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Chronic treatment with glucocorticoids alters rat hippocampal and prefrontal cortical morphology in parallel with endogenous agmatine and arginine decarboxylase levels

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Abstract

In the present study, we examined the possible effect of chronic treatment with glucocorticoids on the morphology of the rat brain and levels of endogenous agmatine and arginine decarboxylase (ADC) protein, the enzyme essential for agmatine synthesis. Seven-day treatment with dexamethasone, at a dose (10 and 50 µg/kg/day) associated to stress effects contributed by glucocorticoids, did not result in obvious morphologic changes in the medial prefrontal cortex and hippocampus, as measured by immunocytochemical staining with β-tubulin III. However, 21-day treatment (50 µg/kg/day) produced noticeable structural changes such as the diminution and disarrangement of dendrites and neurons in these areas. Simultaneous treatment with agmatine (50 mg/kg/day) prevented these morphological changes. Further measurement with HPLC showed that endogenous agmatine levels in the prefrontal cortex and hippocampus were significantly increased after 7-day treatments with dexamethasone in a dose-dependent manner. On the contrary, 21-day treatment with glucocorticoids robustly reduced agmatine levels in these regions. The treatment-caused biphasic alterations of endogenous agmatine levels were also seen in the striatum and hypothalamus. Interestingly, treatment with glucocorticoids resulted in a similar change of ADC protein levels in most brain areas to endogenous agmatine levels: an increase after 7-day treatment versus a reduction after 21-day treatment. These results demonstrated that agmatine has neuroprotective effects against structural alterations caused by glucocorticoids *in vivo*. The parallel alterations in the endogenous agmatine levels and ADC expression in the brain after treatment with glucocorticoids indicate the possible regulatory effect of these stress hormones on the synthesis and metabolism of agmatine *in vivo*.

Keywords

agmatine; arginine decarboxylase; glucocorticoids; hippocampus; medial prefrontal cortex; β-tubulin III

Glucocorticoids are adrenal steroids secreted during stress and their numerous actions are essential to the stress response (Munck *et al.* 1984). However, prolonged glucocorticoid exposure can be pathogenic (Sapolsky *et al.* 1985). Recent work has shown that sustained exposure of experimental animals to glucocorticoids can cause neuronal degeneration or accelerate the neuron loss, especially in the hippocampus (Sapolsky *et al.* 1990; Uno *et al.* 1990; Woolley *et al.* 1990; Watanabe *et al.* 1992), a principal neural target region for glucocorticoids, with high concentrations of both types of corticosteroid receptors (McEwen *et al.* 1986). The prefrontal cortex (PFC) is another target region for glucocorticoid effects, as the PFC has also a rich population of corticosteroid receptors (Chao *et al.* 1989; Herman 1993). Chronic treatments with corticosterone (CORT) produced neuronal impairment in the PFC including the remodeling of pyramidal neurons, significantly reduced distal dendritic spines of neurons and neuronal loss (Wellman 2001; Seib and Wellman 2003; Cerqueira *et al.* 2005). Although the pathophysiological mechanisms regarding neuronal alteration caused by stress and glucocorticoids are still unclear, glucocorticoid-induced excitotoxic condition may be one key factor. These structural alterations consequently impaired the functions contributed by these brain areas (Landfield *et al.* 1981; Cerqueira *et al.* 2005). Given that dysfunctions in the hippocampus and PFC have been implicated to mood disorders and neurodegenerative diseases, it would be meaningful in clinical practice to find an agent to protect these neurons against glucocorticoid-induced neuronal alteration.

Agmatine is an endogenous polyamine which has been proposed as a neurotransmitter or neuromodulator in the brain. In the past decade, many studies revealed that agmatine blocks NMDA receptor (NMDA-R) channels and inhibits all isoforms of nitric oxide synthase (Reis and Regunathan 2000). These characteristics of agmatine may contribute to its functional role in the CNS. In fact, agmatine has been reported to exert neuroprotective action by reducing the size of ischemic infarctions or preventing the loss of cerebella neurons after focal or global ischemia *in vivo* (Gilad *et al.* 1996; Kim *et al.* 2004). Our previous works demonstrated neuroprotective effects of agmatine against neuronal damage caused by glucocorticoids and glutamate in primary neuronal cultures of hippocampus and cell lines (Zhu *et al.* 2003, 2006; Wang *et al.* 2006). Further studies are warranted to explore whether agmatine can protect neurons against neuronal insults caused by glucocorticoids *in vivo*.

Agmatine is mainly synthesized in glia cells following decarboxylation of L-arginine by arginine decarboxylase (ADC) (Tabor and Tabor 1984) and preponderantly localized in neurons (Otake *et al.* 1998). Immunocytochemical studies have demonstrated that agmatine is widely distributed in the brain, but enriched in the hippocampus and frontal cortex (Otake *et al.* 1998; Reis *et al.* 1998). As a possible contributor of neuroprotective mechanisms *in vivo*, endogenous agmatine levels and its synthesis may be influenced by excitatory insults from prolonged treatments with glucocorticoids. This presumption has been tested in the present study to explore the possible regulation of the endogenous agmatine system by glucocorticoids. Rats were chronically treated with glucocorticoids to mimic the effects of stress, and neuroprotective effects of agmatine in the medial prefrontal cortex (mPFC) and hippocampus were examined. Furthermore, the assessment of endogenous agmatine levels and ADC protein in rat brain areas were conducted. The results reveal a potential influence of glucocorticoids on endogenous agmatine biosynthesis.

Materials and methods

Animals and drug treatment

Male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) weighing 250–300 g at the time of initiation of drug treatment were housed on a 12 h light/dark cycle and with food and water provided *ad libitum*. All animal procedures were approved by the UMMC Animal Care and Use Committee and complied with the NIH Guide for the Care and Use of

Laboratory Animals. After an acclimation period of 5 days, rats were randomly assigned to the experimental and placebo groups. Water soluble dexamethasone was administered subcutaneously by means of osmotic mini-pumps (Alza, Palo Alto, CA, USA) for 7 or 21 days. Dexamethasone at the doses of 1.25 (Calvo and Volosin 2001), 40, 200 $\mu\text{g/kg}$ (Stohr *et al.* 1999), or 400 $\mu\text{g/kg}$ (Presse *et al.* 1992) s.c. was reported to restore stress effect in adrenalectomized rats. According to this information and our preliminary study, we used two doses (10 and 50 $\mu\text{g/kg/day}$ dissolved in saline; Sigma, St Louis, MO, USA) for intact rats. Saline instead of dexamethasone was used in mini-osmotic pumps for rats in the control group. In some groups of rats, agmatine (50 mg/kg/day; Sigma) was delivered by mini-pumps together with dexamethasone. CORT was delivered by sustained-release pellets (21-day release pellets; Innovative Research of America, Toledo, OH, USA). It was reported that administration of CORT (1–5 mg/kg/day, s.c.) resulted in an increased plasma CORT levels similar to that in stress animals (Fleshner *et al.* 1995; Stohr *et al.* 1999; Calvo and Volosin 2001). CORT pellet of 35 mg for a 21-day release corresponded to 1.67 mg/day, a dose stated by the manufacturer to result in stress-like CORT levels in blood (Betancur *et al.* 1994). Therefore, in the present study two different CORT pellets (10 and 50 mg) were used for both time schedules (7 and 21 days). The 21-day release pellet of 10 mg CORT would correspond to a dose of 1.90 mg/kg/day for the rat with the weight of 250 g, and pellets of 50 mg would be 9.52 mg/kg/day. Control rats were implanted with placebo pellet (12.5 mg cholesterol). The mini-pumps and pellets were implanted subcutaneously in the interscapular regions of rats using general anesthesia with pentobarbital sodium (50 mg/kg, i.p.; Sigma). On the 8th or 22nd day, all rats for biochemical measurements were killed by decapitation. The rat brain regions used for HPLC measurements were rapidly dissected out on ice and immediately processed for the preparation of HPLC assay. Rat brains used for western blotting were temporarily frozen in 2-methylbutane on dry ice until further dissection. The rats used for immunostainings were transcardially perfused under anesthesia with pentobarbital using 4% *p*-formaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Brains were further stored in 10% sucrose followed by 30% sucrose until sectioning. Brains were sectioned at 30 μm by a sliding microtome.

Measurement of agmatine in brain tissues of rats by HPLC

The amount of agmatine of brain tissues in control and treated rats was measured by HPLC as described earlier (Raasch *et al.* 1995; Feng *et al.* 1997). Brain tissues were homogenized after dissection and re-suspended in phosphate buffer (pH 5.7) containing amino picollinate as internal standard. The cell suspension was then homogenized in 10% trichloroacetic acid, spun down and the supernatant used for HPLC analysis. Samples were derivatized with *o*-phthalaldehyde and injected into a reverse phase column (5 μm) and fluorescence detector. Recover of agmatine was calculated from the added external standard and expressed as $\mu\text{g/g}$ wet tissue.

Detection of ADC protein levels

Arginine decarboxylase protein levels in brain areas were determined by western blotting using a polyclonal antibody raised from rabbits against a unique 17 amino acid ADC peptide by a commercial vender (Sigma-Genosys, Woodlam, TX, USA) as described previously (Iyo *et al.* 2006). The IgG fraction of this antiserum was purified using protein A column (Pierce, Rockford, IL, USA) and the purified IgG was characterized for its specificity by pre-absorption with the antigen peptide (Iyo *et al.* 2006). Rat brain regions were dissected out as previously described (Palkpivits and Brownstein 1998) and homogenized in HEPES buffer (pH 7.4). After centrifugation at 1000 *g*, the supernatants were centrifuged at 30 000 *g* for 20 min and the resulting pellets were solubilized in sodium dodecyl sulfate sample buffer for ADC immunoblotting analysis. Equal amounts of homogenized proteins were loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel for electrophoresis and transferred by electroblotting to a polyvinylidene difluoride membrane. The ADC protein was detected after

exposing to the ADC antibodies at 1/3000 dilution. The membranes were processed for immunoblot analysis using enhanced chemiluminescence detection system (Amersham, Piscataway, NJ, USA). The immunoreactive band was visualized using Kodak ImageStation (New Haven, CT, USA) and quantitated using the image software.

Immunostaining with β -tubulin III

Immunocytochemical staining was performed using a commercial monoclonal antibody to β -tubulin III (β T-III) raised from mouse (Chemicon, Temecula, CA, USA). Brain sections were washed three times in phosphate-buffered saline (PBS) and pre-incubated in 5% bovine serum albumin in PBS supplemented with 0.2% Triton-X 100 for 1 h at 25°C, followed by incubation in primary antibody (1 : 500 dilution, in PBS containing 0.2% Triton-X 100) overnight at 4°C. After washing, sections were then incubated with secondary antibody, biotinylated goat anti-mouse (1 : 200) for 2 h in 25°C, followed by continuous incubation in avidin–biotin complex using ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the instructions of the manufacturer. The signals were visualized by incubating sections in 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.07% NH_4NiSO_4 , and 0.003% H_2O_2 . Some slides were processed to determine the specificity of this immunostaining using the same procedures as described above except that the primary antibody was not applied. No positive immunostaining was found in these slides (data not shown). In order to reduce inter-animal staining variability, each immunohistochemical staining experiment was performed on all tissue sections at the same time by an investigator blinded to the animal group.

Immunohistochemical analysis

β -Tubulin III immunoreactivity was evaluated by the density analysis using ImageJ software (version 1.37) (Rasband, W.S., National Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>, 1997–2006). The CA1 and CA2 fields of the hippocampal formation and the dorsal anterior cingulate cortex (ACd), pre-limbic cortex (PL) and infralimbic cortex (IL) of the mPFC were examined. First, images were acquired in an Olympus BX41 microscope equipped with an Olympus U-TVO digital camera (Olympus, Tokyo, Japan), which is connected to the computer installed with the MagnaFire image software (Goleta, CA, USA). Three area fractions under high power (100 $\times$ oil immersion objective lenses) were captured from the same region of each section, respectively. Then, these grey-scale images were thresholded in ImageJ with a fixed level over the background in order to improve the quality of the images as much as possible. The threshold values for immunoreactive dendrites were obtained by manually sampling the signal intensity in each image, which was visually compared with the original grey-scale images to ensure that the tool effectively resolved all labeled fibers. Thereafter, the intensity of β T-III immunoreactivity (dendrites) in the area fractions was semiquantitatively obtained using the measurement tool of ImageJ and expressed as a percentage of area. The average of the measurements obtained in three rats was considered the value of the area fraction for the hippocampus or the sub-regions of the mPFC.

Statistics

Data are presented as mean $\pm$ SEM values. For the measurements of immunoreactivity, agmatine and ADC protein levels in brain areas, data were analyzed by ANOVA using one-way ANOVA (SigmaStat, SYSTAT Software, Inc, Richmond, CA, USA). In the presence of significant F values, individual comparisons between means were made using the Student's–Newman–Keuls test.

Results

Neuroprotective effect of agmatine on dexamethasone-induced neuronal damage in brain areas

Consistent to our previous study *in vitro* (Zhu *et al.* 2006), only dexamethasone was used in this experiment. Immunocytochemical staining using monoclonal antibody to β T-III was performed on sections of the PFC and hippocampus in rats ($n = 3$ for each group) chronically treated with dexamethasone. Microscopic analysis showed that there were no obvious morphological changes in the hippocampus and mPFC after treatment with dexamethasone either at the doses of 10 and 50 μ g/kg/day for 7 days or at the dose of 10 μ g/kg/day for 21 days. However, treatment with dexamethasone at the dose of 50 μ g/kg/day for 21 days caused noticeable structural alterations in these regions from all treated animals. Compared with the intensive β T-III immunostaining of the hippocampal formation in control animals, the immunostaining of the perikarya of granule cells and apical dendrites extending through the striatum radiatum and stratum lacunosum-moleculare in the CA1 and CA2 was noticeably reduced (Fig. 1). The immunostaining of mossy fibers in the hippocampal CA3 of treated animals was also considerably reduced (data not shown). Under the microscope, CA1 and CA2 pyramidal neurons had disintegrated and apical dendrites were broken or missing. Similarly, the mPFC of rats treated with higher dose of dexamethasone showed a diminution of β T-III immunoreactivity, indicating a possible loss of β T-III-positive neuronal fibers in this region. There were broken dendrites with lost-orientation in the ACd, PL, and IL of the mPFC (Fig. 2), in contrast to a homogeneous and compact pattern in control animals. The simultaneous treatment with both agmatine and dexamethasone prevented these structural changes in both hippocampus and mPFC. Microscopic analysis revealed an almost similar morphological pattern of dendrites in these regions to those in the control animals, indicating a neuronal protective effect of agmatine (Fig 1 and Fig 2).

β -tubulin III immunoreactive intensities were semiquantitatively examined in sections of the hippocampus and mPFC from experimental animals. As shown in Fig. 3, there were significant differences in the β T-III immunoreactivity in the CA1 ($F_{2,14} = 17.43$, $p < 0.01$) and CA2 ($F_{2,11} = 11.50$, $p < 0.01$) of the hippocampus, as well as in the regions of ACd ($F_{2,11} = 23.34$, $p < 0.01$), PL ($F_{2,10} = 9.61$, $p < 0.01$) and IL ($F_{2,10} = 26.41$, $p < 0.001$) of the mPFC from rats of the control, treatment with dexamethasone alone or in combination with agmatine. *Post hoc* analysis revealed that treatment with dexamethasone significantly reduced β T-III immunoreactive intensity in CA1 and CA2 by 22.72% and 23.95% (both $p < 0.05$), respectively, when compared with the control. Treatment with agmatine prevented this reduction in the CA1 and CA2 but this effect did not reach the significant levels, when compared with the dexamethasone treatment (Fig. 3a). Similarly, treatment with dexamethasone significantly reduced β T-III immunoreactivity in the areas of ACd by 48.83% ($p < 0.01$), PL by 34.78% ($p < 0.05$), and IL by 29.75% ($p < 0.01$) of the mPFC. Likewise, treatment with agmatine abolished the effect of dexamethasone treatment on β T-III immunoreactive intensity in the ACd, PL and IL areas of the mPFC (all $p < 0.05$), when compared with the group of dexamethasone treatment (Fig. 3b).

Change of endogenous agmatine level in the PFC and hippocampus after chronic treatment with glucocorticoids

The effect of dexamethasone treatment on endogenous agmatine levels was tested by means of HPLC. CORT was included in this experiment to verify whether the natural glucocorticoid and dexamethasone have similar effects. As shown in Fig. 4, 7 day administration of dexamethasone significantly increased endogenous agmatine concentrations in the PFC ($p < 0.05$, $F_{2,16} = 4.89$) and hippocampus ($p < 0.05$, $F_{2,16} = 5.76$) in a dose-dependent manner. *Post hoc* analysis revealed that the higher dose (50 μ g/kg/day) of dexamethasone increased

endogenous levels of agmatine in the PFC and hippocampus by 25.5% ($p < 0.05$) and 31.5% ($p < 0.05$), respectively, compared with those of control animals. Similarly, there was a significant effect of CORT on endogenous agmatine levels in the PFC ($p < 0.05$, $F_{2,16} = 6.23$) and hippocampus ($p < 0.05$, $F_{2,16} = 5.87$). *Post hoc* analysis revealed that higher dose (9.52 mg/kg/day) of CORT increased agmatine levels by 35.9% and 41.8% (both $p < 0.05$) in these areas, respectively. The lower doses of dexamethasone and CORT did not significantly influence agmatine levels.

In contrast to the 7-day-treatment, 21-day treatment with glucocorticoids produced a pronounced reduction in endogenous levels of agmatine in the PFC ($p < 0.05$, $F_{2,13} = 5.95$ for dexamethasone; $p < 0.05$, $F_{2,13} = 5.32$ for CORT) and hippocampus ($p < 0.05$, $F_{2,13} = 6.87$ for dexamethasone; $p = 0.015$, $F_{2,13} = 7.43$ for CORT) (Fig. 5). *Post hoc* analysis revealed that dexamethasone at the dose of 50 $\mu\text{g/kg/day}$ reduced endogenous agmatine levels in the PFC and hippocampus by 31.1% and 34.8% (both $p < 0.05$), respectively, compared with those of control animals. CORT at the dose of 9.52 mg/kg/day reduced agmatine levels in these two areas by 29.2% ($p < 0.05$) and 56.7% ($p < 0.01$), respectively. The lower doses of glucocorticoids did not significantly affected endogenous levels of agmatine in these areas.

Change of endogenous agmatine levels in the striatum and hypothalamus after chronic treatment with glucocorticoids

The endogenous levels of agmatine in the striatum and hypothalamus were measured to assess whether agmatine levels in other brain areas were affected by the treatment with glucocorticoids. Similar to those observed in the PFC and hippocampus, glucocorticoid treatment for 7 days enhanced endogenous levels of agmatine in the striatum ($p < 0.05$, $F_{2,16} = 4.91$ for dexamethasone and $p < 0.05$, $F_{2,16} = 5.02$ for CORT) and hypothalamus ($p < 0.05$, $F_{2,16} = 7.23$ for dexamethasone and $p < 0.05$, $F_{2,16} = 5.35$ for CORT, Table 1). *Post hoc* test revealed that agmatine levels in the striatum were significantly increased after treatment with dexamethasone or CORT at the higher doses (both $p < 0.05$). While 10 and 50 $\mu\text{g/kg/day}$ dexamethasone significantly increased endogenous agmatine concentrations in the hypothalamus both by 59%, respectively ($p < 0.05$), only the higher dose of CORT showed a similar effect ($p < 0.05$). On the other hand, 21-day treatment with dexamethasone significantly reduced agmatine levels in the striatum ($p < 0.05$, $F_{2,13} = 7.02$) and hypothalamus ($p < 0.05$, $F_{2,13} = 6.03$) in a dose-dependent manner. *Post hoc* test showed in these areas a respective reduction of 51% ($p < 0.05$) and 36.7% ($p < 0.05$) by 50 $\mu\text{g/kg/day}$ dexamethasone. While treatment with CORT for 21 days did not affect agmatine levels in the hypothalamus ($p > 0.05$) (Table 1), same regime with CORT at the dose of 9.52 mg/kg/day resulted in a reduction of agmatine levels in the striatum by 28.6% ($p < 0.05$, $F_{2,13} = 4.54$).

Change of ADC protein levels in brain areas after chronic treatment with glucocorticoids

Arginine decarboxylase protein levels in the selected areas of the rat brain were analyzed by western blotting to examine whether treatment with glucocorticoids affects the expression of this enzyme. Rat brain tissues from each selected brain region used as controls for the dexamethasone- and CORT-treatment groups were pooled, when homogenates were prepared for western blotting.

There was a moderate, though significant, increase of ADC proteins in the PFC after 7-day treatment with dexamethasone ($p < 0.05$, $F_{4,14} = 6.43$) or CORT ($p < 0.05$, $F_{4,14} = 7.64$). *Post hoc* analysis revealed that dexamethasone at the doses of 10 and 50 $\mu\text{g/kg/day}$ increased ADC protein levels in the PFC by 22% ($p > 0.05$) and 34.7% ($p < 0.05$), respectively, compared with those of the control in the same area (Fig. 6). Treatment with CORT at the doses of 1.9 and 9.52 mg/kg/day increased ADC protein by 20.4% ($p > 0.05$) and 40.3% ($p < 0.05$), respectively. Seven-day treatment with dexamethasone at the dose of 50 $\mu\text{g/kg/day}$ significantly increased

ADC protein levels in the hippocampus by 33.1% ($p < 0.05$). The lower dose of dexamethasone, and both doses of CORT did not significantly change ADC protein levels in the hippocampus (Fig. 7).

Consistent with our findings with endogenous levels of agmatine, 21-day-treatment with glucocorticoids produced a dose-dependent reduction of ADC protein in the frontal cortex ($p < 0.05$, $F_{4,14} = 6.21$, Fig. 8) and hippocampus ($p < 0.05$, $F_{4,14} = 5.99$, Fig. 9), respectively. *Post hoc* test revealed that the higher dose dexamethasone increased ADC protein levels in the PFC and hippocampus by 25% ($p < 0.05$) and 37.8% ($p < 0.05$). CORT at doses of 1.9 and 9.52 mg/kg/day reduced ADC levels by 21.8% and 44.4% in the frontal cortex ($p < 0.05$, Fig. 8), and by 24.97% and 24.8% in the hippocampus ($p < 0.05$, Fig. 9), respectively. Western blotting illustrated a parallel alteration in ADC protein levels with those of endogenous levels of agmatine in these two brain areas after chronic treatment with glucocorticoids.

Arginine decarboxylase protein levels in the striatum and hypothalamus were also assessed. All of the treatment regimens, except for the 7-day treatment with CORT in the hypothalamus, resulted in significant alterations in ADC protein levels in these regions in a dose-dependent manner, as shown in Table 2. This result demonstrated that the effect of glucocorticoids on ADC protein expression in these two regions is very similar to that in the PFC and hippocampus, indicating a common regulatory effect of glucocorticoids on the endogenous agmatine system.

Discussion

We have previously reported that agmatine has neuroprotective effects against neuronal damage caused by exposure to glucocorticoids and glutamate in the neuronal cultures of the rat hippocampus (Zhu *et al.* 2006). In this study, we examined whether agmatine plays a neuroprotective role *in vivo*. Our results with the immunocytochemical staining confirmed that agmatine can prevent neuronal impairment in the rat mPFC and hippocampus caused by chronic treatment with glucocorticoids. Our study also demonstrated that chronic treatment with glucocorticoids resulted in a biphasic changes in endogenous agmatine levels in brain areas, with an enhanced agmatine levels after 7-day treatment and decreased levels after 21-day administration. This biphasic change of agmatine levels was paralleled by a similar pattern of changes in ADC protein expression in the same regions, indicating that treatment with glucocorticoids may affect agmatine levels by influencing its synthesis.

The neuroprotective effects of agmatine against neuronal alteration caused by dexamethasone

In the present study, immunostaining with antibody to β T-III was used to identify morphological alterations in the rat brain after chronic treatment with glucocorticoids. β T-III is a neuron-specific cytoskeletal protein. Staining with β T-III is considered as a neuronal marker (Caccamo *et al.* 1989; Svendsen *et al.* 2001; Katsetos *et al.* 2003). Its expression in the brain was highest in the frontal cortex, and moderate in the amygdala and hippocampus (Bu and Lephart 2005). It is well known that essential neuronal functions such as transport and maintenance of cell form are dependent on microtubules (Kimonides *et al.* 1998). In these regards, examining the alteration of β T-III expression can provide a unique insight into the neuronal response to insults and alterations in the composition of the neuronal cytoskeleton, especially those occurred in the PFC and hippocampus. It was reported that a dramatic loss of neurons was detected by immunohistochemical methods with β T-III, when animals received a lesion of the internal capsule involving the cortex and hippocampus (Geisert and Frankfurter 1989). In the present study, the mPFC and hippocampus of rats showed a clear immunoreactive staining of β T-III, especially in the perikarya, dendrites, and axon initial segments of pyramidal cells in CA1, CA2, and CA3, which is consistent to the previous report (McDonald *et al.*

1989). Also, microscopic analysis demonstrated that there were noticeable changes in the mPFC and hippocampus after chronic treatment with dexamethasone, compared with those of control animals. So far in most studies Golgi staining was used to identify the effects of stress or glucocorticoid treatment on the brain morphology of animals (Woolley *et al.* 1990; Wellman 2001; Radley *et al.* 2004, 2005). Our study, the first to use this method to investigate the effects of glucocorticoids on rat brain morphology, provides the evidence for immunostaining with β T-III as a useful method for detection of neuronal structure changes.

In the present study, 21-day treatment with glucocorticoids noticeably reduced the β T-III immunoreactivity in the mPFC and hippocampus, which is consistent with the previous observations (Sapolsky 1985; Sapolsky *et al.* 1985; Wellman 2001; Seib and Wellman 2003; Cerqueira *et al.* 2005) using different methodologies. One advantage of this study is the use of osmotic mini-pump and pellet implantation, respectively, for the delivery of dexamethasone and CORT to ensure consistent elevations over 24-h cycle. By this way, the effects of glucocorticoids at stress-associated doses were effectively achieved. In line with our previous study *in vitro* (Zhu *et al.* 2006), administration of agmatine prevented the glucocorticoid-induced neuronal damage *in vivo* (Fig 1 and Fig 2). As mentioned previously, one of the possible mechanisms of agmatine underlying its neuroprotective effects was related to its ability to block NMDA-R channels, because excitatory amino acids and NMDA-Rs have been showed to play a critical role in glucocorticoid-induced neuronal insults (Watanabe *et al.* 1992; Magarinos and McEwen 1995). While agmatine has potential anti-apoptotic effect as revealed in our *in vitro* study (Zhu *et al.* 2006), further study is needed to assess whether it plays a role *in vivo*.

Possible regulatory effects of glucocorticoids on endogenous agmatine levels and ADC expression in the rat brain

One of the major findings in this study is that endogenous agmatine levels in brain regions were increased by 7-day treatment and reduced by 21-day treatment with glucocorticoids. This result paralleled the morphological analysis data: 7-day treatment did not, but 21-day regimen produced a noticeable reduction of dendrite intensity in the mPFC and hippocampus. Currently, we cannot definitely account for a causal relationship between alteration of endogenous levels of agmatine and glucocorticoid-induced neuronal changes in these regions. However, this possibility is present, considering the administration of exogenous agmatine dramatically prevented the glucocorticoid-induced structural alteration. It can be postulated that stress-associated doses of glucocorticoids stimulated reactions of self-protection mechanisms. As one factor of these mechanisms, increased endogenous agmatine levels in these brain areas contributed its neuronal protection at the initial stage. However, prolonged exposure to glucocorticoids exhausted endogenous agmatine and/or affected its biosynthesis, resulting in the reduced levels of endogenous agmatine. Together with the other impairment of body protection systems, neuronal damage eventually occurred.

Agmatine is formed by decarboxylation of L-arginine catalyzed by ADC. At present, exactly how ADC regulates agmatine synthesis *in vivo* remains poorly understood. Our previous study, however, demonstrated that the down-regulation of ADC activity by specific siRNA leads to lower agmatine production *in vitro* (Iyo *et al.* 2006), indicating that ADC gene is responsible for the production of agmatine in the brain. Our observation was recently confirmed in other types of tumor cell lines from human and rats (Wolf *et al.* 2007). Therefore, one might suppose that stress-associated doses of glucocorticoids possibly act on ADC expression, which in turn influences endogenous agmatine levels. This notion was supported by the measurement of ADC protein levels: 7-day treatment with glucocorticoids increased, but 21-day regimen reduced ADC protein expression in the rat brain regions. However, caution should be taken when interpreting the data of ADC measurements: this parallel changes did not occur in all

brain areas measured in this study. When agmatine levels in these brain areas were uniformly increased or reduced after 7- or 21-day treatments with higher doses of glucocorticoids, corresponded ADC protein expression showed some difference. For example, 7-day treatment with CORT failed to significantly change ADC protein levels in the hippocampus (Fig. 7) and hypothalamus (Table 2). In addition, in some brain areas, the effects of glucocorticoid treatments on ADC protein levels were not dose dependent: such as in the striatum by 7-day treatment with CORT (Table 2) and 21-day treatment with dexamethasone, and in the hypothalamus after 21-day treatment with glucocorticoids (Table 2). Although technical reasons cannot be excluded, currently we do not have a satisfactory explanation. Further studies are warranted to explore the regulatory mechanisms underlying the effect of glucocorticoids on ADC expression. Nevertheless, as glucocorticoid-induced parallel changes in endogenous agmatine and ADC protein levels were observed in the almost all selected brain areas, stress hormones may have general regulatory effects on the endogenous agmatine system.

In conclusion, our present work reveals the neuroprotective effect of agmatine against glucocorticoids in the rat mPFC and hippocampus. The parallel alterations between endogenous agmatine and ADC protein levels after treatment with glucocorticoids indicate that there is a possible regulatory effect of glucocorticoids on agmatine synthesis *in vivo*. This regulation may belong to the adaptation responses of body to stress and underline the physiological role of agmatine in brains.

Abbreviations used

ACd, dorsal anterior cingulate cortex; ADC, arginine decarboxylase; CORT, corticosterone; IL, infralimbic cortex; mPFC, medial prefrontal cortex; NMDA-R, NMDA receptor; PBS, phosphate-buffered saline; PFC, prefrontal cortex; PL, pre-limbic cortex; β T-III, β -tubulin III.

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Fig. 1. Photomicrographs showing the CA1 and CA2 of the rat hippocampus immunostained with β -tubulin III after the treatment with saline (control), dexamethasone (50 μ g/kg/day, DEX), and dexamethasone plus agmatine (50 mg/kg/day) (Dex + Ag) for 21 days. PCL, pyramidal cell layer; SO, stratum oriens; SR, stratum radiatum. Scale bar = 100 μ m for the pictures except for inserts; 20 μ m for inserts.

Fig. 2.

(a) Left: schematic representation of the division of the medial prefrontal cortex (mPFC) in the coronal plane (approximately Bregma +3.24 mm; Paxinos and Watson 2005). Right: the same image of mPFC-infralimbic cortex (IL) showing boundaries between layers. (b) Photomicrographs showing the dorsal anterior cingulate cortex (ACd), pre-limbic cortex (PL) and IL in the rat mPFC immunostained with β -tubulin III after treatment with saline (control), dexamethasone (50 μ g/kg/day, Dex) and dexamethasone plus agmatine (50 mg/kg/day) (Dex + Ag) for 21 days. Arrows indicate apical dendrites immunostained by β -tubulin III. Scale bar = 100 μ m for all pictures.

Fig. 3.

Graphs showing changes of the β -tubulin III immunoreactive intensity in the hippocampus (a) and medial prefrontal cortex (mPFC) (b) among the control, dexamethasone treatment (Dex), and dexamethasone treatment plus agmatine (Dex-Ag) by semiquantitative analysis using ImageJ image software ($n = 3$). * $p < 0.05$ and ** $p < 0.01$, compared with the control. † $p < 0.05$ compared with to the group of dexamethasone plus agmatine (Dex + Ag).

Fig. 4. Endogenous agmatine levels in the prefrontal cortex and hippocampus of rats ($n = 6$) measured by HPLC. Rats were treated with dexamethasone (Dex-1: 10 µg/kg/day and Dex-2: 50 µg/kg/day) or corticosterone (Cort-1: 1.90 mg/kg/day and Cort-2: 9.52 mg/kg/day) for 7 days. * $p < 0.05$ compared with control (C).

Fig. 5. HPLC-measured endogenous agmatine levels in the prefrontal cortex and hippocampus of rats ($n = 5$) treated with dexamethasone or corticosterone for 21 days at the same doses as described in the legend of Fig. 4. * $p < 0.05$ and ** $p < 0.01$ compared with control (C).

Fig. 6.

Upper panel: Photograph of immunoblotted arginine decarboxylase (ADC) in the prefrontal cortex in the control and rats treated with dexamethasone or corticosterone for 7 days at the same doses as described in the legend of Fig. 4. Middle panel: The same membrane was again probed with anti- β -actin antibody after stripping as an internal control. Lower panel: Quantitative analysis by densitometry of bands after western blotting. Results are mean $\pm$ SEM of four independent experiments. * $p < 0.05$ compared with the control (C).

Fig. 7.

Upper panel: Photograph of immunoblotted arginine decarboxylase (ADC) in the hippocampus in the control and rats treated with dexamethasone or corticosterone for 7 days at the same doses as described in the legend of Fig. 4. Middle panel: The same membrane was again probed with anti- β -actin antibody after stripping as an internal control. Lower panel: Quantitative analysis by densitometry of bands after western blotting. Results are mean $\pm$ SEM of four independent experiments. * $p < 0.05$ compared with the control (C).

Fig. 8.

Upper panel: Photograph of immunoblotted arginine decarboxylase (ADC) in the prefrontal cortex in control and rats treated with dexamethasone or corticosterone for 21 days at the same doses as described in the legend of Fig. 4. Middle panel: The same membrane was again probed with anti- β -actin antibody after stripping as an internal control. Lower panel: Quantitative analysis by densitometry of bands after western blotting. Results are mean $\pm$ SEM of four independent experiments. * $p < 0.05$ compared with the control (C).

Fig. 9.

Upper panel: Photograph of immunoblotted arginine decarboxylase (ADC) in the hippocampus in control and rats treated with dexamethasone or corticosterone for 21 days at the same doses as described in the legend of Fig. 4. Middle panel: The same membrane was again probed with anti- β -actin antibody after stripping as an internal control. Lower panel: Quantitative analysis by densitometry of bands after western blotting. Results are mean $\pm$ SEM of four independent experiments. * $p < 0.05$ compared with the control (C).

Table 1

Effects of glucocorticoid treatment on endogenous agmatine levels ($\mu\text{g/g}$ wet tissue) in the striatum and hypothalamus of rats

	7-day treatment ($n = 6$)		21-day treatment ($n = 5$)	
	Striatum	Hypothalamus	Striatum	Hypothalamus
Control	0.43 ± 0.04	0.49 ± 0.08	0.51 ± 0.11	0.49 ± 0.08
Dex-1	0.46 ± 0.10	$0.78 \pm 0.11^*$	0.42 ± 0.05	0.36 ± 0.10
Dex-2	$0.69 \pm 0.06^*$	$0.78 \pm 0.22^*$	$0.25 \pm 0.02^*$	$0.31 \pm 0.02^*$
Control	0.41 ± 0.02	0.43 ± 0.06	0.49 ± 0.07	0.50 ± 0.07
CORT-1	0.49 ± 0.10	0.45 ± 0.12	0.41 ± 0.06	0.55 ± 0.13
CORT-2	$0.65 \pm 0.01^*$	$0.72 \pm 0.11^*$	$0.35 \pm 0.05^*$	0.51 ± 0.05

Results are expressed as mean $\pm$ SEM.

* $p < 0.05$, compared with those of the control. The individual comparisons were performed by the Student's–Newman–Keuls test. CORT, corticosterone.

Table 2

Effects of glucocorticoid treatment on ADC protein levels (arbitrary unit) in the striatum and hypothalamus of rats

	7-day treatment (<i>n</i> = 6)		21-day treatment (<i>n</i> = 5)	
	Striatum	Hypothalamus	Striatum	Hypothalamus
Control	1.11 ± 0.03	1.30 ± 0.01	1.22 ± 0.08	0.86 ± 0.06
Dex-1	1.30 ± 0.08	1.31 ± 0.04	0.84 ± 0.09*	0.36 ± 0.10*
Dex-2	1.53 ± 0.14*	2.31 ± 0.17**	0.80 ± 0.12*	0.31 ± 0.02*
CORT-1	1.57 ± 0.08*	1.44 ± 0.06	0.97 ± 0.11	0.56 ± 0.09*
CORT-2	1.81 ± 0.12*	1.51 ± 0.11	0.78 ± 0.05*	0.39 ± 0.15*

Results are expressed as mean ± SEM.

*

 $p < 0.05$

**

 $p < 0.01$ compared with those of the control. The individual comparisons performed by the Student's–Newman–Keuls test. ADC, arginine decarboxylase; CORT, corticosterone.