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Rapid spinogenesis of pyramidal neurons induced by activation of glucocorticoid receptors in adult male rat hippocampus

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Abstract

Modulation of hippocampal synaptic plasticity by glucocorticoids has been attracting much attention, due to its importance in stress responses. Dendritic spines are essential for memory storage processes. Here, we investigated the effect of dexamethasone (DEX), a specific agonist of glucocorticoid receptor (GR), on density and morphology of dendritic spines in adult male rat hippocampus by imaging of Lucifer Yellow-injected spines in slices. The application of 100 nM DEX (stressful high concentration) induced rapid modulation of the density and morphology of dendritic spines in CA1 pyramidal neurons within 1 h. The total spine density increased from 0.88 spines/μm (control) to 1.36 spines/μm (DEX-treated). DEX significantly increased the density of thin and mushroom type spines, however only a slight increase was observed for stubby and filopodium type spines. Because the presence of 10 μM cycloheximide, an inhibitor of protein synthesis, did not suppress the DEX effect, these responses are probably non-genomic. Western immunoblot analysis demonstrated the localization of classical type GR in Triton-insoluble synaptosomal fractions (enriched in postsynaptic membranes) from hippocampal slices, suggesting a possible action site of DEX at spines.
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It is well accepted that functions and architectures of mammalian brain are altered or modulated under the stressful conditions. At least in part, the influences of stress are elicited by glucocorticoids, produced in adrenal cortex in response to stress. The hippocampus is particularly sensitive to glucocorticoids. The stress-induced increase in corticosterone (CORT), the principal glucocorticoid in rodents, has been known to produce neuronal cell damage in the hippocampus. Exogenous application of a high dose of CORT has been shown to elicit the neuronal atrophy in the hippocampus [1]. Rats exposed to restraint stress for 3 weeks have exhibited neuronal atrophy identical to that seen in rats treated with a high dose of CORT for 3 weeks [2]. In addition to these classical genomic effects, glucocorticoids have modulated rapidly (within 30 min) the neuronal synaptic plasticity, which may occur independently of the regulation of gene transcription (i.e., non-genomic effect) [3,4]. The long-term potentiation (LTP) of young adult

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hippocampal pyramidal neurons has been rapidly suppressed by a high concentration of glucocorticoids [5]. A 30 min application of 1–10 μM CORT has rapidly suppressed the NMDA-induced Ca^{2+} elevation in the CA1 region of adult hippocampal slices [6].

The rapid effect of glucocorticoid on synaptic plasticity has not been well elucidated, except via electrophysiological investigations. In the present study, we focus on the neuronal dendritic spines which are postsynaptic sites of excitatory input and candidates for memory storage apparatus. The rapid modulation of the density and morphology of dendritic spines was investigated following application of dexamethasone (DEX), a specific agonist of glucocorticoid receptor (GR) in hippocampal slices prepared from adult male rats. It appeared that a high dose of DEX application did not decrease the total spine density but did significantly increase the proportion of mushroom and thin type spines.

Materials and methods

Animals

Male Wistar rats (aged 12 weeks) were used in the present study. Rats were purchased from Saitama Experimental Animal Supply. All experiments using animals in this study were conducted according to the Institutional Guidelines.

Imaging and analysis of dendritic spine morphology

Current injection of Lucifer Yellow. Adult male rats were deeply anesthetized with ethyl ether and decapitated. The brains were removed and placed in artificial cerebrospinal fluid (ACSF) at 4 °C. The hippocampus was dissected and 300 μm thick slices were cut at perpendicular transverse to the long axis from the middle third of the hippocampus with a vibratome (Dosaka, Japan). ACSF consisted of (mM): 124 NaCl, 5.0 KCl, 1.25 NaH_2PO_4, 2.0 $MgSO_4$, 2.0 $CaCl_2$, 22 $NaHCO_3$, and 10 glucose, and was equilibrated with 95% O_2/5% CO_2. Hippocampal slices were transferred into an incubating chamber containing ACSF held at 25 °C for 2 h. Slices were then incubated with 100 nM DEX (Sigma, USA) in the presence or absence of other drugs such as RU-486 (Sigma). Slices were then prefixed with 4% paraformaldehyde in PBS at 4 °C for 2–4 h and then placed in PBS at 4 °C. Neurons within slices were visualized by an injection of Lucifer Yellow (Molecular Probes, USA) under Nikon E600FN microscope (Japan) equipped with an infrared camera (Hamamatsu Photonics C2400-79H, Japan) and with a 40× water immersion lens, NA 0.8 (Nikon). Dye injection was performed with glass electrode whose tip was filled with 5% Lucifer Yellow in distilled water under a negative DC current of 10 nA for 15 min, using Axopatch 200B (Axon Instruments, USA). Approximately five neurons within a 100–200 μm depth from the surface of a slice were injected with the dye [7]. After labeling, slices were fixed again with 4% paraformaldehyde overnight and stored in PBS at 4 °C.

Confocal laser microscopy and analysis. The imaging was performed from sequential z-series scans with a MRC-1024 confocal microscope (Bio-Rad, USA). For Lucifer Yellow, the excitation and emission wavelengths were 488 and 515 nm, respectively. For analysis of spines, three-dimensional images were constructed from approximately 40 sequential z-series sections of neurons every 0.5 μm at high zoom (1.5–3.0) with a 60× oil immersion lens, NA 1.4 (Nikon). In each slices, 2–3 neurons with more than 100 spines were analyzed and at least 50 spines were counted on each frame. In total, $N = 5$–9 neurons and $n = 800$–2000 spines were analyzed for each drug treatment. Density and morphology of dendritic spines were analyzed by tracing neurons with Neurolucida (MicroBrightField, USA) [7]. The single apical dendrite was analyzed separately. These dendrites were present within the stratum radiatum, between 100 and 200 μm from the soma. The spine density was calculated from the number of spines along secondary dendrites having a total length of 30–100 μm. While counting the spines in the reconstructed images, the position and verification of spines were aided by rotation of three-dimensional reconstructions and by observation of the images in consecutive single planes. Spine shapes were classified into four categories [8] as follows:

(1) A mushroom spine, which has a large distinguishable head and neck. The head diameter (D) is greater than 0.6 μm, and the total spine length (L) is less than twice the head diameter, i.e., $D > 0.6$ μm and $L < 2D$.
(2) A thin spine, which has a small distinguishable head and an elongated spine neck. $D < 0.6$ μm, $L > 2D$.
(3) A filopodium, which is a long filamentous protrusion with no distinguishable head. L is much longer than the neck diameter (d).
(4) A stubby spine, which does not have a distinguishable head, and has an L value similar to d.

The confocal lateral resolution was approx. 0.18 μm. The applied zoom factor (1.5–3.0) yielded 18.0–9.1 pixels per 1 μm, which was optimal for this resolution. The z-axis resolution was approximately 0.34 μm. Our resolution limits should have allowed the determination of spine number and classification of spine type.

Membrane fractionation and western immunoblot

Postsynaptic membrane-rich fractions were prepared as Triton-insoluble synaptosomal fractions by centrifugation [9]. Approximately 30 hippocampal slices were prepared from adult male rats as described in the previous section of "imaging and analysis of dendritic spine morphology." Then slices were homogenated in 0.32 M sucrose in Hepes buffer (pH 7.3) with 25 mM KCl, 4 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors PMSF (0.5 mM) and leupeptin (0.1 mM), and centrifuged at 830g for 10 min at 4 °C. Supernatant was subjected to centrifugation at 10,000g for 20 min at 4 °C. The retrieved pellet (crude synaptosomal fractions) was treated with 1% Triton X-100 for 30 min at 4 °C, and Triton-insoluble synaptosomal fractions were collected as a pellet by centrifugation at 15,000g for 20 min at 4 °C. These fractions were suspended in 125 mM Tris–HCl buffer (pH 6.8), containing 5 mM 2-mercaptoethanol, 10% sucrose, 6% sodium dodecyl sulfate, and 0.002% bromophenol blue. The fractions were subjected to electrophoresis using a 10% polyacrylamide gel. After transfer to polyvinylidene fluoride membranes (Immobilon-P; Millipore, USA), the blots were probed with antisera against GR (1/3000) [10] for 12–18 h at 4 °C and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling, USA). The membranes were treated with ECL plus Western blotting detection reagents (Amersham, USA), and chemiluminescence photographs were taken with LAS-3000 Image analyzer (Fujifilm, Japan).

Results

Analysis of density and morphology of dendritic spines

We analyzed secondary branches of the apical dendrites in the stratum radiatum of the CA1 region (Fig. 1). Following 1 h treatments with DEX, dendrites

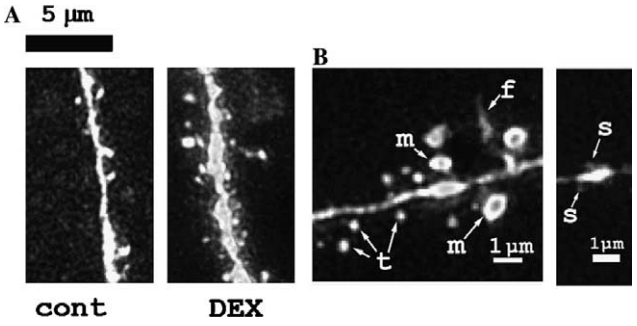

Fig. 1. A confocal micrograph showing spines along the second dendrites in the stratum radiatum regions of hippocampal CA1 pyramidal neurons. (A) Control spines without drug-treatments (cont) and spines after DEX-treatments (DEX). Bar 5 μm. (B) Four different spine types are indicated as: mushroom type (m), thin type (t), filopodium (f), and stubby type (s). Bar 1 μm.

had significantly more spines than control dendrites (i.e., with no DEX treatment), with the spine density increasing from 0.88 spines/μm (control) to 1.36 spines/μm (100 nM DEX) (Figs. 1 and 2). Blocking of glucocorticoid receptors by RU-486 (a specific inhibitor against nuclear glucocorticoid receptor GR) completely suppressed the enhancing effect of DEX on the spine density (Fig. 2). On the other hand, the presence of 10 μM cycloheximide (CHX, Sigma), an inhibitor of protein synthesis, did not suppress the DEX effects (Fig. 2). Blocking of NMDA receptors by MK-801 (Sigma) also completely abolished the DEX-induced spinogenesis.

One hour treatment was chosen to examine the rapid non-genomic effect of DEX, because the enhancing effect by DEX reached a maximal value at 1 h within

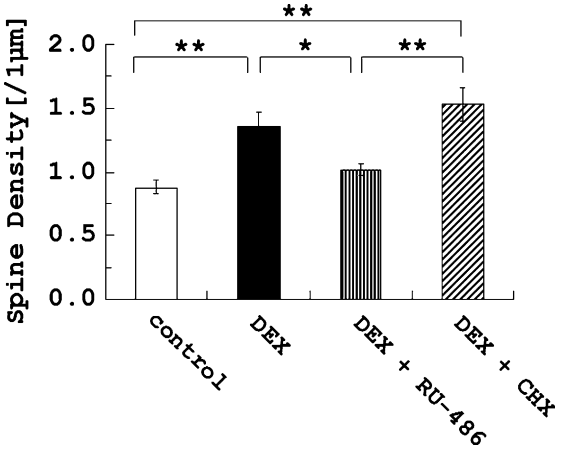

Fig. 2. Effect of DEX treatments on total spine density in hippocampal CA1 neurons. Vertical axis is the average number of spines per 1 μm. A 1 h treatment in ACSF without DEX (control, $N = 9$), with 100 nM DEX (DEX, $N = 5$), with 100 nM DEX and 20 μM RU-486 (DEX + RU-486, $N = 6$), and with 100 nM DEX and 10 μm cycloheximide (CHX) (DEX + CHX, $N = 5$). Results are reported as means ± SEM. The significance of the drug effects was confirmed by using ANOVAs (*$p < 0.05$, **$p < 0.01$). N is the number of neurons, and for each neuron 150–300 spines were analyzed.

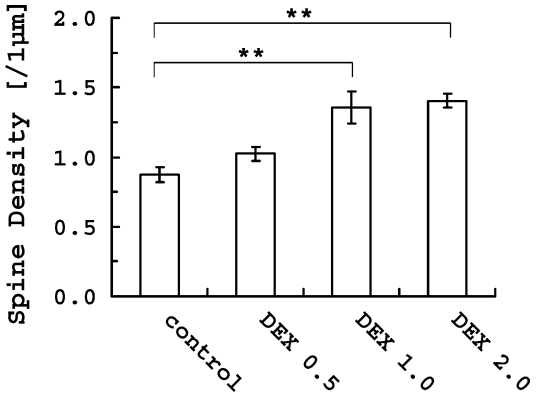

Fig. 3. Time-dependence of DEX treatments on total spine density in hippocampal CA1 neurons. Vertical axis is the average number of spines per 1 μm. A 1 h treatment in ACSF without DEX (control, $N = 9$), a 0.5 h treatment in ACSF with 100 nM DEX (DEX 0.5, $N = 3$), a 1 h treatment in ACSF with 100 nM DEX (DEX 1, $N = 5$), and a 2 h treatment in ACSF with 100 nM DEX (DEX 2, $N = 3$). Results are reported as means ± SEM. The significance of the drug effects was confirmed by using ANOVAs (**$p < 0.01$). N is the number of neurons, and for each neuron 150–300 spines were analyzed.

experimental error. In Fig. 3, time-dependence of rapid effect was shown by treating with 100 nM DEX for 0.5, 1, and 2 h. The enhancing effect on the total spine density was 1.02 spines/μm (0.5 h), 1.36 spines/μm (1 h), and 1.41 spines/μm (2 h). The stressful concentration of DEX was chosen to be 100 nM, because rats subjected to immobilization stress for 1 h have showed approximately 2 μM CORT in blood plasma [11], and DEX action upon GR is almost 10 times stronger than that of CORT at the same concentration [12].

Morphological changes in spines induced by 1 h DEX-treatments were quantitatively determined (Figs.

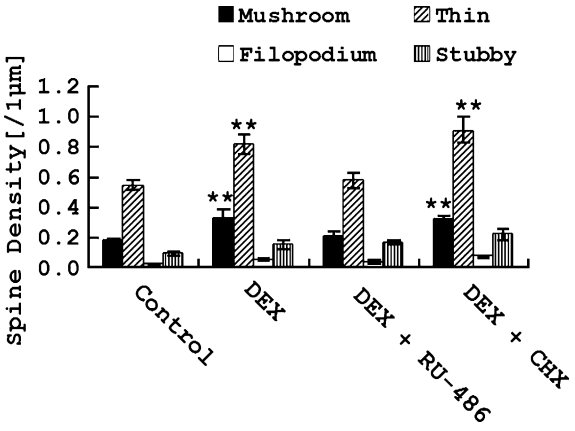

Fig. 4. Density of four subtypes of spines shown in Fig. 2. A 1 h treatment in ACSF without DEX (control), with 100 nM DEX (DEX), with 100 nM DEX and 20 μM RU-486 (DEX + RU-486), with 100 nM DEX and 10 μM CHX (DEX + CHX). In each group, from left to right, (a) mushroom (black column), (b) thin (hatched column), (c) filopodium (open column), and (d) stubby (vertically striped column). Results are reported as means ± SEM. The significance of the drug effects was confirmed by using ANOVAs (**$p < 0.01$).

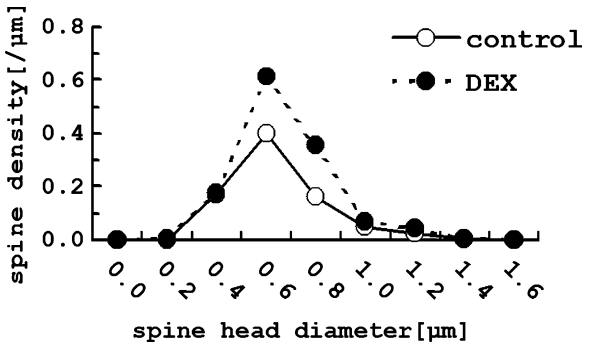

Fig. 5. Spine head diameter of the hippocampal CA1 neurons. A 1 h treatment in ACSF without DEX (open circle), with 100 nM DEX (closed circle).

1 and 4). In control slices, the relative population of spines was approx. 21% for mushroom spine, 64% for thin spine, 3% for filopodium, and 11% for stubby spine. While the densities of filopodia (0.03 spines/μm) and stubby spines (0.10 spines/μm) were not significantly altered (within experimental errors), the densities of mushroom spines and thin spines were increased significantly, from 0.18 and 0.55 to 0.33 and 0.82 spines/μm, respectively (Fig. 4). Average spine head diameter was significantly increased, mainly due to an increase of mushroom spines (Fig. 5).

Western immunoblot analysis of synaptosomal GR

The presence of GR proteins was verified by Western immunoblot analysis. In both Triton-insoluble synaptosomal fractions and hippocampal homogenates, GR-immunoreactivity was observed at an approximately equal position to GR in liver (positive control) (Fig. 6). The electrophoretic mobility of the GR bands indicated a molecular mass of approximately 95 kDa. The strong staining in Triton-insoluble synaptosomal fractions suggested that GR was localized at postsynaptic membrane structures. Triton-insoluble synaptosomal fractions were enriched in the postsynaptic density.

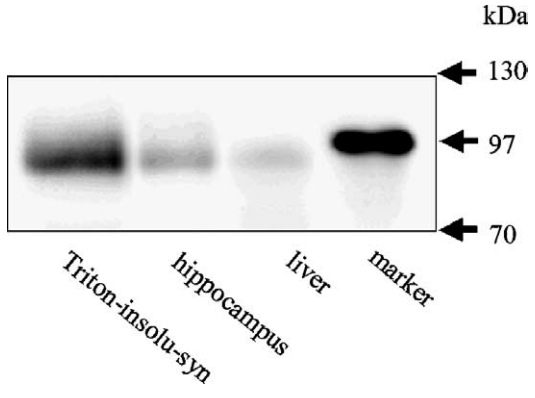

Fig. 6. Western immunoblot analysis of GR in male rat hippocampus. From left to right: GR staining of the Triton-insoluble synaptosomal fractions fraction (Triton-insolu-syn) from hippocampus, whole hippocampus homogenates (hippocampus), liver homogenates (liver), and marker protein of 97 kDa. Liver was used as a positive control. On each lane 20 μg protein was applied.

Discussion

The current study demonstrated that the activation of classical glucocorticoid receptor GR by DEX induced rapid spinogenesis in pyramidal neurons of the adult male hippocampus. Such a rapid modulation of spines is certainly a novel observation and is essential for consideration of the synaptic plasticity affected by stress steroids.

Not only the density change but also the morphology change of dendritic spines were quantitatively observed within only 1 h by DEX treatments. The rapid effect of DEX is probably a non-genomic one, because cycloheximide did not inhibit DEX-induced spinogenesis. Because RU-486 (a specific inhibitor against GR) significantly suppressed rapid spinogenesis, the DEX acted via classical GR. A part of GR molecules, observed in Triton-insoluble synaptosomal fractions, may act at around spines without moving to nuclei. These rapid events are probably very different from the classical intranuclear actions of GR inducing transcriptional processes. Note that according to the classical view of the steroid action, GR is thought to move into nuclei and induce genomic effects through new protein synthesis, requiring 5–24 h for expression [1,2,13,14], which is a much slower process than the current spine change.

Significant distribution of GR-immunoreactivity has been demonstrated in pyramidal neurons in the CA1 and granule neurons in the DG, however only a weak GR-immunoreactivity has been observed in pyramidal neurons in the CA3 [10,15]. The current Western immunoblot analysis demonstrated that GR immunoreactivity was clearly observed in Triton-insoluble synaptosomal fractions which were enriched in postsynaptic membranes of hippocampal slices [9]. Support for extranuclear localization of GR comes from reports that the immunoreactivity of antibodies against GR has been associated with plasma membranes from hippocampal and hypothalamic neurons [16], and that specific CORT binding to neuronal membranes has occurred in different brain areas with moderate affinity ($K_d = 120$ nM) [17,18]. Classical GR has been expressed in the cytoplasm of cultured hippocampal neurons [19]. GR may drive not only classical genomic pathways by moving into nuclei [20] but also non-genomic pathways at around dendritic spines (the current study).

Although the effect of stress on hippocampal function has been attracting much attention, many investigations have focused on the chronic and slow effects of glucocorticoids, which have produced neuronal cell damage in the hippocampus [1,2]. As one of only a few examples of investigations regarding the rapid influence of GR on

memory performance, it has been reported that the acute injection of GR agonist RU-28362, 60 min before retention performance test of water-maze training, has induced the impairment of spatial memory retrieval of rats [21,22].

Interestingly, animal gender may be an important factor in the investigation of the effect of stress steroids. It is reported that stress-induced changes in dendritic spine density in the rat hippocampus have been opposite in the different genders [23,24]. A 1 h stressful event of intermittent tailshocks has increased the density of dendritic spines in males, observed after 24 h, but decreased the spine density in females at diestrus 2 period [23,24]. Therefore, our finding of the increase in spine density upon DEX treatments may be male-specific and may not occur in females.

Prior to the current study, insufficient information has been reported regarding the rapid modulation of spine morphology by glucocorticoids. Rapid actions of corticosterone on synaptic plasticity have mainly been investigated with electrophysiology (e.g., a suppression of LTP after a 30 min preperfusion of CORT in hippocampal slices, upon either tetanic or theta-burst stimulation [25,26]). Although electrophysiological investigations can only detect active spines (containing both AMPA receptors and NMDA receptors), the current single spine morphology imaging can also examine silent spines (containing only NMDA receptors) in addition to active spines. In this sense, single spine imaging provides a definite advantage during investigation of neurodegeneration in hippocampal damage or rescue by drugs such as estrogens, by looking at many different types of spines.

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