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Repeated immobilization stress alters rat hippocampal and prefrontal cortical morphology in parallel with endogenous agmatine and arginine decarboxylase levels

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ABSTRACT

Agmatine, an endogenous amine derived from decarboxylation of L-arginine catalyzed by arginine decarboxylase, has been proposed as a neurotransmitter or neuromodulator in the brain. In the present study, we examined whether agmatine has neuroprotective effects against repeated immobilization-induced morphological changes in brain tissues and possible effects of immobilization stress on endogenous agmatine levels and arginine decarboxylase expression in rat brains. Sprague–Dawley rats were subjected to 2 h immobilization stress daily for 7 days. This paradigm significantly increased plasma corticosterone levels, and the glutamate efflux in the hippocampus as measured by *in vivo* microdialysis. Immunohistochemical staining with β-tubulin III showed that repeated immobilization caused marked morphological alterations in the hippocampus and medial prefrontal cortex that were prevented by simultaneous treatment with agmatine (50 mg/kg/day), i.p.). Likewise, endogenous agmatine levels measured by high-performance liquid chromatography in the prefrontal cortex, hippocampus, striatum and hypothalamus were significantly increased by immobilization, as compared to controls. The increased endogenous agmatine levels, ranging from 92 to 265% of controls, were accompanied by a significant increase of arginine decarboxylase protein levels in the same regions. These results demonstrate that the administration of exogenous agmatine protects the hippocampus and medial prefrontal cortex against neuronal insults caused by repeated immobilization. The parallel increase in endogenous brain agmatine and arginine decarboxylase protein levels triggered by repeated immobilization indicates that the endogenous agmatine system may play an important role in adaptation to stress as a potential neuronal self-protection mechanism.

1. Introduction

Stress is a common experience of daily life. Physical and psychological stressors elicit integrated neuroendocrine and autonomic responses, including an enhanced activity of the hypothalamic–pituitary–adrenal axis with elevated circulating glucocorticoid concentrations. These responses serve to mobilize and redistribute bodily resources to facilitate and maintain physiological homeostasis. While this interaction of hormonal and neuronal systems is a fundamental requirement for survival, there is considerable evidence that high levels of glucocorticoids in the brain resulting from prolonged stress may produce deleterious effects, including damage to neurons (McIntosh and Sapolsky, 1996). In fact, animal studies have shown that prolonged exposure to high levels of corticosterone has diverse effects on the central nervous system, including changes in cellular activity, neurochemistry, and neuronal morphology (Sapolsky et al., 1985; Sapolsky, 1990; Reagan and McEwen, 1997).

It has been known for some time that excitatory amino acids, especially glutamate, are also strongly implicated in stress-induced structural and functional changes in the rat brain. An increased extracellular glutamate level in the rat brain regions caused by both

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acute restraint and swimming stress was reported (Lowy et al., 1993; Moghaddam, 1993). Also *N*-methyl-D-aspartate (NMDA) receptor antagonists can block stress-induced structural changes in the hippocampus (Magarinos et al., 1999). These findings not only suggest a possible role for glutamate in the mechanisms underlying cellular and molecular alterations caused by stress, but also provide an avenue to block or attenuate chronic stress-induced effects by normalizing glutamatergic function during stressful stimuli. Such an approach might have distinct clinical advantages.

Agmatine is an endogenous cationic amine derived from enzymatic decarboxylation of L-arginine by arginine decarboxylase (ADC, EC4.1.1.19) (Tabor and Tabor, 1984). *In vivo,* agmatine is enriched in the hippocampus and other brain regions (Regunathan et al., 1995; Feng et al., 1997; Otake et al., 1998). An important enzyme for biosynthesis of agmatine, ADC is widely expressed in many brain regions of rats and human and this regional distribution may adumbrate some of agmatine's functional effects. In the past decade, many studies have demonstrated that agmatine has neuroprotective effects *in vitro* and *in vivo*. Our previous work has also demonstrated neuroprotective effects of agmatine against cell damage caused by glucocorticoids and glutamate in primary neuronal cultures of the hippocampus (Zhu et al., 2006). The study indicated that agmatine may play a role in the homeostasis during stress, particularly with regard to stress effects that are mediated by glucocorticoids and glutamate.

We have recently reported that chronic treatment of rats with glucocorticoids resulted in morphological changes in the hippocampus and medial prefrontal cortex (mPFC). These morphological changes can be prevented by administration of agmatine (Zhu et al., 2007). To test the hypothesis that the previously observed pathological impacts of chronic exposure to glucocorticoids are similar to the neuronal insults caused from chronic stress, we carried out the present study to determine whether repeated immobilization causes morphological alterations in rat brains and whether agmatine can protect brain tissues from immobilization-induced cytoarchitectural alterations. As glutamate release has been indicated to contribute to the deleterious effects of chronic stress, we also measured extracellular glutamate levels in this animal model. Furthermore, we measured endogenous agmatine and ADC protein levels in brain regions of rats subjected to immobilization stress in order to determine whether repeated immobilization affects endogenous agmatine levels in related brain regions. Our results reveal the neuroprotective effects of agmatine and potential roles of agmatine systems in the adaptation response to chronic stress.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 200–250 g at the time of initiation of immobilization stress were housed on a 12-h light/dark cycle with food and water provided *ad libitum*. All animal procedures were approved by the Animal Care and Use Committee of East Tennessee State University and University of Mississippi Medical Center 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 experimental groups.

2.2. Stress paradigm

The animals were randomly assigned to chronic immobilization or control groups. Immobilization stress was accomplished by taping the four limbs of rats to a wooden board with two metal loops around the neck for 2 h a day (10:00 am to 12:00 pm) for seven consecutive days. Control animals were brought to the same room every day without immobilization (sham). During the actual or sham immobilization sessions there was no access to either food or water.

Four groups of rats were used in the immunostaining study: control, two groups of immobilization stress alone, and immobilization stress plus agmatine treatment. Rats in the control and stress alone groups were daily injected with saline intraperitoneally. Rats in the stress plus agmatine group were administered with agmatine daily (50 mg/kg, i.p.), immediately before beginning of the immobilization paradigm. The choice of agmatine dose is based on a previous study where administration of exogenous agmatine (50 mg/kg, i.p.) to rats increased agmatine levels in the hippocampus and frontal cortex ~2–4-fold at 15–30 min after injection (Feng et al., 2005), demonstrating that agmatine accesses the brain and this dose of agmatine is high enough to raise the agmatine levels in the brain. As our previous study showed that administration of agmatine at the dose of 50 mg/kg did not produce any morphological alteration in the hippocampus and mPFC (Zhu et al., 2008), we did not include this group in the present study. Immediately after the last immobilization session (on the 7th day), rats in the control, one group of immobilization (Immo-1) and immobilization plus agmatine were transcardically perfused under pentobarbital anesthesia using 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Rats in another group of immobilization (Immo-2) were perfused by the same way but 2 h after the last immobilization session. Brains were further stored in 10% sucrose followed by 30% sucrose, and then sectioned at 30 μm using a sliding microtome for subsequent immunostaining.

There were three groups of rats in the studies measuring endogenous agmatine and ADC protein expression: control and two groups of immobilization (similarly, Immo-1 and Immmo-2). As the goal of this experiment was to determine the effects of immobilization on endogenous agmatine and ADC levels, there was no agmatine treatment group in this experiment. After the last immobilization session (on the 7th day), rats in the group of control and Immo-1 were immediately sacrificed by decapitation. Rats in the group Immo-2 were sacrificed 2 h after the last immobilization session. Rat brain regions were rapidly dissected on ice and immediately processed for high-performance liquid chromatography (HPLC) or ADC assays.

2.3. Rat blood sampling and plasma corticosterone determination

On the 7th day after the last session of immobilization stress, rats were sacrificed. The period of time between removing rats from the wooden board to decapitation was strictly held to 15 s or less. Trunk blood from rats was quickly collected into chilled glass tubes, which have been rinsed with a solution of 1.5% EDTA in saline and dried. Animals in the control group were sacrificed by the same procedure. Blood samples were immediately centrifuged at 2500 rpm. Plasma obtained was temporarily stored at −20 °C and plasma corticosterone was later measured by a radioimmunoassay using a commercial kit (ImmuChem radioimmunoassay kit, MP Biomedicals, LLC in Orangeburg, NY (formerly ICN Pharmaceuticals, Costa Mesa, CA)). The assay kit was used according to manufacturer's instructions with the following modification: all reagents are used at half strength and the lowest standard concentration in the kit (25 ng) was serially diluted to yield standards at 12.5, 6.25 and 3.125 ng, respectively. The resulting assay has a sensitivity of 3.125 ng and an IC50 of 65 ng. All samples were run in a single assay with an intra-assay variance of less than 8%.

2.4. Surgery and in vivo microdialysis

This study included two groups of rats: control and immobilization. The surgical procedures and microdialysis sampling were performed using modifications of techniques detailed as previous studies (Feng et al., 2005). Briefly, rats were anesthetized with halothane inhalation, and a CMA/11 microdialysis guide cannula (CMA Microdialysis, N. Chelmsford, MA) was implanted with the tip directed toward the left ventral hippocampus. The coordinates for implantation were (in mm relative to Bregma): lateral 5.0 and posterior 5.2 (Paxinos and Watson, 2005). The cannula tip was lowered 4.5 mm below the surface of the dura. Five stainless steel screws were secured to the skull. An aluminum protective cap was placed around each guide cannula and dental acrylic (Lang Dental, Wheeling, IL) was applied to anchor the assembly to the skull surface. Operated rats were allowed 7 days to recover. All animals used in the study displayed no signs of distress as a result of surgery (i.e., they moved and groomed normally, they ate food and drank water, and there was no sign of bleeding or infection around the surgery area). Microdialysis was initiated by the insertion of microdialysis probes (CMA/11, 2 mm tip) into the guide cannula when the rat was placed in an individual CMA/120 system under freely moving conditions at 8:00 am on the 7th day of stress paradigm. The artificial cerebrospinal fluid (aCSF, composition:140 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 2.7 mM Na_2HPO_4, pH 7.4) was continuously perfused through the probe at 1 μl/min by a CMA pump for an equilibration period of 2 h. A series of four sequential 15 min samples for each rat was taken prior to immobilization to demonstrate a steady baseline. These four samples were averaged and all subsequent values were expressed as a percentage of this pre-immobilization value (% of baseline). Then the rat was subjected to immobilization while dialysis was continued. Dialysate samples were collected and subsequently analyzed by HPLC as described previously (Feng et al., 2005). After completion of microdialysis, rats under anesthesia with pentobarbital were perfused intracardially using 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was examined for probe placement. The rats in which the probe was not in the designated location were not included in statistical data. The *in vitro* recovery of glutamate was determined by the immersion of probes in aCSF containing 10 μM of glutamate and ranged from 20 to 25% for glutamate.