Glucocorticoid receptor-mediated amygdalar metaplasticity underlies adaptive modulation of fear memory by stress

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Abstract

Glucocorticoid receptor (GR) is crucial for signaling mediated by stress-induced high levels of glucocorticoids. The lateral nucleus of the amygdala (LA) is a key structure underlying auditory-cued fear conditioning. Here, we demonstrate that genetic disruption of GR in the LA (LAGRKO) resulted in an auditory-cued fear memory deficit for strengthened conditioning. Furthermore, the suppressive effect of a single restraint stress (RS) prior to conditioning on auditory-cued fear memory in floxed GR (control) mice was abolished in LAGRKO mice. Optogenetic induction of long-term depression (LTD) at auditory inputs to the LA reduced auditory-cued fear memory in RS-exposed LAGRKO mice, and in contrast, optogenetic induction of long-term potentiation (LTP) increased auditory-cued fear memory in RS-exposed floxed GR mice. These findings suggest that prior stress suppresses fear conditioning-induced LTP at auditory inputs to the LA in a GR-dependent manner, thereby protecting animals from encoding excessive cued fear memory under stress conditions.

Introduction

Stress activates the hypothalamus–pituitary–adrenal (HPA) axis, which results in the release of glucocorticoid hormones (cortisol in humans and corticosterone in rodents) from the adrenal cortex. Glucocorticoid hormones can readily enter the brain and bind to specific receptors in regions crucial for memories of stressful experiences, such as the hippocampus and amygdala, thereby enhancing the consolidation of emotionally arousing events (de Quervain et al., 2009; Roozendaal et al., 2009). Two types of receptors mediate the effects of glucocorticoids: type I mineralocorticoid receptor (MR, *Nr3c2*) and type II glucocorticoid receptor (GR, *Nr3c1*). Compared with MR, GR has a lower binding affinity for glucocorticoids and is largely unoccupied at basal levels, and is thus considered to be particularly important in signaling mediated by stress-induced high levels of glucocorticoids (de Kloet et al., 2005; Reul and de Kloet, 1985).

The lateral nucleus of the amygdala (LA) is a key structure underlying auditory-cued fear conditioning (AFC) (LeDoux, 2000). Auditory information, which is critical for AFC, reaches the LA either from the medial geniculate nucleus (MGN) or from the auditory cortex (AC). Long-term potentiation (LTP) in the two auditory inputs to the LA is essential for the acquisition and expression of auditory-cued fear memory (Blair et al., 2001; Rogan et al., 1997; Tsvetkov et al., 2002). In response to stress, GR activation

61 plays a central role in the formation of long-term memory, which is an essential
62 mechanism for learning from stressful events and respond adaptively to similar
63 demands in the future (Finsterwald and Alberini, 2014). In an electrophysiological study
64 using brain slices, glucocorticoid prolonged excitatory synaptic responses in the
65 basolateral complex of the amygdala (BLA) by binding to GR (Karst et al., 2010). In
66 contrast, after an acute stress exposure, application of glucocorticoid suppressed
67 excitatory synaptic responses in the BLA in a GR-dependent manner. This switch in
68 synaptic response to glucocorticoid is referred to as metaplasticity (Abraham and Tate,
69 1997; Schmidt et al., 2013). Prior delivery of behavioral stress has also been shown to
70 suppress subsequent induction of LTP (Kavushansky and Richter-Levin, 2006).
71 However, whether stress-induced amygdalar metaplasticity occurs *in vivo* in a way
72 relevant to the strength of the auditory-cued fear memory, and what role LAGR plays in
73 this neural process are unknown.
74 In this study, we generated LA-selective GR knockout (LAGRKO) mice to investigate
75 the region-specific role of LAGR in mediating the modulatory effects of stress on fear
76 memory. We first compared contextual and auditory-cued fear memory conditioned at
77 different strengths between floxed GR (control) and LAGRKO mice at basal condition.
78 We then investigated the effect of LAGR disruption on the adaptive modulation of fear

79 memory after exposure to acute stress. Finally, using an optogenetic technique to induce
80 LTP and long-term depression (LTD), we investigated how GR-dependent
81 metaplasticity in the LA influenced auditory-cued fear memory in response to prior
82 stress exposure.
83
84 **Results**
85 **Generation of LAGRKO mice**
86 To selectively knockout the GR gene in the LA, we generated two mutant mouse lines.
87 The knock-in line expressed improved Cre (iCre) recombinase (Shimshek et al., 2002)
88 under the control of the gastrin-releasing peptide gene (*Grp*) promoter, and the animals
89 are referred to as Grp-iCre mice (Figure. 1A, B). Grp is abundant in the LA and absent
90 or present at low levels in other subnuclei of the amygdala (Shumyatsky et al., 2002).
91 Thus, *Grp* promoter could be used for driving the expression of Cre recombinase
92 selectively in the LA. To confirm its usefulness, we crossed a Grp-iCre mouse with a
93 CAG-CAT-Z reporter mouse, which carried the chloramphenicol acetyltransferase gene
94 (*CAT*) flanked by two loxP sites and the β-galactosidase gene in sequence (Araki et al.,
95 1995). Cre-mediated recombination between the two loxP sites resulted in the
96 expression of β-galactosidase, which was detected by X-gal staining. As shown in

Figure 1C, strong β-galactosidase expression was detected in the LA and hippocampal CA3 region of Grp-iCre/CAG-CAT-Z mice, indicating the presence of robust Cre-loxP recombination in these brain regions. In addition, sparse and weak expression of β-galactosidase appeared in the accessory basal nucleus of the amygdala and in layer 6 of the cerebral cortex.

Next, we generated a floxed GR ($GR^{loxP/loxP}$) mouse line in which exon 3 of the GR gene (encoding the DNA-binding domain) was flanked by two loxP sites (Supplementary figure 1A, B). Grp-iCre mice were crossed with floxed GR mice to establish the LAGRKO mouse line ($GR^{loxP/loxP}$, Grp-$iCre^{+/-}$). Immunofluorescence staining with a specific antibody against GR revealed a selective disruption of GR proteins in the LA of LAGRKO mice (Figure 2A). There was no significant difference between the two genotypes in the expression level of GR in the central nucleus and basal nucleus of the amygdala. Double immunofluorescence staining with anti-GR and anti-NeuN (a neuronal marker) antibodies showed that GR was undetectable in approximately 70% of LA neurons in LAGRKO mice (floxed GR, 90.88% ± 1.39%; LAGRKO, 20.27% ± 0.95%; Figure 2A, B). The expression level of GR in the hippocampal CA3 region of floxed GR mice was very low, and it was slightly decreased in LAGRKO mice (Figure 2C). There was no significant difference between the two

115 genotypes in the expression level of GR in the cerebral cortex, hippocampal CA1 and
116 CA2 regions, and dentate gyrus (Figure 2C). Collectively, these results indicate the
117 successful establishment of a novel LAGRKO mouse line.
118
119 **Disruption of LAGR impairs auditory-cued fear memory for strengthened**
120 **conditioning**
121 Since GR has a low binding affinity for glucocorticoids and its activation requires high
122 levels of hormones, we investigated the role of LAGR in AFC at different strengths.
123 After moderate conditioning, which included three CS × US pairings, LAGRKO mice
124 exhibited the same freezing levels as floxed GR mice during the training, contextual test,
125 and auditory-cued test (Supplementary figure 2A–D). When the training was
126 strengthened to six CS × US pairings (Figure 3A), LAGRKO mice exhibited
127 significantly lower freezing levels than floxed GR mice during tone presentation in the
128 cued test (p = 0.046; Student's t test; Figure 3D), whereas there were no significant
129 differences between the two genotypes in freezing levels during the training ($F_{1,126}$ =
130 1.19, p = 0.278; two-way repeated measures ANOVA; Figure 3B) and contextual test (p
131 = 0.803; Student's t test; Figure 3C). Corticosterone assays revealed significantly higher
132 plasma corticosterone levels 90 min after training with six CS × US pairings than after

training with three CS × US pairings in both genotypes (floxed GR, $p = 0.016$; LAGRKO, $p < 0.001$; Student's *t* test; Figure 3E). In the open field test, LAGRKO mice did not show any alteration in anxiety level or locomotion (Supplementary figure 3A-C), suggesting that the deficit of auditory-cued fear memory in LAGRKO mice was not the result of altered anxiety and locomotor activity.

Injection of GR-expressing adeno-associated virus into the LA rescues the auditory-cued fear memory deficit in LAGRKO mice

Although we successfully and selectively disrupted GR in the LA of LAGRKO mice, we cannot exclude the possibility that sparse disruption of GR in other brain areas, such as the hippocampal CA3 region, may be associated with behavioral impairment in LAGRKO mice. To confirm that the auditory-cued fear memory deficit can be specifically attributed to the disruption of LAGR, we performed a rescue experiment by injecting a GR-expressing adeno-associated virus (AAV-GR) into the LA of LAGRKO mice. Green fluorescent protein-expressing AAV (AAV-GFP) was used as the control. Fear conditioning was conducted 4 weeks after virus injection (Figure 4A, B). LAGRKO mice injected with AAV-GR and floxed GR mice injected with AAV-GFP exhibited significantly higher freezing levels during the auditory-cued fear memory test

than did LAGRKO mice injected with AAV-GFP ($F_{2,24} = 5.25$, $p = 0.013$; one-way ANOVA followed by *post hoc* Tukey–Kramer test; Figure 4E). There was no significant difference in freezing levels between the three mouse groups during training ($F_{2,168} = 0.60$, $p = 0.522$; two-way repeated measures ANOVA; Figure 4C) and the contextual fear memory test ($F_{2,24} = 0.53$, $p = 0.600$; one-way ANOVA; Figure 4D). This rescue experiment further suggests the specific involvement of LAGR in AFC.

Disruption of LAGR impairs adaptive modulation of conditioned fear in response to prior restraint stress exposure

Fear conditioning is highly susceptible to modulation by prior stress exposure (Cordero et al., 2003; Rodriguez Manzanares et al., 2005). To investigate the involvement of LAGR in mediating the effect of prior stress on subsequent fear conditioning, floxed GR and LAGRKO mice were exposed to a 20-min restraint stress (RS) and conditioned 1 h later (Figure 5A). Mice were conditioned with three CS × US pairings that resulted in equivalent freezing levels in floxed GR and LAGRKO mice in the contextual and auditory-cued fear memory tests (Supplementary figure 2). RS exposure 1 h prior to fear conditioning induced a significant increase in freezing levels during the training ($F_{3,215} = 32.25$, $p < 0.001$; two-way repeated measures ANOVA; Figure 5B) and

169 contextual memory test ($F_{3,43}$ = 5.03, p = 0.004; one-way ANOVA followed by *post hoc*
170 Tukey–Kramer test; Figure 5C) in both floxed GR and LAGRKO mice. In contrast to
171 the facilitating effect of prior RS exposure on contextual fear memory, RS exposure 1 h
172 before fear conditioning induced a significant decrease in auditory-cued freezing in
173 floxed GR mice but not in LAGRKO mice (floxed GR, p = 0.016; LAGRKO, p =
174 0.951; Student's t test; Figure 5D), suggesting this suppressive effect is
175 LAGR-dependent.
176
177 **Optogenetic manipulation of metaplasticity in the LA alters the behavioral effect of**
178 **prior RS exposure on auditory-cued fear memory**
179 We next investigated whether stress-induced changes in synaptic plasticity were
180 responsible for the effect of prior stress on auditory-cued fear memory in floxed GR and
181 LAGRKO mice. It was recently proposed that metaplasticity may play a role in the
182 regulation of learning and memory under stress (Joels et al., 2012; Myers et al., 2014).
183 Therefore, we hypothesized that prior RS exposure would suppress AFC-induced LTP in
184 the LA, leading to the attenuation of auditory-cued fear memory in floxed GR mice, and
185 that LAGR disruption might impair stress-induced metaplasticity in the LA. To test this,
186 we used a recently established technique that allows engineering a memory using an

187 optical LTP or LTD protocol (Nabavi et al., 2014). We injected an AAV vector
188 expressing a variant of light-activated channelrhodopsin 2, oChIEF, into bilateral MGN
189 and AC. Four weeks after virus injection, when oChIEF had reached axon terminals in
190 the LA, mice were exposed to a RS and conditioned 1 h later (Figure 6A, B).
191 Immediately after the first auditory-cued fear memory test (Test 1), which was
192 conducted 24 h after fear conditioning, mice received an optical LTP or LTD protocol
193 and tested 24 h later (Test2). The optical LTP protocol significantly increased freezing
194 levels during the Test 2 compared with Test 1 in floxed GR mice but not in LAGRKO
195 mice (floxed GR, $p = 0.046$; LAGRKO, $p = 0.811$; Student's *t* test; Figure 6C, D). These
196 results support our hypothesis that prior RS exposure suppresses AFC-induced LTP in a
197 GR-dependent manner, thus resulting in reduced auditory-cued fear memory in floxed
198 GR mice. In LAGRKO mice, the optical LTD protocol significantly reduced freezing
199 levels during Test 2 compared with that during Test 1 ($p = 0.010$; Student's *t* test; Figure
200 6E), further supporting the hypothesis that prior stress weakens synaptic strength in the
201 LA leading to reduced cued fear memory.
202 To confirm that the optical LTP or LTD protocols produced the expected synaptic
203 effects, we conducted *in vivo* recordings in the LA of anaesthetized naïve mice
204 expressing oChIEF in auditory regions. Brief light pulses (0.033 Hz) at the recording

site produced field responses, which were potentiated by the LTP protocol ($F_{1,126}$ = 174.06, $p < 0.001$; two-way repeated measures ANOVA; Figure 6F) and depressed by the LTD protocol ($F_{1,126}$ = 376.56, $p < 0.001$; two-way repeated measures ANOVA; Figure 6G). These results suggest that the LTP and LTD protocols used in the behavioral experiments modify synaptic strength in the expected manner.

Discussion

In the present study, we demonstrate that LAGR exclusively mediates auditory-cued fear responses to strengthened conditioning, suggesting a critical role for LAGR in signaling mediated by stress-induced high levels of glucocorticoids. We further demonstrate that LAGR is indispensable for the suppressive effect of prior RS exposure on AFC. The optogenetic manipulation of synaptic strength at auditory inputs to the LA by an LTP protocol significantly increased auditory-cued fear memory in RS-exposed floxed GR mice but not in RS-exposed LAGRKO mice. In contrast, an LTD protocol significantly reduced auditory-cued fear memory in RS-exposed LAGRKO mice. These findings suggest that stress-induced metaplasticity occurs in the LA in a GR-dependent manner, and is responsible for the suppressive effect of prior RS on AFC.

The degree of stress induced by learning itself has been shown to be related to strength of the memory (Cordero et al., 1998; Cordero and Sandi, 1998). In our study, there was no difference in freezing levels between floxed GR and LAGRKO mice conditioned with three CS × US pairings. When the number of CS × US pairings of the conditioning procedure was increased to six, LAGRKO exhibited an impaired auditory-cued fear memory. Concomitantly, in both genotypes, plasma corticosterone levels 90 min after fear conditioning were significantly higher in the group with six CS × US pairings than in the group with three CS × US pairings. It is likely that the increased number of CS × US pairings strengthened the connection between auditory CS and US in the LA, and LAGR, which is activated by high levels of circulating corticosterone, contributes exclusively to this neural process. These findings are in agreement with the lower binding affinity of GR for glucocorticoids, leading to the critical role of GR in signaling mediated by stress-induced high levels of glucocorticoids (Reul and de Kloet, 1985).

Emerging evidence indicates that stress exposure modulates subsequent learning and memory bidirectionally, either through facilitation or impairment (Raio and Phelps, 2015). Glucocorticoid signaling has been thought as a common mechanism for mediating the effects of stress on fear memory (Cordero et al., 2003; Rodriguez

Manzanares et al., 2005). In our behavioral tests, a single RS exposure 1 h before conditioning significantly reduced auditory-cued fear memory in floxed GR mice but not in LAGRKO mice, suggesting that the suppressive effect of prior RS is LAGR-dependent. To date, there are no reports demonstrating the effects of stress 1 h before conditioning on fear memory. One previous report demonstrated that a single acute stress exposure 48 h before fear conditioning had no effect on AFC (Cordero et al., 2003). GRs control the magnitude and duration of stress responses, and they are critical in the negative feedback regulation of the HPA axis in response to acute stress (Laryea et al., 2015). In our study, plasma corticosterone levels were significantly higher 1 h after RS than 24 h after RS (data not shown), and it is possible that the timing of previous stress exposure is a key determinant of the effects of stress on auditory-cued fear memory. Consistent with our in vivo evidence showing the LAGR-dependent suppressive effect of prior RS, Karst *et al.* demonstrated that a long-lasting glutamatergic transmission in BLA neurons triggered by the application of corticosterone was suppressed by RS 1 h before slice preparation and was attenuated by treatment with a GR antagonist (Karst et al., 2010).

Synaptic changes at auditory inputs to the LA play an essential role in the acquisition and expression of auditory-cued fear memory (Blair et al., 2001; Rogan et al., 1997;

Tsvetkov et al., 2002). Exposure to a single stress induces LTP in the BLA (Sarabdjitsingh et al., 2012; Vouimba et al., 2004). Therefore, it is possible that prior RS exposure occludes or suppresses subsequent AFC-induced LTP in floxed GR mice, thereby preventing the formation of excessive cued fear memory. To test this hypothesis, we induced LTP at auditory inputs to the LA by optogenetic stimulation, and found a significant increase in auditory-cued fear responses in RS-exposed floxed GR mice. This is the first evidence supporting a causal link between stress-induced metaplasticity in the LA and adaptive fear response to a salient cue in a stressful condition. Several *in vitro* and *in vivo* electrophysiological studies have demonstrated that the activation of GRs are involved in stress-induced amygdalar metaplasticity (Karst et al., 2010; Kavushansky and Richter-Levin, 2006)**.** In our study, the optogenetic delivery of an LTP protocol failed to change fear responses to auditory cues in RS-exposed LAGRKO mice, supporting the hypothesis that the activation of LAGR is essential for stress-induced metaplasticity in the LA. Weakening synaptic strength by delivering of an LTD protocol significantly reduced fear responses to auditory cues in RS-exposed LAGRKO mice, further suggesting that activation of LAGR after stress exposure suppresses fear conditioning-induced LTP at auditory inputs to the LA, thereby protecting animals from encoding excessive cued fear memory under stress condition.

This study demonstrated that LAGR is indispensible for stress-induced metaplasticity in the LA and for controlling fear responses to salient cues depending on the recent stress history of an animal. Individuals with lower levels of cortisol are more susceptible to developing post-traumatic stress disorder (PTSD) (Yehuda, 2006; Yehuda et al., 1998), suggesting that glucocorticoid signaling dysfunction is involved in the development of PTSD. Our findings contribute to understanding how emotional memory is formed under stress conditions and provide clues for elucidating the pathophysiology and etiology of stress-related disorders, such as PTSD.

Methods

Construction of *Grp-iCre* targeting vector. To construct a codon-improved Cre-recombinase (iCre)-splice gene, the intron sequence from the SV40 t-antigen gene was amplified by PCR and inserted between codons 283 and 284 of iCre. We obtained the bacterial artificial chromosome (BAC) clone containing the gastrin-releasing peptide gene (*Grp*) from the BACPAC Resources Center CHORI (Oakland, CA). Using a counter-selection BAC modification kit (Gene Bridges, Dresden, Germany), we inserted the iCre-splice gene linked to the pgk-neo cassette flanked by two FRT sites into *Grp* gene at the +1 position (+1 corresponding to A of ATG, the initiation site of translation

294 in the *Grp* gene). The modified BAC DNA fragment inserted with the
295 iCre-splice-pgk-neo cassette and containing the ~5.1 kbp upstream (-5079 to -1) and
296 ~4.9 kbp downstream (+2 to +4933) sequences of *Grp* gene were cloned into
297 pMC1DTApMA (Kitayama et al., 2001) to generate the targeting vector pTVGRP-iCre.
298
299 **Construction of *GR^{loxP}* targeting vector.** A BAC clone containing the GR gene was
300 obtained from BACPAC Resources Center CHORI. The 5' arm of ~5.2 kbp (-5344 to
301 -104, +1 corresponding to the first nucleotide of the exon 3 of the GR gene) and the 3'
302 arm of ~5 kbp (+104 to +5092) were subcloned into the pDONR P4-P1R and pDONER
303 P2R-P3 vectors (Invitrogen, Carlsbad, CA), respectively, using the counter-selection
304 BAC modification kit. A 370 bp GR gene fragment containing exon 3, part of intron 2,
305 and part of intron 3 was amplified by PCR and was subcloned between two loxP
306 sequences of a modified pDONR 221 vector containing a pgk-Neo cassette flanked by
307 two FRT sites. To construct the targeting vector, these three plasmids were directionally
308 subcloned into pDEST R4-R3 vector containing the diphtheria toxin gene (MC1-DTA)
309 by MultiSite Gateway LR recombination reaction.
310
311 **ES cell culture and generation of Grp-iCre and GR^{loxP} mice.** The targeting vectors

312 linearized with *NotI* was electroporated into the C57BL/6-derived ES cell line RENKA
313 (Fukaya et al., 2006) as previously described. After the selection with G418 (150
314 μg/mL), the recombinant ES cell clone was identified by Southern blot analysis. The
315 obtained recombinant ES cells were injected into 8-cell-stage embryos of ICR mice.
316 The embryos were cultured to blastocysts and transferred to the uterus of
317 pseudopregnant ICR mice. The resulting chimeric mice were mated with CAG-FLPe
318 mice (provided by RIKEN BRC through the National Bio-Resource Project of the
319 MEXT, Japan) to delete the pgk-neo gene and establish the mutant mouse line. Grp-iCre
320 and GR^{loxP} mice were genotyped using polymerase chain reaction (PCR) amplification
321 of genomic DNA prepared from the mouse tail. The Grp-iCre mice were genotyped
322 using the following primer sequences: Gic1, 5'-GTCGAGAGCTCTGAGGGTTT-3';
323 Gic2, 5'-GATTTCATGATCGGGACACTTACCCA-3'; Gic3,
324 5'-GGTACAGGAGGTAGTCCCTCACATC-3'; NeoP1,
325 5'-GGGGATCCACTAGCGAATTCGAAG-3'; and NeoP2,
326 5'-GGATCCCCTCAGAAGAACTCGTCAA-3'. The GR^{loxP} mice were genotyped
327 using the following primer sequences: GR3'LU,
328 5'-AAAAGAGCAGTGGAAGGTAGTGTG-3' and GR3'LL,
329 5'-TACTTCTGTCCCTTCCCAATGGAG-3'.

Animal care and experimental protocols were approved by the Animal Experiment Committee of the University of Toyama (Authorization No. 2013 MED-18 and MED-68) and carried out in accordance with the Guidelines for the Care and Use of Laboratory Animal of the University of Toyama. Three month-old male mice were used for analyses in a genotype blind manner. The mice were kept in a temperature- and humidity-controlled room under 12 h light/dark cycle (lights on at 7:00 AM) and had ad libitum access to food and water.

Analysis of Cre-mediated recombination efficacy. We used the mutant mice carrying both *Grp-iCre* and *CAG-CAT-Z* transgene heterozygously as the reporter mice. The mice were deeply anesthetized with pentobarbital sodium (100 mg/kg body weight, intraperitoneal injection), and then transcardially perfused with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brains were removed, post-fixed with the 4% PFA overnight, and dipped in 0.1 M PB containing 30% sucrose for 36 h at 4°C. The brains were cut into 1-mm-thick coronal sections and stained for β-galactosidase (β-gal) activity for 18 h in PBS containing 5 mM potassium hexacyanoferrate (III), 5 mM potassium hexacyanoferrate (II), 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Tsujita et

348 al., 1999). After the staining reaction, 20-μm-thick sections were prepared from
349 1-mm-thick sections and mounted on glass slides. Images were obtained using a
350 Keyence BZ-8000 microscope (Keyence, Osaka, Japan).
351
352 **Immunofluorescence staining.** The brains were removed, fixed, and cryoprotected as
353 described above and then cut into 25-μm-thick serial coronal sections using a freezing
354 microtome. For each animal, every fourth section (bregma from -1.4 to -1.8 mm, 5
355 sections per animal) was selected on the basis of the Mouse Brain in Stereotaxic
356 Coordinates (Paxinos and Franklin, 2001) and was prepared for immunofluorescence
357 staining.
358 Free floating brain sections were rinsed with PBS and blocked with Protein Block
359 Serum Free (DAKO Cytomation, Carpinteria, CA) for 10 min at room temperature. The
360 sections were then incubated with primary antibodies (rabbit anti-GR, 1:100, Santa Cruz
361 #H-300; mouse anti-NeuN, 1:100, Chemicon #MAB377) diluted in PBS containing 1%
362 bovine serum albumin (BSA) overnight at 4°C. After washing with PBS, the sections
363 were incubated with Alexa-Fluor-488- and Alexa-Fluor-594-conjugated species-specific
364 secondary antibodies (Invitrogen) for 1 h at room temperature. Then, the sections were
365 washed with PBS, adhered to glass slides and coverslipped. Images were taken using a

366 confocal laser scanning microscope (Leica TCS-SP5, Leica Microsystems, Germany).
367 Three sections per animal were used for quantification of GR-positive and
368 NeuN-positive cells in the LA. All sections were counted by us who were blind to the
369 genotype of the mice.
370
371 **Auditory-cued fear conditioning.** Except for the optogenetic experiment,
372 auditory-cued fear conditioning (AFC) was conducted in a small conditioning chamber
373 (10 × 10 × 10 cm; with transparent walls and a floor made of 14 stainless steel rods)
374 surrounded by a sound-attenuating chest (CL-M3, O'Hara & Co., Ltd, Tokyo, Japan).
375 Mice were placed in the conditioning chamber for 60 s and then presented with a 30-s
376 tone (65 dB, white noise) through a speaker on the ceiling of the sound-attenuating
377 chamber. At the end of the tone presentation, mice were given a foot shock (0.5 mA, 2 s
378 for three CS × US pairings and 0.5 mA, 1 s for six CS × US pairings). Mice were
379 returned to their home cages 30 s after the last foot shock. The contextual and
380 auditory-cued fear memory retention tests were conducted 24 and 48 h after training,
381 respectively. During the contextual memory test, mice were re-exposed to the
382 conditioning chamber for 5 min without the tone and shock presentation. The
383 conditioning chamber was cleaned with 70% ethanol before each trial. For the

auditory-cued memory test, mice were placed in a novel chamber (10 × 10 × 10 cm, with white walls and a flat floor). Freezing was scored for 3 min before delivery of the tone and then scored for 3 min in the presence of the tone. Before each trial, the chamber was cleaned with 4% acetic acid. Freezing responses were analyzed on a Macintosh computer with Image FZC 2.22sr2 (O'Hara & Co., Japan), which is software based on the NIH Image program.

For the optogenetic experiment, mice were anaesthetized with isoflurane and optic fibers were inserted into guide cannulas. Mice were returned to their home cages and left for at least 1 h before AFC. AFC was conducted in a soundproof experimental room (Yamada Co., Japan). Mice were placed in the square conditioning chamber (17.5 × 30 cm, with a transparent front wall, white side and back walls, and a floor of 26 stainless steel rods) for 60 s and then received three pairings of CS (65 dB, 7 kHz, 30 s tone) and US (0.5 mA, 2 s foot shock) with 1 min inter-pairing interval. Mice were returned to their home cages 90 s after the last foot shock. The first auditory-cued fear memory test (Test 1) was conducted in a circular chamber (opaque black walls, diameter 21.5 cm × height 34 cm, with a flat floor) 24 h after conditioning. Immediately after Test 1, mice received the optical LTP or LTD protocol and 24 h later, the second auditory-cued fear memory test (Test 2) was conducted in a novel circular chamber

(opaque reddish walls, diameter 23.5 cm × height 22.5 cm, with a flat floor). Freezing was scored for 3 min before delivery of the tone and then scored for 3 min in the presence of the tone.

Open field test. The open field test was performed to measure locomotion and anxiety in a novel open field using SCANET MV-40 (MELQUEST Co., Toyama Japan). The open field consisted of a square box of 48 cm per side with infrared sensors arranged at 6-mm intervals for the automatic detection of activity. Individual mice were placed at the center of the open field at the initiation of the test session and allowed to freely explore the field for 15 min.

Restraint stress. Mice were placed in an adequately ventilated 50-mL plastic tube (FALCON) for 20 min. They could rotate from a prone to supine position and back again but not turn head to tail.

Corticosterone measurements. Plasma was extracted from blood samples collected from the heart. All blood samples were collected between 2 and 4 p.m. Blood samples were centrifuged at 3,000 rpm for 5 min at 4 °C and extracted plasma was stored at

420 −30°C until assayed for corticosterone using a corticosterone enzyme immunoassay kit
421 (Arbor Assays, Ann Arbor, MI) in accordance with the manufacturer's instructions.
422
423 **Viral vector.** The GR-expressing adeno-associated virus (AAV-GR) and green
424 fluorescent protein-expressing AAV (AAV-GFP) plasmids consisted of the mouse
425 synapsin I promoter followed by cDNA encoding GR or the GFP and a woodchuck
426 hepatitis virus post-transcriptional regulatory element between the inverted terminal
427 repeats of the AAV3 genome. Plasmid AAV-hsyn-oChIEF-tdTomato was purchased
428 from Addgene (Cambridge, MA). Recombinant AAV vectors were produced by
429 transient transfection of HEK293 cells with the vector plasmid, an AAV3 rep and
430 AAV9 vp expression plasmid, and an adenoviral helper plasmid, pHelper (Agilent
431 Technologies, Santa Clara, CA), as previously described (Iida et al., 2013; Li et al.,
432 2006). Recombinant viruses were purified by isolation from two sequential continuous
433 CsCl gradients, and the viral titers were determined by qRT-PCR. Before administration,
434 AAV-GR and AAV-GFP vectors were diluted in phosphate-buffered saline to 5×10^{11}
435 genome copies/100 µL and 1×10^{11} genome copies/100 µL, respectively.
436
437 **Surgery.** Mice were deeply anesthetized with pentobarbital sodium (100 mg/kg body

438 weight, intraperitoneal injection) and placed in a stereotactic frame (Narishige, Tokyo,
439 Japan). AAV-GR or AAV-GFP (0.3 µL per hemisphere) was bilaterally injected into the
440 LA (AP: -1.7 mm; ML: 3.3 mm; DV: −4.1 to −4.2 mm) using glass pipettes (tip
441 diameter 50 µm) connected to a compatible syringe and motorized microinjector
442 (Narishige IMS-10). Mice were allowed to recover in their home cages for 4 weeks
443 before behavioral experiments.
444 For optogenetic experiments, AAV-hsyn-oChIEF-tdTomato was bilaterally injected
445 into the medial geniculate nucleus (AP: −3.1 mm; ML: 1.9 mm; DV: −3.5 mm) and the
446 auditory cortex (AP: −2.7 mm; ML: 4.4 mm; DV: −3.3 mm). A total of 0.5 µL virus per
447 hemisphere was injected. An optic fiber cannula was implanted just above the dorsal tip
448 of the LA (AP: −1.7 mm; ML: 3.4 mm; DV: −4.1 mm) and secured to the skull with
449 screws and dental cement.
450
451 ***In vivo* LTP or LTD induction.** Optical LTP was induced with 10 trains of light (each
452 train consisted of 100 pulses of light, 5 ms each, at 100 Hz) at 90-s inter-train intervals.
453 Optical LTD was induced with 900 pulses of light, 2 ms each, at 1 Hz. After induction,
454 mice were immediately returned to their home cages.
455

***In vivo* recording.** Eight weeks after the injection of AAV-hsyn-oChIEF-tdTomato into the MGN and AC, mice were anesthetized with pentobarbital sodium and then mounted on a stereotaxic frame. The optic fiber was glued to the recording electrode so that the tip of the fiber was 500 μm above the tip of the electrode. The optrode was inserted into the LA and the optic fiber was connected to a 473-nm laser unit. The LTP or LTD induction protocol was identical to those used in the behavioral test. After establishing a stable baseline at the recording site for 21 min (stimulation frequency at 0.033 Hz), *in vivo* LTP or LTD was induced, which was followed by at least 21 min of 0.033 Hz stimulation. Data were analyzed using Clampex 10.7 software. All animals were perfused after the recordings and the position of the recording sites were verified.

Statistical analysis. Statistical analysis was performed using student's *t*-test for the comparison of two groups and one-way ANOVA was used for analyzing experiments consisting of multiple groups. Two-way repeated measures ANOVA was used for analyzing the freezing level during training sessions. Significant ANOVA results were followed by a *post hoc* Tukey–Kramer test for multiple comparisons. All values are presented as mean ± S. E. M. Values of $p < 0.05$ were considered statistically significant.

Statistical analysis was performed using Statcel2. Mice were randomly assigned to experimental groups prior to experimentation. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those generally employed in the field. Animals with viral injections off target or damage during the experiment were excluded from the statistical analysis.

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Agency (JST).

Competing financial interests

S.M. own equity in a gene therapy company (Gene Therapy Research Institution) that commercializes the use of AAV vectors for gene therapy applications. To the extent that the work in this manuscript increases the value of these commercial holdings, S.M. has a conflict of interest. The remaining authors declare no competing financial interests.

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Figure 1

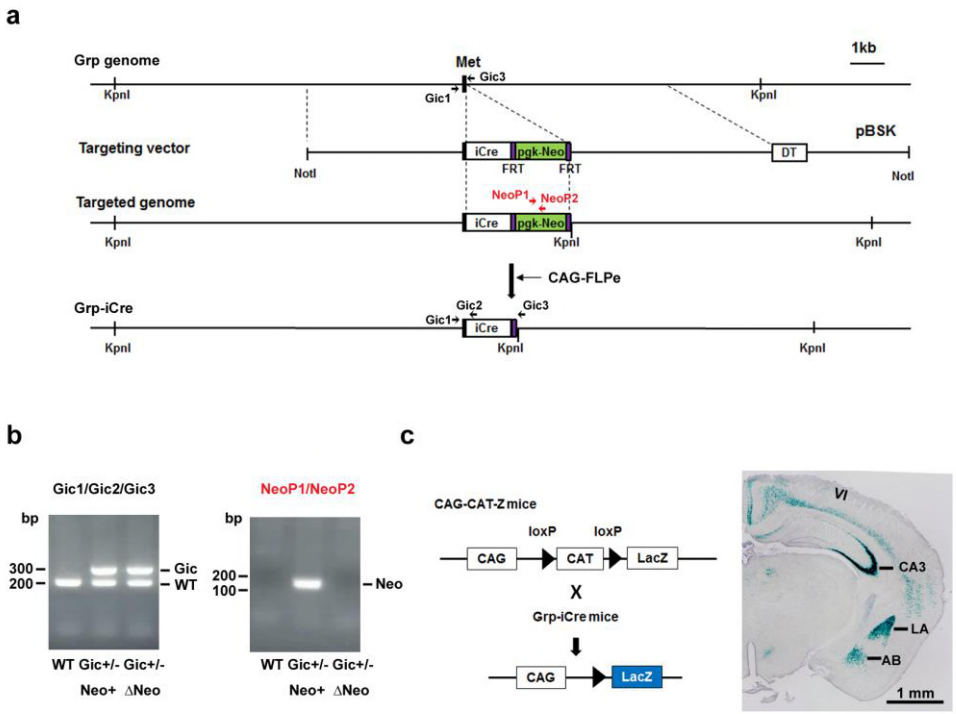

Figure 1. Generation and characterization of Grp-iCre mice. **(A)** Schematic diagram of the gene targeting strategy. iCre and pgk-neo cassettes flanked by two FRT sites were inserted into the gastrin-releasing peptide gene (*Grp*) locus. Met is the translation initiation site of Grp. The location of PCR primers (Gic1, Gic2, Gic3, NeoP1, and NeoP2) used for genotyping are indicated. DT, diphtheria toxin gene; pBSK, pBluescriptII SK. The chimeric mouse obtained was crossed with a CAG-FLPe mouse to delete the pgk-neo cassette and establish the Grp-iCre (Gic) mouse line. **(B)** Genotyping PCR of genomic DNA prepared from WT; $Gic^{+/-}$, Neo^{+}; $Gic^{+/-}$, and ΔNeo mice. **(C)** Cre activity in Grp-iCre mice was examined by crossing Grp-iCre mice with

628 lacZ reporter mice (CAG-CAT-Z) mice. β-galactosidase expression in a
629 Grp-iCre/CAG-CAT-Z mouse stained with X-gal. X-gal staining revealed robust
630 Cre-loxP recombination in the lateral nucleus of the amygdala (LA) and the
631 hippocampal CA3 region, with sparser recombination in the accessary basal nucleus of
632 the amygdala (AB), and in layer 6 of the cerebral cortex (VI).
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Figure 2

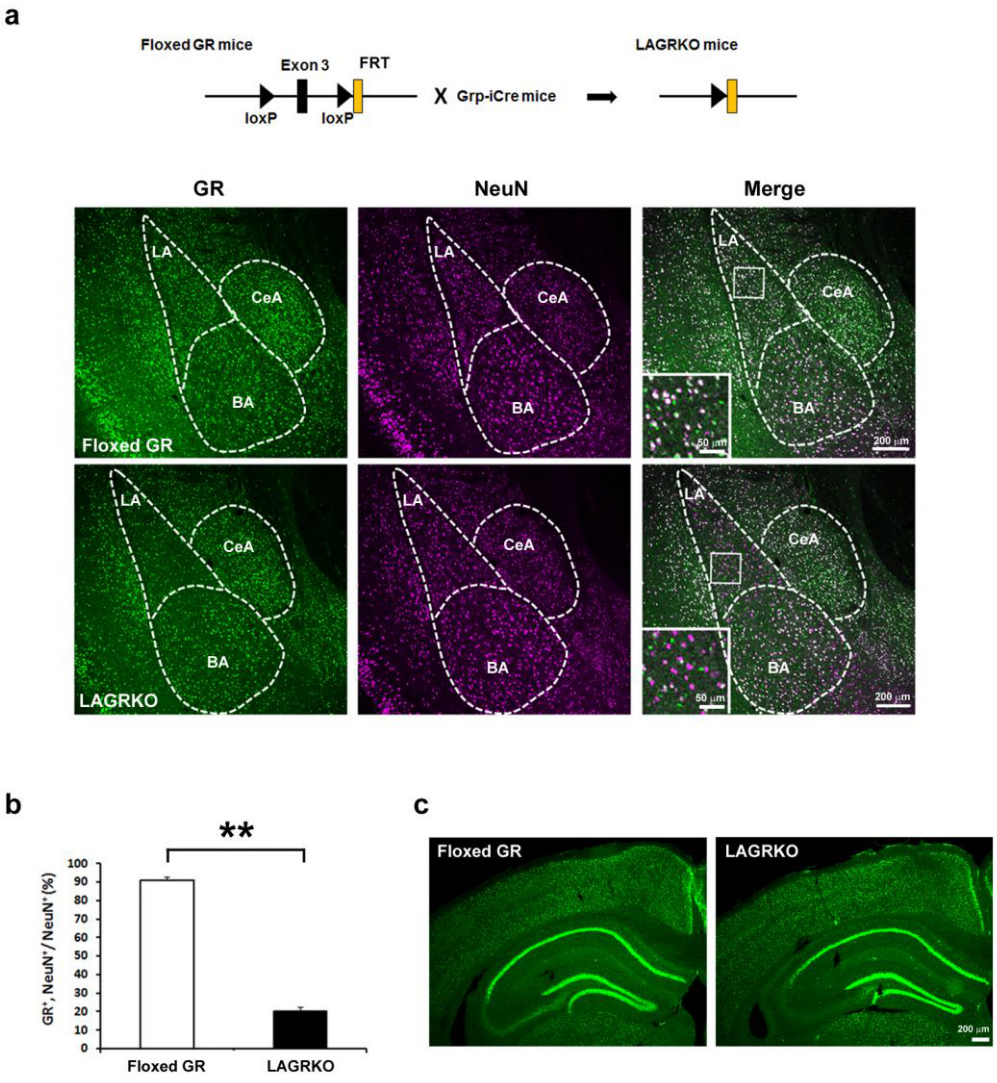

Figure 2. Generation and characterization of lateral amygdala (LA)-selective glucocorticoid receptor (GR) knockout (LAGRKO) mice. **(A)** The LAGRKO mouse line ($GR^{flox/flox}$, Grp-iCre+/-) was established by crossing floxed GR ($GR^{flox/flox}$) and Grp-iCre mice. Double immunofluorescence staining of GR (green, left panels) and NeuN (magenta, middle panels) in coronal brain sections from floxed GR and

652 LAGRKO mice. The overlap of green and magenta signals (white, right panels)
653 indicates the expression of GR in LA neurons in floxed GR mice (upper), which was
654 apparently reduced in LAGRKO mice (lower). Magnified images of the boxed areas are
655 shown in the insets. LA, lateral nucleus of the amygdala; BA, basal nucleus of the
656 amygdala; CeA, central nucleus of the amygdala. **(B)** Quantification of GR^+ and $NeuN^+$
657 cells in the LA of floxed GR and LAGRKO mice (n = 9 sections from 3 mice). Data are
658 presented as mean ± S.E.M. $^{**}p < 0.001$. **(C)** Expression of GR in the cerebral cortex
659 and hippocampus.

Figure 3

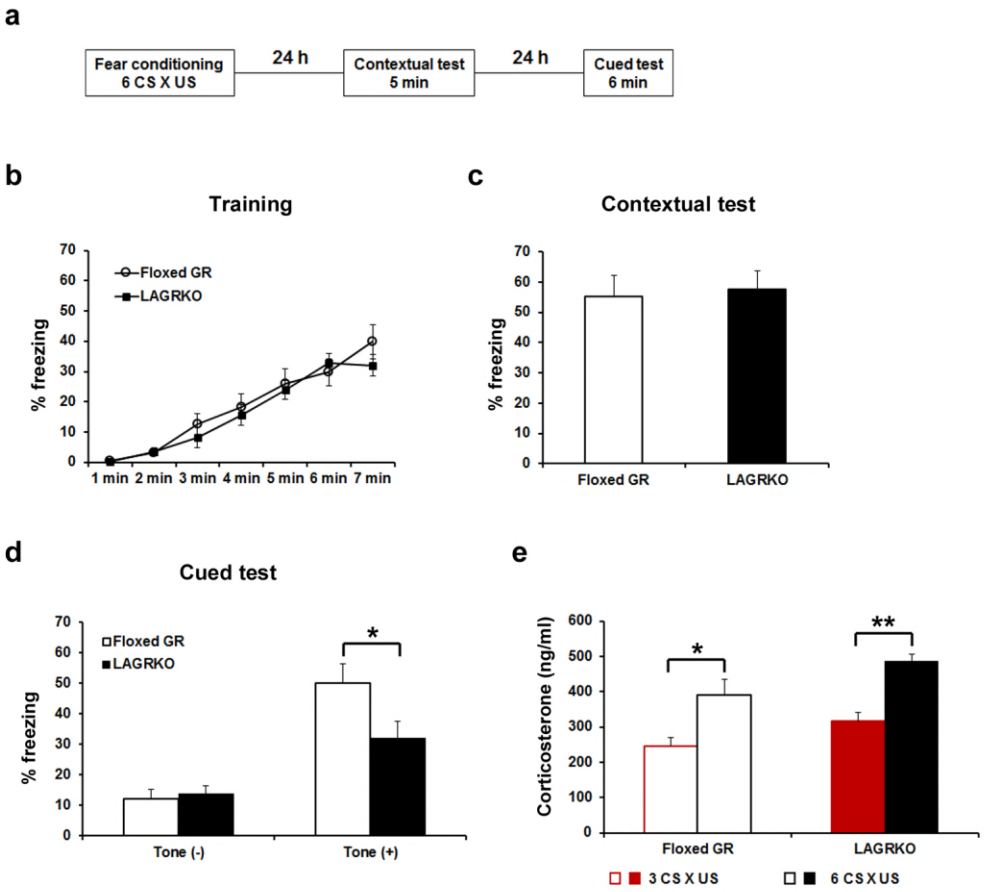

Figure 3. Genetic disruption of LAGR impairs auditory-cued fear memory following strengthened conditioning. **(A)** Experimental protocol for fear conditioning. Mice were trained with a protocol using six CS × US pairings. Contextual and auditory-cued fear memory were tested 24 and 48 h after training, respectively. Freezing levels during the training **(B)**, contextual test **(C)**, and cued test **(D)**. During the cued test, LAGRKO mice (n = 10) exhibited significantly lower freezing levels in the presence of the tone than did floxed GR mice (n = 10). There was no significant difference in freezing levels

668 between the two genotypes during the training and contextual test. **(E)** Plasma
669 corticosterone levels 90 min after training were significantly higher in mice conditioned
670 with six CS × US pairings (n = 7) than in mice conditioned with 3 CS × US pairings (n
671 = 6) in both genotypes. Data are presented as mean ± S. E. M. *$p < 0.05$, *$p < 0.001$.
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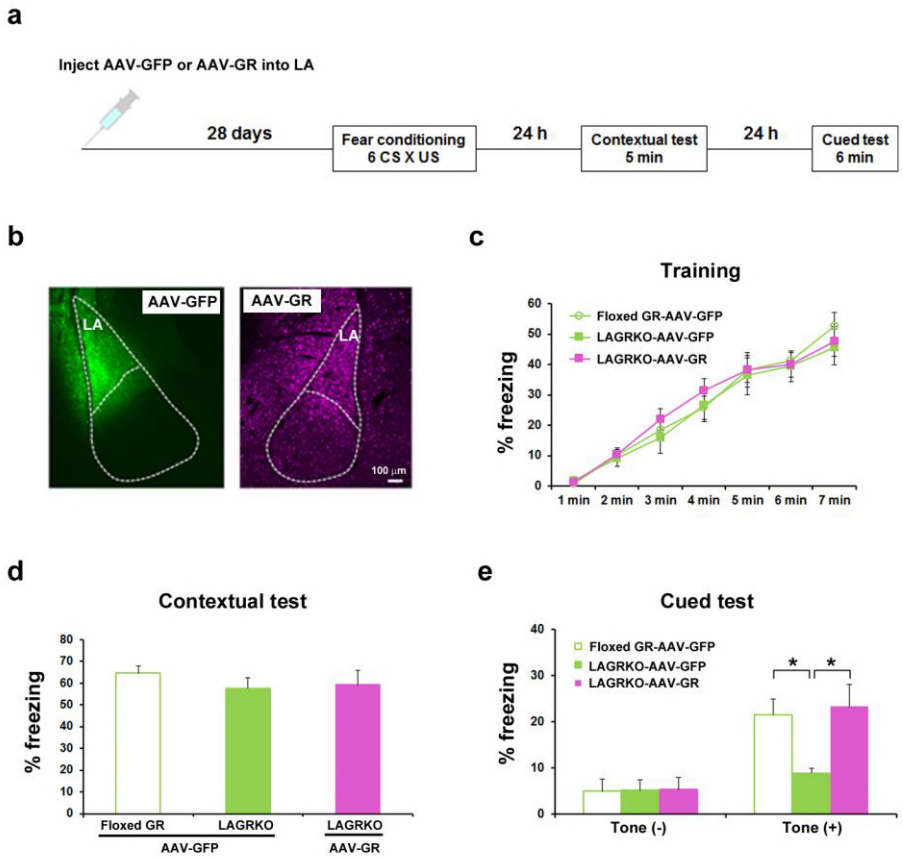
Figure 4



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686 **Figure 4.** Rescue of the auditory-cued fear memory deficit in LAGRKO mice by

687 restoring GR expression levels in the LA. **(A)** The experimental protocol for

688 adeno-associated virus (AAV) vector injection and fear conditioning. **(B)** Representative

689 images of the expression of green fluorescent protein (GFP, green) and GR (magenta) in

690 the LA of LAGRKO mice injected with AAV-GFP or AAV-GR. The expression levels of

691 GFP and GR were assessed after the behavioral test was completed. Freezing levels

692 during the training **(C)**, contextual test **(D)**, and cued test **(E)**. During the cued test,

693 LAGRKO mice (n = 9) injected with AAV-GR and floxed GR injected with AAV-GFP

694 exhibited significantly higher levels of freezing in the presence of the tone than did
695 AAV-GFP-injected LAGRKO mice (n = 9). There were no significant differences
696 between the three groups during the training and contextual tests. Data are presented as
697 mean ± S. E. M. *$p < 0.05$.
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Figure 5

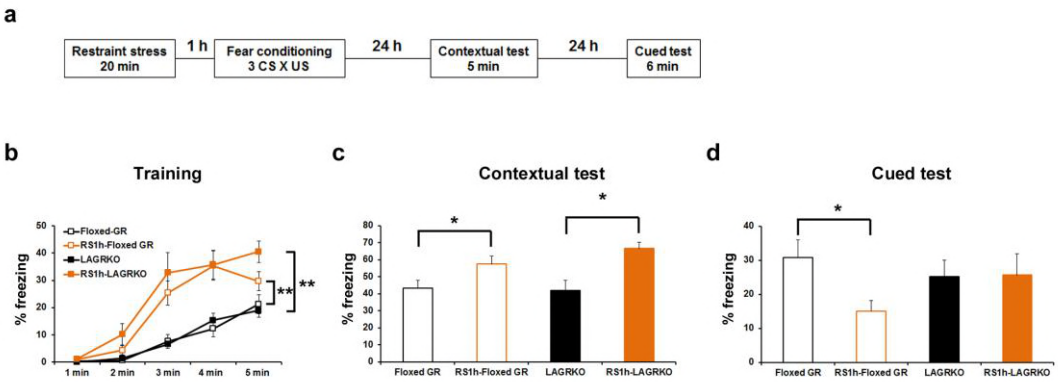


Figure 5. Effect of acute prior stress on auditory-cued fear conditioning. **(A)** The experimental design for prior restraint stress (RS) exposure and fear conditioning. Floxed GR and LAGRKO mice were exposed to a 20-min RS and fear-conditioned 1 h later. Contextual and auditory-cued fear memories were tested 24 and 48 h after training, respectively. **(B)** Mice exposed to RS 1 h before conditioning (brown, RS1h-Floxed GR, n = 11; RS1h-LAGRKO, n = 10) exhibited significantly higher freezing levels than did nonstressed mice (black, Floxed GR, n = 10; LAGRKO, n = 16) during the training session in both genotypes. **(C)** Mice exposed to RS exhibited significantly higher freezing levels than did nonstressed mice during the contextual fear memory test. **(D)** During the cued test, floxed GR mice exposed to RS 1 h before training exhibited significantly lower freezing levels than did the nonstressed floxed GR mice. Previous RS exposure had no effect on auditory-cued fear memory in LAGRKO mice. Data are presented as mean ± S. E. M. *$p < 0.05$; **$p < 0.001$.

Figure 6

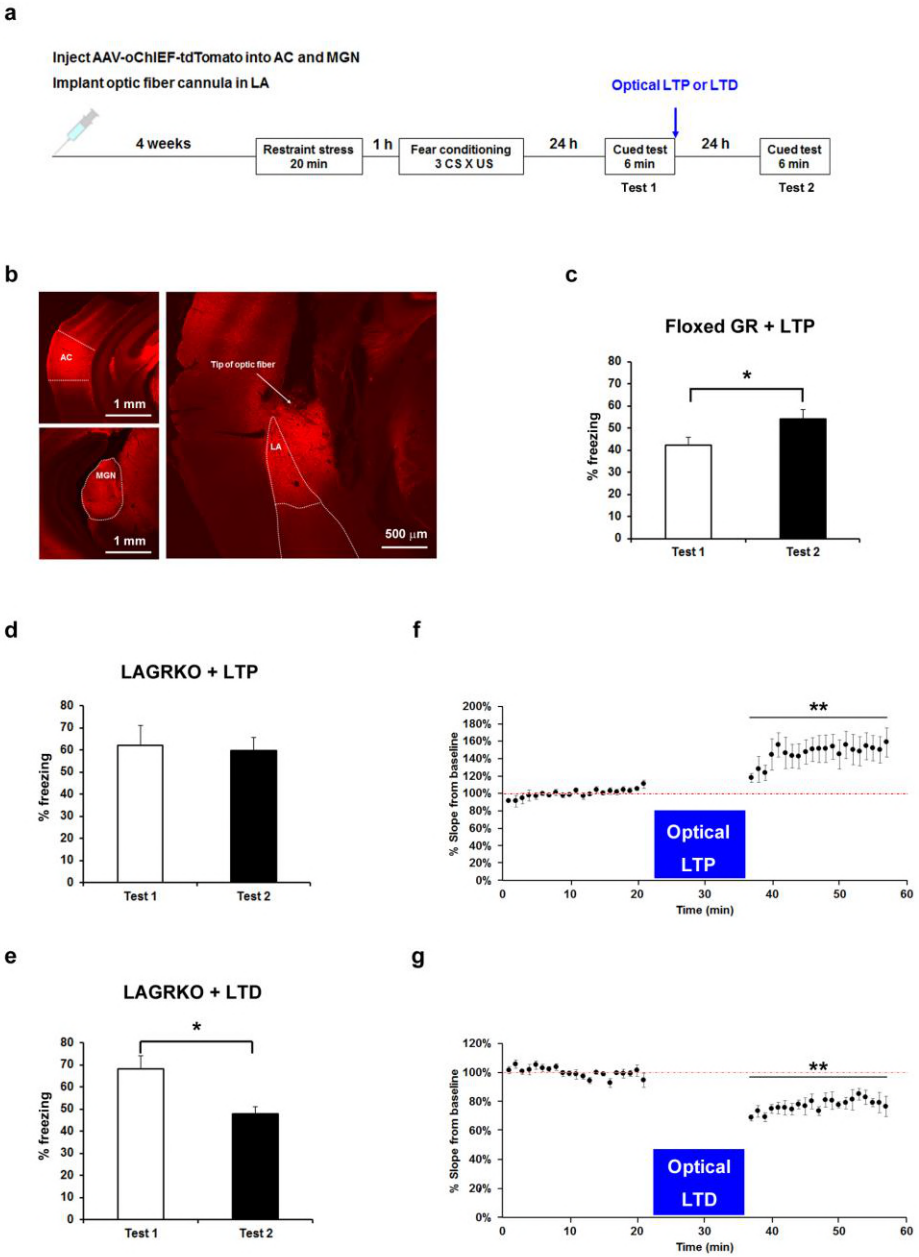
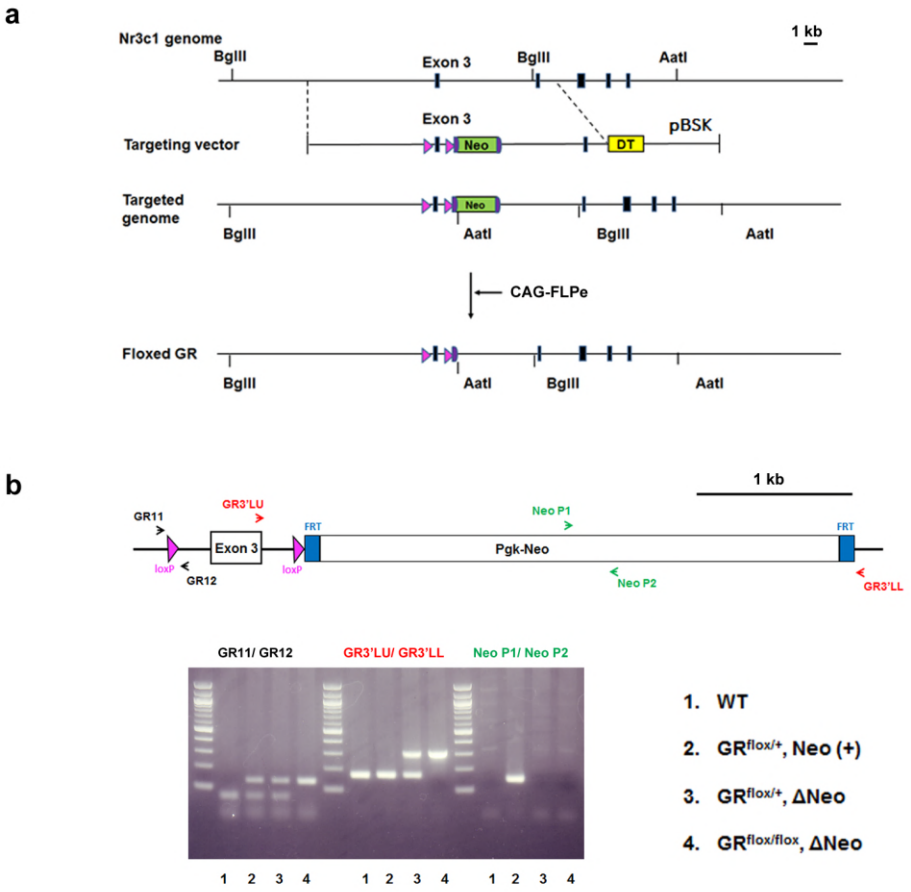


Figure 6. Optogenetic manipulation of LTP or LTD at auditory inputs to the LA alters the effect of acute stress on auditory-cued fear memory. **(A)** Experimental schedule for AAV injection, RS exposure, auditory-cued fear conditioning, and optical LTP or LTD induction. **(B)** Representative fluorescence image of areas expressing

AAV-oChIEF-tdTomato 4 weeks after virus injection in the medial geniculate nucleus (MGN) and auditory cortex (AC). LA, lateral nucleus of the amygdala. **(C)** Floxed GR mice (n = 8) that received immediate optical LTP after the first auditory-cued fear memory test (Test 1) exhibited significantly increased freezing levels in the second auditory-cued fear memory test (Test 2). **(D)** LAGRKO mice (n = 8) that received optical LTP stimulation exhibited the same freezing levels in Tests 1 and 2. **(E)** LAGRKO mice (n = 8) that received LTD stimulation immediately after Test 1 exhibited significantly lower freezing levels in Test 2 than in Test 1. Graphs show the freezing rate during the first 1 min of tone presentation at Tests 1 and 2. **(F, G)** A plot of the average of field EPSP slopes (normalized to the period before optical stimulation) before and after delivering an optical LTP (before LTP, 100.00% ± 0.87%; after LTP, 146.34% ± 3.20%) or LTD (before LTD, 100.00% ± 0.61%; after LTP, 77.35% ± 0.99%). Data are obtained from four mice (two floxed GR and two LAGRKO mice). Data are presented as mean ± S. E. M. *$p < 0.05$, **$p < 0.001$.

Supplementary Figure 1



Supplementary Figure 1. Generation of floxed GR mice. **(A)** Strategy for targeting the GR (*Nr3c1*) gene locus. The coding regions of GR exons are indicated by closed boxes. The construct included two loxP sites and the pgk-neo genes flanked by two FRT sites. DT, diphtheria toxin gene; pBSK, pBluescriptII SK; GR, glucocorticoid receptor. The chimeric mice obtained were crossed with CAG-FLP mice to delete the pgk-neo cassette and establish a floxed GR mouse line. **(B)** Genotyping PCR of genomic DNA prepared from WT, $GR^{flox/+}$, Neo (+), $GR^{flox/+}$, *ΔNeo,* and $GR^{flox/flox}$, *ΔNeo* mice. The

757 locations of PCR primers used are indicated.

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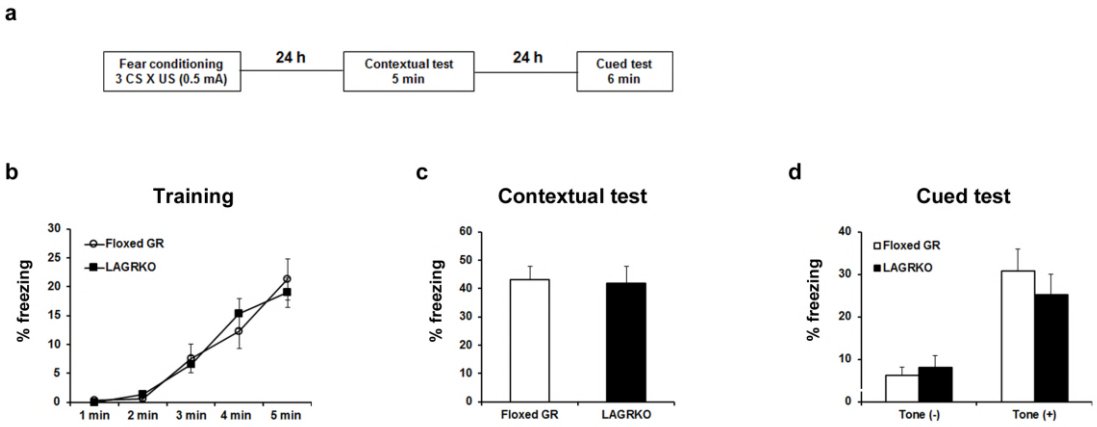
Supplementary figure 2



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761 **Supplementary Figure 2.** LAGRKO mice did not exhibit fear memory deficits with the

762 moderate fear conditioning protocol. **(A)** Experimental design for fear conditioning.

763 Mice were trained with three CS × US pairing protocol. Contextual and auditory fear

764 memories were tested 24 and 48 h after training, respectively. Freezing levels during the

765 training **(B)**, contextual test **(C)**, and cued test **(D)**. Data are presented as mean ± S. E.

766 M. Floxed GR, n = 10; LAGRKO, n = 16. Data are presented as mean ± S. E. M.

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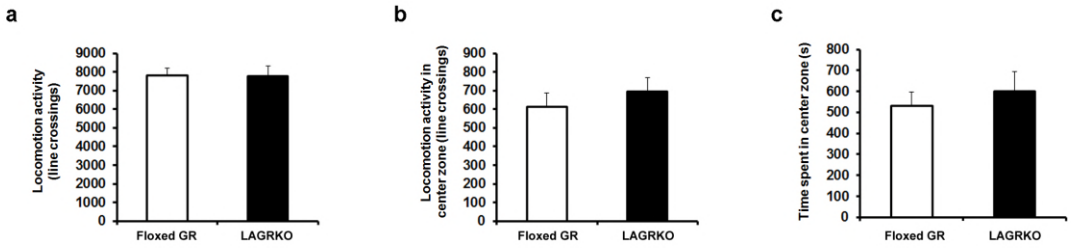
Supplementary figure 3



Supplementary Figure 3. LAGRKO mice exhibited no changes in locomotor activity and anxiety level as determined using the open field test. Distance of travel throughout the apparatus **(A)** and in the center zone of the open field **(B)** and time spent in the center of the open field **(C)** did not differ between the two genotypes. Floxed GR, n = 15; LAGRKO, n = 10. Data are presented as mean ± S. E. M.