubiquitinated target proteins are rapidly degraded by the 26S proteasome (bottom right). (b) Transduction of dissociated hippocampal cells by the AID-fused EGFP. Hippocampal cultures transduced with AAV-hSyn-OsTIR1-P2A-mAID-EGFP vector were immunostained using antibodies against GFP and Tuj1 (a neuronal marker). Scale bars, 50 µm. AAV, adeno-associated virus; AID, auxin-inducible degron; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; SCF, Skp1-Cullin-F-Box protein.

images of immunostained samples were obtained using a BZ-X700 microscope (Keyence, Osaka, Japan).

Indole-3-acetic acid treatment

IAA (IAA sodium salt, Sigma-Aldrich) was diluted in Neurobasal feeding medium to make 0.5 M stock solution. Prewarmed IAA solution was applied in hippocampal neuronal cells and acute slice in a final concentration of 0.5 mM. IAA application was immediately followed by image acquisition.

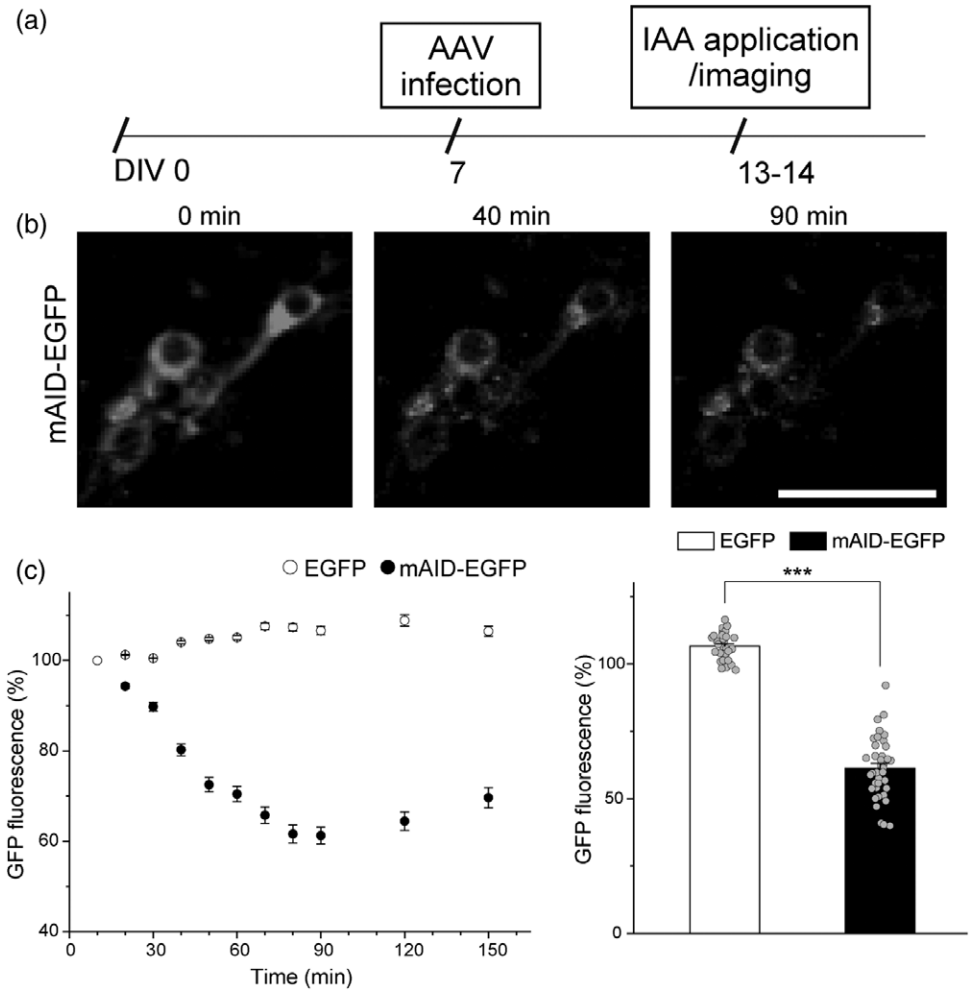
Data acquisition and statistical analysis

For protein degradation assay, images were acquired using an FV1200 scanning confocal microscope (Olympus, Tokyo, Japan) equipped with diode lasers. For imaging primary culture, Z-series images (seven optical sections) were acquired with a 10× water immersion objective lens (0.40 numerical aperture, Olympus). For imaging acute slice, Z-series images (five optical sections) were acquired with a 20× water immersion objective lens. GFP signal intensities within soma were measured with ImageJ (NIH, Bethesda, Maryland, USA). After subtracting background signals, signal intensity at each time point was normalized to the data at 10 minutes for primary cultures and 0 minutes for acute slices, respectively. Student's *t*-test was performed with OriginPro 2018 software (OriginLab, Northampton, Massachusetts, USA).

Results

To test whether the AID system could be applicable in the nervous system, we initially used primary dissociated neurons. To reduce the risk of spontaneous basal degradation

Fig. 2

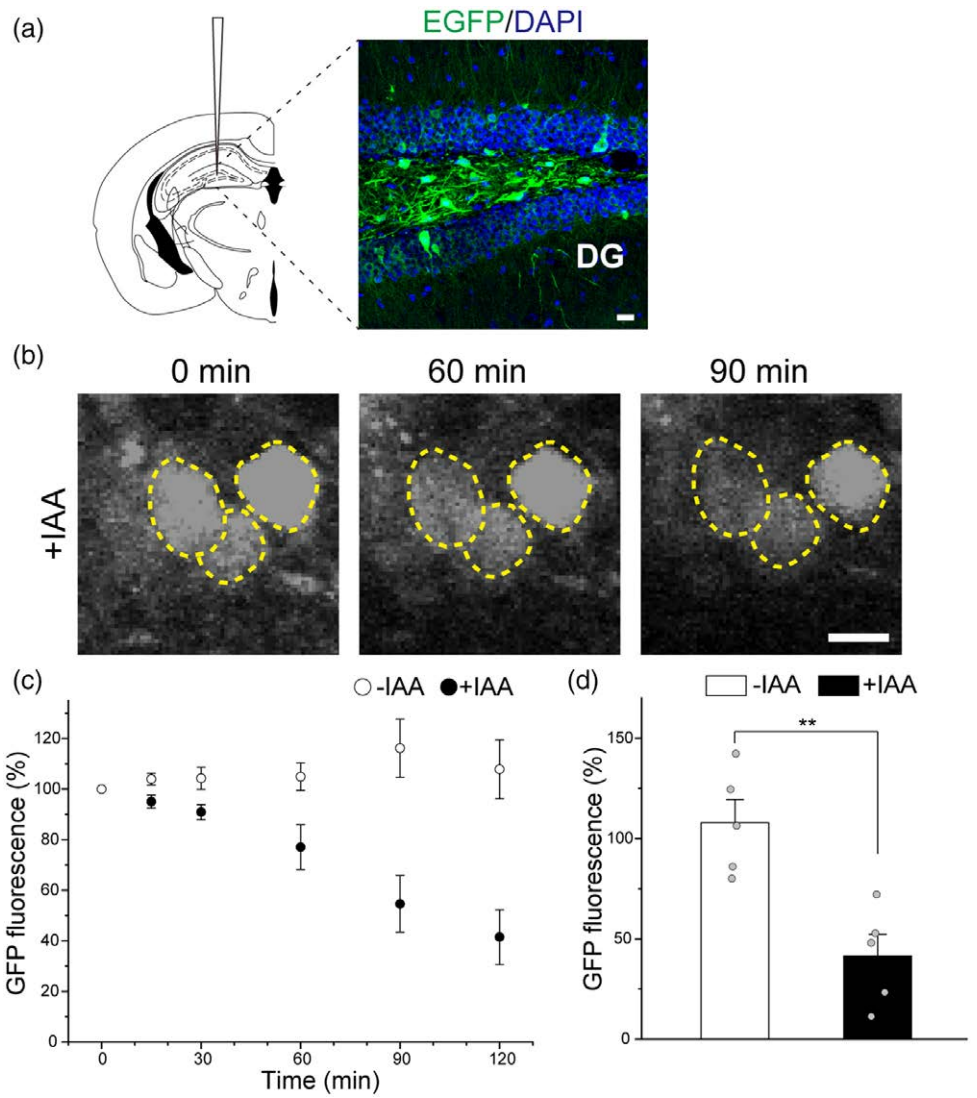


Auxin-induced protein degradation in dissociated hippocampal neurons. (a) Experimental paradigm for the protein degradation assay in dissociated neurons. Dissociated hippocampal neurons were infected with AAV-hSyn-OsTIR1-P2A-mAID-EGFP or AAV-hSyn-EGFP vector at 7 DIV and the degradation assay was performed at 13 or 14 DIV. (b) Representative images of mAID-EGFP expressing dissociated hippocampal neurons before and after IAA treatment. GFP fluorescence decreased upon IAA treatment. Scale bar, 50 μm. (c) Signal intensities of GFP in hippocampal neurons were quantified and plotted with the 10-minute time point sample as 100%. Open circles indicate EGFP and filled circles indicate mAID-EGFP. (d) Signal intensities of EGFP in dissociated hippocampal neurons 90 minutes after IAA application. Error bars indicate the standard error of the mean. $n=30$ and 38 cells for EGFP and mAID-EGFP, respectively. ***$P<0.001$, Student's *t*-test. AAV, adeno-associated virus; DIV, day *in vitro*; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; IAA, indole-3-acetic acid; mAID, mini-AID tag.

of degron-fused proteins suggested by a recent report [25], we switched the culture medium to the serum-free Neurobasal feeding medium one day after plating hippocampal neurons. Two components, TIR1 from *Oryza sativa* (OsTIR1) and proteins fused with a mini-AID tag (mAID), were introduced into neurons by AAV viral vector infection. In this case, EGFP served as the target protein. We generated AAV-DJ carrying *OsTIR1* and *EGFP* fused with *mAID* (pAAV-hSyn-OsTIR1-P2A-mAID-EGFP). Leucine-rich NES sequence was also attached to the C-terminus region of EGFP to promote translocation of EGFP proteins into the cytoplasm. P2A peptide coding sequence was inserted into the middle of *OsTIR1* and *mAID-EGFP* to achieve simultaneous expression of these proteins (Fig. 1a). The human synapsin 1 gene promoter allowed strong expression of these genes specifically in neurons. AAV-DJ containing solution (4.8×10^{13}vg/ml) was applied to dissociated hippocampal neurons prepared from postnatal day 0 pups (see Methods for details). Immunocytochemistry with antibodies against GFP and Tuj1 (a neuronal marker), revealed that mAID-EGFP proteins were present across the entire cytoplasm (Fig. 1b).

We then tested whether degradation of mAID-fused protein in dissociated neurons was triggered by the application of IAA. Dissociated hippocampal neurons prepared from postnatal day 0 pups were infected by the AAV-DJ carrying *OsTIR1* and *mAID-EGFP* at 7 DIV. One week after AAV infection (13–14 DIV), EGFP fluorescent intensities were quantified with fluorescence time-lapse

Fig. 3



Auxin-induced protein degradation in acute brain slices. (a) AAV-hSyn-OsTIR1-P2A-mAID-EGFP vector was injected into dentate gyrus and the degradation assay was performed 3 weeks after the injection. Expression of EGFP in a coronal hippocampal section from the AAV-infected mouse. Scale bar, 20 µm. (b) Representative images of mAID-EGFP expressing hippocampal neurons before and after IAA treatment. GFP fluorescence decreased upon IAA treatment. Neurons are demarked by dashed lines. Scale bar, 10 µm. (c) Signal intensities of mAID-EGFP in hippocampal neurons were quantified and plotted with the 0-minute time point sample as 100%. (d) Signal intensities of GFP in hippocampal neurons 120 minutes after IAA application. Error bars indicate the standard error of the mean. $n=5$ cells per group. $^{**}P<0.01$, Student's t-test. AID, auxin-inducible degron; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; IAA, indole-3-acetic acid.

imaging after IAA treatment to analyze the kinetics of protein degradation (Fig. 2a). We found that fluorescence signals from mAID-EGFP dropped over time (Fig. 2b) and showed 60 % decrease at 90 minutes after IAA application (Fig. 2c and d, $n=30$ and 38 cells for EGFP and mAID-EGFP, respectively. $t(66)=-20.2$, $^{***}P<0.001$, Student's t-test). As a control experiment, we performed the same experiment using EGFP without mAID. As expected, fluorescent signals from EGFP without mAID did not decrease upon IAA treatment. This result indicated that auxin-induced degradation of mAID-fused proteins occurred in *in-vitro* dissociated hippocampal neurons.

We then tested whether the auxin-induced protein degradation occurs in the acute brain slices. We injected the AAV-OsTIR1-P2A-mAID-EGFP into the dentate gyrus of mice (Fig. 3a) and conducted protein degradation assay 3 weeks after AAV infection. Acute brain slices containing dentate gyrus were prepared and EGFP fluorescence signal intensities in hippocampal neurons with (+) or without (–) IAA treatment were measured by time-lapse imaging using confocal microscopy (Fig. 3b and c). We found that EGFP fluorescence decreased to about 50% of its initial value within 120 minutes after IAA application (Fig. 3c and d, $n=5$ cells per group. $t(8)=-4.17$, $^{**}P<0.01$, Student's t-test), suggesting

that auxin-induced degradation of mAID-fused proteins also occurred under acute slice conditions.

Discussion

In the present study, we examined whether conditional protein degradation with the AID system can be transferable to the central nervous system. By time-lapse imaging of EGFP fluorescence, we concluded that auxin-inducible degradation of mAID-tagged proteins occurred in primary culture and acute slice conditions. However, it should be noted that the efficiency and the rate of degradation were not comparable to those observed in other cell lines [18,21]. Why is the AID system only partially working in neurons? One explanation is that the N-terminus fusion of mAID to EGFP was not suitable for efficient protein degradation. It might be possible that protein degradation occurs more efficiently and quickly if mAID was fused to the C-terminus region of the proteins. Another explanation is that the rate of protein synthesis was much higher than that of degradation induced by auxin. Because expression levels of virally induced proteins are thought to be much higher than those of endogenous proteins, it is possible that virally induced mAID-EGFP production overwhelmed protein degradation induced by auxin. It would be interesting to test the AID system at physiological expression levels. To apply this method to endogenous proteins, other knock-in approaches will be required to tag mAID sequence. The recent advancement of genome editing techniques such as the CRISPR/Cas9 system would increase the accessibility of the AID system and facilitate the functional analysis of proteins in a temporally controlled manner [13]. We observed the difference in time-course of protein degradation between dissociated neurons and acute slices. The degradation in dissociated neurons plateaued and recovered at 120 minutes, whereas in the slice, it was still in the phase of declining (Figs. 2c and 3c). We prepared the primary cultures and the acute slices from neonatal and adult mice, respectively. Because the AID system requires endogenous components except for OsTIR1, we speculate that the efficacy of endogenous ubiquitin-mediated protein degradation differs between immature and mature hippocampal neurons. Conditional and reversible control of specific proteins is desirable to elucidate brain functions. Further improvement in the AID system would provide powerful tools to investigate protein functions in the field of neuroscience.

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Conflicts of interest

There are no conflicts of interest.

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