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ORIGINAL ARTICLE

Neuronal nitric oxide synthase is an endogenous negative regulator of glucocorticoid receptor in the hippocampus

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Abstract The hippocampus is rich in both glucocorticoid receptor (GR) and neuronal nitric oxide synthase (nNOS). But the relationship between the two molecules under physiological states remains unrevealed. Here, we report that nNOS knockout mice display increased GR expression in the hippocampus. Both systemic administration of 7-Nitroindazole (7-NI), a selective nNOS activity inhibitor, and selective infusion of 7-NI into the hippocampus resulted in an increase in GR expression in the hippocampus. Moreover, KCl exposure, which can induce overexpression of nNOS, resulted in a decrease in GR protein level in cultured hippocampal neurons. Moreover, blockade of nNOS activity in the hippocampus leads to decreased corticosterone (CORT, glucocorticoids in rodents) concentration in the plasma and reduced corticotrophin-releasing factor expression in the hypothalamus. The results indicate that nNOS is an endogenous inhibitor of GR in the hippocampus and that nNOS in the hippocampus may participate in the modulation of Hypothalamic–Pituitary–Adrenal axis activity via GR.

Keywords nNOS · GR · HPA axis · Hippocampus · Depression

Introduction

Depression is a severe psychiatric disorder with a multitude of symptoms, including lowered mood, anhedonia, low self-esteem and self-confidence [1]. This mood disorder will affect up to 20 % of the population all over the world based on a survey of the World Health Organization [2]. Hyperactivity of the Hypothalamic–Pituitary–Adrenal (HPA) axis has been characterized as a marker of people suffering from depression [3, 4].

The hippocampus is one of the components of the limbic systems in the brain. The hippocampus has been shown as being related with many important functions including learning and memory, mood, and fear [5]. Especially, the hippocampus is the main endogenous place that exerts negative feedback on the HPA axis [6]. However, the inhibitory effect of the hippocampus on the HPA axis is lost in most patients with major depression [7].

The glucocorticoid receptor (GR) is a widely expressed ligand-dependent transcription factor with its highest distribution in the hippocampus. When elevated glucocorticoids after stress arrive at the hippocampus, GR binds to the glucocorticoids and then exerts negative feedback on the HPA axis. Mounting evidence shows that the GR is a critical mediator of the inhibitory modulation of hippocampus on the HPA axis [8]. And importantly, dysfunctional GR signaling in the hippocampus has been shown to be involved in the pathogenesis of hyperactivity of the HPA axis and depressive behavior in mice and humans [9]. Various experiments have revealed that serotonin (5-HT) and noradrenaline (NA) upregulate GR expression [10]. However, the upstream molecules' negative regulating of GR expression has not been revealed.

nNOS is the predominant enzyme synthesizing nitric oxide (NO) in the brain [11]. NO is a free radical with many physiological functions, including neurogenesis, long-term potentiation, and synaptogenesis [12]. NO regulates the function of several molecular by cyclic guanosine monophosphate (cGMP) or cGMP-independent mechanisms

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[13]. Moreover, NO can react with superoxide O_2^- radical to generate peroxynitrite (ONOO-), which is diffusible. ONOO- can nitrate tyrosine residues and consequently modify the functional properties of several proteins [14]. Both GR and neuronal nitric oxide synthase (nNOS) are highly distributed in the hippocampus [15, 16]. Hence, it is possible that hippocampal nNOS-derived NO regulate GR expression via NO-sGC-cGMP pathway or NO-ONOO- pathway or both of them. However, the relationship between the two important molecules, GR and nNOS, in hippocampus under physiological state remains unclear. Here, we report a novel relationship between the two molecules that nNOS acts as an important negative regulator of GR expression in the adult hippocampus. More importantly, hippocampal nNOS is involved in regulating HPA axis activity possibly via modulating GR signal. This finding expands nNOS inhibitors application in curing hippocampal GR impaired function-related disorders including HPA axis hyperactivity and depression.

Materials and methods

Young adult (6–7-week-old) male homozygous nNOS-deficient mice (B6; 129S4-*Nos1tm1Plh*, KO, stock number: 002633) and their wild-type controls of similar genetic background (B6129SF2, WT) (both from Jackson Laboratories; maintained at Model Animal Research Center of Nanjing University, Nanjing, China). All procedures were done in accordance with the NIH guidelines for the care and use of laboratory animals and the Jiangsu Province Institutional Animal Care and Use Committee. 7-nitroindazole (7-NI; 30 mg/kg/d) was intraperitoneally injected and was purchased from Sigma-Aldrich.

Western blot analysis of samples from hippocampal or hypothalamic tissues of animals was performed as described previously [17]. The hippocampus was homogenized in ice-cold lysis buffer containing 100 mmol/L HEPES, 200 mmol/L NaCl, 10 % glycerol, 2 mmol/L $Na_4P_2O_7$, 2 mmol/L dithiothreitol, 1 mmol/L EDTA, 1 mmol/L benzamidine, 0.1 mmol/L Na_3VO_4, 1 mmol/L pepstatin, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μmol/L phenylmethylsulfonyl fluoride at pH 7.4. After lysis for 15 min, samples were centrifuged at 20,000*g* for 15 min. The samples containing equivalent amounts of protein (20 μg) were applied to 8 % acrylamide denaturing gels (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The separated proteins were transferred onto nitrocellulose membranes (Millipore). Blotting membranes were incubated with blocking solution [5 % non-fat dried milk powder dissolved in tris-buffered saline tween-20 (TBST) buffer (pH 7.5, 10 mmol/L Tris–HCl, 150 mmol/L NaCl, and 0.1 % Tween 20)] for 1 h at 22–25 °C, washed three times, and then were incubated with mouse anti-β-Actin (1:1000; Sigma), or mice anti-GR (1:200; Santa Cruz Biotechnology) in TBST overnight at 4 °C. Appropriate horseradish peroxidase-linked secondary antibodies were used for detection by enhanced chemiluminescence (Pierce).

Serial hippocampal sections (40 μm) were made on an oscillating tissue slicer. For nNOS and GR immunofluorescence, the sections were incubated with rabbit anti-nNOS antibody (1:500; Zymed Laboratories) or mice anti-GR antibody (1:500; Santa Cruz Biotechnology) in 0.1 M PBS with 3 % goat serum and 0.3 % Triton X-100, and binding was visualized with a Cy3-conjugated secondary antibody (1:200; Millipore Bioscience Research Reagents).

Primary hippocampal neurons were isolated and cultured as reported [16], with minor modifications. In brief, the hippocampus of embryonic day 18 mice was freshly isolated and enzymatically dissociated in calcium- and magnesium-free HBSS containing 0.125 % trypsin at 37 °C for 10 min. Then the digestion was terminated with DMEM/F12 (1:1) containing 10 % FBS and tissues were triturated with a fire-polished glass pipet. Dissociated cells were centrifuged, resuspended in Neurobasal medium (Invitrogen) containing 2 % B27 supplement, 0.5 mM L-glutamine, 5 IU penicillin, and 5 μg/ml streptomycin, and plated on 10 μg/ml polyornithine-coated dishes (diameter, 3.5 cm) at 5×10^4 cells/cm^2. Half of the medium was replaced with fresh Neurobasal/B27 medium every 4–5 days. Cultured neurons at 10 days in vitro were exposed to KCl at final dose of 50 mM for 24 h and then were collected and homogenized in ice-cold lysis buffer for detecting protein level.

For basal corticosterone level measurement, mice were decapitated between 9:00 and 10:00 a.m. Blood from angulus oculi vessels was collected in heparinized tubes, and corticosterone in plasma was measured with a corticosterone ELISA kit according to the instructions of the manufacturer (Cayman Chemical).

Mice were anesthetized with a solution of chloral hydrate (30 mg/kg, i.p.). Stereotaxic surgery was used to deliver 7-NI into the dentate gyrus (DG) of the hippocampus or the SVZ of the anterior part of the lateral ventricle. 7-NI was dissolved in a solution (DMSO). The 7-NI solution in 2 μl volume (10 μM) or DMSO (2 μl) was infused into the DG (0.2 μl/min) at coordinates 2.3 mm posterior to the bregma, 1.3 mm lateral to the midline, and 2.0 mm below the dura [17].

Results

To address whether nNOS may be involved in the modulation of GR, we compared the sites of GR

expression with the sites of nNOS expression in the adult mice hippocampus. The immunohistochemical results show that nNOS-positive cells mainly exist in CA3, hilus, and "the other region" which is referred as the rest regions excluding CA1, CA3, hilus, and granular cell layer from the hippocampus. About 23.18, 21, and 42.7 % of total numbers of nNOS-positive cells in the whole hippocampus localize in each region, respectively. And, nNOS-positive cells rarely exist in granular cell layer and CA1 (1.6 and 11.52 % of total numbers in the whole hippocampus in each region, respectively) (Fig. 1a, c). Inversely, the fluorescence intensity of GR-positive cells are weak in CA3, hilus and the other region (9.48, 1.65, and 4.21 % of total fluorescence intensity in the whole hippocampus in each region, respectively) and strong in granular cell layer and CA1 (40.65 and 44.01 % of total fluorescence intensity in the whole hippocampus in each region, respectively) (Fig. 1b, c). However, the expression of nNOS and GR does not always co-localize in the same cells because the number of nNOS-positive cells is significantly less than that of GR-positive cells. Therefore, the distribution suggests nNOS may regulate GR by NO due to that NO is diffusible (Fig. 3).

To further determine whether nNOS negatively regulates GR expression, we compared the expression of GR in the hippocampus of nNOS knockout (KO) mice with their wild-type (WT) mice by Western blot and found that GR expression level is significantly higher in nNOS KO mice compared to wild-type mice ($p < 0.05$, t test) (Fig. 2a). Furthermore, we observed that GR expression in the hippocampus is markedly increased in the mice treated with 7-NI (i.p., 30 or 50 mg/kg/d), which is a selective nNOS activity inhibitor, for 7 days ($p < 0.01$ for both dosage, t test) (Fig. 2b). Besides protein level, mRNA level of GR in the hippocampus of mice treated with 7-NI (i.p., 30 or 50 mg/kg/d) for 7 days is markedly increased in comparison to mice treated with DMSO (data not shown). It had been demonstrated that 50 mM KCl exposure induced nNOS overexpression [18], and we confirmed that 50 mM KCl exposure for 24 h resulted in a marked increase of nNOS expression in cultured neurons by Western blotting (data not shown) and an increased NO synthesis by 3-amino,4-aminomethyl-2′7′difluorescein diacetate (DAF-FM) staining [19]. More convincingly, we found that nNOS overexpression by 50 mM KCl exposure for 24 h significantly decreased GR expression in the cultured hippocampal neurons ($p < 0.05$, t test) (Fig. 2c).

However, the intraperitoneal injection may cause systemic effects and lack of selectivity. To exactly determine the role of nNOS in the hippocampus, we delivered 7-NI (2 μl, 10 μM) solution into the bilateral DG of the hippocampus by stereotaxic surgery to inhibit local nNOS activity. Infusion of 7-NI resulted in an increase in GR protein ($p < 0.05$, t test) (Fig. 2d) and mRNA level (data not shown) 7 days later. Meanwhile,

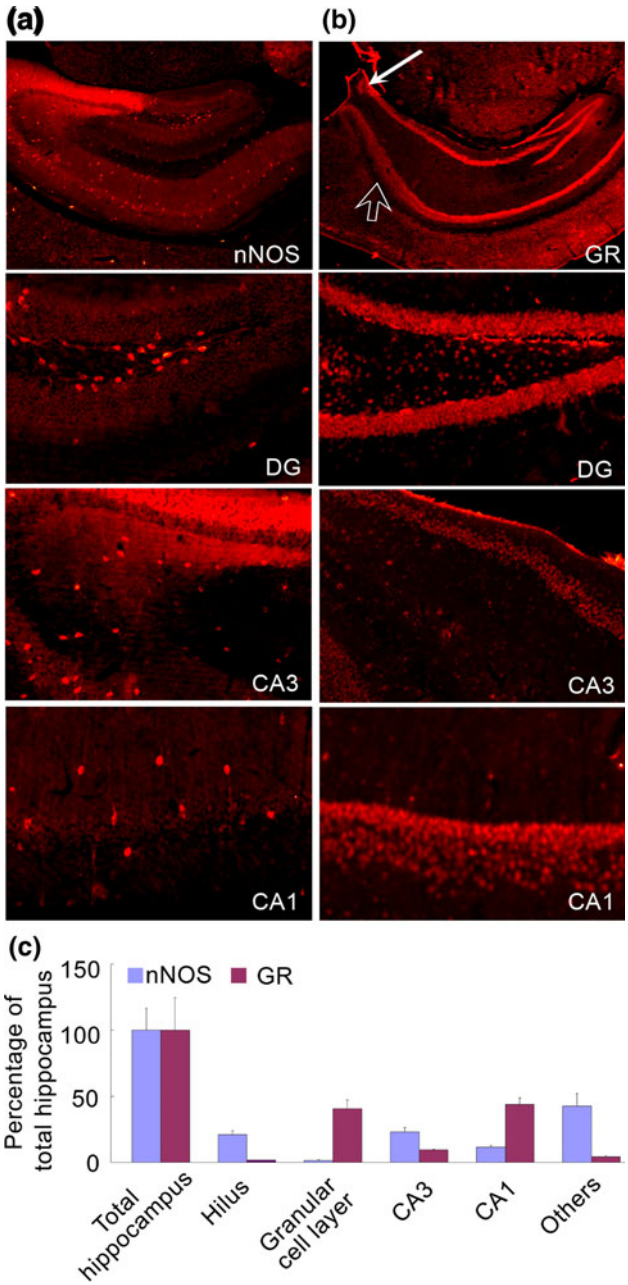

Fig. 1 Distribution of nNOS and GR in adult hippocampus. (**a**) Representative images of nNOS-positive cells in the hippocampus. (**b**) Representative imaging of GR-positive cells in the hippocampus. *Arrow* indicates absence of GR-positive cells in the CA2 same as the previous reports. *Arrow head* indicates fluorescence intensity GR-positive cells are weak in CA3. (**c**) The percentage of nNOS-positive cells numbers of various zones to those of the whole hippocampus and the percentage of GR-positive cells fluorescence intensity of various zones to that of the whole hippocampus. The present data is the mean value of three different mice

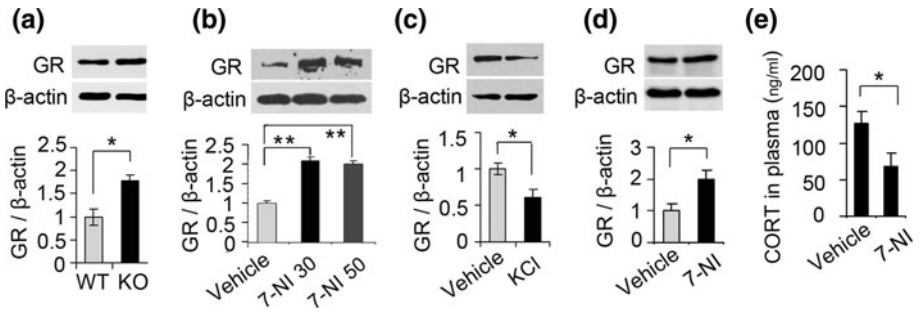

Fig. 2 nNOS represses GR expression in adult hippocampus. (**a**) Immunoblots showing hippocampal GR levels of the nNOS KO and WT mice ($n = 4$). (**b**) Immunoblots showing hippocampal GR levels of the mice treated with 7-NI (30 mg/kg, i.p.) or DMSO for 7 days ($n = 3$). (**c**) Representative imaging of GR levels in the hippocampal neurons exposed to 50 mM KCl for 24 h ($n = 3$). (**d**) Immunoblots showing hippocampal GR levels of the mice infused with 7-NI (2 μl, 10 μM) or DMSO 7 days after surgery ($n = 4$). (**e**) Plasma corticosterone levels of the adult mice infused with 7-NI (2 μl, 10 μM) or DMSO into the DG of hippocampus 7 days after surgery ($n = 6$). *Error bars* denote SEM, $*p < 0.05$, $**p < 0.01$, *t* test

more interestingly, infusion of 7-NI resulted in a marked decrease in the concentration of CORT in the plasma 7 days after stereotaxic surgery (127.3562 ± 15.64966 for DMSO group vs. 67.85979 ± 17.49656 for 7-NI group, $p < 0.05$, *t* test), suggesting reduced activity of HPA axis (Fig. 2e). The synthesis of CRF in the hypothalamus triggers the onset of HPA axis. More convincingly, infusion of 7-NI caused a downregulation of CRF expression in the hypothalamus 7 days after stereotaxic surgery (data not shown).

Discussion

The results of this study are the first to show that nNOS is an endogenous inhibitory factor of GR expression in adult hippocampus under physiological state. Notably, we also found that nNOS in the hippocampus may be an modulator of HPA axis activity through GR. Interestingly, the nNOS enriched regions such as CA3, hilus and the other regions excluding CA1, CA3, hilus, and granular cell layer, there is lack of GR expression. This phenomenon implies that GR

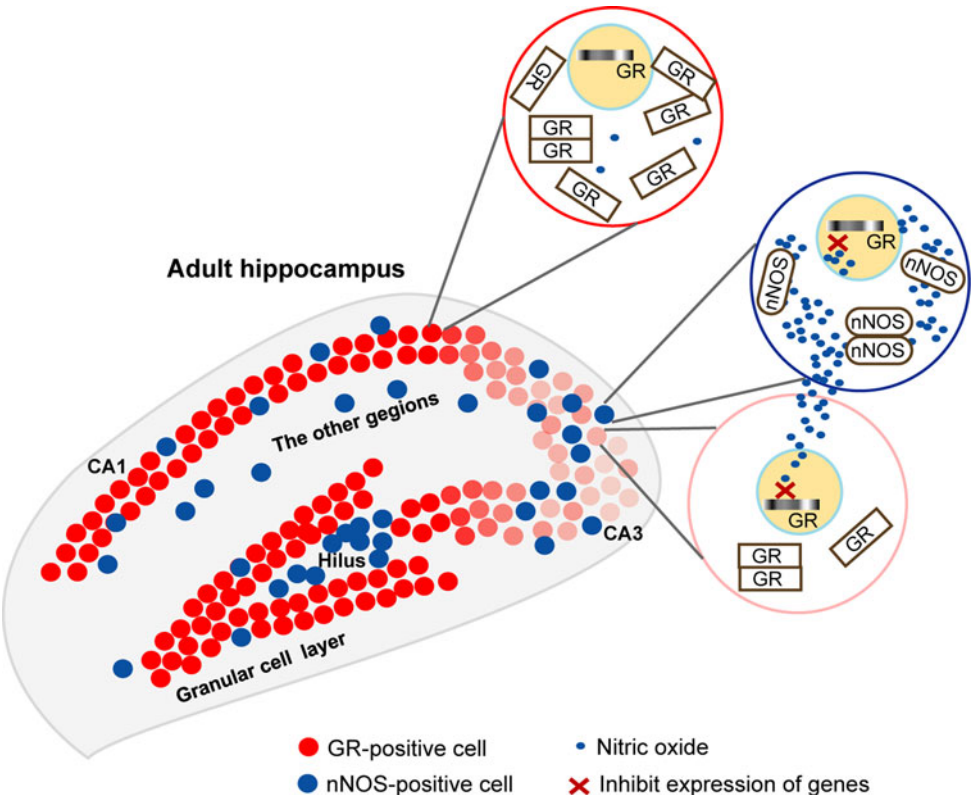

Fig. 3 A model of the relationship between nNOS and GR in adult hippocampus. High amount of NO produced by nNOS repress gene expression of GR in nNOS-expressing cells and in their adjacent cells in CA3, hilus and the other regions. Low amount of NO can not repress gene expression of GR in CA1 and granular cell layer because of rare nNOS-expressing cells in these regions and the limitation of the diffusion ability of NO

expression and function are refrained by nNOS in the hippocampus with important physiological significance in normal life. nNOS may regulate wide signal pathways and functions mediated by GR.

nNOS is the main source of NO in the hippocampus. The action of NO in the hippocampus mainly accounts for the function of nNOS [11]. Galigniana et al. [20] found that NO inhibits GR binging to glucocorticoids by S-nitrosylation of critical -SH groups in GR. And it was reported that treatment with L-NAME (50 mg/kg/day), an inhibitor of NOS, for 7 days increase GR mRNA in the hippocampus [21]. Therefore, nNOS may negatively regulate GR not only at expression level but also at function level (binding affinity to ligand). In the future study, whether hippocampal nNOS represses GR binding function needs to be determined. However, how does nNOS regulate GR expression? NO is the main mediator of the biological effect of nNOS in the central nervous system. We hypothesize that the NO produced by nNOS exerts inhibitory effect of gene expression of GR in the same cell. Besides, the produced NO can diffuse into the adjacent cells and inhibit GR gene expression in these cells (Fig. 3). So, GR expression in those regions such as CA1 and granular cell layer is especially abundant possibly due to the rare number of nNOS-expressing cells in those regions (Figs. 1 and 3).

Previous reports have shown that neurotransmitters including 5-HT and NA upregulate GR mRNA and protein levels in the hippocampus [10]. Moreover, fluoxetine, a 5-HT reuptake inhibitor, increases GR expression in hippocampus. Both 5-HT and fluoxetine treatment decreases nNOS expression in the hippocampus [22], implying that 5-HT and fluoxetine may increase GR expression through nNOS pathway. It is worth noting that we find increased glucocorticoid levels in the plasma following 7-NI infusion into the hippocampus. Consistently, it is reported by Pinnock et al. [21] that treatment with L-NAME, a nonselective NOS inhibitor, for 7 days (50 mg/kg, i.p.) reduces plasma corticosterone significantly. The onset step of HPA axis activation is CRF synthesis and secretion in the hypothalamus. In combination with the evidence that 7-NI infusion into the hippocampus reduced CRF expression in the hypothalamus, increased glucocorticoid levels in the plasma following 7-NI infusion into the hippocampus suggest that nNOS/NO/GR pathway may mediate the negative regulation effect of hippocampus on HPA axis activity. Therefore, 5-HT and fluoxetine may regulate HPA axis activity through 5-HT/nNOS/NO/GR pathway.

Chronic stress-induced GR impairment including reduced expression and decreased ligand-binging ability in the hippocampus is a critical process in the pathology of depression [23]. Our previous research has demonstrated that chronic stress result in nNOS overexpression in the hippocampus, which contributes to the depressive behavior of depression [17]. Here, our finding that nNOS is a repressor of GR in the hippocampus suggest that overexpression of nNOS may mediate chronic stress-induced GR impairment in the hippocampus. More importantly, our results suggest that nNOS in the hippocampus may be involved in the process of the pathophysiology of HPA axis hyperactivity in depression. It has been demonstrated that NOS/NO/cGMP/PKG pathway regulating hippocampal function during pathological conditions such as post-ischemic injury [24]. Therefore, nNOS/NO/cGMP/PKG pathway may mediate chronic stress-induced GR impairment in the hippocampus and HPA axis hyperactivity in depression.

Taken together, the results demonstrate that the nNOS in the hippocampus represses GR expression and is involved in the modulation of HPA axis. The effects of nNOS on the multiple functions mediated by GR needs to be investigated.

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Conflict of interest The author reports no biomedical financial interests or potential conflicts of interest.

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