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Rapid Intracellular Zn^{2+} Dysregulation via Membrane Corticosteroid Receptor Activation Affects In Vivo CA1 LTP

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Abstract

Involvement of membrane mineralocorticoid (MC) and glucocorticoid (GC) receptors in synaptic Zn^{2+} dynamics remains unclear. Here, we tested whether synaptic plasticity is affected by rapid intracellular Zn^{2+} dysregulation via membrane MC and GC receptor activation, in comparison with intracellular Ca^{2+} dysregulation. In anesthetized rats, extracellular Zn^{2+} level was increased under local perfusion of the hippocampal CA1 with 500 ng/ml corticosterone. In vivo CA1 long-term potentiation (LTP) at Schaffer collateral-CA1 pyramidal cell synapses was attenuated by the pre-perfusion with corticosterone prior to tetanic stimulation, and the attenuation was canceled by co-perfusion with CaEDTA, an extracellular Zn^{2+} chelator, suggesting that corticosterone-induced increase in extracellular Zn^{2+} is involved in the subsequent attenuation of LTP. In rat brain slices, corticosterone-induced increases in extracellular and intracellular Zn^{2+} were blocked in the presence of spironolactone, a MC receptor antagonist that canceled corticosterone-induced attenuation of LTP. Mifepristone, a GC receptor antagonist, which canceled corticosterone-induced attenuation of LTP, also blocked corticosterone-induced increase in intracellular Zn^{2+}, but not extracellular Zn^{2+}. Moreover, corticosterone-induced decrease in phosphorylated CaMKII was restored in the presence of CaEDTA or spironolactone. These results indicate that glucocorticoid rapidly induces the increase in intracellular Zn^{2+}, which occurs via membrane MC and GC receptor activations, and decreases phosphorylated CaMKII level, resulting in attenuating LTP. Membrane MC and GC receptors induce intracellular Zn^{2+} dysregulation via differential mechanisms. In contrast, glucocorticoid-induced intracellular Ca^{2+} dysregulation is not crucial for affecting LTP.

Keywords Zn^{2+} · Membrane corticosteroid receptor · Glucocorticoid · Hippocampus · Stress

Introduction

Stress activates the hypothalamo–pituitary–adrenocortical (HPA) system and increases glucocorticoid secretion from the adrenal cortex. Glucocorticoids pass through the brain barrier system and can modify cognitive activity bidirectionally [1–3]. Under stressful circumstances, glucocorticoids are excessively and/or persistently secreted and considered a major factor for stress-related memory disorders [4, 5]. Glucocorticoids (corticosterone in rodents) act on via mineralocorticoid (MC) and glucocorticoid (GC) receptors, which exist on the plasma membrane and in the cytosolic compartment. Many of glucocorticoid actions require time to induce changes in gene expression (> 15–30 min), while glucocorticoids have rapid non-genomic actions via the membrane-bound receptors [6].

The hippocampus is enriched with MC and GC receptors and is a target area under stressful circumstances. Glucocorticoids facilitate glutamate release from the neuron terminals via the rapid action of membrane MC receptors [7, 8]. In the hippocampus, a subclass of glutamatergic neurons, i.e., zincergic neurons, concentrates zinc in the presynaptic vesicles and co-releases Zn^{2+} into the synaptic cleft. Therefore, it is estimated that glucocorticoids facilitate Zn^{2+} release from zincergic neuron terminals under stressful conditions [9] and that Zn^{2+} accumulation in the extracellular compartment plays a key role for cognitive decline in cooperation with glutamate accumulation in the extracellular compartment [10]. Adrenalectomy causes loss of Zn^{2+} in zincergic neuron terminals and decreases seizure-induced neuronal death, suggesting that elevated glucocorticoid concentration exacerbates neuronal death caused by epilepsy [11].

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Synaptic plasticity such as long-term potentiation (LTP) is considered to be a cellular mechanism of memory and has been extensively studied in the hippocampus [12]. Our studies demonstrate that Zn^{2+} released from zincergic Schaffer collateral is required to induce CA1 LTP [13], while excess Zn^{2+} release attenuates CA1 LTP [14]. Zn^{2+} preferentially passes through Ca^{2+}- and Zn^{2+}-permeable GluR2-lacking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors when glutamate accumulates in the extracellular compartment of the CA1 [15–18]. Extracellular glutamate-induced cognitive decline after stimulation with high K^+ is due to intracellular Zn^{2+} dysregulation, but not intracellular Ca^{2+} dysregulation [19].

Acute stress induces a rapid corticosterone rise in the hippocampus and impairs memory formation [20, 21]. Non-genomic actions, especially via membrane MC receptor activation, are involved in the impairment [22–24]. On the other hand, there is no evidence on the relationship between synaptic Zn^{2+} dynamics and membrane MC and GC receptors. The study on the relationship is important to understand cognitive decline under acute stress. Here, we test whether rapid modification of synaptic Zn^{2+} dynamics, which is induced with corticosterone, leads to in vivo aberrant synaptic plasticity, in comparison with that of synaptic Ca^{2+} dynamics. Synaptic Zn^{2+} dynamics is studied focused on the rapid action of membrane MC and GC receptors.

Materials and Methods

Animals

Male Wistar rats (6–9 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and used for the experiments. They were housed under the standard laboratory conditions (23 ± 1 °C, 55 ± 5% humidity) and had access to tap water and food ad libitum. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka that refer to American Association for Laboratory Animals Science and the guidelines laid down by the NIH (NIH Guide for the Care and Use of Laboratory Animals) in the USA.

Chemicals

ZnAF-2DA and ZnAF-2 ($K_d = 2.7 \times 10^{-9}$ M for Zn^{2+}), a membrane-permeable and membrane-impermeable Zn^{2+} indicator, respectively, were kindly supplied from Sekisui Medical Co., LTD (Hachimantai, Japan). ZnAF-2 is selectively bound to Zn^{2+}, but not bound to other divalent cations such as Ca^{2+}, Mg^{2+}, and Cu^{2+} [25]. Calcium orange AM, a membrane-permeable Ca^{2+} indicator was purchased from Molecular Probes, Inc. (Eugene, OR). The indicators were dissolved in dimethyl sulfoxide (DMSO) and then diluted to Ringer solution containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, and 11 mM D-glucose (pH 7.3).

In Vivo Microdialysis

Male rats were anesthetized with chloral hydrate and placed in a stereotaxic apparatus. A microdialysis probe was implanted 3.3 mm posterior to the bregma, 2.2 mm lateral, and 2.2 mm inferior to the dura. The hippocampus was perfused with artificial cerebrospinal fluid (ACSF), which was composed of 127 mM NaCl, 2.5 mM KCl, 0.9 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 21 mM $NaHCO_3$, and 3.4 mM D-glucose (pH 7.3), at 2.0 μl/min for at least 2 h to determine the basal levels of Zn^{2+} and perfused with 500 ng/ml corticosterone in ACSF for 20 min. Forty minutes after corticosterone perfusion, the hippocampus was perfused with 1 mM CaEDTA, an extracellular Zn^{2+} chelator, in ACSF for 30 min. The perfusate was collected every 5 min. To determine extracellular levels of Zn^{2+}, ZnAF-2 (1 μM, 50 μl) was added to the perfusate samples. The fluorescence of ZnAF-2 (Ex/Em; 485/535 nm) was measured using a plate reader ARVO sx (Perkin Elmer, USA).

In Vivo CA1 LTP

Male rats were anesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus. A bipolar stimulating electrode and a monopolar recording attached to a microdialysis probe (Eicom Co., Kyoto) were implanted into the right hippocampal CA1 region. The electrode stimulating the Schaffer collateral was positioned 4.3 mm posterior to the bregma, 3.8 mm lateral, and 2.0–2.5 mm inferior to the dura. The recording electrode was implanted ipsilaterally 3.3 mm posterior to the bregma, 2.2 mm lateral, and 2.0–2.5 mm inferior to the dura. All the stimuli were biphasic square wave pulses (200 μs width), and their intensities were set at the current that evoked 40% of maximum field excitatory postsynaptic potential (fEPSP). Test stimuli were delivered at 20 s intervals to monitor fEPSP. At the beginning of the experiments, input/output curves were generated by systematic variation of the stimulus current to evaluate synaptic potency. After stable baseline recording for at least 30 min, agents in ACSF were perfused via a microdialysis probe. LTP was induced by delivery of high-frequency stimulation (HFS; 4 trains of 100 pulses at 100 Hz separated by 130 s) and recorded for 60 min. Field EPSP slopes for the last 10 min were also averaged and represented as the magnitude of LTP.

In Vitro Zn^{2+} and Ca^{2+} Imaging

Rats were decapitated under anesthesia. The brain was quickly removed and immersed in ice-cold choline-Ringer containing 124 mM choline chloride, 2.5 mM KCl, 2.5 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH 7.3) to suppress excessive neuronal excitation. Coronal brain slices (400 μm) were prepared by using a vibratome ZERO-1 (Dosaka Kyoto, Japan) in an ice-cold choline-Ringer. To assess intracellular levels of Zn^{2+} and Ca^{2+}, brain slices were loaded for 30 min in 10 μM ZnAF-2DA and 10 μM calcium orange AM and then transferred to a chamber filled with Ringer solution to wash out for at least 20 min. The brain slices were transferred to a recording chamber filled with Ringer solution. The fluorescence of ZnAF-2 (laser, 488.4 nm; emission, 500–550 nm) and calcium orange (laser, 561.4 nm; emission, 570–620 nm) was measured in the stratum radiatum of the hippocampal CA1 with a confocal laser-scanning microscopic system (Nikon A1 confocal microscopes, Nikon Corp.). To assess extracellular levels of Zn^{2+}, the brain slices were transferred to a recording chamber filled with 10 μM ZnAF-2 in Ringer solution. The fluorescence of ZnAF-2 was measured in the same manner. Corticosterone (final concentration, 500 ng/ml) was added to the brain slices after measurement of basal fluorescence of ZnAF-2 and calcium orange for 60 s, and the changes in fluorescence were measured for 180 s. Region of interest was set in the stratum radiatum of the CA1. All solutions used in the experiments were continuously bubbled with 95% O_2 and 5% CO_2.

Western Blotting

Male rats were anesthetized with chloral hydrate and placed in a stereotaxic apparatus. A microdialysis probe was implanted 3.3 mm posterior to the bregma, 2.2 mm lateral, and 2.2 mm inferior to the dura. The hippocampus was perfused with ACSF at 2.0 μl/min for at least 2 h and then perfused with 500 ng/ml corticosterone in ACSF, 500 ng/ml corticosterone in ACSF containing 1 mM CaEDTA, or 500 ng/ml corticosterone in ACSF containing 100 μM spironolactone for 20 min. Forty minutes after corticosterone perfusion, rats were decapitated under anesthesia. The brain was quickly removed and immersed in ice-cold choline-Ringer solution. Transverse hippocampal slices (400 μm) were prepared using the vibratome in an ice-cold choline-Ringer. The samples of hippocampal CA1 tissue were separated from the slices and homogenized in Lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 5 mM NaF, 2 mM β-glycerophosphate, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, and 1 mM dithiothreitol. The homogenates were centrifuged at 3000 g for 5 min at 4 °C. The resultant supernatants were added to the sample buffer containing 250 mM Tris–HCl (pH 6.8), 8% sodium dodecyl sulfate (SDS), 40% glycerol, 0.02% Bromophenol blue (BPB), and 4 mM dithiothreitol (3:1) and boiled for 5 min. The samples were used for the western blotting. Protein concentrations were determined using Pierce BCA protein assay Kit (Thermo Scientific, USA). Samples containing the same amount of proteins were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). After performing SDS-PAGE, the gel was transblotted to Immobilon-P membrane (Millipore) and then blocked with Ez Block Chemi (ATTO, Tokyo, Japan) at room temperature for 1 h. The membranes were incubated overnight at 4 °C with anti-phospho Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) antibody (1:5000, Cell signaling, USA) or anti-α-tubulin antibody (1:2500, Cell signaling). After washing the membranes with TBS-T containing 150 mM NaCl, 25 mM Tris–HCl (pH 7.5), and 0.1% Triton X-20, the membrane was incubated with the secondary antibody (1:2500, Cell signaling). Immunoreactive proteins were detected using a LuminoGraph I (ATTO).

Data Analysis

For multiple comparisons, differences between treatments were assessed by one-way ANOVA followed by post hoc testing using the Tukey's test (the statistical software, GraphPad Prism 5). A value of $p < 0.05$ was considered significant. Data were expressed as means ± standard error.

Results

In Vivo Corticosterone-Induced Increase in Extracellular Zn^{2+} and Its Impact on LTP Induction

To assess in vivo dynamics of synaptic Zn^{2+} under the presence of glucocorticoid, the hippocampal CA1 was locally perfused with 500 ng/ml corticosterone. Extracellular Zn^{2+} level in the perfusate, which was determined with ZnAF-2, was increased by corticosterone perfusion, and the increase lasted for more than 30 min after finishing the perfusion (Fig. 1). When CaEDTA was added to the perfusate, the increase in extracellular Zn^{2+} was rapidly canceled by the formation of ZnEDTA in the extracellular compartment.

In vivo CA1 LTP was attenuated by the perfusion with 1 μM $ZnCl_2$ for 20 min prior to LTP induction, but not by the perfusion with 1 μM $ZnCl_2$ for 20 min when LTP was induced (Fig. 2a). CA1 LTP was not attenuated by the perfusion with 50 ng/ml corticosterone for 20 min prior to LTP induction, but attenuated by perfusion with 500 ng/ml corticosterone. The attenuation was canceled by co-perfusion with 1 mM CaEDTA (Fig. 2b). Because the increase in extracellular Zn^{2+} lasted for more than 30 min even after finishing

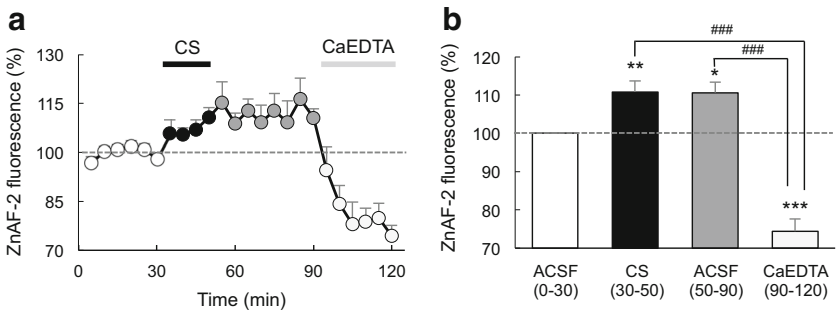


Fig. 1 Corticosterone increases extracellular Zn^{2+} concentration in the hippocampal CA1. **a** The hippocampal CA1 was perfused with 500 ng/ml corticosterone (CS) for 20 min (time, 30–50 min) as shown by the black bar (CS, $n = 15$). Forty minutes after finishing corticosterone perfusion, the hippocampal CA1 was perfused with 1 mM CaEDTA for 30 min (time, 90–120 min) as shown by the light gray bar. Each point and line represents ZnAF-2 fluorescence in the perfusate collected for 5 min that is the ratio (%) of ZnAF-2 fluorescence in each perfusate to basal ZnAF-2 fluorescence in perfusate under the perfusion with ACSF for 30 min. Basal ZnAF-2 fluorescence in perfusate is expressed as 100% in **b**. **b** Each bar and line represents the rate (%) of each averaged ZnAF-2 fluorescence to the basal ZnAF-2 fluorescence. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.005$, vs. ACSF for 0–30 min; $^{\#\#\#}p < 0.005$

500 ng/ml corticosterone perfusion (Fig. 1), the effect of CaEDTA was examined after the corticosterone perfusion. Corticosterone-induced attenuation of CA1 LTP was also canceled by the perfusion with CaEDTA after corticosterone perfusion (Fig. 2c).

Differential Synaptic Zn^{2+} Dynamics Via Membrane MC and GC Receptor Activations and Its Impact on LTP

To clarify the involvement of MC and GC receptors in corticosterone-induced increase in extracellular Zn^{2+}, corticosterone was added to brain slices bathed in Ringer solution containing spironolactone, a MC receptor antagonist, or mifepristone, a GC receptor antagonist. Extracellular Zn^{2+} level was rapidly increased in the stratum radiatum of the CA1 after addition of 500 ng/ml corticosterone. The increase was markedly blocked in the presence of spironolactone, but not in the presence of mifepristone (Fig. 3a, b). Corticosterone-induced rapid increase in intracellular Ca^{2+} was almost completely blocked in the presence of spironolactone, but not in the presence of mifepristone (Fig. 3c, d). Corticosterone-induced increase in intracellular Ca^{2+} was also completely blocked in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor antagonist (Fig. 3e).

Intracellular Zn^{2+} level was also rapidly increased after addition of corticosterone, while the increase was completely blocked in the presence of spironolactone (Fig. 3f, g). Although corticosterone-induced increase in intracellular Zn^{2+} was not significantly blocked in the presence of mifepristone (120 s after addition of corticosterone) (Fig. 3f, g), it was also completely blocked in the presence of mifepristone 180 s after addition of corticosterone (control, 107.1 ± 2.48%; spironolactone, 99.2 ± 1.80%; mifepristone, 99.4 ± 1.80%, $p < 0.05$, vs. control). Corticosterone-induced increase in intracellular Zn^{2+} was also blocked in the presence of CNQX (Fig. 3h).

We tested the significance of rapid increase in intracellular Zn^{2+} induced by MC and GC receptor activations. Corticosterone-induced attenuation of CA1 LTP was canceled by perfusion with spironolactone or mifepristone prior to LTP induction. The perfusion with spironolactone or mifepristone had no effect to LTP induction (Fig. 4a, b).

Impact of Zn^{2+} Influx on Phosphorylated CaMKII Level

To pursue the impact of corticosterone-induced Zn^{2+} influx on LTP induction, we checked the basal expression level of phosphorylated CaMKII in the CA1 after the local perfusion with corticosterone in the same manner. Both levels of phosphorylated CaMKII α and CaMKII β were decreased after perfusion with corticosterone, and both decreases were canceled by co-perfusion with CaEDTA (Fig. 5a). Corticosterone-induced decreases in phosphorylated CaMKII α and CaMKII β were also canceled by co-perfusion with spironolactone (Fig. 5b).

Discussion

Acute stress induces a rapid glucocorticoid rise in the hippocampus, and the activation of membrane MC and GC receptors is involved in cognitive decline via aberrant synaptic plasticity [22–24]. In the hippocampal CA1, rapid changes in CA1 pyramidal cell function emerge via presynaptic and postsynaptic membrane MC receptors: corticosterone increases glutamate release probability pre-synaptically and causes a suppression in potassium current post-synaptically, leading to enhanced CA1 pyramidal cell excitability [26]. Because approximately 50% of the Schaffer collaterals co-release Zn^{2+} with glutamate [27], it is estimated that an increase in corticosterone rapidly modifies extracellular Zn^{2+} dynamics. In anesthetized rats, in the present study, extracellular Zn^{2+} level was increased under local perfusion of the

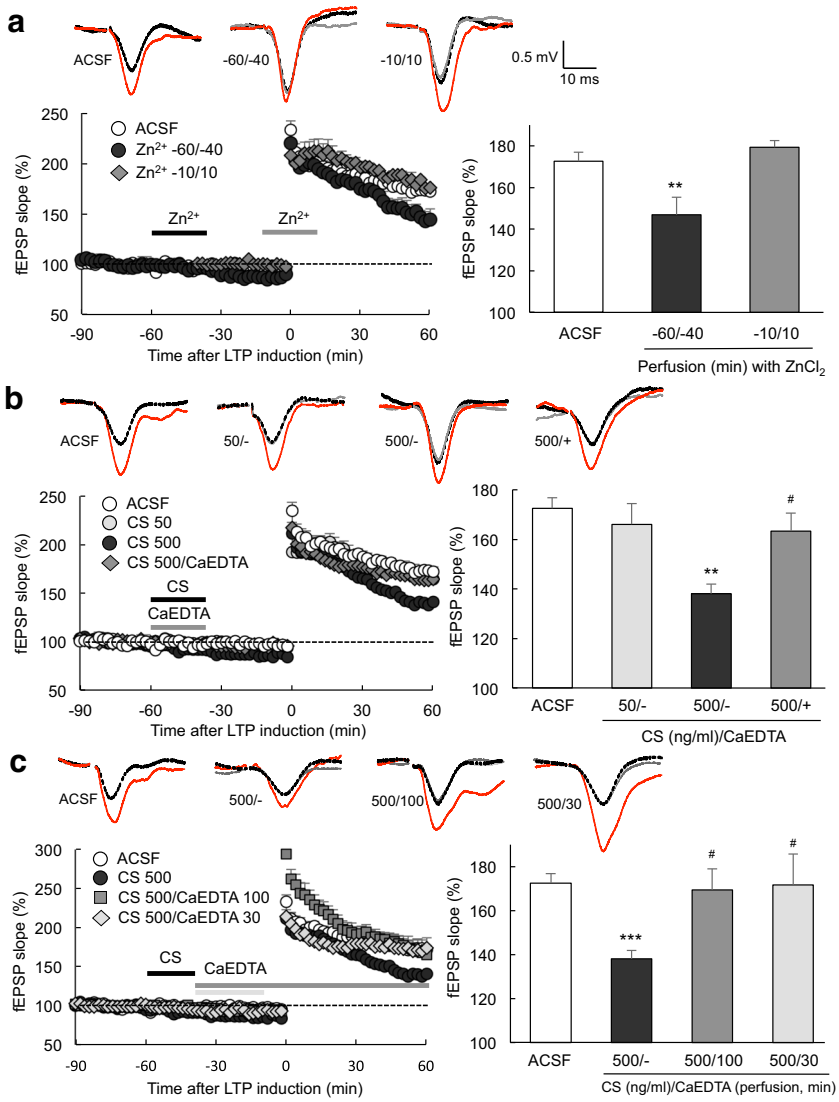
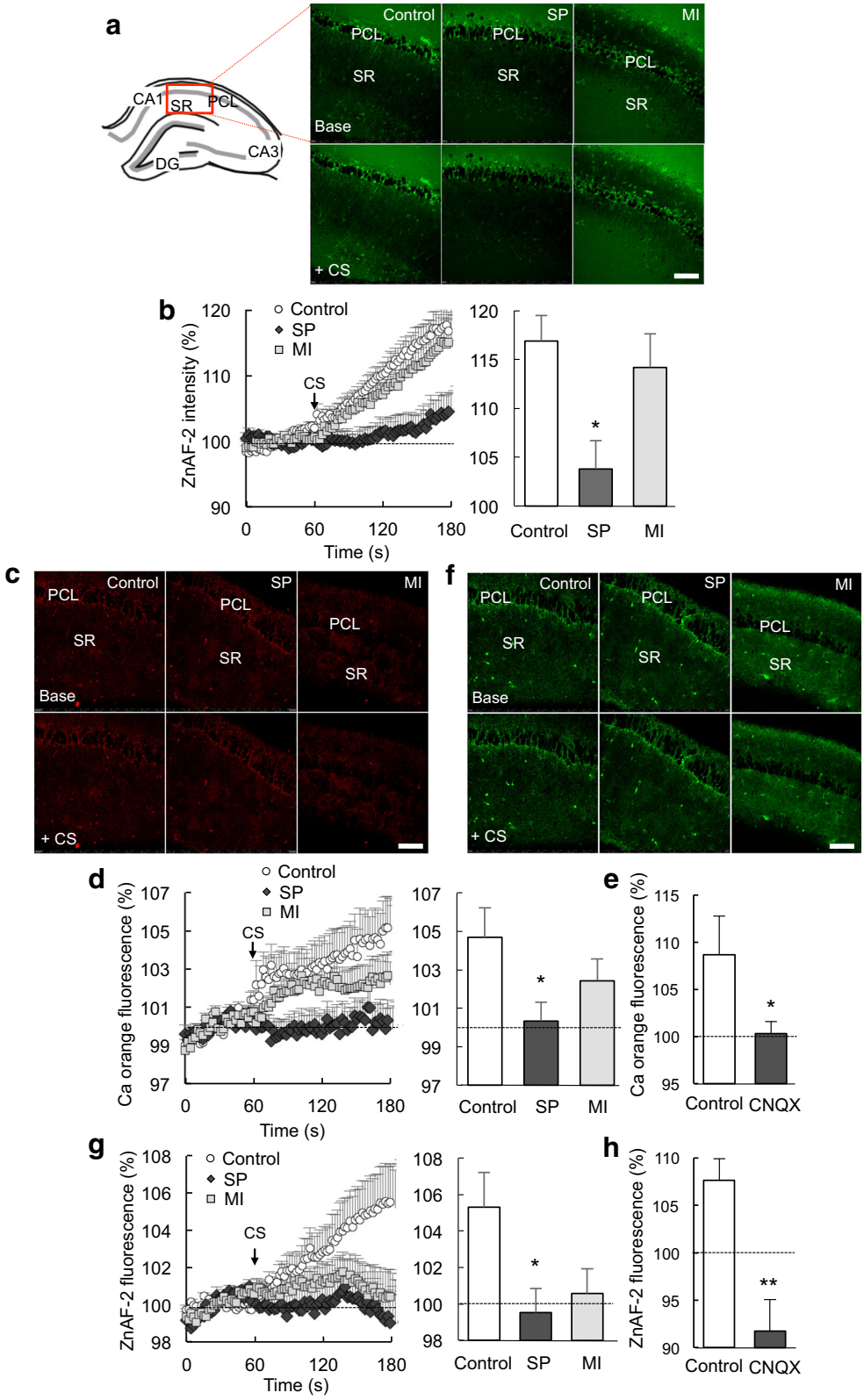


Fig. 2 Extracellular Zn^{2+} chelator rescues corticosterone-induced attenuation of CA1 LTP. **a** LTP was induced under the hippocampal pre-perfusion with 1 μM $ZnCl_2$ in ACSF for 20 min as shown by the black bar ($n = 7$) and the hippocampal perfusion with 1 μM $ZnCl_2$ in ACSF for 20 min as shown by the gray bar ($n = 8$) in anesthetized rats (left). The magnitude of LTP (right). $^{**}p < 0.01$, vs. ACSF ($n = 21$). Representative fEPSP recordings at the time − 70 min (black dotted line), − 50 or − 5 min (gray line) and 50 min (red line) are shown (upper). **b** LTP was induced under hippocampal pre-perfusion with 50 ng/ml corticosterone (CS) ($n = 7$), 500 ng/ml corticosterone ($n = 13$), or 500 ng/ml corticosterone + 1 mM CaEDTA ($n = 12$) as shown by the black and gray bars (left). The magnitude of LTP (right). $^{**}p < 0.01$, vs. ACSF ($n = 21$), $^{\#}p < 0.05$, vs. CS 500. Representative fEPSP recordings at the time − 70 min (black dotted line), − 50 min (gray line), and 50 min (red line) are shown (upper). **c** LTP was induced under hippocampal pre-perfusion with 500 ng/ml corticosterone as shown by the black bar ($n = 13$) and induced under hippocampal perfusion with 1 mM CaEDTA for 100 min ($n = 7$) or for 30 min ($n = 7$), as shown by the gray and light gray bars, respectively, after 500 ng/ml corticosterone pre-perfusion (left). The magnitude of LTP (right). $^{***}p < 0.005$, vs. ACSF ($n = 21$), $^{\#}p < 0.05$, vs. CS 500. Representative fEPSP recordings at the time − 70 min (black dotted line), − 5 min (gray line), and 50 min (red line) are shown (upper)

hippocampal CA1 with corticosterone. CA1 LTP was attenuated by the pre-perfusion with corticosterone prior to tetanic stimulation, and the attenuation was canceled by the co-perfusion with CaEDTA. The perfusion with CaEDTA after corticosterone perfusion was also effective for the cancelation, probably due to the sustained increase in extracellular Zn^{2+} induced with corticosterone. These results suggest that corticosterone-induced increase in extracellular Zn^{2+} is involved in the subsequent attenuation of CA1 LTP. It is likely that corticosterone-induced increase in extracellular Zn^{2+} is mediated by not only rapid non-genomic action via membrane MC receptors but also delayed genomic action.

We tested whether intracellular Zn^{2+} dysregulation via the increase in extracellular Zn^{2+} causes corticosterone-induced attenuation of CA1 LTP, in comparison with intracellular Ca^{2+} dysregulation. In rat brain slices, corticosterone-induced rapid increases in extracellular and intracellular Zn^{2+} were canceled in the presence of spironolactone, a MC



receptor antagonist that canceled corticosterone-induced attenuation of CA1 LTP under condition of co-perfusion, suggesting that the short-term block of corticosterone-induced increase in intracellular Zn^{2+} by spironolactone leads to the cancelation of corticosterone neurotoxicity. Corticosterone-induced rapid increase in intracellular Zn^{2+} was also canceled in the presence of CNQX. The present findings suggest that corticosterone rapidly increases Zn^{2+} release from the

Fig. 3 Corticosterone-induced synaptic Zn^{2+} dynamics is differentially modulated by membrane MR and GR receptors in the hippocampal CA1. **a** The area surrounded with the red line in the CA1 was imaged in brain slices with extracellular ZnAF-2. Extracellular ZnAF-2 fluorescence in the CA1 of brain slices bathed in Ringer solution containing 10 μM ZnAF-2, 100 μM spironolactone (SP) in Ringer solution containing 10 μM ZnAF-2, and 10 μM mifepristone (MI) in Ringer solution containing 10 μM ZnAF-2 was imaged 0 s (base) and 120 s after addition of corticosterone (the final concentration, 500 ng/ml) (+ CS). PCL pyramidal cell layer, SR stratum radiatum. Bar, 50 μm. **b** Corticosterone was added to brain slices bathed in Ringer solution containing 10 μM ZnAF-2, 100 μM SP in Ringer solution containing 10 μM ZnAF-2, and 10 μM MI containing 10 μM ZnAF-2 as shown by the arrow 60 s after measuring the basal level of extracellular ZnAF-2 fluorescence, and the changes in extracellular ZnAF-2 fluorescence were measured for 120 s (control, $n = 10$; SP, $n = 9$; MI, $n = 14$). Each point and line represents the rate of ZnAF-2 fluorescence to the basal ZnAF-2 fluorescence (left). Each bar and line represents the rate (%) of the averaged ZnAF-2 fluorescence of the last 20 s (time 160–180 s) to the basal ZnAF-2 fluorescence, which was expressed as 100% (right). $*p < 0.05$, vs. control. **c**, **d** Intracellular Ca orange fluorescence was imaged in the CA1 of brain slices, which were pre-loaded with calcium orange AM instead of adding ZnAF-2 to Ringer solution. Corticosterone was added to brain slices bathed in Ringer solution, 100 μM SP in Ringer solution, and 10 μM MI in Ringer solution in the same manner as **a**. Control, $n = 26$; SP, $n = 23$; MI, $n = 27$. $*p < 0.05$, vs. control. **e** Corticosterone (the final concentration, 500 ng/ml) was added to brain slices bathed in Ringer solution and 10 μM CNQX in Ringer solution 60 s after measuring the basal level of intracellular Ca orange fluorescence, and the changes in intracellular Ca orange fluorescence were measured in the same manner (control, $n = 11$; CNQX, $n = 5$). $*p < 0.05$, vs. control. **f**, **g** Intracellular ZnAF-2 fluorescence was imaged in the CA1 of brain slices, which were pre-loaded with ZnAF-2DA instead of adding ZnAF-2 to Ringer solution. Corticosterone was added to brain slices bathed in Ringer solution, 100 μM SP in Ringer solution, and 10 μM MI in Ringer solution in the same manner as **a**. Control, $n = 13$; SP, $n = 12$; MI, $n = 13$. $*p < 0.05$, vs. control. **h** Intracellular ZnAF-2 fluorescence was measured in the same manner as **e** (control, $n = 12$; CNQX, $n = 5$). $**p < 0.01$, vs. control

Schaffer collateral via membrane MC receptor activation, followed by the increase in intracellular Zn^{2+} in CA1 pyramidal cells via AMPA receptor activation, which attenuates CA1 LTP (Fig. 6). Corticosterone-induced increase in intracellular Zn^{2+} lasts even in the absence of corticosterone. Nonetheless, the early block of increase in intracellular Zn^{2+} with CaEDTA was effective to cancel corticosterone neurotoxicity as well as the later block, suggesting that corticosterone-induced intracellular Zn^{2+} dysregulation is also mediated by not only rapid non-genomic action via membrane MC receptors but also delayed genomic action.

Mifepristone, a GC receptor antagonist, which canceled corticosterone-induced attenuation of CA1 LTP under condition of co-perfusion, also canceled corticosterone-induced rapid increase in intracellular Zn^{2+}, but not extracellular Zn^{2+} (Fig. 6), suggesting that the short-term block of corticosterone-induced increase in intracellular Zn^{2+} with mifepristone also leads to the cancelation of corticosterone neurotoxicity. Corticosterone triggers, via distinct corticosteroid receptors, time-dependent increases in AMPA receptor surface trafficking and synaptic surface AMPA receptor content. The time-dependent increases are due to membrane MC receptor activation [28]. In the present study, the baseline of fEPSP was not modified by corticosterone perfusion, suggesting that corticosterone does not increase synaptic surface AMPA receptor content at least before LTP induction. On the other hand, corticosterone increases spine density of hippocampal neurons via membrane GC receptor activation [29, 30]. Early spinogenesis via membrane GC receptor activation may require extracellular Zn^{2+} influx into presynaptic and postsynaptic neurons, which leads to membrane GC receptor-mediated increase in intracellular Zn^{2+} (Fig. 6).

The basal concentration of extracellular corticosterone in the brain is approximately 1–2 ng/ml [31], and at least 5 ng/ml corticosterone is required to induce the rapid effect of membrane MC receptor activation on hippocampal glutamate transmission [7]. Because CA1 LTP was not affected by the perfusion with 50 ng/ml corticosterone, it is estimated that extracellular corticosterone level, which might reach 5 ng/ml as the actual concentration, is not enough for glutamate release in the CA1. In contrast, the perfusion with 500 ng/ml corticosterone facilitates glutamate release, followed by the increase in extracellular Zn^{2+}, which induces intracellular Zn^{2+} dysregulation. Corticosterone-induced rapid increase in intracellular Ca^{2+} was blocked by spironolactone, but not by mifepristone, suggesting that corticosterone-induced rapid intracellular Ca^{2+} dysregulation is less crucial for affecting CA1 LTP than rapid intracellular Zn^{2+} dysregulation.

Corticosterone regulates AMPA receptors trafficking including Ca^{2+}-permeable AMPA receptors and facilitates LTP in the hippocampus [32–35]. Newly synthesized LTP-related proteins can be captured at new binding sites via CaMKII, a key molecule for LTP, for structural synapse enlargement, sustaining the potentiated state for a long term. The location of CaMKII is crucial for the construction of the potentiated state [36, 37]. The interplay between the kinase and structural functions of CaMKII is important for defining a time window permissive for synaptic plasticity [38]. It is estimated that Zn^{2+} concentrates in the postsynaptic density (PSD) via intracellular Zn^{2+} signaling after LTP induction, which can be linked with membrane MC and GC receptor activation, and is able to influence the recruitment of ProSAP/Shank proteins to PSDs in a family member-specific manner during the course of synaptogenesis and structural plasticity [39, 40]. Zn^{2+} concentration might transiently reach a few nanomolar in the intracellular compartment for synaptic plasticity (Fig. 6).

Total and phosphorylated CaMKII are increased in the hippocampal CA1 after CA1 LTP induction [41, 42]. In contrast, chronic stress decreases basal levels of phosphorylated CaMKII and then attenuates LTP induction [43]. Zn^{2+} can directly modulate CaMKII activity (Fig. 6) and at high micromolar concentrations (~400 μM), Zn^{2+} turns CaMKII into an increased mobility form on SDS-PAGE in vitro [44]. However, it is improbable in vivo. In the present study,

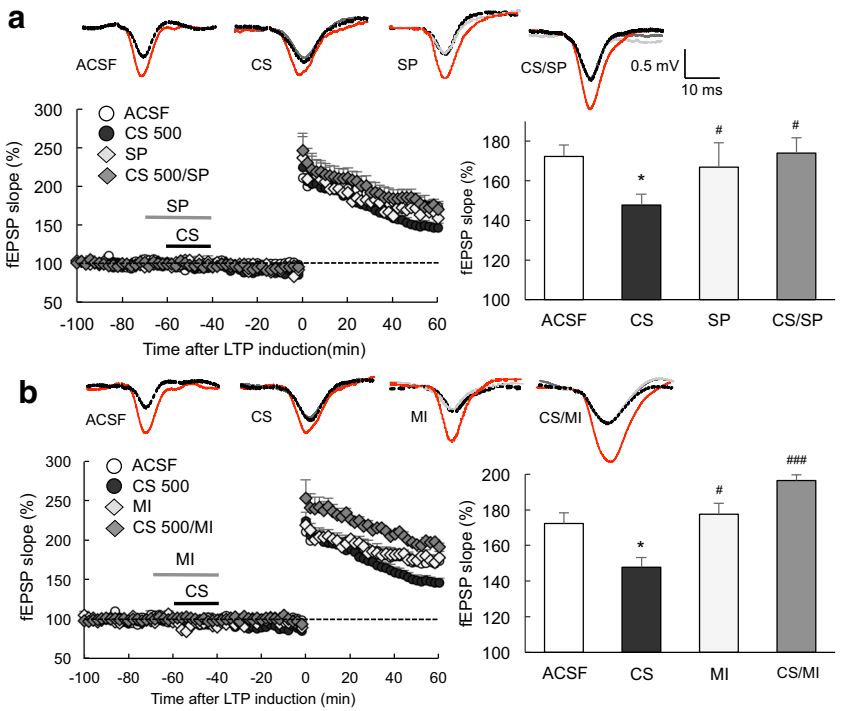


Fig. 4 MC and GC receptor antagonists rescue corticosterone-induced attenuation of CA1 LTP. **a** LTP was induced under hippocampal pre-perfusion with 500 ng/ml corticosterone (CS) in ACSF as shown by the black bar or/and 100 μM spironolactone (SP) in ACSF as shown by the gray bar in anesthetized rats (left). The magnitude of LTP (right). ACSF, $n = 13$; CS, $n = 11$; SP, $n = 6$; SP/CS, $n = 7$. $^{*}p < 0.05$, vs. ACSF ($n = 13$), $^{\#}p < 0.05$, vs. CS 500. Representative fEPSP recordings at the time − 80 min (black dotted line), −50 min (gray line), and 50 min (red line) are shown (upper). **b** LTP was induced under hippocampal pre-perfusion with 500 ng/ml corticosterone in ACSF as shown by the black bar or/and 10 μM mifepristone (MI) in ACSF as shown by the gray bar in anesthetized rats (left). The magnitude of LTP (right). ACSF, $n = 13$; CS, $n = 11$; MI, $n = 4$; MI/CS, $n = 5$. $^{*}p < 0.05$, vs. ACSF ($n = 13$), $^{\#}p < 0.05$, $^{\#\#\#}p < 0.005$, vs. CS 500. Representative fEPSP recordings at the time − 80 min (black dotted line), − 50 min (gray line), and 50 min (red line) are shown (upper)

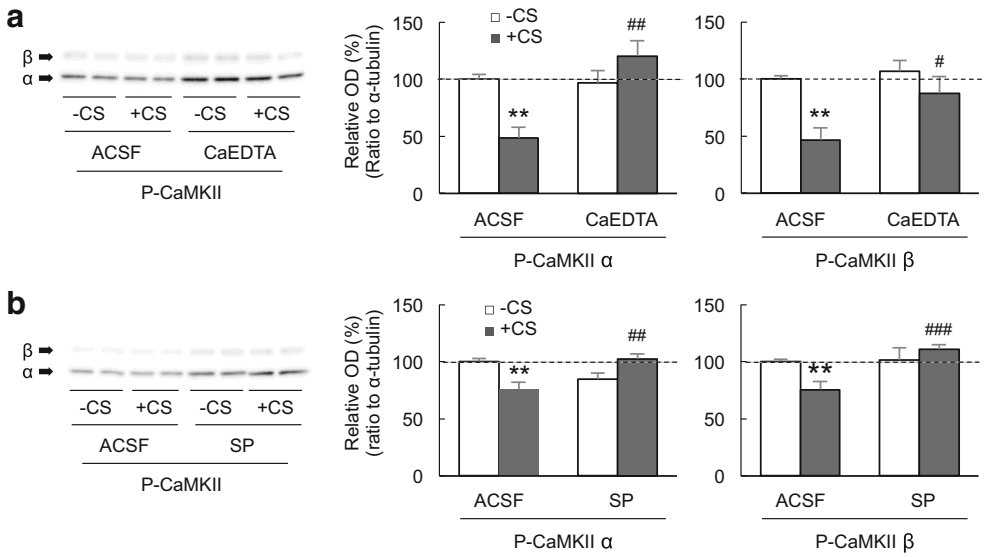


Fig. 5 Corticosterone-induced increase in intracellular Zn^{2+} decreases phosphorylated CaMKII level. The hippocampal CA1 was perfused with ACSF containing 500 ng/ml corticosterone (CS) and ACSF containing 500 ng/ml corticosterone + 1 mM CaEDTA or 100 μM spironolactone (SP) for 20 min. Forty minutes later, phosphorylated CaMKII (P-CaMKII) levels were determined in hippocampal CA1 tissues. Representative images show P-CaMKII α and β protein expressions (left-side). Each bar and line represents the rate (%) of normalized P-CaMKII level by α-tubulin level in hippocampal CA1 tissues to that in hippocampal CA1 tissues perfused with ACSF, which is expressed as 100%. (middle and right). **a** ACSF (control), $n = 14$; CS, $n = 8$; CaEDTA, $n = 12$; CS/CaEDTA, $n = 6$. **b** ACSF, $n = 27$; CS, $n = 24$; SP, $n = 15$; CS/SP, $n = 16$. $^{**}p < 0.01$, vs. ACSF (-CS); $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$, vs. ACSF (+CS)

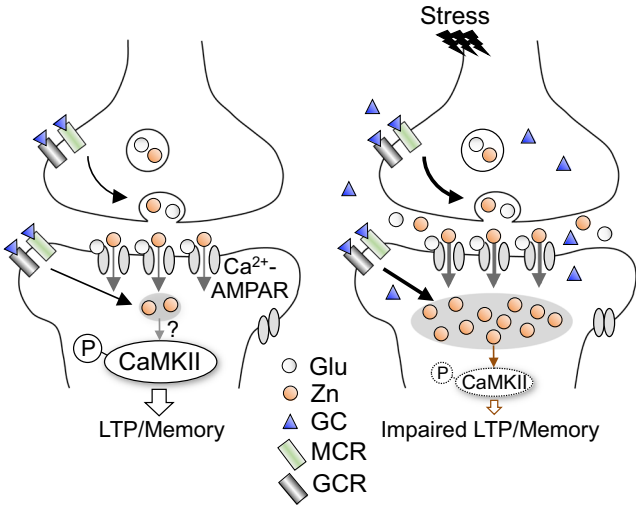


Fig. 6 Schematic diagram on differential actions of membrane MC and GC receptors in synaptic Zn^{2+} dynamics. After acute exposure to corticosterone, extracellular Zn^{2+} is rapidly increased via membrane MC receptor (MCR) activation and taken up into CA1 pyramidal cells, probably through Ca^{2+}-permeable AMPA receptors (Ca^{2+}-AMPAR), followed by excess intracellular Zn^{2+} that decreases phosphorylated CaMKII level. Excess intracellular Zn^{2+} is also induced by membrane GC receptor (GCR) activation. It is likely that membrane MC and GC receptors induce intracellular Zn^{2+} dysregulation via differential mechanisms, followed by affecting synaptic plasticity (right)

intracellular Zn^{2+} concentration may reach low nanomolar (~ 10 nM) under the perfusion with $ZnCl_2$ and corticosterone prior to LTP induction [45]. Corticosterone decreased the basal levels of phosphorylated CaMKII, and the decreases were canceled by co-perfusion with CaEDTA or spironolactone, suggesting that corticosterone-induced rapid influx of extracellular Zn^{2+} via membrane MC receptor activation decreases basal levels of phosphorylated CaMKII (Fig. 6). Although the mechanism on the decreases in phosphorylated CaMKII levels remains unclear, the decreases lead to attenuating CA1 LTP induction. It is possible that low nanomolar Zn^{2+} affects the basal CaMKII phosphorylation process. No effect of the perfusion with 1 μM Zn^{2+} in tetanic stimulation, unlike 1 μM Zn^{2+} prior to tetanic stimulation, on attenuating CA1 LTP might be due to much more increase in intracellular Ca^{2+} by tetanic stimulation [micromolar levels (10~100 μM) vs. the basal level (~ 100 nM)], which might rescue Zn^{2+}-induced decreases in phosphorylated CaMKII levels.

In conclusion, after acute exposure to corticosterone, extracellular Zn^{2+} is rapidly increased via membrane MC receptor activation and taken up into CA1 pyramidal cells, probably through Ca^{2+}-permeable AMPA receptors, followed by intracellular Zn^{2+} dysregulation that decreases phosphorylated CaMKII levels. Intracellular Zn^{2+} dysregulation is also induced by membrane GC receptor activation (Fig. 6). It is likely that membrane MC and GC receptors induce intracellular Zn^{2+} dysregulation via differential mechanisms, followed by affecting CA1 LTP. In contrast, glucocorticoid-induced intracellular Ca^{2+} dysregulation is not crucial for affecting CA1 LTP.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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