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Exercise Prevents Memory Consolidation Defects Via Enhancing Prolactin Responsiveness of CA1 Neurons in Mice Under Chronic Stress

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Abstract

We investigated the effects of regular exercise on chronic stress-induced memory consolidation impairment and its underlying mechanism. We focused on prolactin (PRL)-modulated calcium-permeable (CP)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs) in neurons in the CA1 stratum lacunosum-moleculare (SLM) area of the dorsal hippocampus. Regular exercise protected against memory retention defects and prevented dendritic retraction in apical distal segments of hippocampal CA1 neurons, as indicated by enhanced dendritic ramification, dendritic length, spine density, and synaptic protein levels following chronic stress. Regular exercise normalized synaptic CP-AMPAR assembly in the hippocampal CA1 SLM area, as evidenced by an enhanced ratio of GluR1 to GluR2 during chronic stress. This alteration in AMPARs was critical to memory retention, whereby memory retention was blunted by local blockage of CP-AMPARs in the SLM of naïve and exercised mice. Regular exercise improved PRL responsiveness in the hippocampal CA1 region during chronic stress, which led to increased binding of PRL to its receptor (PRLR) and PRL-dependent enhancement in phosphorylated signal transducer and activator of transcription 5 levels. The improvement in PRL responsiveness contributed to memory retention during chronic stress, as the protective action of exercise on memory persistence during stress was abolished by PRLR knockdown in the hippocampal CA1 area. Finally, in primary hippocampal cultures, repeated treatment with corticosterone led to decreased AMPAR-mediated Ca^{2+} influx, which was restored by PRL treatment. The above findings suggest a protective role for exercise against chronic stress-evoked defects in memory consolidation via PRL-modulated incorporation of CP-AMPARs into hippocampal CA1 synapses.

Keywords Chronic stress · Regular exercise · Memory consolidation · Prolactin · Calcium-permeable AMPA receptor · Hippocampus

Introduction

Chronic stress is a well-known cause of cognitive- and/or mood-related behavioral deficits. Chronic stress-elicited failure of long-term memory formation is especially closely related to abnormal synaptic plasticity in the dorsal hippocampal CA1 area [1]. Memory retention is closely correlated with synaptic structure, including dendritic outgrowth and ramification of synapses [2]. Chronic stress or corticosteroid treatment results in pyramidal neuron remodeling, including decreased dendritic arbors, dendritic outgrowth, and spine density in the hippocampal CA1 subfield [3, 4]. Chronic stress-evoked abnormalities in dendritic remodeling may lead to disruption of synaptic efficacy through altered protein turnover and receptor trafficking in synapses.

Mechanistically, local glutamatergic activity-dependent Ca^{2+} influx plays a regulatory role in neuronal excitability, long-term potentiation (LTP), and synaptic plasticity [5, 6]. In particular,

Yea-Hyun Leem and Jin-Sun Park contributed equally to this work.

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Ca^{2+} influx through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) is critical for synaptic plasticity, including hippocampal N-methyl-D-aspartate receptor (NMDAR)-dependent LTP; AMPAR-mediated Na^{2+} entry induces postsynaptic depolarization, which causes Mg^{2+} efflux and opening of NMDARs; consequent NMDAR-mediated Ca^{2+} influx causes further membrane localization of AMPARs, which allows additional Ca^{2+} influx in the neurons that are involved in memory processing in the hippocampal CA1 synapses [6–8]. Apart from NMDAR-dependent LTP, activation of AMPARs lacking GluR2 enables permeability to Ca^{2+} and plays important roles in synaptic regulation, including LTP, long-term depression, and homeostatic synaptic plasticity [9, 10]. For example, insertion of GluR2-lacking Ca^{2+}-permeable AMPARs in the hippocampal CA1 induced NMDAR-independent LTP and learning [11–13]. It has been reported that chronic stress leads to maladaptive alterations in activity-dependent synaptic Ca^{2+} influx or conductance of AMPARs [14, 15], which may lead to affective behaviors.

Prolactin (PRL) is a versatile pituitary hormone contributing to behavioral and physiological alterations associated with reproduction and immunomodulation [16–18]. This hormone acts as a neuropeptide and has a regulatory role in maternal behavior, sexual and feeding behavior, the sleep/wake cycle, neurotransmitter metabolism, and stress response in the central nervous system [17, 19, 20]. Several reports have specifically demonstrated the anti-stress and anti-depressive actions of PRL. For example, elevated plasma PRL levels and decreased dopamine D2 receptor mRNA expression in the arcuate nucleus were observed in stress-non-reactive animals with reduced anhedonic phenotypes, but not in stress-reactive animals [21]. Moreover, PRL contributes to hippocampal neurogenesis-dependent memory tasks [22, 23]. However, the role for PRL in memory consolidation-related processes in the CA1 region is obscure.

Regular exercise resulting in neurophysiological adaptation is beneficial to multiple brain functions such as cognition and mood [24–26]. The beneficial actions of exercise on hippocampal-dependent memory tasks is responsible for hippocampal neurogenesis and brain-derived neurotrophic factor (BDNF) release, which is involved in learning and memory processes [27–29]. With regard to hippocampal LTP, evidence has increasingly suggested that regular exercise enhanced LTP under various noxious stimuli. For example, 5-week swimming rescued impaired LTP through induction of hippocampal BDNF in an Alzheimer's disease (AD) mouse model [30]. Ecstasy, an amphetamine derivative, disrupted LTP and BDNF expression in the dentate gyrus, which was prevented by 1-month treadmill running [31]. Treadmill running for 6 weeks enhanced LTP and dendritic complexity in the hippocampal CA1 region of 3-month- and 18-month-old mice [32]. Collectively, these previous studies suggest that regular exercise enhances LTP thereby protecting against brain defects. In fact, regular exercise is a powerful factor affecting the neuroendocrine system, and leads to dramatic changes in the levels of hormones and neurotransmitter metabolism, including that of dopamine, serotonin, and thyrotropin-releasing hormone (TRH) [33–36]. These neuropeptides can affect PRL secretion by acting as PRL-releasing (such as TRH and serotonin) or PRL-inhibiting factors (such as dopamine) [37]. Monoamine neurotransmitters metabolism, including that of dopamine and serotonin, is especially strongly associated with chronic stress-related mood and affective disorders, and is often targeted in therapeutic strategies. However, there is little information on the contribution of exercise-adapted PRL responsiveness to memory consolidation and related synaptic plasticity in chronically stressed dorsal hippocampal CA1 synapses.

In the present study, we aimed to provide a mechanistic understanding of the potential role of regular exercise in memory consolidation and the PRL-modulated synaptic plasticity of hippocampal CA1 neurons during chronic stress. Therefore, we explored the effects of regular exercise on memory retention, structural plasticity, and calcium-permeable (CP)-AMPARs in hippocampal CA1 synapses under chronically stressful conditions. We also investigated the impact of PRL on cellular and behavioral changes using PRL agonists or knockdown of PRL in vivo and in vitro.

Materials and Methods

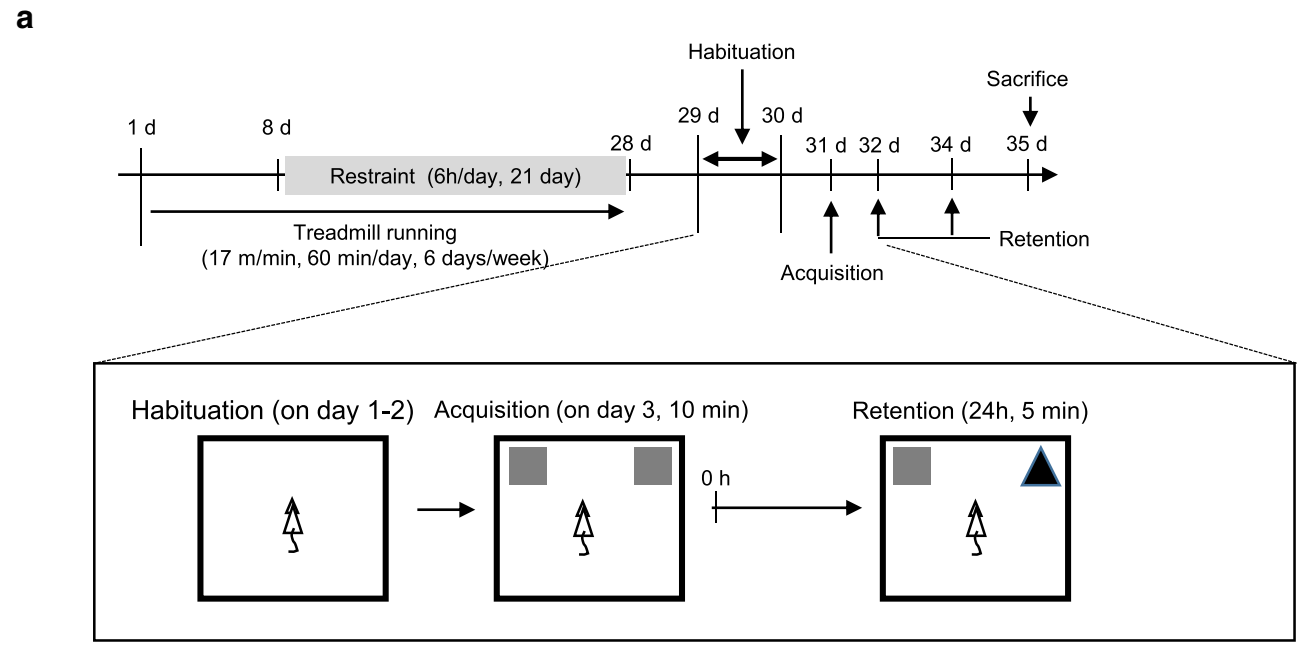
Experimental Mice

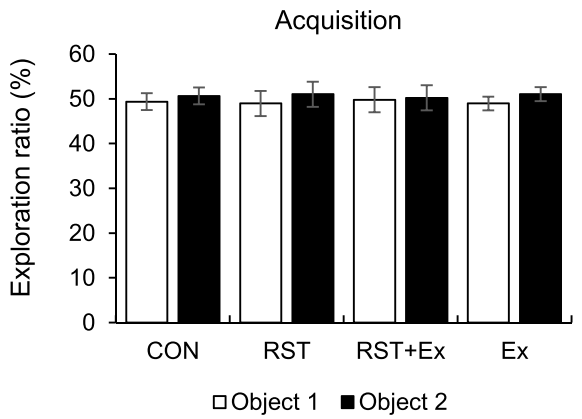
Male 7-week-old C57BL/6 mice were obtained from Daehan Biolink, Co., Ltd. (Eumsung, Chungbuk, Korea) and housed in clear plastic cages under specific pathogen-free conditions and a 12:12-h light-dark cycle (lights on at 08:00 and off at 20:00). The mice had free access to standard irradiated chow (Purina Mills, Seoul, Korea). The Animal Care and Use Committee of Ewha Womans University approved all of the experimental procedures involving animals.

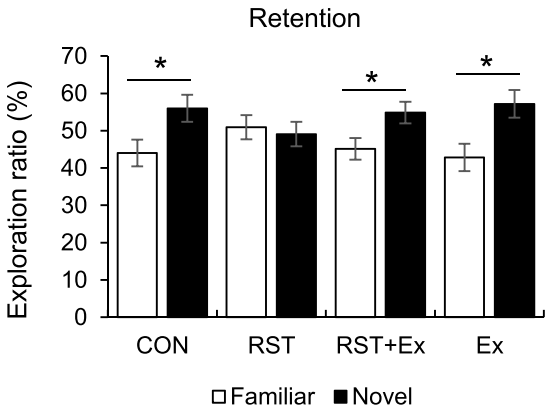
Experimental Design

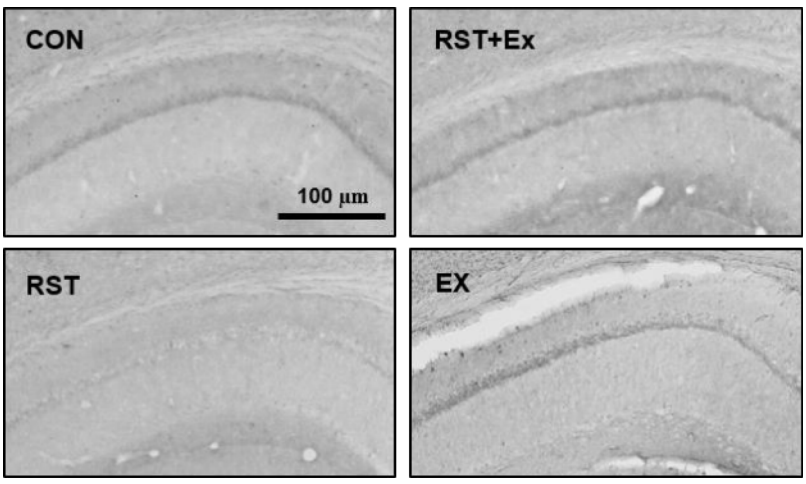
In experiment 1 (Fig. 1), mice were divided into four groups (control, CON; restraint stress, RST; exercise combined with

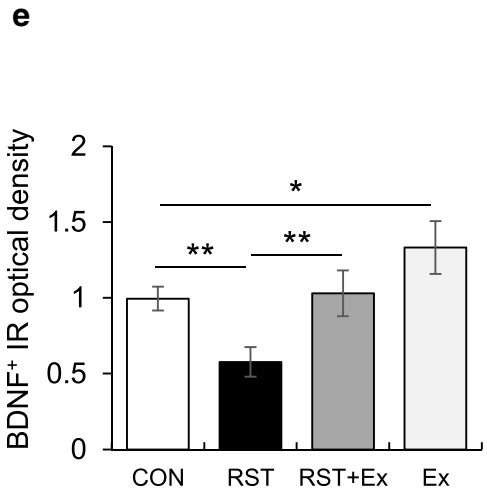
Fig. 1 Four weeks of treadmill running recovered chronic restraint stress-induced failure of memory consolidation and decreased BDNF levels in the dorsal hippocampal CA1. **a** The experimental procedure. **b**, **c** Quantitative analysis of object exploration in the acquisition and retention phases during the NOR test (each group $N = 10$). **d**, **e** Photomicrographs and quantification of the immunoreactivity for BDNF (each group $N = 8$). For statistical analysis, data were subjected to independent t test (for NOR test) and one-way analysis of variance, followed by Newman-Keuls tests (for BDNF expression). The data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$


a
Habituation
Sacrifice
1 d
8 d
29 d
30 d
28 d
31 d
32 d
34 d
35 d
Restraint (6h/day, 21 day)
Treadmill running
(17 m/min, 60 min/day, 6 days/week)
Retention
Acquisition
Habituation (on day 1-2)
Acquisition (on day 3, 10 min)
Retention (24h, 5 min)
0 h


b
Acquisition
Exploration ratio (%)
60
50
40
30
20
10
0
CON
RST
RST+Ex
Ex
Object 1
Object 2


c
Retention
Exploration ratio (%)
70
60
50
40
30
20
10
0
*
*
*
CON
RST
RST+Ex
Ex
Familiar
Novel


d
CON
RST+Ex
100 μm
RST
EX


e
BDNF+ IR optical density
2
1.5
1
0.5
0
*
**
**
CON
RST
RST+Ex
Ex

restraint stress, RST + Ex; and exercise, Ex). The exercise protocol and chronic restraint stress were performed as described previously [31]. To induce chronic stress by restraint, 8-week-old mice were individually placed in well-ventilated 50-mL conical tubes, which prevented forward or backward movement; whereas, control mice were left undisturbed in their home cages. Restraint was conducted at 6 h per day from 1000 to 1600 for 21 days. All mice were acclimated to treadmill running (Myung Jin Instruments Co., Seoul, Korea) by pre-exercise (Pre-Ex) at 12 m per minute for 20 min per day (0% grade) from set-time of 1800 for 3 days prior to the principal exercise procedure. Subsequently, treadmill exercise was performed for 60 min (12 m/min, 10 min; 16 m/min, 40 min; 12 m/min, 10 min; 0% grade) for 4 weeks. Non-exercised mice were placed on the treadmill that was turned off for 60 min once a day. The treadmill speed was increased gradually so as not to increase stress to the experimental animals. Mice underwent behavioral tests (each group $N = 10$), followed by sacrifice for immunohistochemical analysis of BDNF expression 1 day after the novel object recognition (NOR) test (each group $N = 8$).

In experiment 2 (Figs. 2 and 3), we aimed to understand the architectural characteristics of hippocampal CA1 neurons during chronic stress with/without regular exercise. We thus analyzed neuronal structure using Golgi-Cox staining (each group $N = 8$) and synaptosomal synaptic proteins in the stratum lacunosum-moleculare (SLM) CA1 area of the dorsal hippocampus in independent experiments (each group $N = 8$).

In experiment 3 (Fig. 4), to investigate the indispensable role of CP-AMPARs in memory retention, IEM-1460 (Tocris Bioscience, Bristol, UK), which is a selective and potent CP-AMPAR blocker, was microinjected into the SLM CA1 area of the dorsal hippocampus 10 min after the acquisition phase of the NOR. This was followed by evaluation of the exploration ratio in the NOR test (each group $N = 7$–8).

In experiment 4 (Figs. 5 and 6), we aimed to elucidate the potential role of the PRL response to regular exercise in the hippocampal CA1 region in chronically stressed mice. We first measured the serum PRL level and the binding affinity of PRL to its receptor in mice subjected to regular exercise or chronic stress at the indicated time points. PRL responsiveness to stress and exercise was then measured by assessing the phosphorylation of signal transducer and activator of transcription 5 (STAT5) after an intraperitoneal injection of ovine prolactin (25 μg/mouse; Sigma-Aldrich, MO, USA). To explore the role of the PRL response to exercise in memory consolidation, we performed a microinjection of siRNA against the PRL receptor (siRNA-PRLR) into the hippocampal CA1 4 h after the second habituation phase of the NOR test during chronic stress (each group $N = 8$).

In experiment 5 (Fig. 7), we verified the regulatory role of prolactin in AMPAR assembly and Ca^{2+} influx in hippocampal neurons following repeated exposure to a stress hormone. Primary hippocampal cells were treated with prolactin (30 ng/mL, Sigma-Aldrich) for 1 h after 6-h treatment with 0.3 μM corticosterone (CORT) (Sigma-Aldrich), a 2 h of interval between the treatments. After 120 h, we assessed neuronal synaptic protein levels and Ca^{2+} influx.

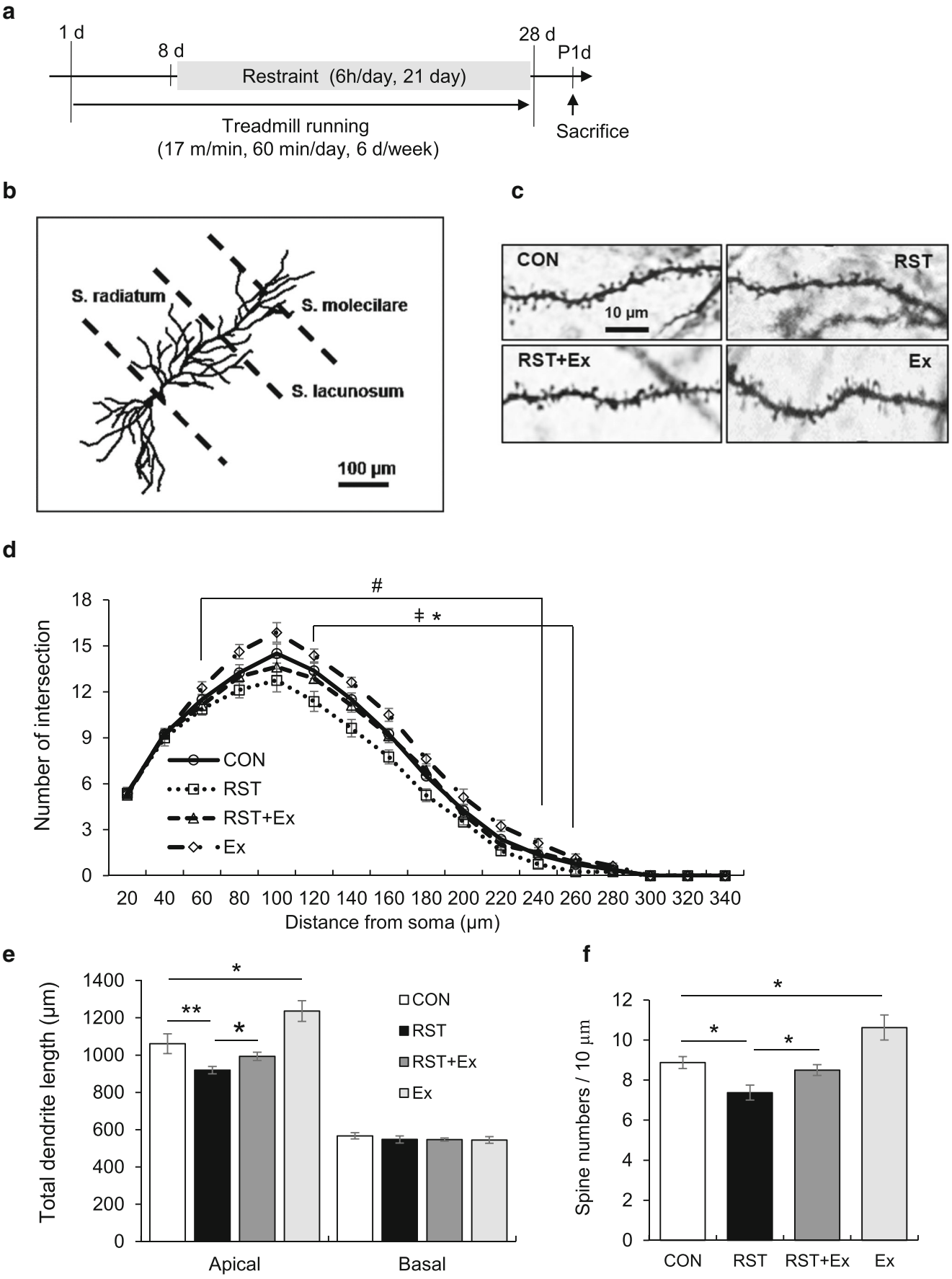
NOR Test

The NOR test was performed as previously described, with modification [38]. Mice were handled and habituated to the empty open-field arena (35 × 35 × 19, the arena was black inside) on days 1 and 2 (twice/day, 15 min/trial). On day 3 (acquisition session), the mice were placed in the arena with two identical objects (Falcon tissue culture flasks filled with sand) and were allowed to explore the objects for 10 min (5 min/trial, twice/day, 15-min inter-trial interval). Following a 1-day delay, one of the familiar objects used before was replaced with a novel object (interlocking tower of Lego pieces with different shapes and colors) for 5 min. The exploration ratio (%) was expressed as the percentage of time spent with each object divided by the total exploration time.

Stereotaxic Injections into the Hippocampal CA1 Area

Mice were anesthetized with 250 mg/kg body weight of tribromoethanol. IEM-1460 (30 μM, 1 μL) or vehicle was bilaterally microinjected at a speed of 0.2 μL/min using a 31 G needle (anterior-posterior [AP], − 1.65 mm; medial-lateral [ML], ± 1.25 mm; and dorsal-ventral [DV], − 1.65 mm). One volume of diluted siRNA-negative control (sense: 5′-GGA ACU GGA AAG AGU UCU UUU-3′, antisense: 5′-AAG AAC UCU UUC CAG UUC CUU-3′, Dhamarcon Inc., CO, USA) or siRNA-PRLR (sense: 5’-CAC CUA UGA AUG UCC AGA CUU-3′, antisense: GUC UGG ACA UUC AUA GGU GUU-5′, Accession No. NM_011169.5, Dhamarcon Inc.) was mixed with 2.5 volumes of lipofectamine (Lipofectamine RNAiMAX Transfection Reagent; Invitrogen, Waltham, MA, USA) in 5% sucrose. A total 1 μL of the mix containing the siRNA-negative control (4.15 ng) or siRNA-PRLR (4.10 ng) was bilaterally

Fig. 2 Treadmill running prevented chronic stress-induced dendritic retraction in the apical distal segments of neurons in the dorsal hippocampal CA1. **a** The experimental procedure. **b**–**f** Quantitative analysis of the dendritic morphologies of neurons in the CA1. Photomicrographs showing reconstructions of **b** Golgi-stained neurons and **c** a dendritic spine. **d** Quantitative analyses of the dendritic intersections (*CON vs. RST: 120–260 μm, $p < 0.05$; RST vs. RST + Ex: 120–260 μm, $p < 0.05$; #CON vs. Ex: 60–240 μm, $p < 0.05$), **e** total dendritic length, and **f** dendritic spine number. Statistical analysis was performed by two-way repeated-measures analysis of variance (for dendritic intersection, each group $N = 8$) and one-way analysis of variance, followed by Newman-Keuls tests (for dendritic length and dendritic spine number, each group $N = 8$). The data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$


a
1 d
8 d
28 d
P1d
Restraint (6h/day, 21 day)
Treadmill running
(17 m/min, 60 min/day, 6 d/week)
Sacrifice
b
S. radiatum
S. molecilare
S. lacunosum
100 μm
c
CON
RST
10 μm
RST+Ex
Ex
d
Number of intersection
Distance from soma (μm)
CON
RST
RST+Ex
Ex
#
‡ *
e
Total dendrite length (μm)
Apical
Basal
f
Spine numbers / 10 μm
CON
RST
RST+Ex
Ex

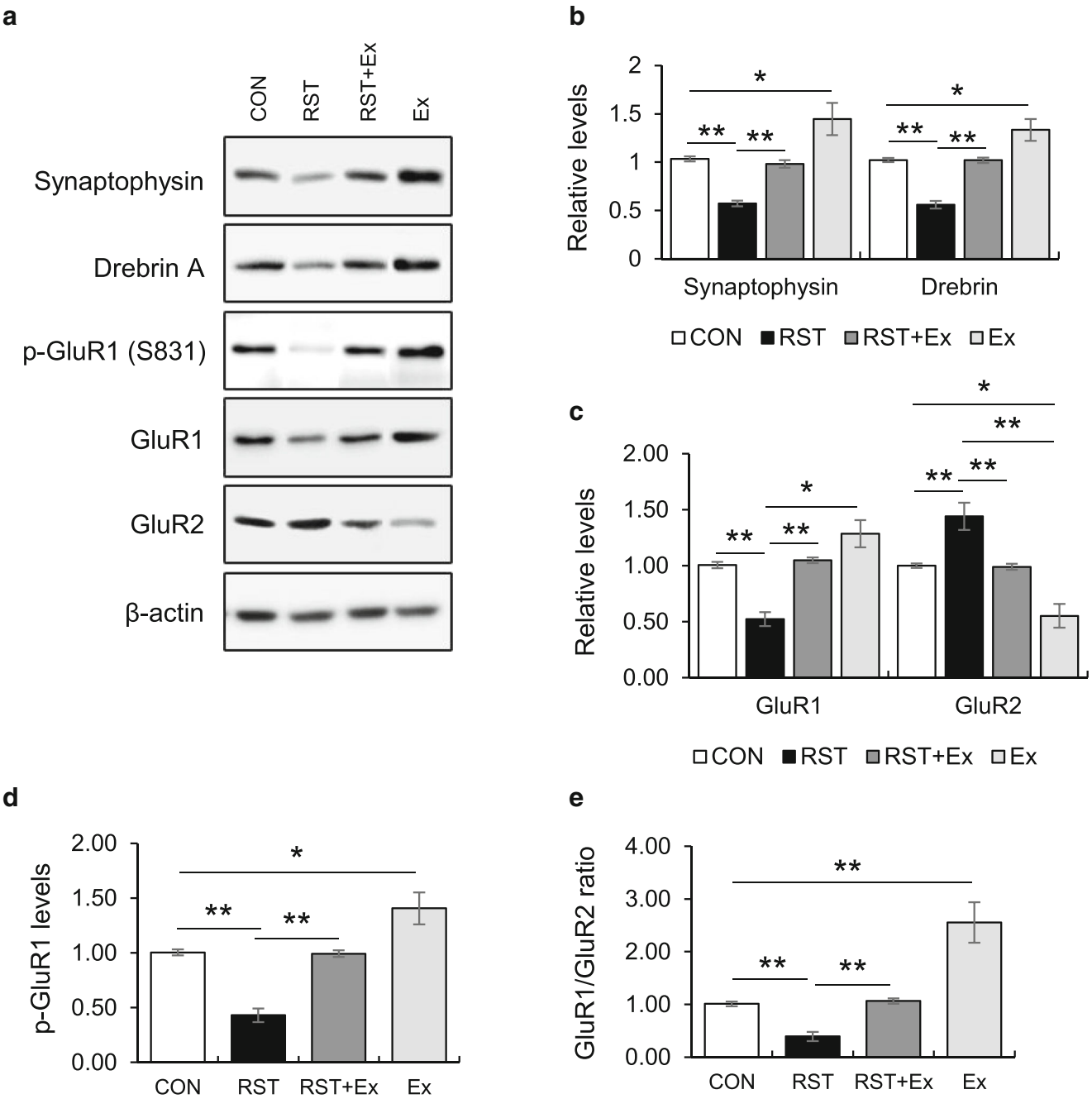


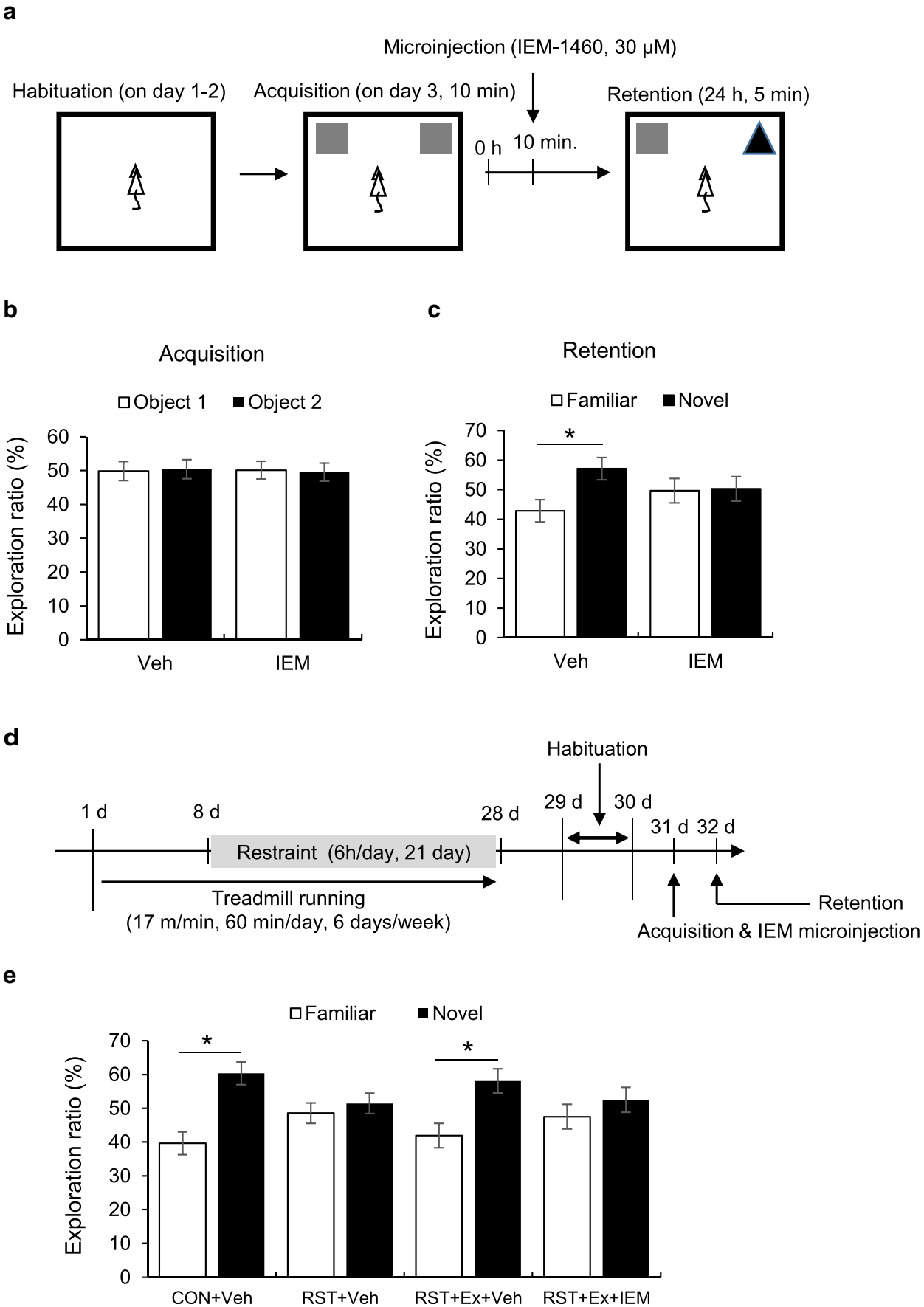
Fig. 3 Treadmill running reversed chronic stress-induced changes in synaptic proteins and AMPAR behavior in the dorsal hippocampal CA1. **a** Western blot data for SYP, drebrin A, pGluR1, GluR1, and GluR2. **b**–**e** Quantitative analysis of each protein. For statistical analysis, data were subjected to one-way analysis of variance, followed by Newman-Keuls tests (each group $N = 8$). The data are presented as the mean ± SEM. $*p < 0.05$, $**p < 0.01$

microinjected (AP, − 1.94 mm; ML, ± 1.8 mm; DV, − 1.45 mm) at a speed of 0.2 μL/min using a 31 G needle.

Golgi Staining, Neuronal Reconstruction, and Morphometric Analyses

Golgi-Cox staining of brain tissue was performed using a NovaUltra™ Golgi-Cox Stain Kit (IHC World, Woodstock,

Fig. 4 Memory persistence was blunted by the local blockage of CP-AMPARs using IEM-1460 in the SLM of the dorsal hippocampal CA1 in naïve and exercised mice. **a** The experimental procedure. **b** Quantitative analysis of the time spent exploring each object in the acquisition session of the NOR test in naïve mice. **c** Quantitative analysis of the time spent exploring each object in the retention session of the NOR test in naïve mice. **d** The experimental procedure. **e** Quantitative analysis of the time spent exploring each object in the retention session. For statistical analysis, data were subjected to independent *t* test (each group $N = 7$–8). The data are presented as the mean ± SEM. $*p < 0.05$


a
Microinjection (IEM-1460, 30 μM)
Habituation (on day 1-2)
Acquisition (on day 3, 10 min)
Retention (24 h, 5 min)
0 h
10 min.
b
Acquisition
Object 1
Object 2
Exploration ratio (%)
Veh
IEM
c
Retention
Familiar
Novel
Exploration ratio (%)
Veh
IEM
d
Habituation
1 d
8 d
28 d
29 d
30 d
31 d
32 d
Restraint (6h/day, 21 day)
Treadmill running
(17 m/min, 60 min/day, 6 days/week)
Retention
Acquisition & IEM microinjection
e
Familiar
Novel
Exploration ratio (%)
CON+Veh
RST+Veh
RST+Ex+Veh
RST+Ex+IEM

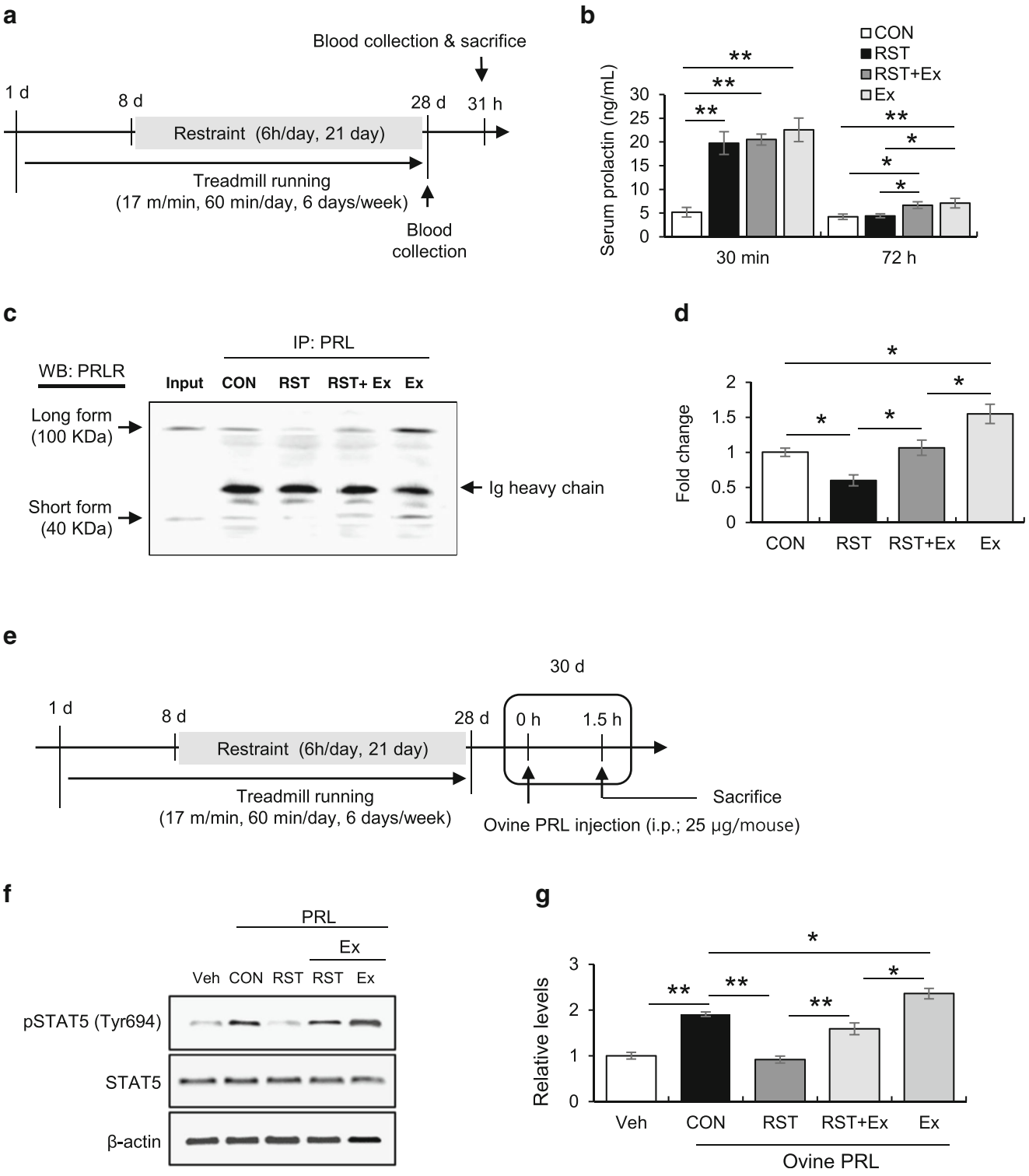


Fig. 5 Regular exercise increased basal serum PRL levels and prevented the chronic stress-induced defects of PRL responsiveness in the dorsal hippocampal CA1. **a** The experimental procedure. **b** Quantitative analysis of serum PRL levels (each group $N = 8$). **c** Western blot data showing co-immunoprecipitation of PRL and PRLR (each group $N = 8$). **d** Quantitative analysis of the binding affinity of PRL to its cognate receptor. **e** The experimental procedure. **f** Western blot data for p-STAT5 levels (each group $N = 8$). **g** Quantitative analysis of the western blot. For statistical analysis, data were subjected to one-way analysis of variance, followed by Newman-Keuls tests. The data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$

MD, USA) according to the procedure suggested by the manufacturer. The protocols for Golgi staining, reconstruction, and morphometric analysis have been described in a previous publication by our group [39]. We examined pyramidal neurons in the dorsal hippocampal CA1 region. For each brain, approximately four to eight neurons that appeared to be completely filled were selected based on the degree of preservation and visibility of the soma and dendritic trees. For neuronal reconstruction, each neuron was traced using the Sholl analysis and NeuronJ plugin in ImageJ (National Institutes of Health, Image Engineering, Bethesda, MD, USA).

Synaptosome Extraction and Western Blot Analyses

Immediately after decapitation, the brain was removed from the skull, and 500-μm-thick transverse hippocampal slices were prepared using the Mouse Brain Slicer (ZIVIC Instruments, PA, USA). SLM tissue was dissected manually using a fine forceps under ×6 magnifying glass from the CA1 area in the hippocampal slices (between − 1.43 and − 2.46 mm from the bregma). Tissues dissected from the mice were pooled (2–3 mice) and homogenized. The procedures for synaptosome fractionation and western blot analysis have been described in a previous publication by our group [36]. The optical density of each band was measured using ImageJ. Anti-STAT5 (1:1000) and anti-phosphorylated STAT5 (pSTAT5) (Tyr694; 1:1000) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-drebrin (1:1000), anti-GluR1 (1:1000), anti-phosphorylated GluR1 (pGluR1) (Ser831, 1:500), anti-PRL (1:500), anti-PRLR (1:500), and anti-β-actin (1:5000) were purchased from Abcam (Cambridge, UK). Anti-GluR2 (1:1000) was obtained from EMD Millipore Corporation (Billerica, MA, USA), and anti-synaptophysin (SYP) (1:1000) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Co-immunoprecipitation

For the prolactin immunoprecipitation assay, 1 mg of protein lysates from the hippocampal CA1 was incubated with anti-prolactin antibody (5 μL/mL; Abcam, CA, USA) overnight at 4 °C, and immune complexes were captured by incubating in 50% protein G-Sepharose resin slurry for 4 h at 4 °C. Protein G complexes were precipitated by centrifugation, and pellets were washed three times in binding buffer and boiled in 5 μL of 5× reducing SDS sample buffer for 5 min at 95 °C. Finally, immunoprecipitated samples were immunoblotted using an anti-prolactin receptor antibody (1: 500) with 8% SDS-PAGE.

Primary Hippocampal Culture

Primary hippocampal cell cultures were prepared from E17 ICR mice. The procedure for hippocampal cell culture has been described in a previous publication by our group [31]. Dissociated single cells were plated in a solution containing RF media (DMEM with 10% FBS, 1× penicillin/streptomycin, 1.4 mM L-glutamine, and 0.6% glucose) in 12-well or 96-well plates for 24 h. On day in vitro (DIV) 1, cells were incubated in Neurobasal Medium with 1× B27, 1× penicillin/streptomycin, and 1× L-glutaMax. The medium was changed every 2 days. Cultures from DIV 7 were used experimentally. Each in vitro experiment was performed in four independent cultures.

Ca^{2+} Influx Assay

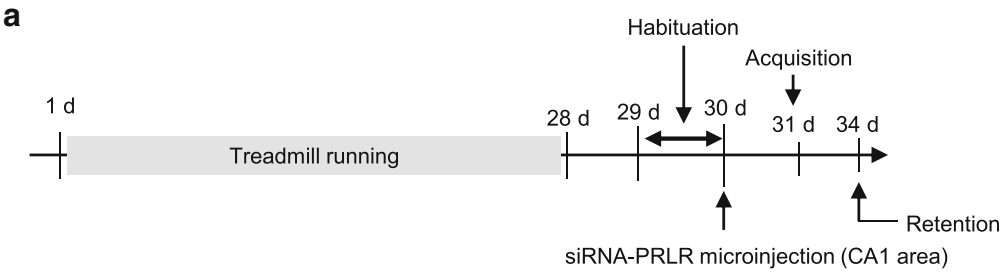
Ca^{2+} influx in response to s-AMPA treatment was analyzed using FLUOFORTE® Calcium Assay Kit according to the procedure suggested by the manufacturer (Enzo Life Sciences, Inc.). Briefly, on DIV 12 of CORT treatment (0.3 μM), the media were removed and replaced with 100 μL FLUOFORTE® Dye-loading solution in 96-well plates (2×10^4 cells/well). After 1 h incubation at room temperature, 20 μL of s-AMPA (a selective AMPA agonist, 100 μM; Tocris Bioscience) dissolved in ddH_2O was added and the fluorescence (excitation = 490 nm/emission = 525 nm) was measured for 60 s at 4-min intervals using the FLx800™ Fluorescence Microplate Reader (BioTek instrument, VT, USA). The Ca^{2+} influx efficiency index was calculated using the following formula: Ca^{2+} influx efficiency index = (drug of total relative fluorescence unit/vehicle of total relative fluorescence unit) × 100.

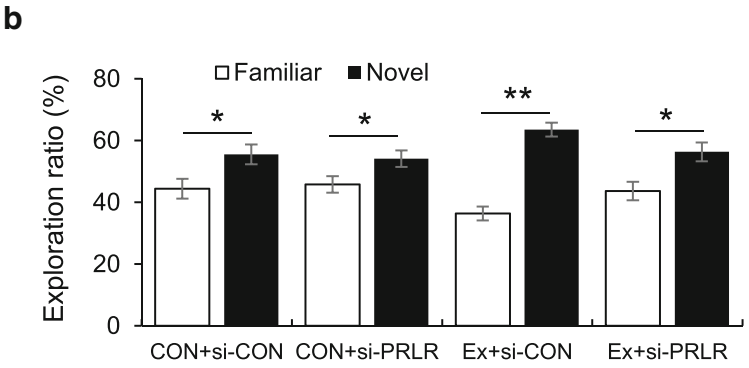
Immunohistochemical Analyses

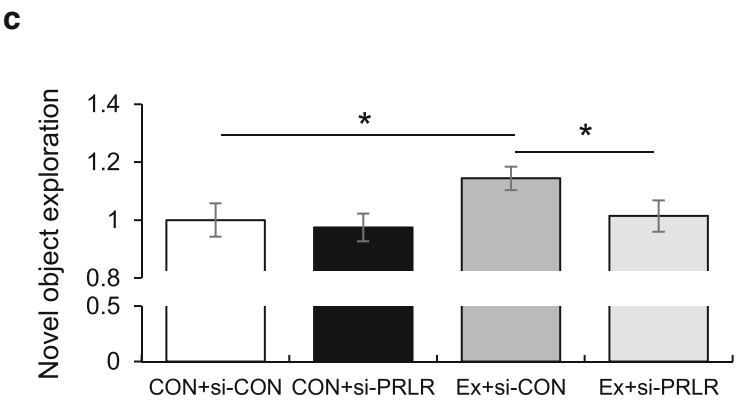
Every fifth section from the region between bregma − 1.82 and − 2.18 mm was analyzed. The immunohistochemistry protocol has been described in a previous publication from our group [31]. Anti-BDNF primary antibody was obtained from Abcam (Cambridge, MA, USA; rabbit polyclonal, 1: 2000). Immunoreactivity was assessed in digital images using ImageJ.

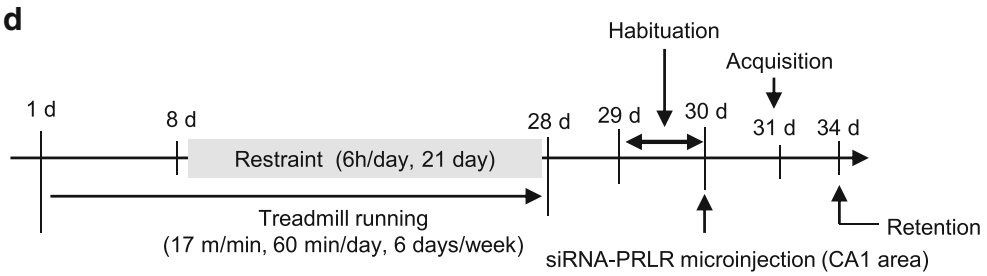
Statistical Analysis

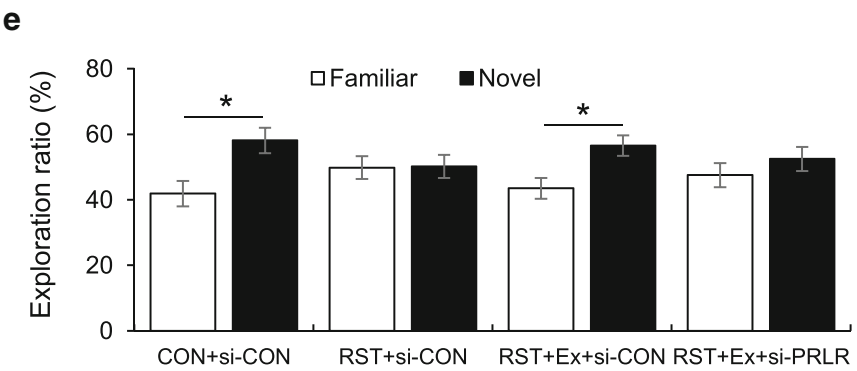
Statistical analysis was performed using SPSS (SPSS for Windows, version 24; IBM Corporation, Armonk, NY, USA). One-way analysis of variance was conducted for the data of BDNF IR, dendritic length, spine number, serum prolactin level, and western blot, two-way repeated-measures analysis of variance for those of dendritic intersection and Ca^{2+}-influx, and independent t tests for the results of NOR test to assess significance. Post hoc comparisons were made using Newman-Keuls tests. All values are reported as mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.


a
Habituation
Acquisition
1 d
28 d
29 d
30 d
31 d
34 d
Treadmill running
Retention
siRNA-PRLR microinjection (CA1 area)


b
Familiar
Novel
Exploration ratio (%)
80
60
40
20
0
*
*
**
*
CON+si-CON
CON+si-PRLR
Ex+si-CON
Ex+si-PRLR


c
Novel object exploration
1.4
1.2
1
0.8
0.5
0
*
*
CON+si-CON
CON+si-PRLR
Ex+si-CON
Ex+si-PRLR


d
Habituation
Acquisition
1 d
8 d
28 d
29 d
30 d
31 d
34 d
Restraint (6h/day, 21 day)
Treadmill running
(17 m/min, 60 min/day, 6 days/week)
Retention
siRNA-PRLR microinjection (CA1 area)


e
Familiar
Novel
Exploration ratio (%)
80
60
40
20
0
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*
CON+si-CON
RST+si-CON
RST+Ex+si-CON
RST+Ex+si-PRLR

◀**Fig. 6** Regular exercise prevented the chronic stress-induced decline in memory retention, which was blocked following PRLR knockdown in the CA1 of chronically stressed mice. **a** The experimental procedure. **b** Quantitative analysis of the time spent exploring each object in the retention session. **c** Quantitative analysis of the novel object exploration between groups in the retention session. **d** The experimental procedure. **e** Quantitative analysis of the time spent exploring each object in the retention session. For statistical analysis, data were subjected to independent *t* test (each group $N = 8$). The data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$

Results

Regular Treadmill Running Prevented Chronic Stress-Induced Memory Consolidation Impairments and Led to Recovery of BDNF Levels in the Dorsal Hippocampal CA1

We first measured memory consolidation and BDNF expression in the hippocampal CA1 after chronic stress and regular exercise using the protocol described in Fig. 1a. In the NOR test, the exploration of the two objects did not differ significantly during the acquisition phase in any of the mice (CON $t_{18} = -0.46$, $p > 0.05$; RST $t_{18} = -0.48$, $p > 0.05$; RST + Ex $t_{18} = -0.10$, $p > 0.05$; Ex $t_{18} = -0.90$, $p > 0.05$) (Fig. 1b). In the 24-h retention test, the time spent exploring the novel object was greater than that spent exploring the familiar object in the CON, RST + Ex, and Ex groups, while the exploration of the two objects did not differ significantly in the RST mice (CON $t_{18} = -2.22$, $p < 0.05$; RST $t_{18} = 0.37$, $p > 0.05$; RST + Ex $t_{18} = -2.26$, $p < 0.05$; Ex $t_{18} = -2.60$, $p < 0.05$) (Fig. 1c). Immunohistochemistry revealed that the level of BDNF in the hippocampal CA1 area was decreased following chronic stress, which was prevented by treadmill running ($F_{3,\ 16} = 16.74$, $p < 0.01$) (Fig. 1d, e). Furthermore, Ex mice had higher levels of BDNF than the CON mice.

Regular Treadmill Running Reversed Chronic Stress-Induced Structural Remodeling in Hippocampal CA1 Area

To understand the structural properties of hippocampal CA1 neurons following chronic stress and regular exercise, we examined alterations in the structural properties of these neurons. Representative images of the reconstructed neurons (Fig. 2b) and dendritic spines (Fig. 2c) are shown following the experimental procedure described in Fig. 2a. The number of dendritic intersections in the apical distal segments (120–260 μm from the somata) of the CA1 neurons was decreased following chronic stress. Treadmill running (RST + Ex) recovered the numbers of intersections in the same segments, and exercise alone (Ex) enhanced the intersection number when compared with CON mice in a broader distance range (80–260 μm) from the somata (group × distance, $F_{48,\ 448} = 2.39$, $p < 0.01$; group, $F_{3,\ 28} = 32.82$, $p < 0.01$; distance, $F_{16,\ 448} = 1038.87$, $p < 0.01$) (Fig. 2d). The apical layer dendrites of hippocampal CA1 neurons in the RST mice were significantly shorter than those in the CON mice. Exercise (RST + Ex) normalized this change in dendrite length while under stress (apical: $F_{3,\ 28} = 10.86$, $p < 0.01$; basal: $F_{3,\ 28} = 0.34$, $p > 0.05$) (Fig. 2e). It also led to recovery of the chronic stress-induced reduction in spine number ($F_{3,\ 28} = 10.53$, $p < 0.05$) (Fig. 2f). Moreover, the dendritic length and spine numbers in Ex group were longer and higher, respectively, than in CON mice.

Regular Treadmill Running Reversed Chronic Stress-Induced Changes in the Synaptosomal Protein Contents of the Dorsal Hippocampal CA1 SLM Area

Given that the structural change in the apical distal segment of the CA1 neuron was observed following chronic stress and regular exercise, we explored alterations in synaptic proteins in the hippocampal CA1 SLM area to understand the functional properties of the structural changes. Exercise led to amelioration of the chronic stress-evoked decrease in SYP and drebrin A levels in synaptosomal fractions from SLM tissue (SYP: $F_{3,\ 16} = 16.32$, $p < 0.01$; drebrin A: $F_{3,\ 16} = 26.25$, $p < 0.01$) (Fig. 3a, b). GluR1 and pGluR1 (Ser831) protein levels were significantly lower in RST mice than in CON mice, and exercise led to recovery of this change in GluR1 and pGluR1 levels (pGluR1: $F_{3,\ 16} = 23.88$, $p < 0.01$; GluR1: $F_{3,\ 16} = 20.46$, $p < 0.01$) (Fig. 3a, c, d). The opposite result was observed for GluR2 ($F_{3,\ 16} = 19.34$, $p < 0.01$) (Fig. 3a, c). Exercise restored the chronic stress-elicited decline in GluR1/GluR2 ratio ($F_{3,\ 16} = 21.27$, $p < 0.01$) (Fig. 3e). Exercise alone (Ex) significantly enhanced SYP, drebrin A, and pGluR1 protein levels when compared with CON mice. Furthermore, Ex mice showed reduced GluR2 protein levels and augmented GluR1/GluR2 ratios.

Blocking CP-AMPARs in the SLM Led to Deficits in Memory Persistence

To elucidate the role of CP-AMPARs in the hippocampal CA1 SLM area in memory consolidation in naïve mice, we investigated memory retention after treatment with IEM-1460, a specific inhibitor of CP-AMPARs (Fig. 4a). In the acquisition session, there was no difference in exploration of the two objects between groups (vehicle: $t_{12} = -0.06$, $p > 0.05$; IEM: $t_{12} = 0.91$, $p > 0.05$) (Fig. 4b). In the 24-h retention test, the exploration of the novel object was greater than that of the familiar object in mice microinfused with vehicle (Veh), but there was no significant difference in the exploration of the two objects in mice treated with IEM-1460 (vehicle: $t_{12} = -$

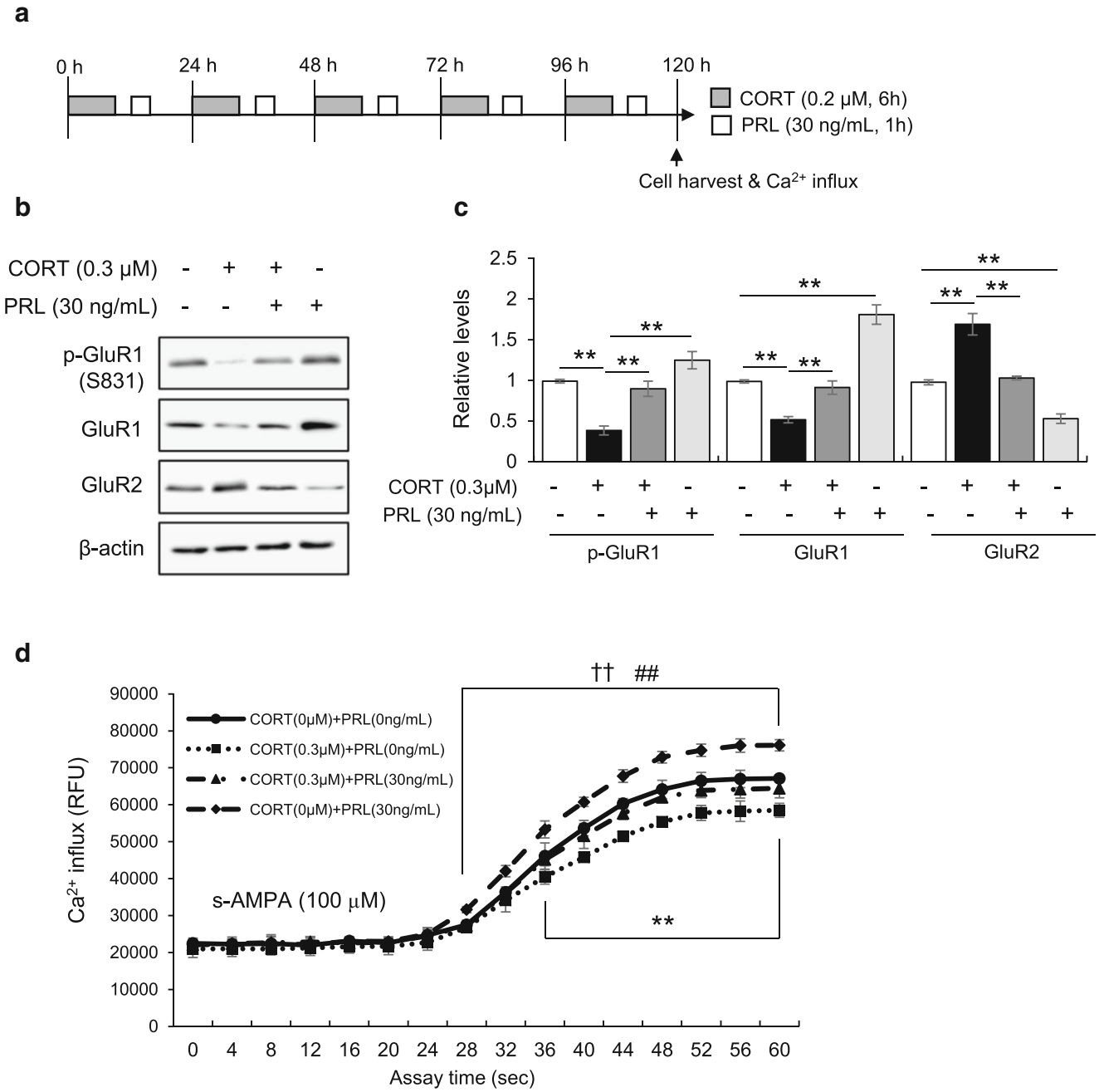

Fig. 7 Ovine PRL peptide reversed the repeated CORT treatment-induced alteration in AMPAR phenotypes and AMPAR-mediated Ca^{2+} influx of primary hippocampal neurons. **a** The experimental procedure. **b**, **c** Western blot data (**b**) and quantitative analysis (**c**) of pGluR1, GluR1, and GluR2 levels. $*p < 0.05$, $**p < 0.01$. **d** Quantitative analysis of Ca^{2+} influx. **, ††, and ## denote the following differences: CORT (0) + PRL (0) vs. CORT (0.3) + PRL (30), CORT (0) + PRL (30) vs. CORT (0) + PRL (30), and CORT (0.3) + PRL (30) vs. CORT (0.3) + PRL (0), respectively, $p < 0.01$. Statistical analysis was performed by two-way repeated-measures analysis of variance (for Ca^{2+} influx, independent experiment $N = 4$) and one-way analysis of variance, followed by Newman-Keuls tests (for proteins expression, independent experiment $N = 4$). Data are presented as the mean ± SEM

2.49, $p < 0.05$; IEM: $t_{12} = -0.01$, $p > 0.05$) (Fig. 4c). We next studied the beneficial effects of exercise on CP-AMPARs during chronic stress (Fig. 4d). Exploration of the novel object was comparable to that of the familiar object in RST + Veh mice (RST + Veh: $t_{14} = -0.06$, $p > 0.05$) (Fig. 4e). Novel object exploration was significantly higher than that of the familiar object in the CON + Veh and RST + Ex + Veh groups, while the exploration of the two objects did not differ significantly in RST + Ex mice treated with IEM-1460 (CON+Veh: $t_{12} = -2.96$, $p < 0.05$; RST + Ex + Veh: $t_{12} = -2.27$, $p < 0.05$; RST + Ex + IEM: $t_{14} = -0.89$, $p > 0.05$) (Fig. 4e). The above data suggest the importance of CP-AMPARs in the

hippocampal CA1 SLM area in memory consolidation in response to chronic stress and regular exercise.

Regular Exercise-Elicited Restoration of Memory Consolidation Depended on the Responsiveness of Hippocampal CA1 Neurons to PRL in Chronically Stressed Mice

Serum PRL levels were profoundly increased 30 min after the last exposure to stress and/or exercise; 72 h later, serum PRL levels in the RST + Ex and Ex groups were significantly higher as compared to those in the CON and RST groups (post-exposure 30 min: $F_{3,\ 28} = 15.71$, $p < 0.01$; post-exposure 72 h: $F_{3,\ 28} = 3.84$, $p < 0.05$) (Fig. 5a, b), but not those in the acute exercise group (Fig. S1). The binding affinity of PRL to its receptor in the dorsal hippocampal CA1 area was significantly reduced 72 h after the last exposure to stress, while that in Ex mice (RST + Ex) was significantly elevated when compared to that in RST mice ($F_{3,\ 12} = 13.86$, $p < 0.01$) (Fig. 5c, d). The PRL-binding affinity of Ex mice was higher than CON mice. To assess the effects of regular exercise on PRL responsiveness in the hippocampal CA1 area during chronic stress, we determined the phosphorylation levels of STAT5, which is a specific marker of the PRL response (Fig. 5e). The ratio of pSTAT5 (Tyr 694) immunoreactivity to that of STAT5 in the hippocampal CA1 area in CON, RST + Ex, and Ex groups was significantly elevated 90 min after the intraperitoneal injection of PRL. The same elevation was not observed in the RST group ($F_{4,\ 15} = 34.07$, $p < 0.01$) (Fig. 5f, g).

Regular Exercise-Elicited Restoration of Memory Consolidation Was Abolished by the Local Knockdown of PRLR in CA1 Neurons in Naïve and Chronically Stressed Mice

Given that exercise enhanced PRL responsiveness of hippocampal CA1 area, we explored the role of PRL responsiveness in memory retention by reducing PRL responsiveness via microinjection of siRNA-PRLR into the CA1 in naïve mice. Exploration of novel object was higher than that of familiar object in mice regardless of the treatment with siRNA-PRLR (CON + si-CON: $t_{14} = -2.61$, $p < 0.05$; CON + si-PRLR: $t_{14} = -2.37$, $p < 0.05$; Ex + si-CON: $t_{14} = -8.94$, $p < 0.05$; Ex + si-PRLR: $t_{14} = -3.18$, $p < 0.05$) (Fig. 6a, b); however, regular exercise-induced enhancement of memory retention was blunted by a local decrease in PRL responsiveness in naïve mice; the time spent exploring the novel object in the Ex + si-PRLR group of mice was significantly lower than that of the Ex + si-CON group of mice ($F_{3,\ 24} = 3.52$, $p < 0.05$) (Fig. 6c). Next, regular exercise-elicited restoration of long-term memory in chronically stressed mice was suppressed by the local knockdown of PRLR in the CA1 (CON + si-CON: $t_{14} = -2.77$, $p < 0.05$; RST + si-CON: $t_{14} = -0.06$, $p > 0.05$; RST + Ex + si-CON: $t_{14} = -2.73$, $p < 0.05$; RST + Ex + si-PRLR: $t_{14} = -0.89$, $p > 0.05$) (Fig. 6d, e). The decrease in the levels of PRLR in the hippocampal CA1 area did not affect memory consolidation under normal conditions (Fig. S2), implying that intact memory persistence may be regulated by other mechanisms, including hippocampal neurogenesis or Schaffer collateral (SC) pathway-mediated processes. These results suggest an ameliorative effect of regular exercise on chronic stress-elicited impairment of memory persistence through restoration of PRL responsiveness in the hippocampal CA1 area.

PRL Treatment Altered AMPAR Assembly and Enhanced Ca^{2+} Influx in Response to CORT in Primary Hippocampal Cultures

To elucidate the modulatory actions of PRL on the calcium permeability of AMPARs in response to repeated exposure to CORT, AMPAR-mediated Ca^{2+} influx and the synaptosomal levels of GluR1 and GluR2 were measured in response to CORT and PRL in a hippocampal culture system (Fig. 7a). PRL ameliorated the reductions in the levels of GluR1 and pGluR1 in cells repeatedly treated with CORT (pGluR1: $F_{3,\ 12} = 22.25$, $p < 0.01$; GluR1: $F_{3,\ 12} = 51.55$, $p < 0.01$) (Fig. 7b, c). Changes in the levels of GluR2 were in contrast to those in the levels of GluR1 ($F_{3,\ 12} = 41.32$, $p < 0.01$) (Fig. 7b, c). Finally, we explored the relationship between PRL and AMPAR-mediated Ca^{2+} influx in vitro. (s)-AMPA treatment enhanced Ca^{2+} influx in a dose-dependent manner. This increase was blocked by pretreatment with NBQX, which is a specific AMPAR antagonist (Fig. S3). Further, the (s)-AMPA-induced increase in Ca^{2+} influx was not suppressed by pretreatment with MK801 or UBP301, which are antagonists of NMDARs and kainate receptors, respectively (Fig. S3). These results indicate that (s)-AMPA acts as a specific AMPAR agonist. Repeated CORT treatment significantly reduced Ca^{2+} influx in response to (s)-AMPA in dose-dependent manner (Fig. S4), although the same was not observed after a single bout of CORT treatment. PRL restored the (s)-AMPA-mediated decrease in Ca^{2+} influx in neurons repeatedly treated with CORT (group × time, $F_{45,\ 180} = 15.56$, $p < 0.01$; group, $F_{3,\ 12} = 88.20$, $p < 0.01$; time, $F_{15,\ 180} = 2099.30$, $p < 0.01$) (Fig. 7d).

Discussion

Here we reported that regular exercise ameliorated chronic stress-induced deficits in hippocampal-dependent memory consolidation, architectural changes, and disruption of CP-AMPAR incorporation into temporoammonic (TA)-CA1 synapses by improving PRL responsiveness. Of the different

hippocampal substructures, the CA1 area is considered a key structure for the formation of temporal-involved memory consolidation. Exercise regimen ameliorated the chronic stress-induced decrease in BDNF protein levels in the hippocampal CA1 area, consistent with previous findings [28, 40, 41]. Considering the observation, our data indicate that regular exercise has protective effects on synaptic plasticity-related abnormalities in the hippocampal CA1 area induced by chronic stress. Further, regular exercise alone enlarged neuronal dendrites and enhanced BDNF levels in the CA1. Thus, the chronic restraint stress paradigm, exercise regimen, and behavioral tests used here are considered to be valid for exploration of the effects of regular exercise on stress-related memory persistence and the underlying mechanisms in hippocampal CA1 synapses.

Memory consolidation from a labile state to a stable and solid state requires structural remodeling, including the growth or pruning of synapses, as well as molecular and cellular modifications, for synaptic efficacy [2, 42]. Chronic stress led to dendritic retraction distal to the soma in the apical layer of dorsal hippocampal CA1 neurons, consistent with previous studies in different stress models [3, 14]. Moreover, synaptic density measured using the levels of synaptic SYP (a presynaptic marker) and drebrin A (a postsynaptic marker) in the SLM area corresponded well to the structural changes. These results imply that chronic stress is likely to affect the plasticity of input path-specific structures of dendrites, namely the selective retraction of dendrites in the TA-CA1 path rather than those in the SC in the dorsal hippocampal CA1 region. Regular exercise prevented chronic stress-induced dendritic atrophy in hippocampal CA1 neurons, suggesting that the recovery of this pathway may be a crucial requirement of the protective action of exercise. Some studies have reported that running increases dendritic complexity and spine density in the hippocampal CA1 area and entorhinal cortex, consistent with increased BDNF expression [43, 44]. These studies support our finding that regular exercise restored the dendritic structure of hippocampal CA1 neurons by increasing BDNF levels during chronic stress, namely that exercise-induced BDNF increases leads to enhanced structural plasticity.

Dendritic geometry affects the intrinsic excitability, input signal transduction and strength, and synaptic connectivity of neurons [45, 46]. The characteristics of AMPARs, including synaptic recruitment and calcium permeability, play crucial roles in synaptic plasticity, such as LTP, primarily at excitatory glutamatergic synapses [5–8]. Especially, permeability of AMPAR to calcium is determined by the presence/absence of a GluR2 subunit. Unlike AMPARs containing GluR2, those lacking GluR2 are Ca^{2+}-permeable, of high single-channel conductance, and inwardly rectifying. CP-AMPARs lacking GluR2 are more prominent in neurons at the early developmental stage of life and play a role in neonatal synaptic function; however, they also exist in mature neurons [47, 48]. NMDAR-dependent Na^{2+} and Ca^{2+} entry at post-synapse triggers delivery of CP-AMPARs to the synapse, which facilitates long-lasting increase in the excitatory postsynaptic potential (EPSP), the underlying mechanism of LTP; thus, CP-AMPARs are required for NMDAR-dependent LTP [6–8]. Moreover, CP-AMPARs are closely related to NMDAR-independent LTP based on the finding of high-frequency stimulation-induced fEPSP potentiation in hippocampal CA1 slices treated with NMDAR antagonist, D-APV [13] and D,L-AP5 [49] in mice lacking GluR2, which suggests that NMDAR-independent LTP is dependent on the calcium permeability of AMPARs. Enhanced LTP was accompanied by synaptic recruitment of CP-AMPARs at early age (1 month) in the PS1ΔE9 transgenic mouse model of AD [50]. Intracellular delivery of Aβ 1-42 led to rapid increase in AMPAR-mediated synaptic transmission, which was reversed by a CP-AMPAR inhibitor [51]. These previous findings suggest that CP-AMPARs contribute to normal physiological function and are important for preventing neurotoxicity and noxious pathological conditions. Some studies have shown that chronic stress leads to decreases in AMPAR-mediated fEPSP and GluR1 levels in hippocampal CA1 neurons [14, 52]. Furthermore, potentiated AMPAR-mediated synaptic excitation and the enhanced AMPAR expression in CA1 neuronal synapses was observed after treatment with antidepressants or ketamine [53, 54]. Synaptic incorporation of AMPARs and the Ca^{2+} permeability in the hippocampal CA1 SLM area was shown to be important in memory retention in this work. This observation is partly supported by the previous studies that chronic stress-induced impairment of memory retention was attributed to the decrease in synaptic transmission and expression of glutamate receptors, including AMPAR and NMDAR, through ubiquitin/proteasome-mediated degradation of GluR1 and NR1 [55, 56]. With regard to hippocampal LTP, regular exercise prevented or rescued impairment of hippocampal LTP under noxious conditions such as AD, drug addiction, and aging. For example, regular exercise rescued disrupted LTP and expression change of neuroplasticity-related proteins in the hippocampal subregions, as suggested by the findings of exercise-induced increase in the decline of hippocampal fEPSP slope and synaptic plasticity-enhancing molecules such as BDNF in PS1ΔE9 transgenic mice [30], ecstasy-treated mice [31], and aged mice [32]. Moreover, treadmill running has been reported to upregulate AMPAR levels and lead to enhanced EPSPs in the hippocampus, processes that are required for the facilitation of LTP [57, 58]. The above findings support our hypothesis that CP-AMPAR incorporation into TA-CA1 synapses plays a critical role in the protective effects of exercise against chronic stress, thereby preserving memory consolidation via strengthening AMPAR-mediated synaptic excitatory transmission.

PRL has anti-stress effects, including inhibition of the activity of the hypothalamic-pituitary-adrenal (HPA) axis [59,

60]. Resilient rodents have been shown to have higher plasma levels of PRL than stress-reactive rodents [21], consistent with our data showing regular exercise-adapted increase in basal serum PRL levels. Similar to the PRL-enhancing effect of antidepressant [21, 61, 62], serotonin-induced PRL secretion is regarded as a hormonal probe for serotonergic activity in response to exercise. This is evidenced by increased PRL levels following the enhanced availability of tryptophan (a serotonin precursor) in the brain during exercise and the suppression of serotonin-induced PRL secretion following lesioning of dorsal raphe nuclei [57, 63, 64]. Based on previous studies, our results showing an exercise-induced increase in basal serum PRL levels may be attributed to facilitating PRL stimulatory input such as 5-HT. Here we also show the PRL responsiveness in the CA1 region played a crucial role in regular exercise-induced protection of memory persistence during chronic stress. A study showed the decrease in BDNF levels in *prlr*$^{-/-}$ retina [65], implying the strong association of PRL response with BDNF expression. The PRLR-activating effect of exercise has been a topic of speculation: first, exercise-adapted increase in basal serum PRL levels may enhance tonic activity of hippocampal PRL receptors; second, increased PRL levels in response to exercise may prevent dysregulated HPA-axis-induced disruption of hippocampal PRL receptor function against chronic stress, thereby normalizing the hippocampal PRLR sensitivity. We also found that PRL restored the reduction in AMPAR-mediated Ca^{2+} influx induced by repeated treatment with CORT in hippocampal neurons, implying that PRL modulates the permeability of AMPARs to Ca^{2+} in hippocampal neuronal synapses in response to chronic stress. Therefore, regular exercise-adapted tonic enhancement of the PRL response may produce neuroplasticity-promoting conditions, such as enhanced BDNF expression and AMPAR-mediated Ca^{2+} influx, in opposition to the effects of chronic stress. Taken together, these results suggest a crucial role for the sensitivity of the PRL response in the hippocampal CA1 area in memory consolidation during chronic stress. This may be a specific mechanism underlying the protective action of regular exercise against chronic stress.

In conclusion, our results demonstrate that regular exercise protected memory consolidation, prevented dendritic retraction, and re-distributed synaptic CP-AMPARs in the hippocampal CA1 SLM area during chronic stress by improving PRL responsiveness. Therefore, regular exercise may be a promising therapeutic and managing strategy for chronic stress-related affective disorders such as those involving impaired memory processes.

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