# Title Page

**Title: Autophagy enhances memory erasure through synaptic destabilization**

**Abbreviated title:** Autophagy enhances memory destabilization

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## Abstract

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42    There is substantial interest in memory reconsolidation as a target for the treatment of

43    anxiety disorders such as post-traumatic stress disorder (PTSD). However, its applicability is

44    restricted by reconsolidation-resistant conditions that constrain the initial memory

45    destabilization. In this study, we investigated whether the induction of synaptic protein

46    degradation through autophagy modulation, a major protein degradation pathway, can enhance

47    memory destabilization upon retrieval and whether it can be utilized to overcome these

48    conditions. Here, using male mice in an auditory fear reconsolidation model, we showed that

49    autophagy contributes to memory destabilization and its induction can be utilized to enhance

50    erasure of a reconsolidation-resistant auditory fear memory that depended on α-amino-3-

51    hydroxy-5-methyl4-isoxazolepropionic acid receptor (AMPAR) endocytosis. Using male mice in

52    a contextual fear reconsolidation model, autophagy induction in the amygdala or in the

53    hippocampus enhanced fear or contextual memory destabilization, respectively.  The latter

54    correlated with AMPAR degradation in the spines of the contextual memory-ensemble cells.

55    Using male rats in an *in vivo* long-term potentiation reconsolidation model, autophagy induction

56    enhanced synaptic destabilization in an N-methyl-D-aspartate receptor-dependent manner. These

57    data indicate that induction of synaptic protein degradation can enhance both synaptic and

58    memory destabilization upon reactivation and that autophagy inducers have the potential to be

59    used as a therapeutic tool in the treatment of anxiety disorders.

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## Significance Statement

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61      It has been reported that inhibiting synaptic protein degradation prevents memory

62  destabilization. However, whether the reverse relation is true and whether it can be utilized to

63  enhance memory destabilization is still unknown. Here we addressed this question on the

64  behavioral, molecular and synaptic levels, and showed that induction of autophagy, a major

65  protein degradation pathway, can enhance memory and synaptic destabilization upon

66  reactivation. We also show that autophagy induction can be utilized to overcome a

67  reconsolidation-resistant memory, suggesting autophagy inducers as a potential therapeutic tool

68  in the treatment of anxiety disorders.

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## Introduction

Retrieval of long-term memories (LTM) can induce a destabilization process that returns them into a labile state, which is followed by a protein synthesis-dependent reconsolidation process that serves to strengthen or update the original memories (Nader et al., 2000; Besnard et al., 2012; Finnie and Nader, 2012; Inaba et al., 2015; Lee et al., 2017). Blocking reconsolidation has been suggested as a tool to weaken traumatic memories in anxiety disorders such as post-traumatic stress disorder (PTSD). However, the initial destabilization step is challenging when memories are formed under extremely stressful conditions, and it would require pharmacological assistance (Tronson and Taylor, 2007; Pitman, 2011; Besnard et al., 2012). It has been reported that inhibition of synaptic protein degradation, through blocking the ubiquitin-proteasome system, prevents memory destabilization (Lee et al., 2008). However, whether induction of synaptic protein degradation can be utilized to enhance memory destabilization is yet to be tested.

Macro-autophagy, hereafter referred to as autophagy, is a major protein degradation pathway where a newly synthesized isolation membrane sequesters a small portion of the cytoplasm to form a multilamellar vesicle called an autophagosome. To degrade the entrapped contents, autophagosomes fuse into the endosome-lysosome system (Mizushima and Komatsu, 2011; Yamamoto and Yue, 2014). The process of autophagosome synthesis is orchestrated by molecular machinery consisting of the autophagy-related genes (Atg) found in yeast, and their mammalian homologs (Mizushima et al., 2011; Ohsumi, 2014). In the brain, autophagy plays an important role in neurodegenerative diseases (Yamamoto and Yue, 2014) and is essential for the development of a healthy brain (Hara et al., 2006; Komatsu et al., 2006; Liang et al., 2010). It has been suggested that neurons may have adapted autophagy to suit their complex needs

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92    including contribution to synaptic function (Bingol and Sheng, 2011; Mizushima and Komatsu,

93    2011; Shehata and Inokuchi, 2014; Yamamoto and Yue, 2014). In line with this idea,

94    autophagosomes are found not only in the neuron's soma and axons but also in the dendrites

95    (Hollenbeck, 1993; Shehata et al., 2012), Also, autophagy contributes to the degradation of the

96    endocytosed γ-aminobutyric acid receptors (GABAR) in *Caenorhabditis elegans* and of the α-

97    amino-3-hydroxy-5-methyl4-isoxazolepropionic acid receptors (AMPAR) upon chemical long-

98    term depression (LTD) in cultured neurons (Rowland et al., 2006; Shehata et al., 2012). Both

99    GABAR and AMPAR play pivotal roles in the synaptic plasticity models of LTD and long-term

100   potentiation (LTP), which are causally correlated with memory (Kessels and Malinow, 2009;

101   Squire and Kandel, 2009; Nabavi et al., 2014). Moreover, the regulation of autophagy intersects

102   with protein synthesis regulation at the mammalian target of rapamycin (mTOR) and the

103   phosphatidylinositol-3-monophosphate kinase (PI3K) and by careful consideration of the

104   discrepancy in the effects of the mTOR and PI3K modulators on memory processes, autophagy

105   is suggested to play a role in memory reconsolidation (Chen et al., 2005; Gafford et al., 2011;

106   Shehata and Inokuchi, 2014). In the present study, we tested the hypothesis that autophagy could

107   play a role in synaptic and memory destabilization and therefore, the induction of autophagic

108   protein degradation can be utilized to enhance erasure of reconsolidation-resistant fear memories.

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## Materials & Methods

### Animals

112   All procedures involving the use of animals were conducted in compliance with the guidelines of

113   the National Institutes of Health (NIH) and were approved by the Animal Care and Use

114   Committee of the University of Toyama, Japan. Eight-week-old male Wistar ST rats were

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115    purchased for electrophysiological experiments, and 8-week-old male C57BL/6J mice were

116    purchased for behavioral experiments. The c-fos-tTA mice were purchased from the Mutant

117    Mouse Regional Resource Center (stock number: 031756-MU). The progeny for the c-fos-tTA

118    line was generated using *in vitro* fertilization of eggs from C57BL/6J mice, as described

119    previously (Ohkawa et al., 2015). All animals were purchased from Sankyo Labo Service Co.

120    Ltd. (Tokyo, Japan). Rats and mice were maintained in separate rooms on a 12 h light-dark cycle

121    at $24 \pm 3°C$ and $55 \pm 5\%$ humidity. They were given food and water ad libitum and housed with

122    littermates until surgery.

123    **Drugs and peptides**

124    Anisomycin (Sigma Aldrich Japan Co., Tokyo, Japan) was dissolved in a minimum quantity of

125    HCl, diluted with phosphate buffered saline (PBS), and adjusted to pH 7.4 with NaOH (Ani).

126    Ifenprodil tartrate (Sigma Aldrich Co., Japan) and trifluoperazine dihydrochloride (Sigma

127    Aldrich Japan Co.) were dissolved in PBS. Spautin-1 (Sigma Aldrich Co., Japan) was dissolved

128    in DMSO and diluted with equal volume of saline. The retro-inverso Tat-beclin 1 peptide D-

129    amino acid sequence (RRRQRRKKRGYGGTGFEGDHWIEFTANFVNT; synthesized by

130    GenScript through Funakoshi Co., Ltd., Tokyo, Japan) was dissolved in either PBS (tBC) or

131    anisomycin solution (Ani+tBC). The control D-Tat peptide D-amino acid sequence

132    (YGRKKRRQRRR; EMC microcollections, Tübingen, Germany) was dissolved in PBS (D-Tat).

133    The Tat-GluA2$_{3Y}$ peptide L-amino acid sequence (YGRKKRRQRRRYKEGYNVYG, AnaSpec,

134    Fremont, CA) and its control Tat-GluA2$_{3A}$ peptide L-amino acid sequence

135    (YGRKKRRQRRRAKEGANVAG; AnaSpec), were both dissolved in PBS (GluA2$_{3Y}$ or

136    GluA2$_{3A}$, respectively). All peptides were aliquoted into single experiment volumes and stored at

137    $-80°C$.

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**Stereotactic surgery and drug infusion in mice**

Mice were 8–10 weeks old at the time of surgery. They were anesthetized with isoflurane, given

an intraperitoneal injection of pentobarbital solution (80 mg/kg of body weight), and then placed

in a stereotactic apparatus (Narishige, Tokyo, Japan). Mice were then bilaterally implanted with

a stainless guide cannula (PlasticsOne, Roanoke, VA, USA). For targeting the CA1, the guide

cannula was positioned 1.8 mm posterior, 1.55 mm lateral, and 1.5 (C57BL/6J) or 1.0 mm (c-

fos-tTA mice) ventral to the bregma. For targeting the BLA, the guide cannula was positioned

1.5 mm posterior, 3.3 mm lateral, and 3.4 mm ventral to the bregma. For targeting the LA, the

guide cannula was positioned 1.7 mm posterior, 3.4 mm lateral, and 2.6 mm ventral to the

bregma. After surgery, a cap or dummy cannula (PlasticsOne) was inserted into the guide

cannula, and mice were allowed to recover for at least 7 days in individual home cages before the

experiment. Mice in the NoFS condition were not cannulated. All drug infusions were done

under isoflurane anesthesia, using an injection cannula with a 0.25 mm internal diameter

(PlasticsOne), and extending beyond the end of the guide cannula by 0.5 mm for the CA1, or by

1.5 mm for the BLA and LA. The drug infusion rate was 0.2 µl/minute for the CA1 in C57BL/6J

mice, or 0.1 µl/minute for the CA1 in c-fos-tTA mice, and the BLA and LA. Following drug

infusion, the injection cannula was left in place for 2 minutes to allow for drug diffusion. For the

reconsolidation experiments, immediately after retrieval 1 µl of drug solution was injected into

the CA1 in C57BL/6J mice, or 0.5 µl was injected into the CA1 in c-fos-tTA mice, the BLA and

LA. In all of these reconsolidation experiments, 1 µl of drug solution contained either PBS, 125

µg of Ani, 20 µg of tBC, or 125 µg Ani + 20 µg tBC. For autophagy inhibition, 0.5 µl of a

solution containing 8.3 µg Spautin-1 or vehicle was injected into LA. For blocking AMPA

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160     receptor endocytosis, 0.5 µl of solution containing 20 ng of $GluA2_{3Y}$ or $GluA2_{3A}$ was injected

161     into the LA.

**Lysate preparation and immunoblot analysis**

163     Drugs were infused into the CA1 or amygdala of one hemisphere of the C57BL/6J mice, as

164     described above. Four hours later, their brains were removed and cut into 1-mm slices, placed on

165     ice, and the hippocampus or amygdala from each hemisphere was dissected under a binocular

166     microscope, rapidly frozen on dry ice, and stored at $-80°C$. Samples were then sonicated in

167     RIPA buffer (50 mM TrisHCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium

168     deoxycholate, 0.1% SDS, and 50 mM NaF) containing a protease inhibitor mixture (cOmplete

169     ULTRA tablets, Roche Diagnostics GMBH, Mannheim, Germany) and a phosphatase inhibitor

170     mixture (PhosSTOP tablets, Roche Diagnostics GMBH). Samples were then centrifuged at

171     14000 rpm for 15 minutes at $4°C$, and supernatants were stored at $-30°C$ until use. Measurement

172     of protein concentration, immunoblotting for LC3 detection (ab48394; Abcam, Tokyo, Japan),

173     visualization and quantitation were performed as previously described (Shehata et al., 2012).

**Contextual fear conditioning (CFC)**

175     All behavioral sessions were conducted during the light cycle, in a dedicated soundproof

176     behavioral room (Yamaha Co., Shizuoka, Japan), described here as Room A. The CS context

177     was a square chamber (Chamber A) with a plexiglass front, off-white side- and back-walls

178     (length $175 \times$ width $165 \times$ height 300 mm) and a floor consisting of stainless steel rods

179     connected to an electric shock generator. The distinct context was a circular chamber (Chamber

180     B) with opaque reddish walls (diameter 235 mm $\times$ height 225 mm) and a smooth gray floor. One

181     day before the experiment, mice were left undisturbed on a waiting rack for 2 h for habituation

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182    purposes. On the day of the experiment, mice were left undisturbed on the waiting rack for at

183    least 30 minutes before and after each session, and during the experiment. In each session, one

184    mouse in its home cage was moved into Room A. During the conditioning or reconditioning

185    sessions, mice were placed in Chamber A and allowed to explore for 148 sec, before receiving

186    one footshock (2 sec, 0.4 mA). They then remained for 30 sec, before being moved back to their

187    home cages and returned to the waiting rack. During the retrieval session (T1), mice were placed

188    back into Chamber A for 3 minutes, then immediately subjected to isoflurane anesthesia and

189    drug infusion. Mice in the NoFS condition were manipulated identically, with the exceptions that

190    the shock generator was turned off. During the test sessions, mice were placed back into

191    Chamber A (T2 and T4) for 5 minutes, and 1 h later into Chamber B (T3) for 5 minutes. Mice

192    remained on the waiting rack during the 1 h interval. In all behavioral sessions, chambers were

193    cleaned with 70% ethanol and water between each mouse, and kept odorless to the experimenter.

**Auditory fear conditioning (AFC)**

195    Different chambers were used for each auditory fear conditioning session. Context exploration

196    and conditioning were performed in Chamber A. Retrieval sessions were performed in a circular

197    chamber (Chamber C) with opaque black walls (diameter 215 mm × height 340 mm) and a

198    smooth gray floor. Test sessions were performed in a circular chamber (Chamber D) with opaque

199    reddish walls (diameter 235 mm × height 310 mm) and a smooth gray floor. After recovery from

200    surgery, a maximum of six mice were moved with their home cages on racks in the maintenance

201    room to a soundproof (Yamaha Co.) waiting room (Room B). Mice were left undisturbed for at

202    least 15 minutes before and after each session and during the experiment. In each session, one

203    mouse in its home cage was moved into Room A. During the context exploration sessions, mice

204    were placed in Chamber A and allowed to explore for 5 minutes per day for 2 days. During the

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205    conditioning sessions, mice were placed in Chamber A for 2 minutes, and then received one or

206    three tones (30 sec, 65 dB, 7 kHz), co-terminating with a shock (2 sec, 0.4 mA), with an interval

207    of 30 sec. After the last shock, mice remained for 30 sec, and were then returned to their home

208    cages and to Room B. During the retrieval sessions, mice were placed into Chamber C for 2

209    minutes before receiving a tone (30 sec, 65 dB, 7 kHz), then 30 sec later, mice were subjected to

210    isoflurane anesthesia and drug infusion before being returned to Room B. For autophagy

211    inhibition or blocking of AMPA receptor endocytosis, mice were subjected to isoflurane

212    anesthesia and drug infusion 75 minutes prior to the retrieval sessions. During test sessions, mice

213    were placed in Chamber D for 2 minutes, and then received a tone (30 sec, 65 dB, 7 kHz).

214    **Behavioral analysis**

215    All experiments were conducted using a video tracking system (Muromachi Kikai, Tokyo,

216    Japan) to measure the freezing behavior of the animals. Freezing was defined as a complete

217    absence of movement, except for respiration. We started scoring the duration of the FR after 1

218    sec of sustained freezing behavior. All behavioral sessions were screen recorded using Bandicam

219    software (Bandisoft, Seoul, Korea). Occupancy plots representing the maximum occupancy of

220    the mouse center in the defined context space during each session were generated by analyzing

221    the screen recorded movies using ANY-maze software (Stoelting Co., Wood Dale, IL, USA).

222    Mice were assessed as completely amnesic when they: (1) showed at least a 50% decrease in

223    freezing level after drug infusion compared with the level before treatment, and (2) showed a

224    freezing level in the CS or distinct contexts within the 95% confidence interval of the freezing

225    level of the NoFS condition (used as a reference for normal mouse behavior). Animals were

226    excluded from behavioral analysis when showing abnormal behavior after surgery or the cannula

227    was misplaced in position.

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**Plasmid construction, lentivirus preparation, and infection**

For plasmid construction, mCherry (Clontech, Palo Alto, CA, USA) was amplified by PCR using

the following primers, sense: gggggatccgccaccatggtgagcaagggcgaggagg; antisense:

ggggtcgaccccgggctacttgtacagctcgtcc. The resulting fragment was then used to replace the EYFP

fragment at the BamHI-Sall sites in pBS-TRE3G-EYFP to produce the pBS-TRE3G-mCherry

plasmid. The pBS-TRE3G-EYFP plasmid is a pBluescript II SK+ plasmid (Stratagene, La Jolla,

CA, USA) containing the third generation tTA-responsive TRE3G promoter sequence, derived

from pTRE3G-IRES (Clontech, 631161) fused to EYFP. Finally, the TRE3G-mCherry fragment

was subcloned into the STB plasmid using the SpeI/XbaI-XmaI sites to produce the pLenti-

TRE3G-mCherry plasmid, which was used for the lentivirus preparation as previously described

(Ohkawa et al., 2015). The viral titer was approximately $5 \times 10^9$ IU/ml. Virus infection into CA1

of the c-fos-tTA mice (18–20 weeks old) was performed during the surgery for drug cannula

fixation. Lentivirus (0.5 µl/site) was introduced through an injection cannula inserted into the

guide cannula and left protruding by 0.5 mm. The injection rate was 0.1 µl/minute, and the

cannula was left in place for 20 minutes after the end of the injection, before being slowly

withdrawn.

**Labeling of the memory-ensemble cells**

Labeling of the memory-ensemble cells associated with contextual fear was performed in a

similar manner to the experiments on CFC. The experiment was performed on lentivirus-injected

c-fos-tTA mice, maintained since weaning on food containing 40 mg/kg doxycycline. Two

weeks after lentivirus infection, mice were subjected to the waiting rack for 2 h for habituation

purposes. One day later doxycycline was removed and mice were maintained on normal food.

Two days after doxycycline removal, mice were subjected to a CFC session as mentioned above.

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251  Six hours later, the feed for the mice was changed to food containing 1000 mg/kg doxycycline.

252  The retrieval session and drug infusion were performed as mentioned above. One day after drug

253  infusion, the mice were deeply anesthetized with an overdose of pentobarbital solution, and

254  perfused transcardially with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS (PFA). The

255  brains were removed, further post-fixed by immersion in PFA for 16–24 h at 4°C, equilibrated in

256  30% sucrose in PBS for 36–48 h at 4°C, and then stored at –80°C.

257  **Immunohistochemistry**

258  Double labeling primary antibodies from the same host species (rabbit) were used for GluA1 and

259  mCherry staining. Several incisions where made to label the right side of the brains, and they

260  were then cut into 50 μm coronal sections using a cryostat and transferred to 12-well cell culture

261  plates (Corning, NY, USA) containing PBS. After washing with PBS, the floating sections were

262  treated with blocking buffer (5% normal donkey serum; S30, Chemicon by EMD Millipore,

263  Billerica, MA USA) in 0.3% Triton X-100-PBS (TPBS) at room temperature (RT) for 1 h. They

264  were then treated with anti-GluA1 antibody (1:500; AB1504; EMD Millipore) in blocking buffer

265  at 4°C for 36-40 h. After three 10-minute washes with 0.1% PBST (the procedure for further

266  mentions of washing in this paragraph), sections were incubated with donkey anti-rabbit IgG-

267  AlexaFluor 488 secondary antibody in blocking buffer (A21206, Molecular Probes, Invitrogen,

268  Carlsbad, CA, USA) at RT for 4 h. After washing, sections were incubated with 5% normal

269  rabbit serum (Jackson ImmunoResearch Inc., West Grove, PA, USA) in 0.3% TPBS at RT for 1

270  h. Following washing, sections were incubated with 4% Fab Fragment Donkey Anti-Rabbit IgG

271  (Jackson ImmunoResearch) in 0.3% TPBS at RT for 2 h. Sections were then washed and treated

272  with anti-mCherry antibody (1:500; 632496; Clontech) in blocking buffer at 4°C for 36-40 h.

273  After washing, sections were incubated with donkey anti-rabbit IgG-AlexaFluor546 secondary

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274    antibody in blocking buffer (A10040, Molecular Probes) at RT for 4 h. Sections were then

275    washed and treated with DAPI (1 μg/ml, Roche Diagnostics, 10236276001), then washed three

276    times with PBS. Sections were then mounted on glass slides with ProLong Gold antifade reagent

277    (Invitrogen).

278    **Confocal microscopy and analysis of puncta**

279    Images were acquired using a Zeiss LSM 780 confocal microscope (Carl Zeiss, Jena, Germany).

280    First, a Plan-Apochromat 5× objective lens was used to check for the treatment side, and then

281    low magnification images of the CA1 radiatum were acquired for each selected hemisphere

282    using a Plan-Apochromat 20× objective lens. High magnification images for dendrites and spines

283    were acquired using a Plan-Apochromat 63×/1.4 oil DIC objective lens. All acquisition

284    parameters were kept constant within each magnification. To detect GluA1 puncta and the

285    mCherry-labeled dendrites and spines, high resolution (4096 × 4096) images were acquired by

286    collecting z-stacks (5 slices at 0.6 μm thickness, and 0.3 μm interval). After performing a digital

287    zoom (7×), maximum intensity projection images were created with ZEN 2.1 Black (Carl Zeiss)

288    and further processed with Gamma correction at $\gamma = 1.5$ (for mCherry-labeled spines and Total

289    GluA1 puncta) or $\gamma = 5$ (for GluA1 strong signal). ImageJ software (NIH, Bethesda, Maryland,

290    USA) was used to apply a constant threshold to the green channel to create binary images for

291    both Total GluA1 puncta (GluA(Tot)[+]) and GluA1 strong signal (GluA1(Str)[+]). Both puncta

292    were automatically counted using the Analyze particles function with a particle size of > 50

293    pixel$^2$ for GluA(Tot)[+] or > 100 pixel$^2$ for GluA1(Str)[+], and a circularity of 0.2–1.0. Any fused

294    puncta were manually separated before automatic counting. Overlaps between the GluA1(Str)[+]

295    puncta and mCherry[+] spines were manually counted, guided by the green and red color

296    thresholding in ImageJ. The mCherry[+] only spines did not overlap with any GluA1(Tot)[+] puncta.

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297     Three hemispheres were analyzed for each of the PBS or Ani+tBC treatments from four mice.

298     For each hemisphere, data from four analyzed maximum intensity projection images were

299     averaged.

**Stereotactic surgery and drug infusion in rats**

301     Previously described surgical procedures were used with some modifications (Okubo-Suzuki et

302     al., 2016). Rats were 8–10 weeks old at the time of surgery. In brief, a bipolar stimulating

303     electrode and a monopolar recording electrode, both made of tungsten wire, were stereotaxically

304     positioned to stimulate the perforant pathway (angular bundle), while recording in the dentate

305     gyrus. The stimulating electrode was positioned $7.5 \pm 0.3$ mm posterior, $4.4 \pm 0.3$ mm lateral,

306     and $4.7 \pm 0.3$ mm ventral to the bregma. The recording electrode was positioned ipsilaterally 4.0

307     $\pm 0.3$ mm posterior, $2.5 \pm 0.3$ mm lateral and $3.8 \pm 0.3$ mm ventral to the bregma. For

308     intracerebroventricular (ICV) infusion, a stainless-steel guide cannula (Eicom, Tokyo, Japan)

309     was positioned ipsilaterally $0.7 \pm 0.3$ mm posterior, $1.6 \pm 0.3$ mm lateral, and 4.0 mm ventral to

310     the bregma. After surgery, a dummy cannula (Eicom), which extended 1.0 mm beyond the end

311     of the guide cannula, was inserted into the guide cannula. Rats were allowed to recover for at

312     least 10 days in individual home cages before the experiment. ICV drug infusion was performed

313     on unanesthetized freely moving rats, using an injection cannula (Eicom) that extended 0.5 mm

314     beyond the end of the guide cannula, with an infusion rate of 1 µl/minute. Following drug

315     infusion, the injection cannula was left in place for 5 minutes to allow for drug diffusion.

**In vivo electrophysiology on freely moving rats**

317     The LTP experiments were modified from those previously described (Okubo-Suzuki et al.,

318     2016). After recovery from surgery, the input/output curves were determined as a function of

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319     current intensity (0.1–1.0 mA), and the intensity of the stimulus current required to elicit the

320     maximum fEPSP slope (MAX) was determined for each animal. The stimulus current intensity

321     was set to elicit 50% of MAX. Three days later, 400 Hz or 8 Hz stimulations were induced. The

322     400 Hz stimulation used for LTP induction consisted of 10 trains with 1 minute inter-train

323     intervals, with each train consisting of five bursts of 10 pulses at 400 Hz, delivered at 1 s

324     interburst intervals, giving a total of 500 pulses. The later 8 Hz stimulation, which was

325     performed as a reactivation stimulation, consisted of 128 pulses at 8 Hz. The fEPSP slope was

326     monitored by delivering test pulses at 0.05 Hz for 15 minutes before (PreStim), and 5 minutes

327     after (PostStim) stimulation, over the following 4 days. For testing the dependency of the

328     stimulation on protein synthesis, 5 µl PBS or 5 µl of a solution containing 400 µg of Ani, were

329     infused directly after the PostStim recording. For LTP reconsolidation experiments, LTP (400 Hz

330     stimulation) was induced 3 days after MAX, and 1 day later, the 8 Hz reactivation stimulation

331     was performed. As mentioned above, the fEPSP slope was monitored both before (PreLTP) and

332     5 minutes after LTP induction (PostLTP), and before (PreReact) and 5 minutes after (PostReact)

333     the 8 Hz reactivation stimulation. Immediately after PostReact recording, rats received a 5 µl

334     IVC drug infusion, containing either PBS, 400 µg of Ani, 100 µg of tBC, or 400 µg of Ani  +

335     100 µg of tBC, as described above. For inhibition of NMDAR-2B, 5 µl of a solution containing 5

336     µg of ifenprodil tartrate was IVC infused immediately before the PreReact recording. The fEPSP

337     slope was monitored over the following 3 days. Rats were excluded when showing abnormal

338     behavior after surgery, LTP was not induced from the first trial, or the cannula or the electrodes

339     were misplaced in position.

340     **Experimental Design and Statistical Analysis**

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341     In figure legends, n refers to the number of animals per treatment condition unless otherwise

342     indicated. All experiments were performed at least three times with lots of 3-6 animals each.

343     Treatments were counterbalanced for each lot. Animals were blindly and randomly allocated for

344     each treatment condition. Statistical analysis was performed using Prism 6.01 or InStat 3.1

345     (GraphPad Software, San Diego, CA, USA). Data from two conditions were compared using

346     two-tailed unpaired Student t tests. Multiple-condition comparisons were assessed using

347     ANOVA with post hoc tests as described the results section. *P* values were considered significant

348     if less than 0.05. Quantitative data are presented as mean ± SEM.

349

## Results

### Autophagy contributes to fear memory destabilization

352         To modulate autophagy activity within the time window of reconsolidation, we

353     pharmacologically targeted the Beclin1 protein, which is part of the Beclin1-Atg14L-Vps34 lipid

354     kinase complex that is involved in the autophagosome synthesis. This will specifically modulate

355     autophagy activity without affecting endocytosis, mTOR, or PI3K activity (Vanhaesebroeck et

356     al., 2010; Liu et al., 2011; Shoji-Kawata et al., 2013; Marsh and Debnath, 2015; De Leo et al.,

357     2016). For autophagy induction, we used the cell-permeable tat-beclin1 peptide (tBC), which is

358     composed of the human immunodeficiency virus-1 transduction domain attached to the

359     necessary and sufficient peptide sequence of the beclin1 protein (Shoji-Kawata et al., 2013). The

360     tBC peptide induces autophagy in the brains of mice neonates when systemically injected (Shoji-

361     Kawata et al., 2013), and induces autophagy in the amygdala of adult mice when directly infused

362     as monitored through the conversion of the light chain protein 3 (LC3), an autophagosome-

363     specific marker, from its inactive form (LC3-I) to the lipidated active form (LC3-II). For

364     autophagy inhibition, we used Spautin-1 (Spautin), which promotes the degradation of the

365     Beclin1-Atg14L-Vps34 complex through inhibiting the ubiquitin-specific peptidases that target

366     the beclin1 subunit of the complex (Liu et al., 2011). Infusion of spautin into the amygdala

367     inhibited both the basal and the tBC-induced autophagic activity (one-way ANOVA, LC3-

368     II/LC3-I: $P = 0.007$ and Total LC3: $P = 0.505$; Tukey's post-hoc test; Fig. 1*A,B*).

369         To examine the effect of autophagy modulation on memory destabilization, we employed a

370     reconsolidation model of fear conditioning. Fear conditioning is an associative learning

371     paradigm, in which animals learn to associate a specific auditory cue (auditory fear conditioning,

372     AFC) or context (contextual fear conditioning, CFC), which is a conditional stimulus (CS), with

373     a foot shock, an unconditional stimulus (US). When animals are subjected to the CS, they recall

374     the fear memory, resulting in a freezing response.

375         When a one tone-footshock pair (1FS-AFC) was used for conditioning in the AFC

376     paradigm, anisomycin (Ani) infusion into the lateral amygdala (LA) after tone retrieval led to a

377     significant decrease in the tone-elicited freezing response compared with the vehicle-infused

378     condition (Fig. 1*C-E*), in agreement with previous reports (Nader et al., 2000; Suzuki et al.,

379     2004; Mamiya et al., 2009). Ani produced a retrieval-specific retrograde amnesia as Ani

380     administration without the retrieval session had no effect on tone fear memory (Fig. 1*E*).

381     Inhibiting autophagy through Spautin infusion into the LA before retrieval partially blocked the

382     Ani amnesic effect, indicating that autophagy contributes to the memory destabilization process

383     (two-way ANOVA, F = 4.224, $P = 0.0115$; Bonferroni's post-hoc test for within condition

384     comparison and Newman-Keuls test for between conditions comparison; Fig. 1*C,D*). Ani

385     administration alone resulted in almost complete fear memory amnesia of the weak AFC training

18

386    (1FS-AFC), leaving no space for a further decrease in the tone-elicited freezing response.

387    Therefore, autophagy induction combined with Ani (Ani+tBC) did not show any additional

388    amnesic effect over Ani administration alone (two-way ANOVA, $F_{(2, 22)} = 1.594$, $P = 0.2257$;

389    Holm-Sidak's post-hoc test; Fig. 1C,*E*).

390

391    **Autophagy overcomes a reconsolidation-resistant condition that is AMPAR endocytosis-**

392    **dependent**

393         Next, we examined the effect of autophagy induction on stronger AFC training by

394    increasing memory strength using three tone-FS pairs (3FS-AFC), generating a reconsolidation-

395    resistant condition. In the 3FS-AFC, Ani infusion into the LA after retrieval did not show any

396    significant effect on the tone-elicited freezing response in comparison with the vehicle-infused

397    condition. By contrast, Ani+tBC infusion after retrieval significantly reduced the tone-elicited

398    freezing response levels, indicating that autophagy induction enhances memory destabilization

399    beyond the fear memory reconsolidation-resistant condition. Without the retrieval session,

400    Ani+tBC administration in the 3FS-AFC had no effect on auditory fear memory, indicating that a

401    retrieval-specific process is necessary for the autophagy-enhancing effect on memory

402    destabilization (two-way ANOVA, $F_{(3, 30)} = 3.476$, $P = 0.0281$; Bonferroni's post-hoc test for

403    within condition comparison and -Kramer test for between conditions comparison; Fig. 2*A,B*).

404    Collectively, the results obtained from both the inhibition and the induction of autophagy

405    indicate a causal relationship between autophagy activity and memory destabilization.

406         We attempted to elucidate how autophagy modulates memory destabilization? As AMPAR

407    are endocytosed after memory retrieval, we hypothesized that the autophagosome may fuse with

408    endosomes carrying AMPAR and dictate their fate to lysosomal degradation (Rao-Ruiz et al.,

19

409    2011; Shehata et al., 2012; Shehata and Inokuchi, 2014). Therefore, blocking endocytosis would

410    block the autophagy effect on memory destabilization. The neural activity-dependent

411    endocytosis of AMPAR relies on the carboxy-tail of GluA2, and the use of the synthetic peptide

412    Tat-GluA2$_{3Y}$ is well-established in attenuating activity-induced, but not constitutive, GluA2-

413    dependent synaptic removal of AMPARs (Kim et al., 2001; Lee et al., 2002; Ahmadian et al.,

414    2004; Scholz et al., 2010). In the 3FS-AFC, Tat-GluA2$_{3Y}$ peptide infusion into the LA before

415    retrieval completely blocked the Ani+tBC amnesic effect, while the control mutant peptide Tat-

416    GluA2$_{3A}$ had no effect (two-way ANOVA, $F_{(3, 35)} = 4.787$, $P = 0.0067$; Bonferroni's post-hoc

417    test for within condition comparison and Tukey-Kramer test for between conditions comparison;

418    Fig. 2*A,C*). These data indicate that AMPAR endocytosis is upstream to the autophagy induction

419    effect on memory destabilization enhancement.

420

421    **Autophagy enhances retrograde amnesia of fear memory in CFC when targeted to the**

422    **amygdala**

423        We further investigated the autophagy induction effect on CFC as another reconsolidation

424    paradigm. In CFC, the CS is a specific context, and the memory of the details of that context

425    triggers a freezing response that is greater than that triggered by any other distinct context

426    (Fanselow, 2000). Typically, inhibition of protein synthesis after CS retrieval leads to a certain

427    degree of retrograde amnesia (Besnard et al., 2012; Finnie and Nader, 2012). To assess the

428    degree of the retrograde amnesia, we compared it with that of a reference condition exposed to

429    the same contexts without receiving any shock (NoFS). After CFC, an Ani infusion into the

430    baso-lateral amygdala (BLA) after memory retrieval led to a decrease in the freezing response in

431    comparison with the vehicle-infused condition (Fig. 3*A,B*) (Suzuki et al., 2004; Mamiya et al.,

20

432    2009). Nevertheless, the freezing response after Ani administration was significantly higher than

433    that in the NoFS condition, in both the specific and distinct contexts, implying that the resultant

434    retrograde amnesia was only partial. After Ani+tBC administration, the average freezing

435    response dramatically reduced, reaching no statistical significant difference from the NoFS

436    condition in both contexts (two-way ANOVA, $F (8, 102) = 10.19$, $P = 0.0001$; Bonferroni's post-

437    hoc test for within condition comparison and Tukey-Kramer test for between conditions

438    comparison; Fig. 3*B*). In addition, we assessed the complete amnesia for each mouse. In the

439    Ani+tBC administered condition, 5 out of 12 mice were regarded as completely amnesic, in

440    contrast, only 1 out of 12 mice in the Ani administered condition (see Materials & Methods

441    section details) (Fig. 3 *C,D*). When the completely amnesic mice were subjected to a

442    reconditioning session, they regained the freezing response to levels matching the pre-amnesic

443    freezing levels, indicating an intact capacity for fear expression (one-way ANOVA, T1 and T2:

444    $P = 0.0001$; within Ani+tBC complete group: $P = 0.0021$; Tukey's post-hoc test; Fig. 3*E*).

445    Altogether, these behavioral data indicate that induction of autophagy enhanced the amnesic

446    effect of protein synthesis inhibition after retrieval and resulted in an enhanced level of

447    retrograde amnesia.

448

449    **Autophagy enhances retrograde amnesia of contextual memory when targeted to the**

450    **hippocampus and AMPAR degradation in the spines of the memory-ensemble cells**

451         We next examined the generality of the autophagy induction effect on other brain areas by

452    targeting the CA1 region of the hippocampus. The tBC peptide induced autophagy in the

453    hippocampus of adult mice when directly infused (one-way ANOVA, LC3-II/LC3-I: $P = 0.0253$

454    and Total LC3: $P = 0.88$; Tukey's post-hoc test; Fig. 4*A,B*). As with the results from the BLA,

21

455    Ani infusion into the CA1 after memory retrieval led to a decrease in the freezing response (two-

456    way ANOVA, F (8, 106) = 9.027, *P* = 0.0001; Bonferroni's post-hoc test for within condition

457    comparison and Tukey-Kramer test for between conditions comparison; Fig. 4*C,D*) (Mamiya et

458    al., 2009). As the hippocampal CA1 region encodes mainly spatial and contextual (CS)

459    information, while the fear (FS or US) memory itself is encoded by the amygdala (LeDoux,

460    2000; Maren et al., 2013), Ani+tBC infusion into CA1 significantly decreased the

461    discrimination between the specific and distinct contexts (two-way ANOVA, F (3, 44) = 7.211, *P*

462    = 0.0005; Holm-Sidak's post-hoc test for within condition comparison and Newman-Keuls test

463    for between conditions comparison; Fig. 4*E,F*) without affecting the fear memory itself in the CS

464    context (Fig. 4*D,E*). The same result was obtained when another autophagy inducer,

465    trifluoperazine (TFP), was combined with Ani (two-way ANOVA, F (3, 18) = 5.328, *P* = 0.0084;

466    Bonferroni's post-hoc test for within condition comparison and Tukey-Kramer test for between

467    conditions comparison; Fig. 4*G*). These results indicate that the enhanced memory

468    destabilization resulting from induction of autophagy is not restricted to one brain area, and that

469    the behavioral outcome of autophagy induction differs in accordance with the main information

470    encoded in the target brain area.

471       We tested the involvement of autophagy in the degradation of the endocytosed AMPAR

472    after retrieval utilizing the CFC model and benefiting from the dendrite orientation in the CA1

473    radiatum. We quantified the level of AMPAR co-localizing with the spines of the neurons

474    holding the memory trace after the amnesic treatments. To label the memory-ensemble cells in

475    the CA1 region, lentivirus expressing mCherry under the control of the tetracycline response

476    element was injected into c-fos-tTA transgenic mice, which had been maintained on a diet

477    containing doxycycline, except for the period spanning 2 days before and 6 h after the CFC

22

478    session (Fig. 5*A,B*) (Reijmers et al., 2007; Ohkawa et al., 2015). Following retrieval, vehicle or

479    Ani+tBC was unilaterally infused into the CA1, and changes in the GluA1, an AMPAR subunit,

480    staining and its overlap with the mCherry spines (representing the spines of the memory-

481    ensemble cells) were checked 1 day later, reflecting their status at the test 2 session (Fig. 5*B,C*).

482    The GluA1 puncta were classified into strong or weak signals according to their fluorescent

483    intensity, and reflecting the level of AMPAR enrichment. The total GluA1 puncta, the ratio of

484    strong signals to the total GluA1 puncta, and the number of mCherry-only spines did not

485    significantly differ between the two conditions (unpaired Student's t-tests; Fig. 5*D,E, I-K*). Only

486    the overlap between the GluA1 strong signals and mCherry spines was significantly lower in the

487    Ani+tBC condition compared to the vehicle condition and not significantly different from the

488    chance level (two-way ANOVA, $F(1, 4) = 12.4$, $P = 0.0244$; Bonferroni's post-hoc test; Fig. 5*F-*

489    *H*). This decrease in AMPAR enrichment in the spines of ensemble-cells to the chance level is in

490    accordance with the behavioral data showing a decrease in contextual memory and hence loss of

491    context discrimination (Fig. 4*E*).

492

493    **Autophagy destabilizes synaptic plasticity in a LTP reconsolidation model**

494         Finally, we tested the effect of autophagy induction on synaptic destabilization using an *in*

495    *vivo* LTP system in rats, in which a protein synthesis-dependent long-lasting LTP was induced in

496    the dentate gyrus by 400 Hz high frequency stimulation of the perforant path (unpaired Student's

497    t-test, 400 Hz: $P = 0.0371$; Fig. 6*A,B*) (Fukazawa et al., 2003). To model synaptic

498    reconsolidation, the perforant path was reactivated by a protein synthesis-dependent 8 Hz

499    stimulation (unpaired Student's t-test, 8 Hz: $P = 0.0086$; Fig. 6*B*) 1 day after LTP induction; this

500    to resensitize the LTP to the protein synthesis inhibitor Ani, thereby mimicking behavioral

23

501    reconsolidation (Fig. 6*C*) (Okubo-Suzuki et al., 2016). Ani treatment significantly decreased the

502    field excitatory postsynaptic potential (fEPSP) slope 1 day after 8 Hz reactivation compared with

503    the vehicle condition. However, this effect was only partial, as the fEPSP slope was still higher

504    than the baseline level before LTP induction. Following Ani+tBC treatment, LTP destabilization

505    was almost complete and the fEPSP slope was not significantly higher than the baseline level.

506    This enhancement of synaptic destabilization was not observed when Ani administration was

507    combined with the unfused Tat peptide (D-tat) (two-way ANOVA, F (20, 235) = 4.279, *P* =

508    0.0001; Holm-Sidak's post-hoc test; Fig. 6*D,E*). These data indicate that induction of autophagy

509    enhanced the synaptic destabilization triggered by the 8 Hz reactivation. Furthermore, behavioral

510    reconsolidation is dependent on NMDA receptors (NMDAR), and GluN2B-containing NMDAR

511    (NMDAR-2B) is required for memory destabilization after recall (Ben Mamou et al., 2006;

512    Milton et al., 2013). In our synaptic reconsolidation model, ifenprodil, a selective NMDAR-2B

513    antagonist, blocked the LTP-destabilizing effect of Ani administration, mimicking behavioral

514    reconsolidation. Also, ifenprodil completely blocked the LTP-destabilizing effect of Ani+tBC

515    administration (two-way ANOVA, F (10, 105) = 0.5138, *P* = 0.8771; Holm-Sidak's post-hoc

516    test; Fig. 6*F,G*). These data indicate that NMDAR-2B was involved in physiological

517    destabilization in our *in vivo* synaptic reconsolidation model, and demonstrate that the effect of

518    enhanced autophagy on synapse destabilization is downstream of NMDAR-2B.

519

## Discussion

521        Our results indicate that autophagy contributes to memory destabilization and that its

522    induction enhances memory destabilization, including a reconsolidation-resistant one, and the

523    degradation of the endocytosed AMPAR in the spines of memory ensemble-neurons. Also,

524    autophagy induction enhances synaptic destabilization in an NMDAR-dependent manner (Fig.

24

525   7*A,B*). A consistent finding through our study is that autophagy induction alone, through tBC

526   administration after reactivation, did not show any significant amnesic effect in the 3FS-AFC,

527   CFC, and synaptic reconsolidation models. This indicates that, regardless of the degree of

528   destabilization, if the protein synthesis is not compromised within a certain time window

529   following reactivation, the synthesized proteins have the capacity to regain synaptic plasticity

530   and memory (Fig. 7*B*). This demonstrates the capacity of protein synthesis in the restabilization

531   of synapses and the reinstating of specific memories.

532   AMPAR are heterotetrameric complexes composed of various combinations of four

533   subunits (GluA1–4), with the GluA1/2 and GluA2/3 tetramers being the two major subtypes

534   (Wenthold et al., 1996). The amount of synaptic GluA2-containing AMPARs correlates with

535   LTM maintenance and strength (Yao et al., 2008; Migues et al., 2010; Migues et al., 2014; Dong

536   et al., 2015). Blocking the endocytosis of GluA2-containing AMPARs inhibits the induction of

537   LTD, but not LTP, without affecting basal synaptic transmission (Ahmadian et al., 2004;

538   Brebner et al., 2005; Dalton et al., 2008; Scholz et al., 2010). More relevant is its involvement in

539   memory destabilization, where it does not affect the acquisition or the retrieval of conditioned

540   fear memory (Rao-Ruiz et al., 2011; Hong et al., 2013). These reports are in agreement with our

541   hypothesis that autophagy works through dragging endosomes carrying AMPAR to lysosomal

542   degradation, as evidenced by our demonstration that GluA2-dependent AMPAR endocytosis is a

543   prerequisite for autophagy to affect memory destabilization. Additionally, GluA2-dependent

544   AMPAR endocytosis correlates with the decay of LTP and the natural active forgetting of LTM

545   (Hardt et al., 2014; Dong et al., 2015; Migues et al., 2016), which suggests that autophagy may

546   play a role in the forgetting of consolidated memories through the gradual synaptic loss of

547   AMPAR overtime, and, hence, memory loss.

25

548    The GluA1 subunit acts dominantly over other subunits to determine the direction of

549    AMPAR to the surface, and is correlated with synaptic potentiation, LTP, and fear memory

550    (Ehlers, 2000; Shi et al., 2001; Malinow and Malenka, 2002; Lee et al., 2004; Rumpel et al.,

551    2005). A mouse model lacking GluA1 subunit expression exhibits impaired hippocampus-

552    dependent spatial memory (Reisel et al., 2002; Sanderson et al., 2007). CFC recruits newly

553    synthesized GluA1-containing AMPAR into the spines of the hippocampal memory-ensemble

554    cells in a learning-specific manner (Matsuo et al., 2008). Inhibitory avoidance, a hippocampus-

555    dependent contextual fear-learning task, delivers GluA1-containing AMPARs into the CA1

556    synapses in the dorsal hippocampus, where they are required for encoding contextual fear

557    memories (Mitsushima et al., 2011). Inhibition of the cAMP response element-binding protein, a

558    key transcription factor implicated in synaptic plasticity and memory, is associated with a

559    specific reduction in the AMPAR subunit of GluA1 within the postsynaptic densities, and

560    impaired CFC (Middei et al., 2013). These reports are in agreement with our use of spine

561    enrichment with GluA1-containing AMPAR as a molecular reflection of synaptic and contextual

562    memory strength in the CA1 region of the hippocampus.

563    D-cycloserine, an NMDAR agonist, prepares resistant memories for destabilization (Bustos

564    et al., 2010). Noteworthy, d-cycloserine also enhances memory update (fear extinction) by

565    increasing GluA2-containing AMPAR endocytosis, and augments NMDAR-2B-dependent

566    hippocampal LTD (Duffy et al., 2008; Bai et al., 2014). Therefore, autophagy might be a

567    potential downstream mechanism by which d-cycloserine facilitates destabilization.

568    In the present study using the CFC paradigm, manipulation of BLA neurons led to

569    complete retrograde amnesia, while manipulation of CA1 neurons led to a generalization of fear.

570    Therefore, targeting of the proper brain region is necessary to achieve the desired behavioral

26

571    response. This highlights the importance of targeting the fear memory to successfully alleviate

572    PTSD symptoms, rather than any other associated memory within the entire network.

573         We showed here that autophagy destabilizes resistant memories formed under stressful

574    conditions, suggesting autophagy as a potential target for clinical applications. Owing to the

575    growing interest in finding autophagy inducers for several applications, many FDA-approved

576    autophagy inducers already exist, including known anti-psychotic and anti-depressant drugs, and

577    more specific ones are on their way (Levine et al., 2015; Morel et al., 2017). This increases the

578    feasibility of using autophagy inducers for future therapeutic applications, including PTSD

579    treatment.

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581

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761

762    **Legends**

763

764    **Figure 1. Autophagy contributes to fear memory destabilization.**

765    *A,* Representative LC3 immunoblot from mouse amygdala lysates showing autophagy induction

766    by Tat-beclin 1 (tBC) and inhibition by Spautin-1 (Spautin). The lipidated active form (LC3-II) is

767    an autophagosome-specific marker. *B,* Quantitation of the immunoblot signal intensity represented

768    as percentage relative to a Veh/PBS sample (n = 4 mice/condition). *C,* Design for the one tone-

769    footshock pair auditory fear conditioning (1FS-AFC) experiments. *D,* Average percentage freezing

770    during tone at T1 and T2 showing that blocking autophagy significantly decreased the amnesic

771    effect of Ani (n = 10-11 mice/condition). *E,* Average percentage freezing during tone at T1 and

772    T2 showing that, when the amnesic effect of anisomycin (Ani) was complete, autophagy induction

773    did not have any further effect. No reactivation (NR) control showed no amnesic effect. Note: no

774    injections were done before T1. (n = 7-9 mice/ condition). Error bars represent mean ± SEM; * $P$

775    $< 0.05$; ** $P < 0.01$; **** $P < 0.0001$. PBS: phosphate buffered saline; Veh: Vehicle.

776

777    **Figure 2. Autophagy overcomes a reconsolidation-resistant condition in an AMPAR**

778    **endocytosis-dependent manner.**

779    *A,* Design for the three tone-FS pairs auditory fear conditioning (3FS-AFC) experiments. The

780    experiment was carried out either with no injection before T1 or with injection of Tat-GluA2

781    peptides: GluA2$_{3Y}$, for blocking AMPA receptor endocytosis, or GluA2$_{3A}$, as a negative control.

782    *B,* Average percentage freezing during tone at T1 and T2 showing that Ani combined with

783    autophagy induction (Ani+tBC) showed significant retrograde amnesia while the Ani alone

784    condition showed no amnesic effect (n = 7-10 mice/condition). *C,* Average percentage freezing

36

785    during tone at T1 and T2 showing that blocking AMPAR endocytosis abolished the amnesic effect

786    of autophagy induction (n = 8-11 mice/condition). Error bars represent mean ± SEM; * $P < 0.05$;

787    ** $P < 0.01$. Ani: anisomycin; NR: no reactivation (no T1); PBS: phosphate buffered saline; tBC:

788    Tat-beclin 1.

789

790    **Figure 3. Autophagy enhances fear memory destabilization in contextual fear conditioning**

791    **(CFC) when targeted to amygdala.**

792    *A,* Design for the CFC reconsolidation and reconditioning experiments. *B,* Average percentage

793    freezing during retrieval (T1), and after the drugs were infused into the BLA when tested in the

794    conditional stimulus context (T2) and in a distinct context (T3). Freezing levels at both T2 and

795    T3 is showing a significant enhancement of anisomycin (Ani) amnesic effect on fear memory

796    when combined with autophagy induction (n = 10-12 mice/condition). *C,* Plot of individual mice

797    freezing level at T2 against their freezing level at T3, for the assessment of complete fear

798    amnesia after CFC. The red cross and yellow dashed lines represent a hypothetical point

799    calculated from double the standard deviation for the freezing of the no foot shock (NoFS)

800    condition at T2 and T3, where most of mice received Ani combined with Tat-beclin (Ani+tBC)

801    treatment behaved as the NoFS condition. *D,* Individual data for the complete and incomplete

802    amnesic mice of the Ani+tBC condition compared with the Ani alone condition. The dashed red

803    line is a complete amnesic mouse in the Ani only condition. *E,* Average percentage freezing for

804    the complete and incomplete amnesic mice of the Ani+tBC condition compared with the NoFS

805    condition. The complete amnesic mice showed a normal freezing response one day after a

806    reconditioning session (T4) (n = 5-10 mice/condition). Error bars represent mean ± SEM; * $P <$

807    0.05; ** *P* < 0.01; *** *P* < 0.001; **** *P* < 0.0001; n.s. = not significant. BLA: baso-lateral

808    amygdala; PBS: phosphate buffered saline.

809

810    **Figure 4. Autophagy enhances context memory destabilization in contextual fear**

811    **conditioning (CFC) when targeted to hippocampus.**

812    *A,* Representative immunoblot from hippocampal lysates collected 4 h after unilateral drug

813    infusion into CA1.  *B,* Quantitation of the signal intensity represented as percentage relative to a

814    control PBS sample (n = 4–5 per condition). *C,* Design for the CFC reconsolidation experiment.

815    *D,* Average percentage freezing during retrieval (T1), and after the drugs were infused into the

816    hippocampal CA1 region when tested in the conditional stimulus context (T2) and in a distinct

817    context (T3). Combined anisomycin and autophagy induction (Ani+tBC) showed higher freezing

818    levels at T3 compared to Ani alone condition (n = 10-14 mice/condition). *E,* Data from *D*

819    represented as the freezing level at T2 or T3 relative to T1, showing the loss of context

820    discrimination after Ani+tBC combined treatment. *F,* A representative occupancy plot for a

821    mouse per condition at T3 from *D*. *G,* Freezing level at T2 or T3 relative to T1 after autophagy

822    induction by infusion of TFP into the hippocampal CA1 region alone or combined with Ani. As

823    with tBC, TFP combined with Ani resulted in loss of context discrimination (n = 5-6

824    mice/condition). Error bars represent mean ± SEM; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001; ****

825    *P* < 0.0001; n.s. = not significant. PBS: phosphate buffered saline.

826

827    **Figure 5. Autophagy enhances AMPA receptor degradation in the spines of memory-**

828    **ensemble cells.**

38

829  *A,* Lentivirus-mediated labeling of the spines of the memory-ensemble cells with mCherry in the

830  c-fos-tTA transgenic mice. *B,* Experimental design for checking the effect of autophagy

831  induction on α-amino-3-hydroxy-5-methyl4-isoxazolepropionic acid receptor (AMPAR)

832  expression and distribution in memory-ensemble cells. *C,* Images showing immunohistochemical

833  staining for mCherry (red), and endogenous GluA1 (green) in an Ani+tBC treated hemisphere

834  (scale bar, 400 µm). *D-E,* Low magnification images (*D*; scale bar, 50 µm) and higher

835  magnification maximum intensity projection images (*E*; scale bar, 20 µm) showing that

836  anisomycin combined with Tat-beclin (Ani+tBC) treatment did not affect the overall AMPA

837  receptor signals compared to PBS control. *F-G,* Representative dendrites for each treatment

838  condition showing less co-localization of the mCherry-stained spines (mCherry$^+$) with the

839  GluA1-strongly-stained puncta (GluA1(Str)$^+$) in the Ani+tBC condition than in the PBS

840  condition (scale bar, 500 nm; insets are shown in *G*; co-localization is indicated by yellow

841  arrowheads, and arrows indicate mCherry$^+$-only spines). *G,* Higher magnification images for two

842  spines per condition (scale bar, 200 nm). *H,* Quantitation for the co-localization of mCherry$^+$

843  spines with the GluA1(Str)$^+$ puncta per total puncta counted and the chance level (red line). The

844  overlap between mCherry$^+$ spines and the GluA1(Str)$^+$ decreased to chance level after Ani+tBC

845  treatment (n = 3 hemispheres/condition; four images/hemisphere). *I-K,* No significant difference

846  between PBS- or Ani+tBC-injected hemispheres in: *I,* The total GluA1 puncta (GluA1(Tot)$^+$), or

847  total counted puncta. *J,* The mCherry-labeled spines (mCherry$^+$). *K,* The ratio of the GluA1

848  puncta with a strong signal (GluA1(Str)$^+$) or in the mCherry$^+$ spines with GluA1(Tot)$^+$ puncta (n

849  = 3 hemispheres/condition; four images/hemisphere). Error bars represent mean ± SEM; * $P <$

850  0.05;; n.s. = not significant. DOX: doxycycline; PBS: phosphate buffered saline.

851

39

852    **Figure 6. Autophagy induction enhances synaptic destabilization of long-term potentiation**

853    **(LTP) in freely moving rats.**

854    *A,* Diagrams and images of hematoxylin-stained slices for the stimulation electrode, the

855    recording electrode, and the drug injection cannula. Arrows indicate corresponding scars. *B,* In

856    the *in vivo* LTP, both 400 and 8 Hz stimulation were protein synthesis-dependent. Anisomycin

857    (Ani) or phosphate buffered saline (PBS) was infused 5 minutes after the 400 or 8 Hz stimulation

858    and percentage field excitatory postsynaptic potential (fEPSP) slope was calculated on day 2

859    relative to the pre-stimulation level on day 1 (n = 5–7 rats/condition). *C,* Design for the LTP

860    reconsolidation experiment; fEPSP was recorded immediately before (pre) and after (post) LTP

861    induction and reactivation, and for 3 consecutive days after intracerebroventricular drug infusion.

862    *D,* Percentage fEPSP slope relative to the preLTP level showing that autophagy induction using

863    Tat-beclin 1 (tBC), but not the unfused control peptide D-tat, significantly enhanced synaptic

864    destabilization compared to anisomycin (Ani) only treatment. Note: no injections were given

865    before reactivation (n = 10-11 rat/condition). *E,* Representative waveform traces and enlarged

866    portion of slope (inset) for each treatment from *D*. *F,* Percentage fEPSP slope relative to the

867    preLTP level when ifenprodil (IFN), a N-methyl-D-aspartate (NMDA) receptor blocker, was

868    injected before 8 Hz reactivation. IFN completely blocked the synaptic destabilization effect of

869    the Ani only and the Ani+tBC treatments (n = 8 rats/condition). *G,* Representative waveform

870    traces and enlarged portion of slope (inset) for each treatment from *F*. Error bars represent mean

871    ± SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; n.s. = not significant.

872

873    **Figure 7. Models for the effect of autophagy on synaptic and memory destabilization.**

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874    *A,* Molecular mechanism for the effect of autophagy induction on synaptic and memory

875    destabilization. Memory retrieval leads to NMDAR activation, which stimulates both autophagy

876    and the endocytosis of AMPAR in the activated neurons. Autophagosomes (AP) fuse with the

877    endosomes carrying internalized AMPARs, forming autolysosomes (AL), and dictate their fate to

878    lysosomal degradation (green arrows). During the reconsolidation process, newly synthesized

879    proteins, including AMPARs, are delivered to the synaptic surface replacing the degraded ones

880    (red arrows). In the present study, ifenprodil (IFN) was used to block NMDA receptor activation,

881    GluA2$_{3Y}$ peptide to block endocytosis of AMPARs, Tat-beclin-1 (tBC) peptide to induce

882    autophagy, and anisomycin (Ani) to inhibit protein synthesis.

883    *B,* Model explaining the time line of synaptic and memory strength changes by autophagy. After

884    recall, consolidated synaptic plasticity and memory strength are usually physiologically

885    destabilized and return to a labile state, after which a protein synthesis-dependent reconsolidation

886    process is required for restabilization (black line). The labile state (destabilization) is inferred from

887    the decreased synaptic strength and the retrograde amnesia produced by the protein synthesis

888    inhibition (red line). Autophagy induction when combined with protein synthesis inhibition leads

889    to a greater decrease in synaptic strength and enhanced retrograde amnesia, indicating enhanced

890    destabilization (blue line). Autophagy induction alone does not affect synaptic or memory strength
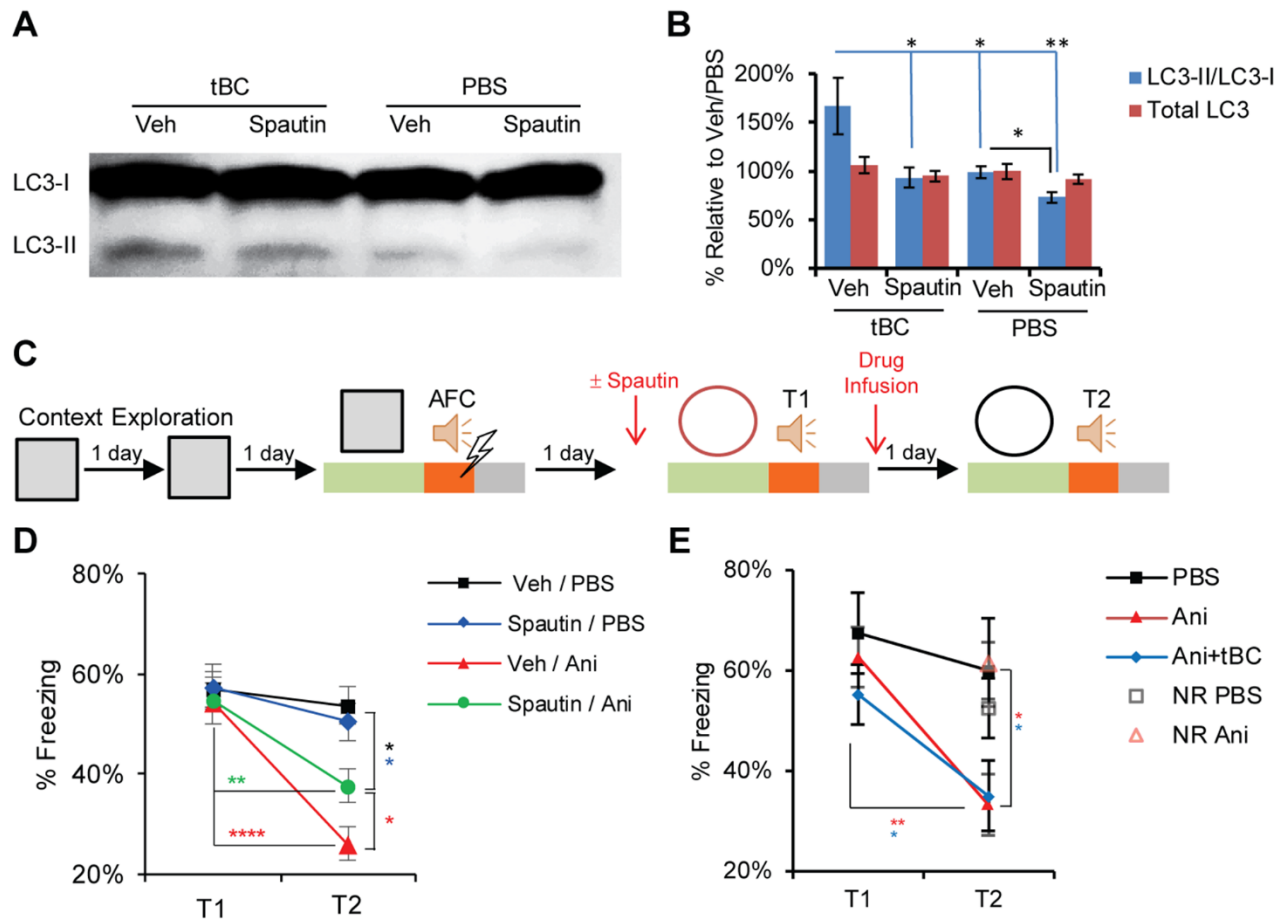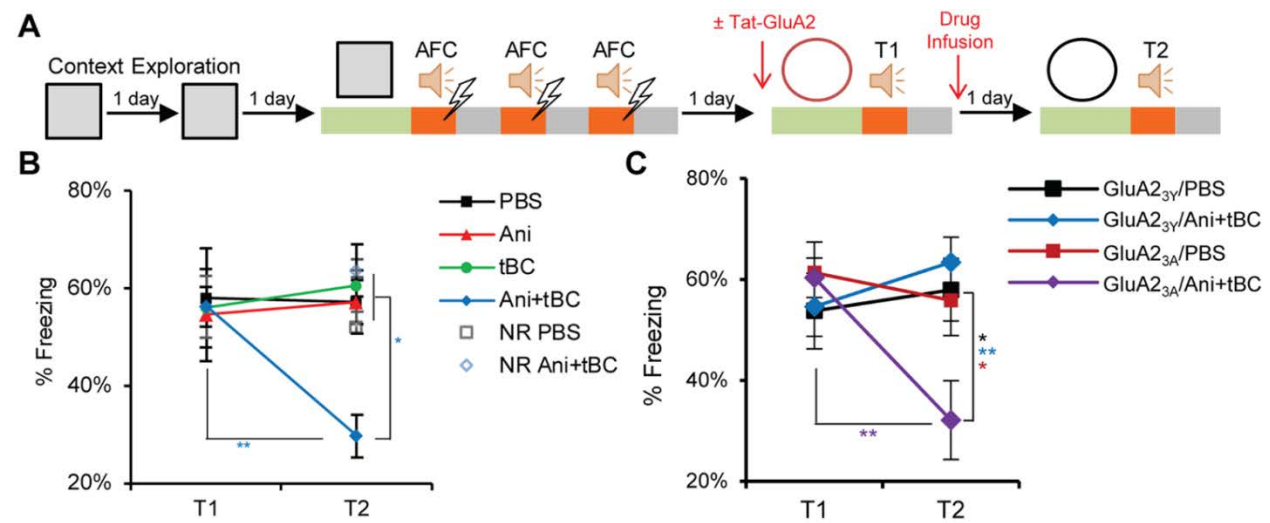
891    (green line).
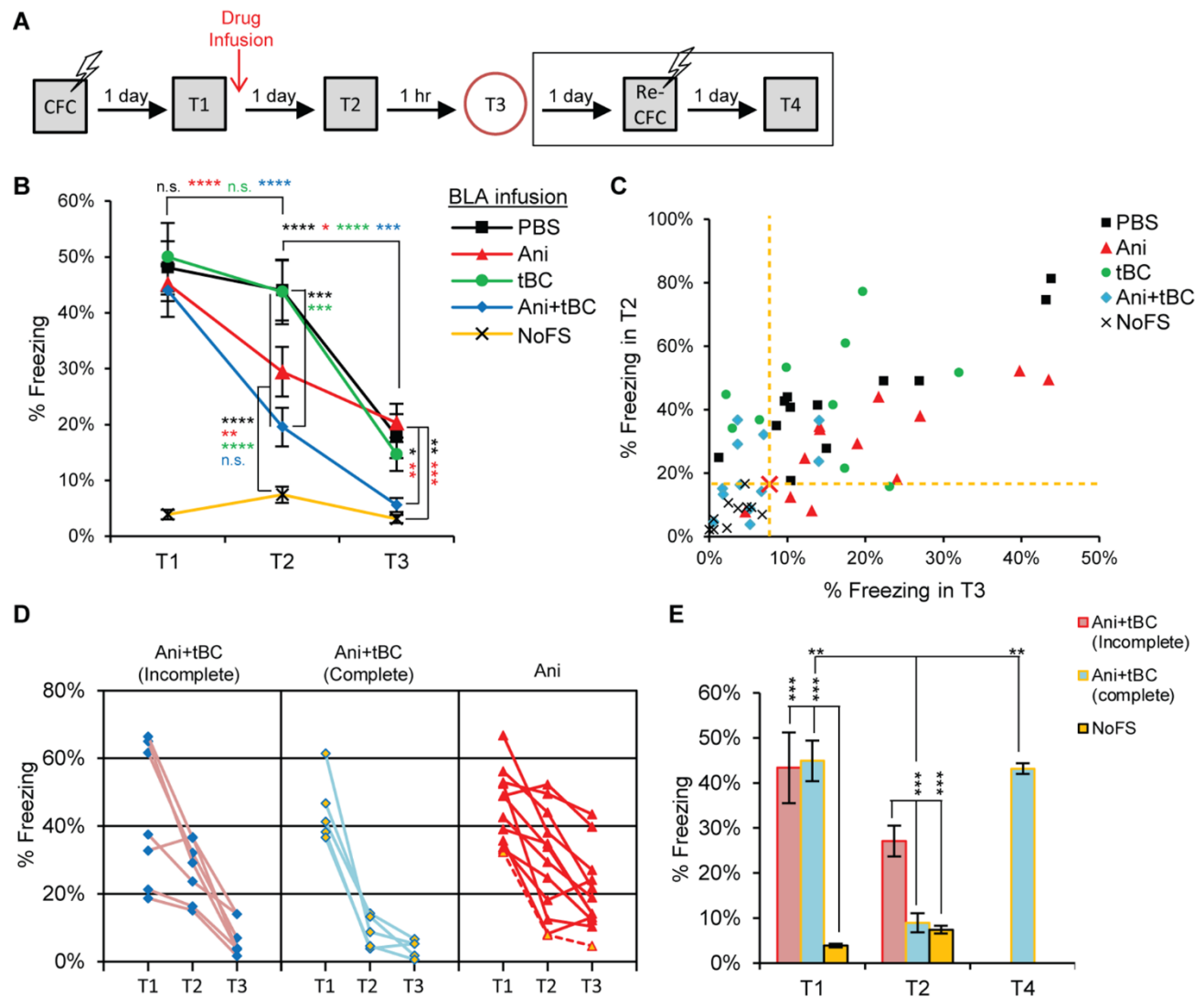
892    **Illustrations**

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894    **Figure 1**

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897    **Figure 2**

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900 **Figure 3**

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903 **Figure 4**

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906    **Figure 5**



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909 **Figure 6**



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911 **Figure 7**

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