Supplementary Materials and Methods

Production of the lentivirus[1]

The lentivirus was generated by transient transfection of HEK293T cells (ATCC, Manassas, VA) using the Effectene reagent (QIAGEN, Valencia, CA). The viral supernatants were collected twice, at 48 h and 72 h post-transfection, passed through a 0.2-μm filter, and concentrated by centrifugation using polyethylene glycol and NaCl. Aliquots were stored in phosphate-buffered saline (PBS) in the presence of 8 μg/mL polybrene, and stored frozen at −80°C until use. Each viral titre was determined with a p24 enzyme-linked immunosorbent assay (ELISA) (Cell Biolabs, Inc., San Diego, CA), showing on average approximately 0.2 mg/mL of p24. The glucocorticoid response element-enhanced green fluorescence protein (GRE-EGFP) reporter in the presence of corticosterone (CORT; 100 nM; Sigma, St. Louis, MO) resulted in 4×10^5 EGFP transduction units (TU)/100 ng human immunodeficiency virus (HIV) p24.

Animals

In this study, we used male Wistar rats weighing 280–300 g (Japan SLC, Inc., Hamamatsu, Japan). The animals were individually housed in transparent plastic cages with wire grid covers under controlled temperatures (22–24℃) with a 12-h light/dark cycle (lights on from 08:00 to 20:00). The rats were given at least 1 week to adapt to their environment before the experiments. The Institutional Animal Care and Use Committee at the Korea Basic Science Institute (KBSI-AEC 1109) reviewed and approved this study. All animal procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* issued by the Laboratory Animal Resources Commission of KBSI.

Stereotaxic injection of lentiviral-based reporters

Rats were anesthetized by intraperitoneal (ip) injection with a combination of zolazepam (50 mg/mL; Zoletil 50, Vibac, Carros, France) and xylazine (10 mg/mL; Rompun, Bayer Sverige AB, Germany) and placed into a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The GRE-Luc or ΔGRE-Luc lentiviral reporters (Supplementary Figs. 1) were injected into the left hippocampal CA1 region based on coordinates from the bregma and dura mater according to the atlas of Paxinos and Watson (anterior −3.6, lateral –2.0, ventral −2.9)[2]. Using a microsyringe nanopump (Leica Microsystems, Richmond Hill, ON, Canada) and a 10-μL Hamilton syringe, a 5-μL viral solution was injected at a rate of 0.2 μL/min. The syringe was then held in place for 10 min before it was withdrawn. Following the procedure, the rats were allowed at least a 7-day recovery period in their home cages before the imaging experiments. After completing the experiments, the brains were removed and coronally sectioned (40-μm thickness). Each section was stained with Cresyl violet to confirm that the injection sites were located in the dorsal CA1 region of the hippocampus (data not shown). Rats with injection sites outside of this region were excluded from imaging analyses.

Experimental design for hippocampal GR activity analysis

Rats injected with one of the lentiviral reporters (GRE-Luc or ΔGRE-Luc) were tested in three independent experiments. In the first experiment, following 2 weeks of recovery from surgery, the rats were randomly assigned to either a non-stress (NS) or acute stress (AS) group with a 2-h immobilization condition (IMO, typically between 09:00 and 11:00). In the second experiment, 7 days after lentiviral injection, rats were adrenalectomized (ADX) bilaterally and after another 7 days were randomly assigned to two groups; one group was subjected to 2-h IMO stress and the second was subcutaneously injected with CORT (3.0 mg/kg in sesame oil, Sigma) at a volume adjusted to 1 mL/kg body weight.[3] In the third

experiment, rats were exposed daily to 2-h IMO stress for 21 consecutive days during which they were randomly assigned to receive ip injection of either fluoxetine (CS+F; 10 mg/kg, dissolved in saline solution, Daewoo Pharmaceuticals, Busan, Korea) or 0.9% saline (CS; 1 mL/kg). On the test day, reporter activity was assessed using the IVIS 200 imaging system (Xenogen Corporation, Alameda, CA) before and after the 2-h IMO stress exposure, and then every 2 h after the stress exposure for 6–10 h.

Imaging of in vivo and ex vivo GR activity

We recently reported *in vivo* visualization of luciferase activity using the bioluminescence imaging (BLI) technique.[4-6] The current study was performed based on these previous reports. All animals received an ip injection of 150 mg/kg D-luciferin (Biosynth International, Naperville, IL) dissolved in Dulbecco's PBS, and were then anesthetized in an induction chamber with 2.5% isoflurane in 100% oxygen at a flow rate of 1.0 L/min for 10 min. For the *in vivo* BLI analyses, 3 live rats were imaged simultaneously for 5 min using the IVIS system with a 2.0% mixture at 0.5 L/min, and the regions of interest were quantified with photon flux (p/s) using Living Image software v4.2 (Xenogen Corporation). The data represent BLI signals from an individual rat, combined from at least two independent studies. The BLI signals at each time point were divided by the averaged signal of the negative control (ΔGRE-Luc) recorded at the same time point in the same batch of experiments. For the *ex vivo* BLI analyses, after completing the acute stress, rats were injected with luciferin 15 min prior to euthanization. The brain was removed and dissected into 1-mm coronal or sagittal segments using a slicer matrix (ASI Instruments, Warren, MI). Twenty-five minutes after the luciferin injection, serial segments were imaged with a 5-min exposure time.

In vitro assay of the lentivirus

African green monkey kidney fibroblasts (COS-1) and rat immortalized hippocampal neurons (H19-7) were purchased from ATCC (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin under 5% CO_2 at 37℃ for COS-1 cells, or at 34℃ for H19-7 cells. Primary hippocampal neurons were isolated from Wistar rats on postnatal day 4; the neurons were isolated using the method described by Brewer,[7] with some modifications. Isolated cells were cultured under 5% CO_2 at 37℃ on 6-well plates coated with poly-D-lysine in Neurobasal/B27 media (Invitrogen, Grand Island, NY). A 50% glutamate-free medium was changed on day 4, and then once per week thereafter. The cells were used for experiments after 6 days of culture. For the antagonism assay, following infection with the GRE-Luc reporter, COS-1 cells were transiently transfected with GR or mineralocorticosteroid receptor (MR) vectors using Effectene (QIAGEN). Cells were incubated with RU486 (dissolved in 70% EtOH, Sigma) or spironolactone (dissolved in 70% EtOH, Sigma) 3 h prior to 36-h treatment with cortisol for COS-1 cells, or CORT for rat hippocampal neurons. After these treatments, luciferase activity was measured using an IVIS 200 system (Xenogen Corporation), as described previously.[8] Reporter activity was analysed using Living Image software v4.2 (Xenogen Corporation) and was normalized for transfection efficiency with total protein (BCA Protein Assay-RAC; Pierce, Rockford, IL). Results were expressed as fold activity per milligram of cellular protein.

Corticosterone assay

A subset of the rats was used to examine serum CORT, using previously described methods.[8] Briefly, trunk blood was collected at the indicated time points (n = 10/group), and serum was obtained by

centrifugation at 1000 *g*. Serum CORT levels were determined using commercially available Enzyme Immunoassay (EIA) kits (R&D Systems, Inc., Minneapolis, MN).

Forced swim test

After imaging, the forced swim test (FST) was conducted in a transparent Plexiglas cylinder (50 × 20 cm) filled to a 30-cm depth with 23–25℃ water as described previously.[8] The first FST session lasted 15 min followed 24 h later by a 5 min second FST session, which measure the acquisition for the first 5 min and retention of the immobility response for the total 5 min as an index of 'depressive-like' behaviours in rodents.[9, 10] A rat was considered immobile when it floated without attempting to swim. The duration of the immobile period was measured by two blinded observers for up to 5 min.

Western blot analysis

A subset of rats from the CS (n = 10) and CS+F (n = 10–12) groups were examined in a western blot analysis conducted immediately and 2 h after the stress ended. The left hippocampus was homogenized in cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulphate [SDS], 0.25% sodium deoxycholate, and 5 mM *N*-ethylmaleimide), with phosphatase and protease inhibitor cocktails (Roche Molecular Biochemicals, Indianapolis, IN). Samples (10–50 μg) were resolved using 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membranes were incubated with primary antibodies and then with horseradish-conjugated secondary antibody; immunoreactivity was visualized using enhanced chemiluminescence. The following primary antibodies were used: anti-GR (1:1000; Santa Cruz, San Diego, CA), anti-phospho-GR (S224) (1:500; Abcam, Cambridge, UK), anti-phospho-GR (S232) (1:500; Cell Signaling Technology, Danvers, MA), and anti-β-actin (1:10,000; Sigma). The band intensities in the western blot analysis were determined using the ImageJ programme (open source ImageJ software available at http://rsb.info.nih.gov/ij/).

Immunofluorescence and confocal imaging

The brain was sectioned coronally (30-μm thick) and processed for immunofluorescence staining with primary antibodies specific for GR (1:500; Santa Cruz Biotechnology) and neuronal nuclei (NeuN; 1:500; Santa Cruz Biotechnology).[8] Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1:3000; Invitrogen, Paisley, UK) before washing and mounting. Images were acquired on a confocal laser scanning microscope (LSM-780 NLO; Carl Zeiss, Oberkochen, Germany) equipped with a C-Apochromat 20×/0.5 W objective lens. Using ZEN 2010 software (version 6.0; Carl Zeiss), the nuclear GR intensity was quantified as the mean of the nuclear area with the same size as that defined by DAPI, and was corrected by subtracting the background signal in which the image intensity did not indicate cells. We confirmed GR and MR expression in primary hippocampal neurons by staining with GR (1:500; Santa Cruz) and MR (1:500; Santa Cruz Biotechnology) specific antibodies.

Statistical analyses

Statistical analyses were performed using Prism 4 software (GraphPad Software, San Diego, CA). A two-way repeated measures analysis of variance (ANOVA) was used to examine the serum CORT level in the chronic stress experiment, mean GR activity, and nuclear GR intensity using the within-subject factors of stress and time (h). A one-way repeated measures ANOVA was performed on the serum CORT levels in the acute stress experiment. Other statistical analyses, including Student's *t*-tests and one-way ANOVAs,

are discussed under the appropriate categories in the figure legends. Relationships between behaviour and GR activity or phosphorylated GR proteins were evaluated with Pearson's correlations. All *post hoc* analyses included a Bonferroni correction. Data are expressed as the mean ± standard error of the mean (SEM); $P < 0.05$ was considered statistically significant.

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6. Lee MS, Her S. Direct quantification of PTD transduction using real-time monitoring. *Methods Mol Biol* 2013; **991:** 307-314.

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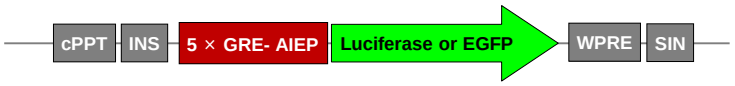
9. Jefferys D, Funder J. The effect of water temperature on immobility in the forced swimming test in rats. *Eur J Pharmacol* 1994; **253:** 91-94.

10. Jefferys DE, Funder JW. The thyroadrenal axis, food deprivation and retention of a behavioural response. *Eur J Pharmacol* 1993; **239:** 189-193.

Supplementary Figures

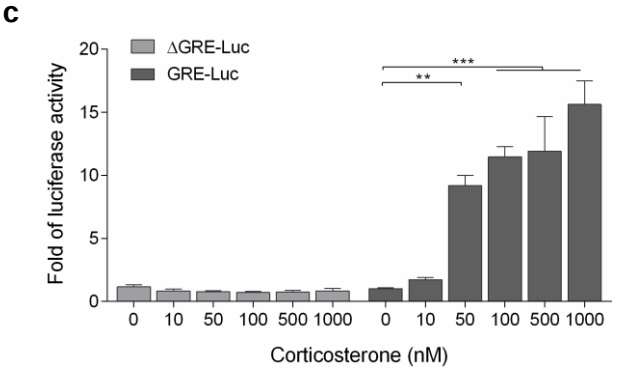
a



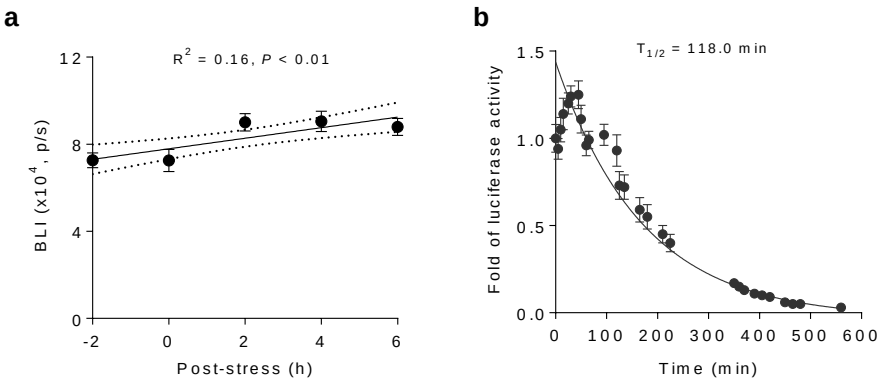
b

Designation	Promoter	Reporter	Function
GRE-Luc	5 × GRE/minimal promoter	Firefly luciferase	GR activity for BLI
GRE-EGFP	5 × GRE/minimal promoter	Enhanced green fluorescent protein	GR activity for IHC
ΔGRE-Luc	minimal promoter	Firefly luciferase	Negative control for the GRE-Luc
LV-Luc	EF1-α promoter	Firefly luciferase	Positive control for luciferase activity

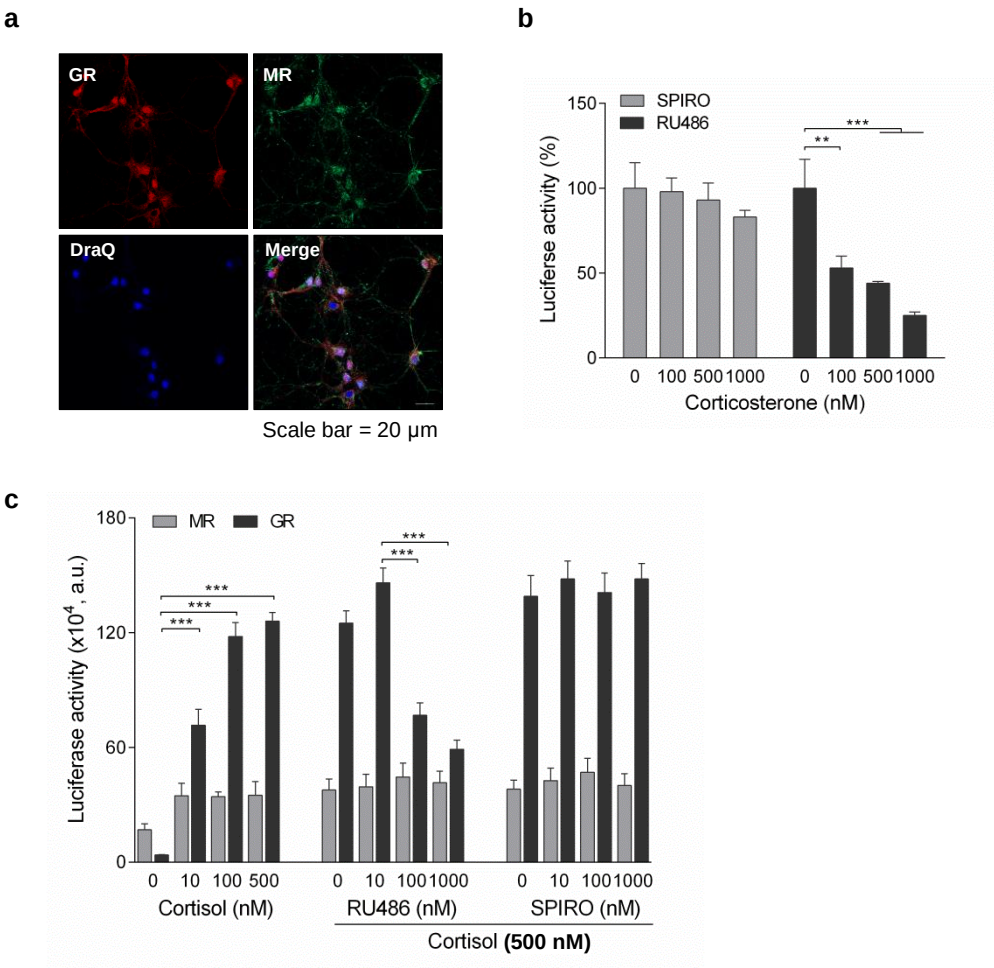
c



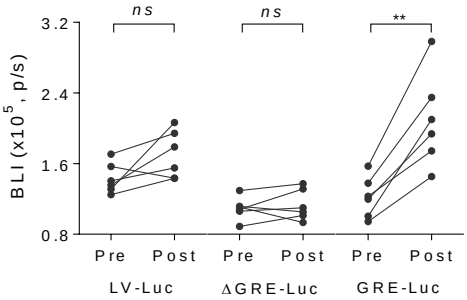
Supplementary Figure 1. Lentiviral reporters used in this study. (a) Lentiviral reporter constructs. (b) List of the reporter vectors and their functions. (c) Responses of GRE-Luc or ΔGRE-Luc reporter to CORT in H19-7 hippocampal neuronal cells. To evaluate the effect of CORT on GR transcriptional activity, H19-7 cells were infected with the GRE-Luc or ΔGRE-Luc reporter, and then were incubated for 36 h in the presence of indicated doses of CORT. Luciferase activity was measured using IVIS 200. There were significant inductions of luciferase activity in the GRE-Luc reporter ($F_{5,48} = 16.53$, $P < 0.001$), but not in the ΔGRE-Luc reporter ($F_{5,48} = 1.36$, $P = 0.26$). Data are expressed as a relative fold change of luciferase activity normalized to the amount of cellular protein (μg) and represent the mean ± SEM ($n = 3$ experiments in triplicate). $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Bonferroni's multiple comparisons *post hoc* test. cPPT, central polypurine tract; INS, chicken β-globin insulator; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; SIN, self-inactivating; BLI, bioluminescent imaging; IHC, immunohistochemistry.



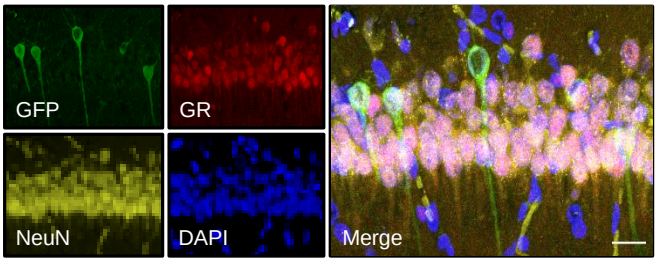
Supplementary Figure 2. Time-dependent increase in luciferase activity. (a) Temporal increase in the BLI signal *in vivo*. Rats were stereotaxically microinjected with the ΔGRE-Luc reporter into the hippocampal CA1 region. After 14 days, the BLI signals were monitored pre-stress (-2 h) and every 2 h after acute stress by IVIS 200 (n = 14). Results illustrate increased luciferase signals in a time-dependent manner (R^2 = 0.16, P < 0.01). Linear regression (solid line) that lies within the 95% confidence intervals (dashed line) analysis was applied to evaluate the relationship between time and the BLI signals. The data are presented as the mean ± SEM. (b) *In vitro* half-life ($T_{1/2}$) of luciferase activity. H19-7 hippocampal neuronal cells infected with the GRE-Luc reporter were cultured with 500 nM CORT and subsequently incubated with 100 μg/mL cycloheximide to block new protein synthesis. A time-dependent decrease of luciferase was observed, showing that the estimated half-life of luciferase is 118 min. The data are presented as the mean ± SEM, n = 3 experiments in triplicate. The fitted curve and half-life of luciferase activity was obtained using non-linear regression analysis. These results indicate that the time-dependent increase of the *in vivo* luciferase signal is due to the prolonged half-life of luciferase activity.



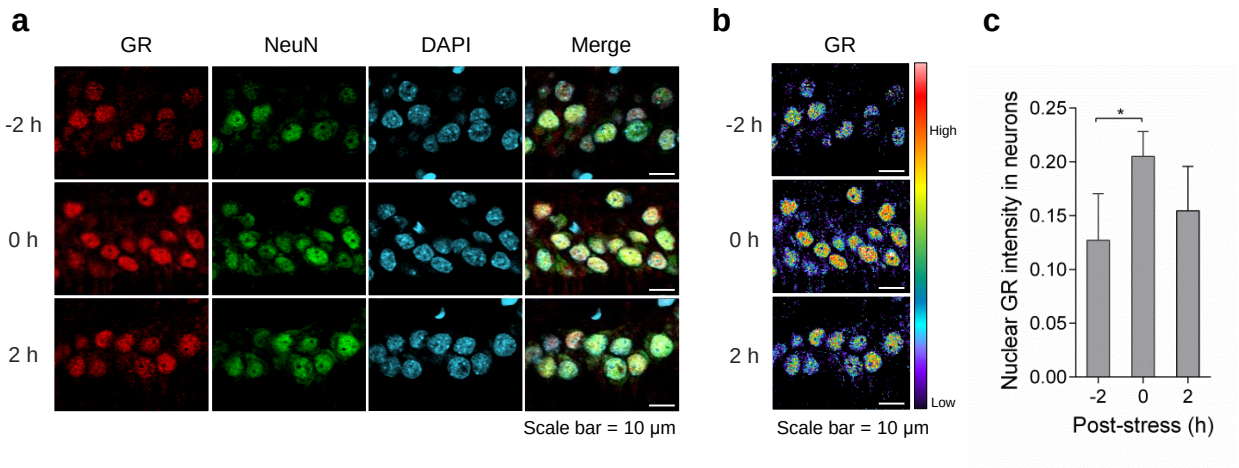
Supplementary Figure 3. Evaluation of GR specificity *in vitro*. (a) Confocal images from primary hippocampal neuronal cultures. Cells were stained with anti-GR (red) and anti-MR (green) to visualize the expressions of GR and MR, respectively. DraQ (blue) was used for nuclei staining. (b) Effects of RU486 or spironolactone (SPIRO) on the GR activity of GRE-Luc reporter in the primary hippocampal neuronal cells. After infection with the GRE-Luc reporter, The cells was treated with the indicated doses of CORT in the presence of the indicated doses of RU486 or SPIRO for 48 h (n = 3 experiments in triplicate). Treatment with RU486 inhibited luciferase activity in a dose-dependent manner ($F_{3,32} = 11.85$, $^{***}P < 0.001$ for RU486; $F_{3,32} = 0.57$, $P = 0.64$ for SPIRO). (c) Antagonistic effects of the GRE-Luc reporter in COS-1 cells. Cells transiently expressing GR or MR were infected with GRE-Luc reporter (n = 3 experiments in sextuplicate). The same procedures as in the hippocampal neuronal cells were performed except for cortisol treatment instead of CORT. Cortisol increased luciferase activity in the COS-1 cells transiently expressing GR in a dose dependent manner ($F_{3,68} = 88.72$, $P < 0.001$), and this effect was blocked by pretreatment with RU486 ($F_{3,68} = 39.68$, $P < 0.001$ for RU486). However, the COS-1 cells transiently expressing MR showed neither an agonist effect of cortisol ($F_{3,68} = 2.47$, $P = 0.07$) nor an inhibitory effect by SPIRO ($F_{3,68} = 0.21$, $P = 0.89$ for RU486; $F_{3,68} = 0.36$, $P = 0.78$ for SPIRO). Data are shown as the mean ± SEM and expressed as luciferase activity (a.u.) normalized to the amount of cellular protein (μg). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{**}P < 0.01$ by one-way ANOVA with Bonferroni's multiple comparisons test. a.u., arbitrary units.



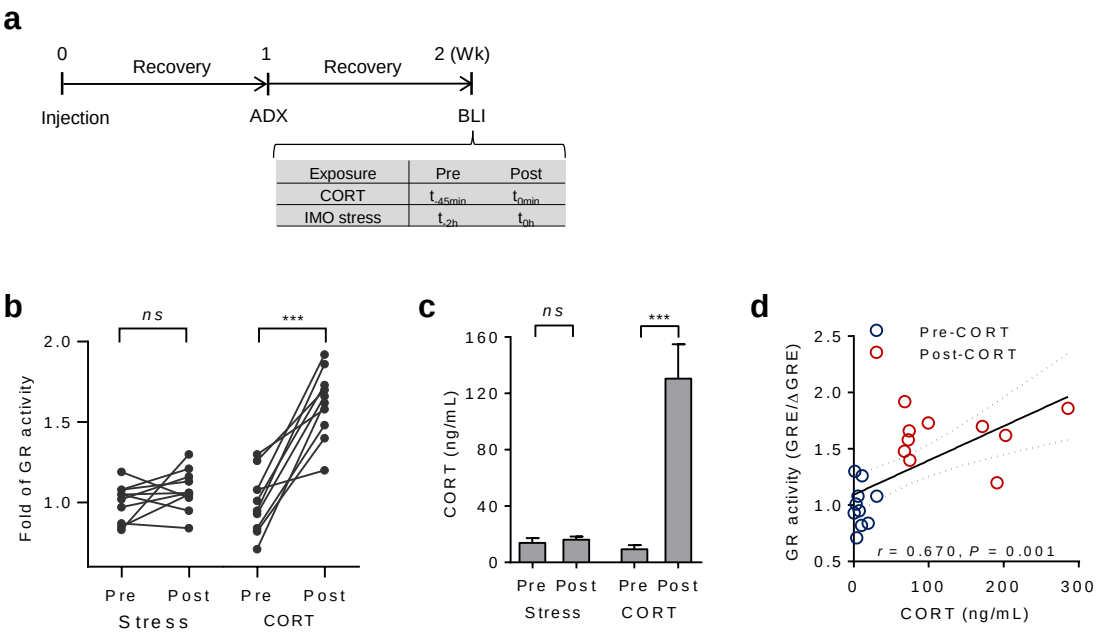
Supplementary Figure 4. Individual stress response of lentiviral reporters *in vivo*. Rats were stereotaxically microinjected with three lentiviral reporters (left, LV-Luc; middle, ΔGRE-Luc; right, GRE-Luc) into the hippocampal CA1 region. After 14 days, the BLI signals were monitored before (pre-stress, Pre) and after (post-stress, Post) the 2-h IMO stress exposure by IVIS 200 (n = 6/group). Acute stress significantly increased GRE-Luc reporter activity (t = 6.04, p < 0.01), but not LV-Luc (t = 2.22, p = 0.08), and ΔGRE-Luc (t = 0.67, p = 0.54), reporter activities. $^{**}P$ < 0.01 by Student's t-test*; ns*, non-significant.



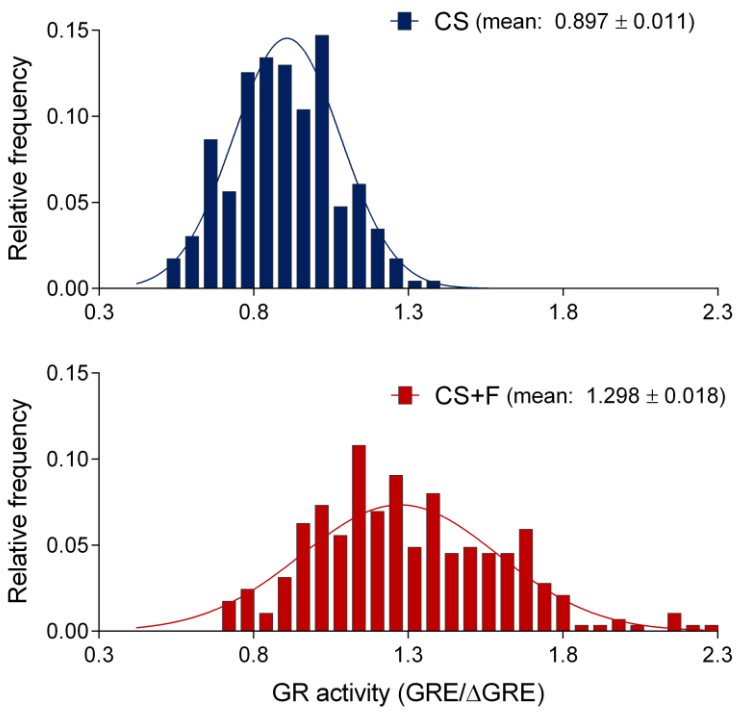
Supplementary Figure 5. Cellular location of GR activation in the hippocampal CA1 region by the GRE-EGFP reporter. Two-weeks after injection, rats were subjected to a 2-h IMO stress and subsequently sacrificed for immunohistochemical assessment. Hippocampal sections were stained with GR (red), NeuN (yellow), and DAPI (blue) to detect the location of EGFP expression (green). GR activation was determined in $NeuN^+$ hippocampal CA1 neurons in which expressions of EGFP were co-localized with GRs. Scale bar, 20 μm.



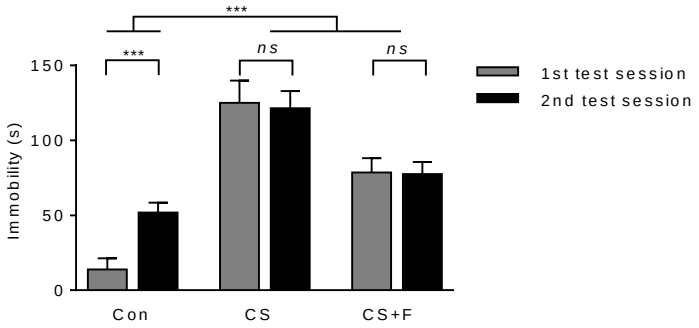
Supplementary Figure 6. GR nuclear translocation in acute stress. (a) Representative confocal images of hippocampal sections stained for GR (red), NeuN (green), and DAPI (cyan). (b) Rainbow pseudocolour images for GR intensity. Fluorescence intensities are represented linearly on a rainbow scale with red being the maximum signal and black being the lowest signal. (c) Quantification of nuclear GR intensity in neurons at indicated time point. Data are presented as the mean ± SEM (n = 4/each time point). $^{*}P < 0.05$ by one-way ANOVA followed by Bonferroni's multiple comparison test as the *post hoc* test. Scale bar for all images, 10 μm.



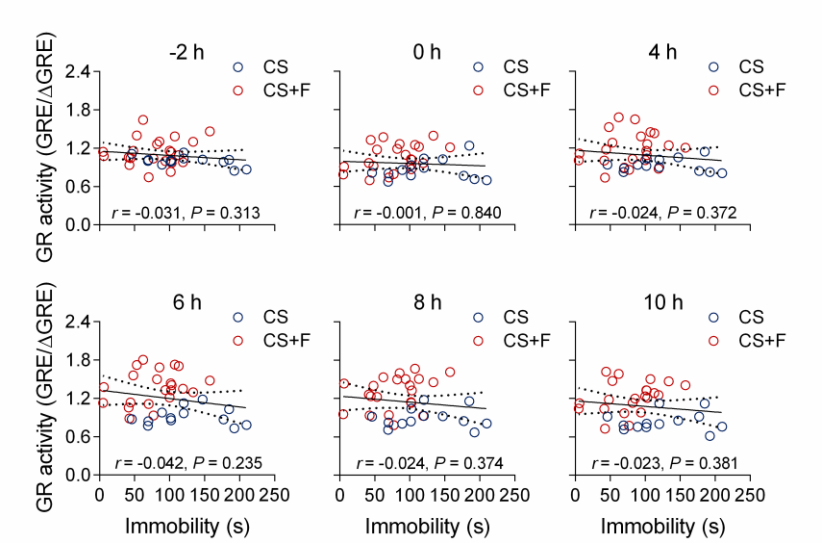
Supplementary Figure 7. Effect of exogenous CORT pulse on GR activity in adrenalectomized (ADX) rats. (a) Experimental design for ADX experiments (n = 10/group). GR activity (b) and CORT levels (c) of ADX rats subjected to 2-h of IMO stress or CORT (3.0 mg/kg, ip). The ADX models (n = 10) were confirmed by acute stress in which no induced CORT was observed (t = 0.53, p = 0.60). (d) Correlation analysis of the CORT level with GR activity at pre- and post-treatment with CORT from independent experiments. Scatter plots with fitted linear regression lines and a 95% confidence interval (dashed lines) for that line are shown for 20 pairs conducted between the CORT level and GR activity at pre- and post-treatment with CORT. A significant linear correlation was detected between the CORT level and GR activity.



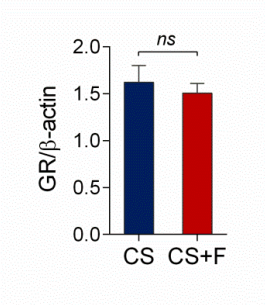
Supplementary Figure 8. Comparison of the frequency distribution of the individual GR activity in the CS and CS+F groups. The distribution for the CS group is clustered at a lower range of GR activity than the CS+F group in which the distribution is dispersed in a wide range of GR activity. The best-fitted normal distribution curve is also depicted.



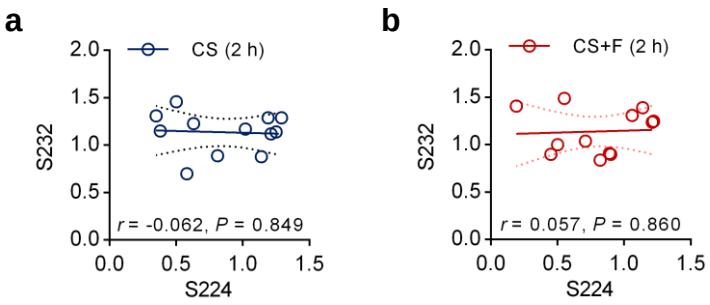
Supplementary Figure 9. Retention of the first immobility time in the Con (*n* = 10), CS (*n* = 17), and CS+F (*n* = 24) groups. The first session lasted 15 min followed 24 h later by a 5-min second FST test. The comparisons were made between the first 5 minutes of the first 15 min session (gray bar) and the total 5 min of the second session (black bar). There were significant differences between the CS and CS+F groups ($F_{2,96}$ = 585.5, $P < 0.001$) in terms of the latency to immobility in the first (t = 14.69, $P < 0.001$) and second sessions (t = 15.20, $P < 0.001$). However, fluoxetine treatment did not demonstrate the same retention effect (t = 1.81, first *vs* second) with the level of immobility as shown in the saline-treated group (t = 1.06, first *vs* second), while the intact control appeared to retain the acquired immobility response by 3.73-fold (t = 8.30, $P < 0.001$). The data are presented as the mean ± SEM. $^{***}P < 0.001$ by two-way ANOVA with Bonferroni's multiple comparisons test. *ns*, non-significant.



Supplementary Figure 10. Correlation analysis of depressive-like behaviour with GR activity at each time point. Scatter plots with fitted linear regression lines and a 95% confidence interval (dashed lines) for that line are shown for 41 pairs conducted between the GR activity at each time point and the immobility duration of the FST.



Supplementary Figure 11. Effect of fluoxetine treatment on GR expression levels. The levels of GR protein obtained from the hippocampus at 2 h post-stress were not different between the CS group and CS+F group (t = 0.622, P = 0.546 vs. CS). The data are presented as the mean ± SEM (n = 12/group). *ns*, non-significant.



Supplementary Figure 12. Pearson correlation between S224 and S232 in the (a) CS and (b) CS+F groups at 2-h post-stress. Linear regression analysis (solid line) within the 95% confidence intervals (dashed lines) was applied (n = 12/group). There is no significant relationship between S224 and S232 of either of the groups.