Title: Auxin-mediated rapid degradation of selective proteins in hippocampal neurons

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Abstract

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 (AID) 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 AID system could be applied to the nervous system. Using adeno-associated viruses, we simultaneously introduced EGFP fused with an AID tag, and an F-box family protein, TIR1 from *Oryza sativa* (OsTIR1) into hippocampal neurons. In dissociated hippocampal neurons, EGFP fluorescence signals rapidly decreased when adding a plant hormone, auxin. Further, auxin-induced EGFP degradation was also observed in hippocampal acute slices. Taken together, these results open the door for neuroscientists to manipulate protein expression levels by the AID-system in a temporally-controlled manner.

17 **Keywords**

18 **auxin-inducible degron, rapid protein degradation, ubiquitin-proteasome system,**

19 **neurons**

20 **Introduction**

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

29 To achieve precise temporal control of protein expression, a variety of
30 systems have been invented that target posttranslational protein degradation using
31 cell-permeable small molecules [8-13]. The auxin-inducible degron (AID) system has
32 been developed by modifying a plant-specific ubiquitin-proteasome pathway [14].
33 Skp1-Cullin-F-Box protein (SCF) complex catalyzes polyubiquitination of proteins
34 destined for degradation by the 26S proteasome. The SCF complex is composed of the
35 core CUL1-RING complex and an F-box substrate-recognition subunit. Although the
36 SCF complex is highly conserved across species, a type of F-Box protein determines

37 the specificity of substrate recruited to the SCF complex. TIR1 is an F-box protein that
38 is only preserved within plant species and recognizes a degron sequence (hereafter
39 called the AID tag) conserved in the AUX/IAA family proteins only in the presence of
40 phytohormone auxin for degradation via the ubiquitin-proteasome pathway [15-17].
41 The AID system has been developed by introducing two components, the
42 plant-specific TIR1 and the AID tag, the latter of which is fused with a protein of
43 interest to promote the degradation of AID-tagged proteins through the binding of
44 TIR1. To date, the AID system has been employed for conditional ablation of specific
45 proteins in a variety of cells [14, 18-20]. Previous studies demonstrated that the AID
46 system achieves not only conditional, but also rapid degradation of target proteins on a
47 time scale of several ten minutes [14, 18, 21]. In this study, we examined whether the
48 AID system can be used for manipulating specific protein levels in the nervous system.

49 **Materials and methods**

50 **Animals**

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

56 **DNA construction and AAV vector production**

57 Adeno-associated virus (AAV) vector was generated as described previously
58 [22]. pAAV-hSyn-OsTIR1-P2A-mAID-EGFP construct was synthesized by replacing
59 coding region of the pAAV-hSyn-EGFP (Addgene plasmid #50465) with the
60 OsTIR1-P2A-mAID-EGFP-NES sequence OsTIR1-p2A-mAID-EGFP-NES sequence
61 was amplified by PCR from pAY8 using following primers; 5'
62 GGATCCGCCACCATGACATACTTTCCTGAAGAGGTCGTC 3' and 5'
63 GCTTTGTACGGAATTGGGAGGTGTGGGAGGAGGTTTT 3'. The PCR product

64 was subcloned into the NcoI and EcoRI sites of the pAAV-hSyn-EGFP. HEK293T
65 cells were transfected with the pAAV-hSyn-OsTIR1-P2A-mAID-EGFP and two AAV
66 helper plasmids encoding serotype DJ (Cell Biolabs, San Diego, CA, USA) using
67 polyethylenimine (Polysciences, Warrington, PA, USA). Three days after
68 transfection, AAVs were collected from HEK293T cells and purified using AAVpro
69 Purification Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol.
70 The AAV titer was determined to be 4.8×10^{13} vg/ml by real-time PCR using ITR2
71 primers [23].

72 **Primary culture of hippocampal neuronal cells**

73 Dissociated hippocampal neurons were prepared from postnatal day (PD) 0
74 C57BL/6J mice as previously described [24]. Mice were anesthetized by hypothermia
75 and quickly decapitated. Hippocampal tissue was dissected and minced in pre-warmed
76 Hank's Balanced Salt Solution (HBSS) and treated with 0.25% Trypsin/EDTA at 37
77 °C. After 15 min of incubation, the tissue was treated with DNaseI (Sigma-Aldrich, St
78 Louis, Missouri, USA) at room temperature (RT) for 5 min and washed with HBSS
79 three times. HBSS was replaced with Neurobasal plating medium [Neurobasal

80 Medium containing B27 Supplement (1:50), 0.5 mM Glutamine Solution, 25 μ M
81 Glutamate, Penicillin/Streptomycin (1:200), 1 mM HEPES, 10% horse serum
82 (heat-inactivated and filter-sterilized, Gibco, Inc., Grand Island, NY, USA)]. Tissue
83 was triturated using fire-polished Pasteur pipettes and filtered through a 40- μ m-pore
84 cell strainer (Corning, New York City, NY, USA). Hippocampal cells were plated on
85 poly-D-Lysine coated glass base dishes (35 mmf with a window of 12 mmf, 0.15 mm
86 thick glass; IWAKI) at a density of 8.0×10^4 cells/well, and placed in a 37 °C, 5% CO₂
87 incubator. At 1 day *in vitro* (DIV), Neurobasal plating medium was replaced with
88 Neurobasal feeding medium (Neurobasal Medium containing B27 Supplement (1:50),
89 0.5 mM Glutamate Solution, Penicillin/Streptomycin (1:200), 1 mM HEPES). At 2
90 DIV, the medium was replaced with fresh feeding medium containing a final
91 concentration of 5 μ M cytosine β -D-arabinofuranoside (AraC; Sigma-Aldrich) for 24
92 hours to inhibit the growth of non-neuronal cells. The medium was replaced with fresh
93 feeding medium 24 hours after adding AraC. After 3 DIV, half of the Neurobasal
94 medium was replaced with fresh Neurobasal feeding medium every 4 days. 1 μ l of the
95 diluted AAV (4.8×10^{12} vg/ml) was dropped into the culture at 7 DIV and the
96 degradation assay was performed at 13 or 14 DIV.

97 **Hippocampal acute slice preparation**

98 Adult mice were anesthetized by isoflurane and fixed in a stereotaxic frame.

99 The skull was exposed and a glass micropipette containing the AAV was inserted to

100 dentate gyrus (AP = -2 mm; ML = +1.3 mm; DV = -2.05 mm). 500 nl of AAV was

101 injected at 50 nl per min using a syringe pump (KD Scientific, Tokyo, Japan). 3 weeks

102 after the AAV injection, a posterior brain block was cut into 300- μ m thick coronal

103 slices using a Vibratome VT1200S (Leica Microsystems, Wetzlar, Germany) in

104 ice-cold oxygenated artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 2.5mM KCl,

105 1.2 mM NaH_2PO_4 , 24 mM NaHCO_3 , 5 mM HEPES, 13 mM glucose, 2 mM CaCl_2 .

106 Slices were briefly transferred to an interface chamber containing oxygenated aCSF at

107 RT. Slices were placed onto 35 mm glass base dish filled with Neurobasal feeding

108 medium during the degradation assay.

109 **Immunocytochemistry**

110 Cells were fixed in 4% paraformaldehyde in PBS for 10 min at RT. After

111 fixation, cells were incubated in PBS containing 0.1% Triton X-100 for 15 min at RT.

112 After washing twice with PBS, cells were blocked in a solution of PBS containing 5%

113 normal donkey serum for 30 min at RT. Primary antibodies used are as follows: mouse
114 anti-Tuj1 antibodies (Covance, 1:1,000); chicken anti-GFP antibodies (Abcam,
115 1:1,000). Fluorescent images of immunostained samples were obtained using a
116 BZ-X700 microscope (Keyence, Osaka, Japan).

117 **Data acquisition and statistical analysis**

118 Images were acquired using an FV1200 scanning confocal microscope
119 (Olympus, Tokyo, Japan) equipped with diode lasers. For imaging primary culture,
120 Z-series images (7 optical sections) were acquired with a 10 × water immersion
121 objective lens (0.40 numerical aperture, Olympus). For imaging acute slice, Z-series
122 images (5 optical sections) were acquired with a 20 × water immersion objective lens.
123 GFP signal intensities within soma were measured with ImageJ (NIH, Bethesda, MD,
124 USA). After subtracting background signals, signal intensity at each time point was
125 normalized to the data at 10 min (100%). Statistical analyses were performed with
126 OriginPro (OriginLab) software.

127 **IAA treatment**

128 IAA (indole-3-acetic acid sodium salt, Sigma-Aldrich) was diluted in
129 Neurobasal feeding medium to make 0.5 M stock solution. Pre-warmed IAA solution
130 was applied in hippocampal neuronal cells and acute slice in a final concentration of
131 0.5 mM.

132 **Results**

133 To test whether the AID system could be applicable in the nervous system, we
134 initially used primary dissociated neurons. To avoid spontaneous basal degradation of
135 degron-fused proteins suggested by a recent report [25], we cultured hippocampal
136 neurons in culture media without serum. Two components, TIR1 from *Oryza sativa*
137 (OsTIR1) and proteins fused with a mini-AID tag (mAID), were introduced into
138 neurons by AAV viral vector infection. We generated AAV-DJ carrying *OsTIR1* and
139 *EGFP* fused with *mAID* (pAAV-hSyn-OsTIR1-P2A-mAID-EGFP). Leucine-rich
140 nuclear export signal (NES) sequence was also attached to the C-terminus region of
141 EGFP to promote translocation of EGFP proteins into the cytoplasm. P2A peptide

142 coding sequence was inserted into the middle of *OsTIR1* and *mAID-EGFP* to achieve
143 simultaneous expression of these proteins (Fig. 1A). AAV-DJ containing solution (4.8
144 $\times 10^{13}$ vg/ml) was applied to dissociated hippocampal neurons prepared from PD0
145 pups (See Methods for details). Immunocytochemistry with antibodies against GFP
146 and Tuj1 (a neuronal marker), revealed that mAID-EGFP proteins were present across
147 the entire cytoplasm and that ectopic expression of mAID-EGFP proteins did not affect
148 the morphology of the dissociated neurons (Fig. 1B).

149 We then tested whether degradation of mAID-fused protein in dissociated
150 neurons was triggered by application of indole-3-acetic acid (IAA). Dissociated
151 hippocampal neurons prepared from PD0 pups were infected by the AAV-DJ carrying
152 *OsTIR1* and *mAID-EGFP* at 7 days *in vitro* (7 DIV). One week after AAV infection
153 (13-14 DIV), EGFP fluorescent intensities were quantified with fluorescence
154 time-lapse imaging after IAA treatment to analyze the kinetics of protein degradation
155 (Fig. 2A). We found that EGFP fluorescence dropped over time (Fig. 2B) and showed
156 the weakest signal intensity 90 min after IAA application (Figs. 2C and D, $n = 30$ and
157 38 cells for EGFP and mAID-EGFP, respectively. $***p < 0.001$, Student's *t*-test). In
158 contrast, fluorescent signals from EGFP without mAID did not decrease upon IAA
159 treatment. This result indicated that auxin-induced degradation of mAID-fused proteins

160 occurred in *in vitro* dissociated hippocampal neurons.

161 We then tested whether the auxin-induced protein degradation occurs in the
162 acute brain slices. We injected the AAV-OsTIR1-P2A-mAID-EGFP into the dentate
163 gyrus of mice (Fig. 3A) and conducted protein degradation assay 3 weeks after AAV
164 infection. Acute brain slices containing dentate gyrus were prepared and EGFP
165 fluorescence signal intensities in hippocampal neurons with (+) or without (-) IAA
166 treatment were measured by time-lapse imaging using confocal microscopy (Figs. 3B
167 and C). We found that EGFP fluorescence decreased to about 50% of its initial value
168 within 120 min after IAA application (Figs. 3C and D, $n = 5$ cells per group, $**p <$
169 0.01 , Student's t -test). This result implies that the AID system is partially working.

170 Discussion

171 In the present study, we examined whether conditional protein degradation
172 with the AID system can be transferable to the central nervous system. By time-lapse
173 imaging of EGFP fluorescence, we concluded that auxin-inducible degradation of
174 mAID-tagged proteins occurred in primary culture and acute slice conditions.

175 However, it should be noted that the efficiency and the rate of degradation were not
176 comparable to those observed in other cell lines [18, 21]. Why is the AID system only
177 partially working in neurons? One explanation is that the N-terminus fusion of mAID
178 to EGFP was not suitable for efficient protein degradation. It might be possible that
179 protein degradation occurs more efficiently and quickly if mAID was fused to the
180 C-terminus region of the proteins. Another explanation is that the rate of protein
181 synthesis was much higher than that of degradation induced by auxin. Because
182 expression levels of virally-induced proteins are thought to be much higher than those
183 of endogenous proteins, it is possible that virally-induced mAID-EGFP production
184 overwhelmed protein degradation induced by auxin. It would be interesting to test the
185 AID system combined with a knock-in approach to achieve physiological expression
186 levels of mAID-fused proteins. Conditional and reversible control of specific proteins
187 is desirable to elucidate brain functions. Further improvement in the AID system
188 would provide powerful tools to investigate protein functions in the field of
189 neuroscience.

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195 **Abbreviations**

196 AID, auxin-inducible degron; EGFP, enhanced green fluorescent protein; SCF
197 complex, Skp1-Cullin-F-Box protein complex; AAV, adeno-associated virus; DIV,
198 day *in vitro*; IAA, indole-3-acetic acid sodium salt

199 **LEGENDS**

200 **Figure 1. An auxin-inducible degron system for rapid protein depletion in**
 201 **neurons.**

202 **(A)** Schematic drawings of AAV vector for a protein degradation assay using the AID
 203 system (top). Virally expressed TIR1 proteins are incorporated into SCF complex
 204 (bottom left). In the presence of auxin, TIR1 interacts with AID-fused target proteins
 205 and promotes polyubiquitination by the E2 ligase (bottom middle). Ubiquitinated
 206 target proteins are rapidly degraded by the 26S proteasome (bottom right). **(B)**

207 Transduction of dissociated hippocampal cells by the AID-fused EGFP. Hippocampal
 208 cultures transduced with AAV-hSyn-OsTIR1-P2A-mAID-EGFP vector were
 209 immunostained using antibodies against GFP and Tuj1 (a neuronal marker). Scale bars,
 210 50 μ m.

211 **Figure 2. Auxin-induced protein degradation in dissociated hippocampal neurons.**

212 **(A)** Experimental paradigm for the protein degradation assay in dissociated neurons.
 213 Dissociated hippocampal neurons were infected with
 214 AAV-hSyn-OsTIR1-P2A-mAID-EGFP or AAV-hSyn-EGFP vector at 7 DIV and the
 215 degradation assay was performed at 13 or 14 DIV. **(B)** Representative images of
 216 mAID-EGFP expressing dissociated hippocampal neurons before and after IAA
 217 treatment. GFP fluorescence decreased upon IAA treatment. Scale bar, 50 μ m. **(C)**
 218 Signal intensities of GFP in hippocampal neurons were quantified and plotted with the
 219 10 min time point sample as 100%. Open circles indicate EGFP and filled circles
 220 indicate mAID-EGFP. **(D)** Signal intensities of EGFP in dissociated hippocampal
 221 neurons 90 min after IAA application. Error bars indicate the SEM. $n = 30$ and 38 cells
 222 for EGFP and mAID-EGFP, respectively. $***p < 0.001$, Student's t -test.

223 **Figure 3. Auxin-induced protein degradation in acute brain slices.**

224 **(A)** AAV-hSyn-OsTIR1-P2A-mAID-EGFP vector was injected into dentate gyrus and
 225 the degradation assay was performed 3 weeks after the injection. Expression of EGFP
 226 in a coronal hippocampal section from the AAV-infected mouse. Scale bar, 20 μ m.
 227 DG, dentate gyrus. **(B)** Representative images of mAID-EGFP expressing
 228 hippocampal neurons before and after IAA treatment. GFP fluorescence decreased
 229 upon IAA treatment. Neurons are demarked by dashed lines. Scale bar, 10 μ m. **(C)**
 230 Signal intensities of mAID-EGFP in hippocampal neurons were quantified and plotted
 231 with the 0 min time point sample as 100%. **(D)** Signal intensities of GFP in
 232 hippocampal neurons 120 min after IAA application. Error bars indicate the SEM. n =
 233 5 cells per group. $**p < 0.01$, Student's *t*-test.

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A **B**

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