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# Exogenous agmatine has neuroprotective effects against restraint-induced structural changes in the rat brain

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## Abstract

Agmatine is an endogenous amine derived from decarboxylation of arginine catalysed by arginine decarboxylase. Agmatine is considered a novel neuromodulator and possesses neuroprotective properties in the central nervous system. The present study examined whether agmatine has neuroprotective effects against repeated restraint stress-induced morphological changes in rat medial prefrontal cortex and hippocampus. Sprague-Dawley rats were subjected to 6 h of restraint stress daily for 21 days. Immunohistochemical staining with β-tubulin III showed that repeated restraint stress caused marked morphological alterations in the medial prefrontal cortex and hippocampus. Stress-induced alterations were prevented by simultaneous treatment with agmatine (50 mg/kg/day, i.p.). Interestingly, endogenous agmatine levels, as measured by high-performance liquid chromatography, in the prefrontal cortex and hippocampus as well as in the striatum and hypothalamus of repeated restraint rats were significantly reduced as compared with the controls. Reduced endogenous agmatine levels in repeated restraint animals were accompanied by a significant increase of arginine decarboxylase protein levels in the same regions. Moreover, administration of exogenous agmatine to restrained rats abolished increases of arginine decarboxylase protein levels. Taken together, these results demonstrate that exogenously administered agmatine has neuroprotective effects against repeated restraint-induced structural changes in the medial prefrontal cortex and hippocampus. These findings indicate that stress-induced reductions in endogenous agmatine levels in the rat brain may play a permissive role in neuronal pathology induced by repeated restraint stress.

### Keywords

agmatine; arginine decarboxylase; hippocampus; prefrontal cortex; repeated restraint stress; β-tubulin III



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## Introduction

Stress is one of the physiological challenges that can alter brain functions. During stress, enhanced activity of the hypothalamic-pituitary-adrenal axis results in an elevated level of circulating glucocorticoids. Although homeostasis of glucocorticoids within the physiological range sets the level of arousal and influences vital signs, prolonged and excessive glucocorticoid secretion can result in endangering effects on the structural integrity of brain neurons. A growing body of evidence has demonstrated that chronic stress can cause structural alterations in the brain. For example, repeated restraint stress induced dendritic atrophy of CA3 pyramidal neurons in the rat hippocampus (Watanabe *et al.*, 1992; Magarinos & McEwen, 1995; Sunanda *et al.*, 1995; Galea *et al.*, 1997). A similar pattern of dendritic alteration was observed in the medial prefrontal cortex (mPFC) after repeated restraint stress (Cook & Wellman, 2004; Radley *et al.*, 2004). Likewise, sustained social stress, although a different stressor from restraint, also produced prominent neuronal damage in CA3 and CA1 of the hippocampus in primate models (Uno *et al.*, 1989). Collectively, these results indicate that structural alterations in the brain are a common pathological consequence of chronic stress.

Morphological changes in the mPFC and CA3 of the hippocampus resulting from repeated restraint appear to be mostly reversible (Conrad *et al.*, 1999; Radley *et al.*, 2005). However, dendrites are a major site of synaptic connectivity, which determines many functional properties of neurons (Koch & Segev, 2000). The pronounced dendritic changes caused by stress, although apparently not permanent based on current understanding, probably result in important functional changes. In fact, morphological alterations in the mPFC caused by chronic restraint stress have been suggested to be a biological component of impairment of attention that is symptomatic of depression and anxiety disorders (Liston *et al.*, 2006). The detailed mechanisms by which chronic stress induces morphologic alterations in the hippocampus and mPFC are unclear, as is the relationship between structural changes and functional consequences. Hence, additional research is necessary to facilitate our understanding of the pathophysiology and behavioral consequences of chronic stress.

Agmatine is an endogenous polyamine derived from enzymatic decarboxylation of L-arginine by arginine decarboxylase (ADC) (Tabor & Tabor, 1984). *In-vivo* agmatine is mainly synthesized in glia and present in relatively high concentrations in the hippocampus and other brain areas (Regunathan *et al.*, 1995; Feng *et al.*, 1997). As an important enzyme for biosynthesis of agmatine, ADC is widely expressed in many brain areas of rats and human (Feng *et al.*, 1997; Iyo *et al.*, 2006). In the past decade, many studies have demonstrated that agmatine can block *N*-methyl-D-aspartate receptor channels (Yang & Reis, 1999) and voltage-gated $Ca^{2+}$ channels (Weng *et al.*, 2003; Zheng *et al.*, 2004), and inhibit all isoforms of nitric oxide synthase (Tassone *et al.*, 2002). Furthermore, our previous work has demonstrated neuroprotective effects of agmatine against cell damage caused by glucocorticoids and glutamate in primary neuronal cultures of the hippocampus (Zhu *et al.*, 2006). These latter studies raise the possibility that agmatine plays a role in the homeostasis during stress, particularly with regard to physiological effects of stress that are mediated by glucocorticoids and glutamate.

The goals of the present study were to test whether agmatine has neuroprotective effects *in vivo* during chronic stress, and to determine the effect of chronic stress on endogenous agmatine levels and ADC protein expression. Immunostaining with an antibody to β-tubulin III (βT-III), high-performance liquid chromatography (HPLC) and western blotting were performed in brain tissues from rats subjected to repeated restraint stress or simultaneous treatment with exogenous agmatine. Our results reveal neuroprotective effects of agmatine in this animal stress model, as well as a dynamic regulation of endogenous agmatine and ADC protein levels by stress in several rat brain areas.

## Materials and methods

### Animals

Male Sprague-Dawley rats, weighing 200–250 g at the beginning of the experiment, were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). 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. Rats were maintained on a 12-h light/dark cycle (lights on at 07:00 h) with *ad-libitum* access to food and tap water. After an acclimation period of 5 days, rats were randomly assigned to experimental groups.

### Restraint stress paradigm and agmatine treatment

For the stress model, rats were kept in plastic rodent restrainers for 6 h (10:00–16:00 h) daily for 21 days. The restrainer completely restricted the lateral and front-to-back movements of the rat. Control animals were brought to the same room every day without restraint. During restraint sessions, all animals had no access to either food or water. Body weights of all experimental animals were recorded periodically.

Four groups of rats were used in this study: control rats, control rats treated with agmatine, rats exposed to daily restraint stress, and rats exposed to daily restraint stress plus daily agmatine treatment. Rats in the control and stress alone groups were injected daily with saline intraperitoneally. Rats in the control plus agmatine treatment and stress plus agmatine treatment groups were administered 50 mg/kg, i.p. agmatine daily, immediately before beginning the restraint procedure. The choice of agmatine dose is based on a previous study that demonstrated that administration of exogenous agmatine (50 mg/kg, i.p.) to rats increased agmatine levels in the hippocampus and frontal cortex by approx. two- to fourfold at 15–30 min after injection (Feng *et al.*, 2005), demonstrating that agmatine, at least at this dose, accesses the brain.

For immunostaining experiments and ADC assays, brain sections or tissues from rats of all four treatment groups were studied. For HPLC measurement of agmatine, only the control and restraint stress groups of rats were studied. The goal of this latter experiment was to determine effects of stress on endogenous agmatine levels. Agmatine levels were not analysed in the restraint plus agmatine group of rats because the ability of agmatine injections to increase brain agmatine levels has been previously reported by our group (Feng *et al.*, 2005).

For the immunostaining experiments, after the last restraint session (on the 21st day), rats were immediately transcardially perfused under anesthesia with pentobarbital (40 mg/kg, i.p.) using 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Brains were further stored in 10% sucrose followed by 30% sucrose and then sectioned at 30 μm using a sliding microtome for immunostaining. For HPLC analyses and ADC assays, rats were killed by decapitation immediately after the last restraint session (on the 21st day). Rat brain areas were rapidly dissected on ice and immediately processed for HPLC and ADC assays.

### Rat blood sampling and plasma corticosterone determination

Rats were killed on the 21st day immediately after the last 6-h restraint session. The period of time between removing rats from the restrainer to decapitation was strictly held to 10 s or less. Trunk blood from rats was quickly collected into chilled glass tubes, which had been rinsed with a solution of 1.5% EDTA in saline and dried. Animals in the control and control plus agmatine groups were killed by the same procedure. Blood samples were immediately centrifuged at 800 ***g***. Plasma obtained was temporarily stored at −20 °C and plasma corticosterone was later measured by a radioimmunoassay using a commercial kit (ImmuChem radioimmunoassay kit, ICN Pharmaceuticals, Costa Mesa, CA, USA).

### Measurement of agmatine by HPLC

The amount of agmatine in brain tissues in the control and stressed rats was measured by HPLC as described earlier (Raasch *et al.*, 1995; Feng *et al.*, 1997). Rats were killed by decapitation. Brain tissues were homogenized after dissection and resuspended in the phosphate buffer (pH 5.7) containing amino picollinate as an internal standard. The cell suspension was then homogenized in 10% trichloroacetic acid and centrifuged. The supernatant was used for HPLC analysis. Samples were derivatized with *o*-phthalaldehyde and loaded onto a reverse phase column (5 μm) connected with a fluorescence detector. The recovery of agmatine was calculated from an added external standard and expressed as μg/g wet weight.

### Detection of ADC protein levels

The ADC 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 as described previously (Iyo *et al.*, 2006). The IgG fraction of this antiserum was purified using a protein A column (Pierce, Rockford, IL, USA) and the purified IgG was characterized for its specificity by pre-absorption with an antigen peptide (Iyo *et al.*, 2006). Rats were killed by decapitation. Rat brain regions were dissected and homogenized in HEPES buffer (pH 7.4). After centrifugation at 1000 ***g***, the supernatants were centrifuged at 30 000 ***g*** for 20 min. The resulting pellets were solubilized in sample buffer containing sodium lauryl sulfate for ADC immunoblotting analysis. Equal amounts of homogenized proteins were loaded on a 10% sodium lauryl sulfate-polyacrylamide gel for electrophoresis and transferred by electroblotting to a polyvinylidene difluoride membrane. The ADC protein was detected after exposure to the ADC antibody at a dilution of 1 : 3000. Membranes were processed for immunoblot analysis using the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ, USA). Immunoreactive bands were visualized using the Kodak Imagestation (New Haven, CT, USA). The measurement of western blots was carried out in five rats from each group by analysing protein band densities using imaging software (Molecular Dynamics IQ Solutions, Molecular Dynamics, Inc., Sunnyvale, CA, USA). A linear standard curve was created from optical densities of bands with a dilution series of total proteins prepared from saline-treated rats on each blot. Optical density values of sample bands (ADC) were compared with those of the standard curve to ensure that detection was in the linear range of measurement. Optical density values of ADC immunoreactivities were further normalized by dividing by optical density values for β-actin immunoreactivities determined on the same blot.

### Immunohistochemical staining with βT-III

Immunohistochemical staining was performed using a monoclonal antibody against βT-III raised from mice (Chemicon, Temecula, CA, USA). Brain sections were washed three times in phosphate-buffered saline and pre-incubated in 5% bovine serum albumin in phosphate-buffered saline supplemented with 0.2% Triton-X 100 for 1 h at room temperature (25 °C), followed by incubation with the primary antibody (1 : 500 dilution in phosphate-buffered saline containing 0.2% Triton-X 100) overnight at 4 °C. After washing, sections were incubated with the secondary antibody, a biotinylated goat anti-mouse antibody (1 : 200) for 2 h at room temperature (25 °C), followed by continuous incubation with the avidin–biotin complex using the ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. βT-III immunoreactivity was visualized by incubating sections in 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.07% $NH_4NiSO_4$ and 0.003% $H_2O_2$. Staining for βT-III was then analysed microscopically using MagnaFire SP imaging software (Optromics, Goleta, CA, USA). 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 interanimal 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

The βT-III immunoreactivity for dendrites 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). Care was taken to ensure that the identical plane of sectioning from animals of different groups was used to ensure uniformity and exclude the possibility that the present results were an artifact of the sectioning plane. Images were acquired in an Olympus BX41 microscope (Tokyo, Japan) equipped with an Olympus U-TVO digital camera connected to a computer with MagnaFire image software (Goleta, CA, USA). Three digital microscopic images under higher power (100× oil immersion objective lenses) were randomly captured at the CA1, CA2 and CA3 of the same hemisphere of hippocampus and the dorsal anterior cingulated cortex (ACd), prelimbic cortex (PL) and infralimbic cortex (IL) of the mPFC. The image size of the view field was ~75 $\mu m^2$. These gray-scale images were thresholded in ImageJ with a fixed level over the background. The threshold values for immunoreactive dendrites were obtained by manually sampling the signal intensity in each image, which was visually compared with the original gray-scale images to ensure that the tool effectively resolved all labeled fibers. Thereafter, the intensity of βT-III immunoreactivity (dendrites) in the area was semiquantitatively obtained using the measurement tool of ImageJ and the target (above threshold) area was expressed as a percentage of the sampled area. Two sections (around Bregma −3.24 mm for the hippocampus and near Bregma +3.24 mm for the mPFC) from each animal were examined. Therefore, for each animal, about six measurements were obtained from each of the CA1, CA2, CA3 and mPFC sub-field regions. The average of the measurements (percent areas) obtained in six rats from each group was a reflection of staining intensity for the hippocampus or the sub-regions of the mPFC.

### Statistics

Data are presented as means ± SEM. For measurements of agmatine levels in brain areas, a Student's *t*-test was used. Data from ImageJ analysis and ADC measurements were analysed using two-way anova (SigmaStat, SYSTAT Software, Inc, Richmond, CA, USA). In the presence of significant *F*-values, individual comparisons between the means were made using the Student-Newman-Keuls test.

## Results

### Effects of agmatine on repeated restraint stress-induced morphological alterations in the hippocampus and mPFC

Compared with the control animals, rats subjected to repeated restraint stress showed a reduced rate of body weight gain (data not shown), consistent with a previous report (Magarinos & McEwen, 1995). Also, to define the efficacy of chronic stress in inducing hormonal modification, plasma corticosterone was measured in samples collected immediately after 6-h restraint at the 21st day at the end of the 3-week restraint stress. Plasma corticosterone levels were significantly elevated in the rats subjected to repeated restraint stress ($P < 0.01$; Fig. 1), as compared with control rats. Treatment with agmatine did not influence basal levels of plasma corticosterone and did not affect the increased levels of cortcosterone caused by stress.

Morphological analysis using immunohistochemical staining with βT-III demonstrated a marked structural change in neuronal cytoskeleton in the hippocampus of stressed rats. As shown in Fig. 2, there was a reduction of immunoreactive staining of dendrites in CA1 and CA2 regions. These less intensively immunostained dendrites appeared twisted or broken and displayed a disoriented morphology. In contrast, dendrites in control animals showed a

homogeneous distribution with intensive immunostaining. Immunostaining of mossy fibers in the hippocampal CA3 of stressed animals was also considerably reduced (Fig. 2). Similarly, in the mPFC of the control animals, immunostained dendrites appeared as long, branching processes. However, in the sections of rats subjected to repeated restraint stress, the density of staining was weaker in the ACd, PL and IL fields of the mPFC (Fig. 3), as compared with their respective controls. These morphological changes could be associated with dendritic degeneration and/or retraction from the cortical or pyramidal neurons. In control rats treated with agmatine (50 mg/kg, i.p.; Fig 2 and Fig 3), there were no obvious morphological changes in the mPFC and hippocampus. In contrast, treatment with agmatine in the stressed rats diminished the immunostaining loss in these brain regions. Sections from these stressed animals treated with agmatine showed strong cytoplasmic staining that extended down proximal dendrites in the hippocampus, showing clear apical dendrite distribution in the mPFC, a morphology similar to that of the control rats receiving no stress. As shown in Fig. 5, βT-III-immunoreactive neuronal cell bodies and neurites were clustered around striatal fiber bundles in the striatum of the four groups of rats. Stress and treatment with agmatine produced no noticeable structural changes in the striatum. In the hypothalamus, βT-III immunostaining showed a homogeneous distribution of stained neuronal cell bodies and neurites in the sections from all four groups of rats. Similar to the striatum, no effects of stress or agmatine were observed in the hypothalamus (Fig. 6).

To statistically compare the effects of chronic stress on the hippocampus and mPFC, βT-III immunoreactivities for dendrites were semiquantitatively evaluated. Using stress and agmatine treatment as factors, a two-way anova analysis revealed significant effects of stress on βT-III immunoreactivities of the CA1 ($F_{1,36} = 8.45$, $P < 0.05$), CA2 ($F_{1,36} = 24.56$, $P < 0.001$) and CA3 ($F_{1,36} = 12.07$, $P < 0.05$) in the hippocampus, as well as those of the areas of the ACd ($F_{1,36} = 13.22$, $P < 0.01$), PL ($F_{1,36} = 17.22$, $P < 0.01$) and IL ($F_{1,36} = 22.91$, $P < 0.001$) fields of the mPFC. There was a significant interaction of treatment with agmatine and stress on βT-III immunoreactivities in the hippocampus ($F_{1,36} = 5.37$, $P < 0.05$) and mPFC ($F_{1,36} = 6.48$, $P < 0.05$) (Fig. 4). Post-hoc analysis revealed that repeated restraint stress significantly reduced the βT-III-immunoreactive intensity in CA1, CA2 and CA3 by 26.8% ($P < 0.05$), 34.8% ($P < 0.01$) and 26.3% ($P < 0.05$), respectively, as compared with the control group. Treatment of control rats with agmatine did not change the basal levels of βT-III immunoreactivities in these regions but it prevented the reduction of immunoreactive intensities in the CA1, CA2 and CA3 (all $P < 0.05$), as compared with the restraint group (Fig. 4A). Similarly, repeated restraint stress significantly reduced the βT-III-immunoreactive intensity in the following areas of the mPFC: ACd by 32.10% ($P < 0.01$), PL by 26.51% ($P < 0.05$) and IL by 27.63% ($P < 0.05$). Likewise, treatment with agmatine abolished the effect of repeated restraint on the βT-III-immunoreactive intensity in the ACd, PL and IL areas of the mPFC (all $P < 0.05$), as compared with the restraint group receiving saline (Fig. 4B).

### Effects of repeated restraint stress on endogenous agmatine levels in the rat brain

To investigate the potential relationship between endogenous agmatine and the stress-induced brain morphological alterations, endogenous agmatine levels were measured in the rat prefrontal cortex and hippocampus by HPLC. To assess whether chronic stress also affects agmatine levels in other brain regions, agmatine levels in the striatum and hypothalamus were measured. As shown in Fig. 7, endogenous agmatine levels in the prefrontal cortex and hippocampus of repeated restraint rats were significantly reduced by 29.1% ($P < 0.05$) and 75% ($P < 0.01$), respectively, as compared with those of control rats. Similarly, endogenous agmatine levels in the striatum and hypothalamus in stressed rats were significantly reduced by 56.1% ($P < 0.05$) and 50.5% ($P < 0.05$), respectively.

### Effects of repeated restraint stress on ADC protein levels in brain regions

Agmatine is synthesized from arginine by ADC (Tabor & Tabor, 1984). Therefore, ADC protein levels were measured in rat brain regions to test whether ADC expression was affected by repeated restraint stress and whether exogenous agmatine administration influenced ADC expression. Western blotting data were analysed by two-way ANOVA using stress and agmatine treatment as factors. Semiquantitative analysis of western blots revealed that repeated restraint stress significantly increased ADC protein levels in all four brain regions (prefrontal cortex: $F_{1,19} = 5.237$, $P < 0.05$, Fig. 8A; hippocampus: $F_{1,19} = 6.99$, $P < 0.01$, Fig. 8B; striatum: $F_{1,19} = 7.16$, $P < 0.01$, Fig. 9A; hypothalamus: $F_{1,19} = 5.91$, $P < 0.05$, Fig. 9B). There was a significant interaction between the treatment with exogenous agmatine and stress on ADC protein levels in the prefrontal cortex ($F_{1,19} = 5.745$, $P < 0.05$) and striatum ($F_{1,19} = 18.109$, $P < 0.001$). However, this interaction did not reach statistical significance in the hippocampus ($F_{1,19} = 0.872$, $P > 0.05$) and hypothalamus ($F_{1,19} = 1.045$, $P > 0.05$). Post-hoc analysis revealed that injection of agmatine had no significant effect on the ADC levels in the no-stress rats ($P > 0.05$) but agmatine treatment significantly abolished the increase of ADC protein levels induced by repeated restraint stress in the prefrontal cortex ($P < 0.05$, Fig. 8A) and striatum ($P < 0.01$, Fig. 9A). Injection of agmatine did appear to block the stress-induced increase of ADC protein levels in the hippocampus (Fig. 8B) and hypothalamus (Fig. 9B). Nevertheless, the comparison of ADC protein levels in the hippocampus and hypothalamus in the stress vs. stress plus agmatine groups did not demonstrate a statistically significant difference. It indicates that the reduced endogenous agmatine levels induced by stress (Fig. 7) may trigger a compensatory increase of ADC protein levels and injection of exogenous agmatine did modestly attenuate the increase of ADC protein levels in these regions, especially in the prefrontal cortex and striatum.

## Discussion

Repeated restraint stress induced structural alterations in the rat mPFC and hippocampus, as revealed by a significant reduction of βT-III immunoreactivity. These structural alterations were diminished by concomitant treatment of stressed rats with exogenous agmatine. Further biochemical measurements using HPLC and western blotting demonstrated that repeated restraint stress resulted in a significant decrease of endogenous agmatine levels and a pronounced increase of ADC protein expression in the mPFC and hippocampus, as well as in the striatum and hypothalamus. Interestingly, increased ADC protein levels induced by stress were abolished by the concomitant administration of exogenous agmatine. It can be inferred from these results that the endogenous agmatine system plays an important role in modulating the biological response to chronic stress. Stress-related reductions in endogenous agmatine may increase the susceptibility of certain brain regions to stress-induced neuronal insult.

Repeated restraint stress causes significant reductions in apical dendritic length and branch number in pyramidal neurons of the CA1, CA3 and dentate gyrus in the rat hippocampus (Watanabe *et al.*, 1992; Magarinos & McEwen, 1995; Sunanda *et al.*, 1995; Galea *et al.*, 1997; Sousa *et al.*, 2000), and induces a reduction in apical dendritic length and numbers (Cook & Wellman, 2004; Radley *et al.*, 2004), as well as axospinous synapse loss in the rat mPFC (Radley *et al.*, 2006). The present study demonstrated a restraint stress-induced reduction of immunoreactive dendrites in the mPFC and hippocampus, consistent with previous studies. Caution must be exercised, however, as there are methodological differences between the present study and works published previously. Golgi impregnation methods were used in previous studies (Watanabe *et al.*, 1992; Magarinos & McEwen, 1995; Sunanda *et al.*, 1995; Galea *et al.*, 1997; Sousa *et al.*, 2000), a method that is well established. In contrast, immunostaining with βT-III was used in the present study to examine the neuronal morphology. βT-III is a neuron-specific cytoskeletal protein (McDonald *et al.*, 1989). Immunostaining with

βT-III has been previously used as a neuronal marker (Caccamo *et al.*, 1989; Svendsen *et al.*, 2001; Katsetos *et al.*, 2003) and has been used to demonstrate neuronal loss following lesions (Geisert & Frankfurter, 1989). Using this method, alterations induced by glucocorticoids were detected in neuronal structures *in vitro* (Zhu *et al.*, 2006) and *in vivo* (Zhu *et al.*, 2007). Analysis of immunostaining in the present study reveals that both repeated restraint and immobilization result in marked morphological alterations in the hippocampus and mPFC. Taken together, these findings suggest that β-III immunostaining is a useful method for detection of changes in neuronal morphology induced by stress.

Atrophy of apical dendrites in the rat hippocampus CA3 and mPFC resulting from repeated restraint stress was reversible (Conrad *et al.*, 1999; Radley *et al.*, 2005), as identified by Golgi staining. Whether this form of stress results in permanent neuronal damage remains questionable (Conrad *et al.*, 1999). We have not tested whether the structural alterations in the mPFC and hippocampus identified by β-T-III immunostaining are reversible. Obviously, this work is essential. Nevertheless, the present findings with βT-III immunostaining provide empirical evidence for striking stress-induced neuronal changes in rat brain induced by restraint stress.

Despite extensive efforts, the molecular mechanisms underlying chronic stress-induced neuronal damage have not been fully elucidated. There is considerable evidence that stress-induced increases of corticosterone in brain tissues are factors that mediate neuronal effects of repeated stress (Moghaddam *et al.*, 1994; Magarinos & McEwen, 1995). In fact, prolonged corticosterone exposure significantly reduces hippocampal neuron numbers in both rats and primates (Sapolsky *et al.*, 1985, 1990; Woolley *et al.*, 1990). Consistent with this notion, the present study showed a significantly high level of plasma corticosterone in rats subjected to repeated restraint even on the 21st day. Although the hypothalamic-pituitary-adrenal axis can undergo habituation to a new stressor after chronic stress (Magarinos & McEwen, 1995; Bhatnagar & Dallman, 1998), numerous follow-up studies (Suarez *et al.*, 1998; Mizoguchi *et al.*, 2001; Makatsori *et al.*, 2003; Weiss *et al.*, 2004; Gameiro *et al.*, 2005) demonstrated that chronic stress, including a stress regimen the same as that used in the current study (Watanabe *et al.*, 1992; Madrigal *et al.*, 2003; Retana-Marquez *et al.*, 2003), significantly increases plasma corticosterone levels, as compared with those of control animals. One point worth noting in the present study is that the time interval between removing rats from restrainers to decapitation was kept very short (≤ 10 s). This rapid conscious decapitation causes a rapid cessation of neuronal activity without antecedent stress (Vanderwolf *et al.*, 1988; Holson, 1992; Vahl *et al.*, 2005). Therefore, the increased plasma corticosterone levels were not a result of a new stress response secondary to handling and transport but instead reflected hormone levels at the end of the chronic restraint stress. Whereas repeated stress may result in a habituation or partial habituation manifested as a relatively lower corticosterone level than that occurring in the initial days of stress and a rapid decrease in elevated plasma corticosterone levels after the cessation of stress (Mizoguchi *et al.*, 2001), corticosterone levels in response to chronic stress are still higher relative to non-stressed animals and to basal levels pre-stress. This elevated corticosterone may contribute to hippocampal dendritic changes caused by chronic stress.

The neurotransmitter glutamate also plays an important role in the stress response. A modest increase in glucocorticoid concentrations that occurs between the trough and peak part of the normal circadian cycle doubles extracellular glutamate concentrations, whereas a rise in glucocorticoids induced by stress produces a fourfold increase in glutamate (Stein-Behrens *et al.*, 1994). In fact, glucocorticoid-induced hippocampal damage can be prevented by blocking *N*-methyl-D-aspartate receptors (Armanini *et al.*, 1990). Agmatine carries a guanidino group that enables it to block heteromeric *N*-methyl-D-aspartate receptor channels (Yang & Reis, 1999). Furthermore, agmatine can block voltage-gated calcium channels with high potency in cultured rat hippocampal neurons (Weng *et al.*, 2003; Zheng *et al.*, 2004). These specific

characteristics of agmatine may account for its ability to diminish the injurious effects of repeated restraint stress on mPFC and hippocampal neurons as observed in the present study. Recent studies demonstrate that agmatine may have antiapoptotic properties (Zhu *et al.*, 2006) that may also contribute to its putative neuroprotective effects observed in the current study. Our present study also showed that treatment with agmatine of both control and stressed animals did not result in any significant effects on basal or stress-induced increases of plasma corticosterone. Hence, agmatine does not appear to affect the release and metabolism of corticosterone during stress. The neuroprotective effects of agmatine against stress-induced morphological alterations are probably dependent on the other mechanisms mentioned above.

Repeated restraint stress-induced structural alterations in the mPFC and hippocampus were accompanied by significant reductions of endogenous agmatine levels in these regions (Fig. 7). These findings point to a causal relationship between these structural changes and the loss of neuroprotective actions of endogenous agmatine, particularly given that administration of exogenous agmatine antagonized stress-induced neuronal abnormalities (Fig 2 and Fig 3). However, at present, it is unknown exactly how repeated restraint stress caused the reduction of endogenous agmatine and how reduced endogenous agmatine levels play a role in stress-induced structural alterations. One possible explanation is that endogenous agmatine may be depleted during the restraint stress paradigm, after which protection via blockade of *N*-methyl-D-aspartate receptors and calcium channels may be lost. In support of such a view is the finding of significantly increased concentrations of putrescine, a metabolic product of agmatine and also a product of ornithine catalysed by ornithine decarboxylase (Tabor & Tabor, 1984), in the frontal cortex and hippocampus of rats and mice after acute and chronic restraint stress (Gilad & Gilad, 2002;Hayashi *et al.*, 2004) or after chronic immobilization (Sohn *et al.*, 2002). Increases in putrescine concentrations have been explained as a result of stress-induced activation of ornithine decarboxylase (Gilad & Gilad, 2003). However, it cannot be ruled out that some part of putrescine comes from the increased metabolism of agmatine during chronic stress.

It is important to note that repeated restraint stress significantly reduced endogenous agmatine levels in all of the four brain regions measured. However, we did not find any noticeable architectural alterations in the striatum and hypothalamus. This paradoxical phenomenon may be because agmatine's neuroprotective effects are consistent with regions that display robust synaptic plasticity. Similar examples of a selective effect include those of glucocorticoids and glutamate. Glucocorticoids are the main stress hormones released during stress. Glucocorticoids exert effects throughout the brain (McEwen *et al.*, 1968), whereas exposure to glucocorticoids results in neuronal damage only in the hippocampus (Sapolsky *et al.*, 1985, 1985) and mPFC (Wellman, 2001). Furthermore, stress-induced glutamate release has been recognized to play an important role in stress-induced cellular architectural changes. However, whereas stress (including restraint) causes significantly elevated levels of glutamate in the hippocampus, mPFC, striatum and hypothalamus (Moghaddam, 1993; Bland *et al.*, 1999; Engelmann *et al.*, 2002), architectural changes have only been found in the hippocampus and mPFC. Therefore, lower agmatine levels in the stress-unaltered striatum and hypothalamus are not in conflict with its neuroprotective effect in the hippocampus and mPFC, where administration of agmatine did prevent the neuronal changes caused by repeated restraint stress.

As an endogenous polyamine, agmatine is derived from enzymatic decarboxylation of L-arginine by ADC (Tabor & Tabor, 1984). However, whether the ADC gene is responsible for the biosynthesis of agmatine in mammalian tissues is still a matter of debate. Although one study questioned the production of agmatine *in vivo* (Coleman *et al.*, 2004), another report confirmed that the decarboxylation of arginine produces agmatine in rat liver (Nissim *et al.*, 2002). We have previously identified a mammalian cDNA and amino acid sequence that encodes ADC (Zhu *et al.*, 2004). Recently, we demonstrated the presence of this enzyme in

brain regions including the prefrontal cortex and hippocampus in rats and human (Iyo *et al.*, 2006). Interestingly, after silencing of ADC by applying small interfering RNA directed to the N-terminal regions of ADC cDNA, the mRNA and protein levels of ADC in cultured neurons/C6 cells were down-regulated, resulting in reduced ADC activity and agmatine production (Iyo *et al.*, 2006). This experiment not only confirmed the contribution of the ADC gene to the agmatine biosynthesis in mammalian cells but also revealed some of the regulatory characteristics of mammalian ADC. The present study demonstrates that a stress-induced reduction of endogenous agmatine levels is paralleled by an elevation of ADC protein expression, which may be a compensatory mechanism to respond to an increased demand for agmatine. The fact that elevated ADC protein levels were abolished by administration of exogenous agmatine further indicates that ADC is a regulated enzyme *in vivo* and that ADC may play a crucial role in determining the availability of agmatine in brain tissues.

In summary, the present study provides evidence of neuroprotective properties of agmatine against repeated restraint stress-induced structural alterations in the rat mPFC and hippocampus. Neuronal structural alterations were paralleled by reduced endogenous agmatine levels in these brain areas and were reversible upon exogenous agmatine administration. The activation of ADC expression following chronic stress may be an important homeostatic adaptation of the brain. Taken together, the present study suggests that agmatine and ADC may be one component of a defensive system involved in protection against chronic stress-induced neuronal damage in the brain. As there is a strong relationship between stress, mood disorders and neurodegenerative diseases, clarification of the neuroprotective role of agmatine as well as the mechanisms involved could have a significant impact on treatment strategies for these diseases.

## Acknowledgments

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## Abbreviations

ACd, dorsal anterior cingulated cortex; ADC, arginine decarboxylase; βT-III, β-tubulin III; HPLC, high-performance liquid chromatography; IL, infralimbic cortex; mPFC, medial prefrontal cortex; PL, prelimbic cortex.

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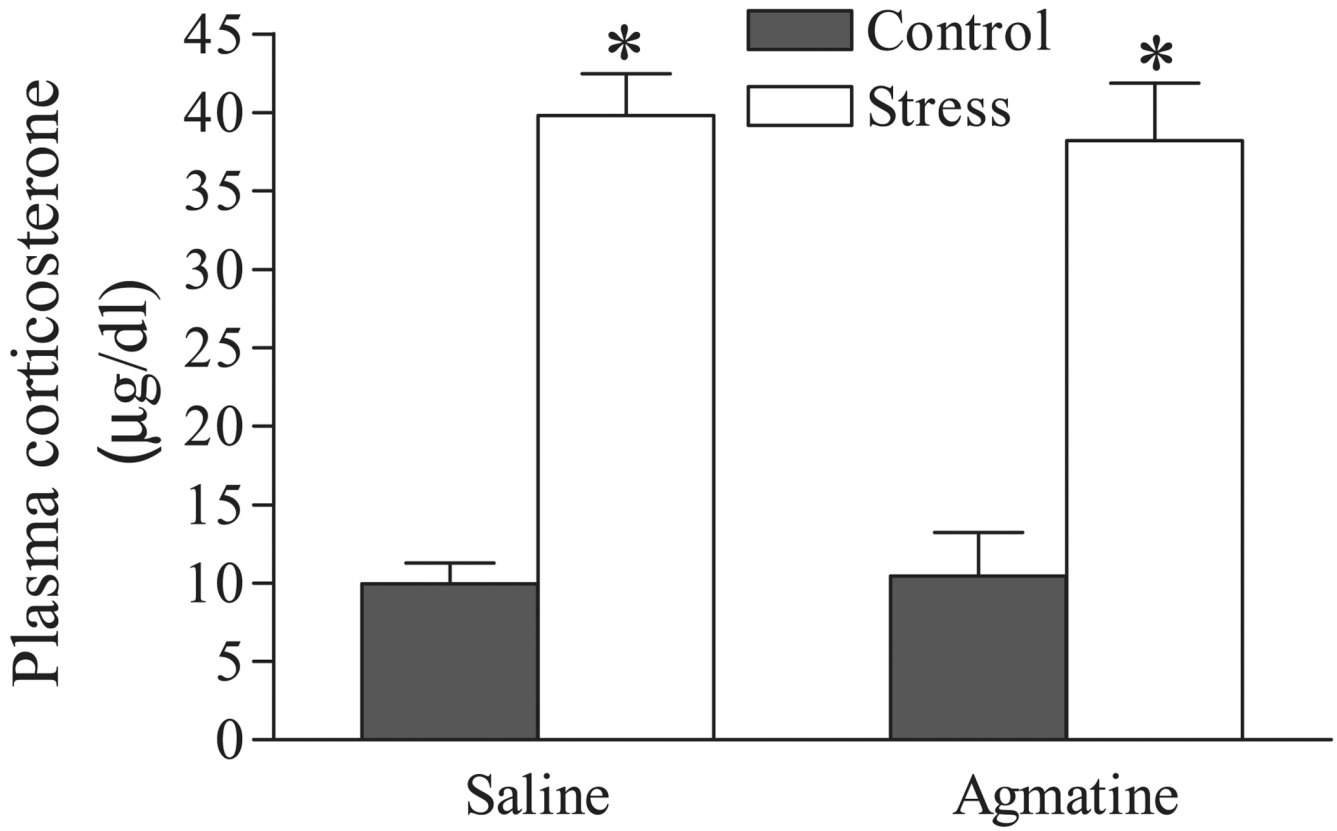


**FIG. 1.**

Plasma corticosterone concentrations were measured in rats of the control, control plus agmatine, restraint and restraint plus agmatine groups. The rats in the control and restraint groups were injected with saline ($n = 8$). The trunk blood was collected on the 21st day immediately after the end of the 6-h restraint. *$P < 0.01$, as compared with the control.

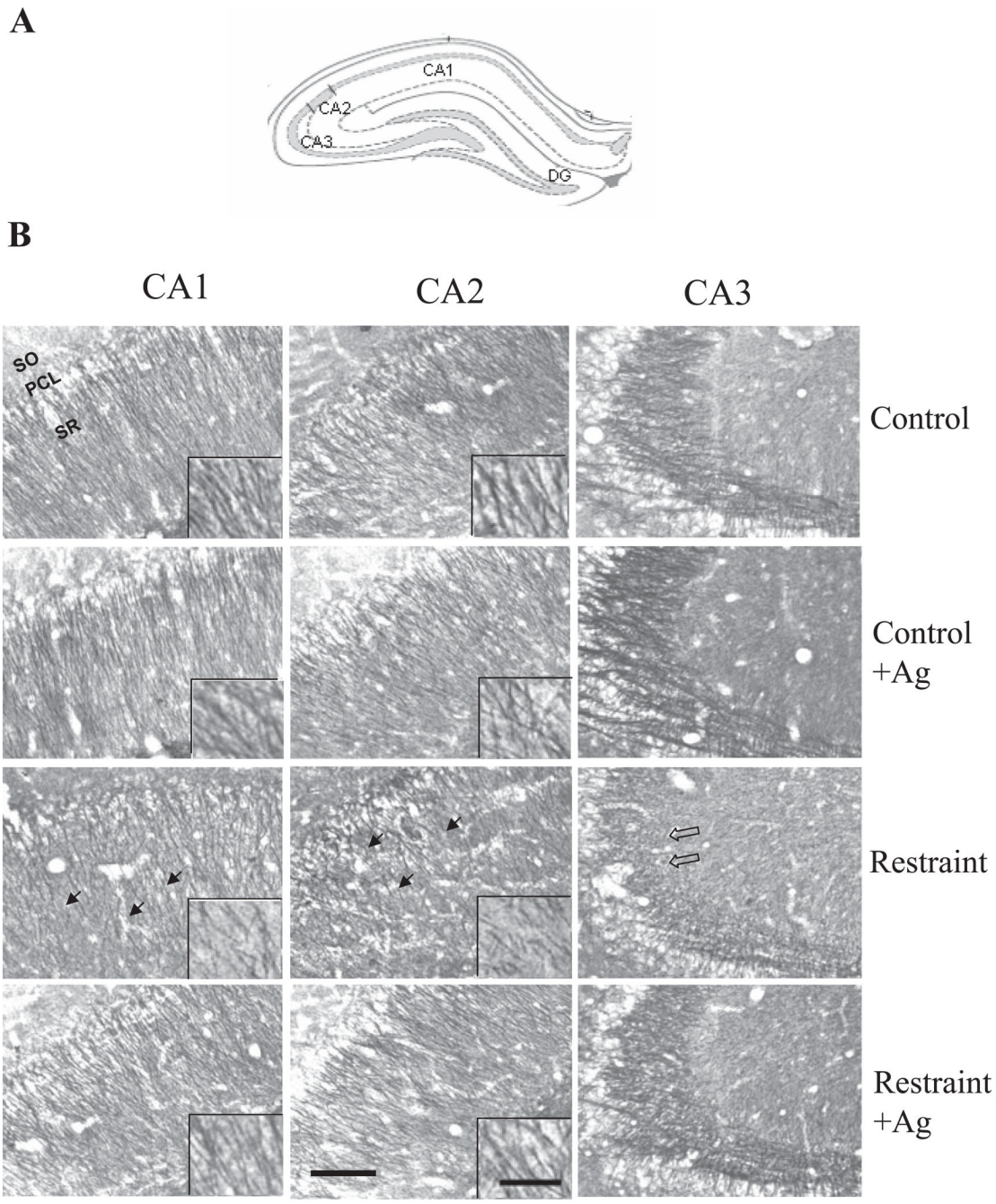


**FIG. 2.**

(A) Schematic representation of the hippocampal sub-fields in the coronal plane (approximately Bregma −3.24 mm; Paxinos & Watson, 2005). (B) Photomicrographs showing the CA1, CA2 and CA3 of the rat hippocampus immunostained with βT-III after the injection of saline (Control), agmatine alone (50 mg/kg/day, i.p., Control + Ag), repeated restraint plus saline (Restraint) and repeated restraint stress plus agmatine (50 mg/kg/day, i.p., Restraint + Ag) for 21 days ($n$ = 6 for each group). PCL, pyramidal cell layer; SO, stratum oriens; SR, stratum radiatum. Solid arrows point to areas that showed less intensity of immunostaining in the CA1 and CA2 of stressed animals, compared with those of the control and restraint plus agmatine groups. The open arrows point to an area in the CA3 with reduced mossy .bers in

stressed animals. Higher magnifications are shown in the lower right corner of each low-power photomicrograph. Scale bars:100 μm except for inserts; 20 μm for inserts.

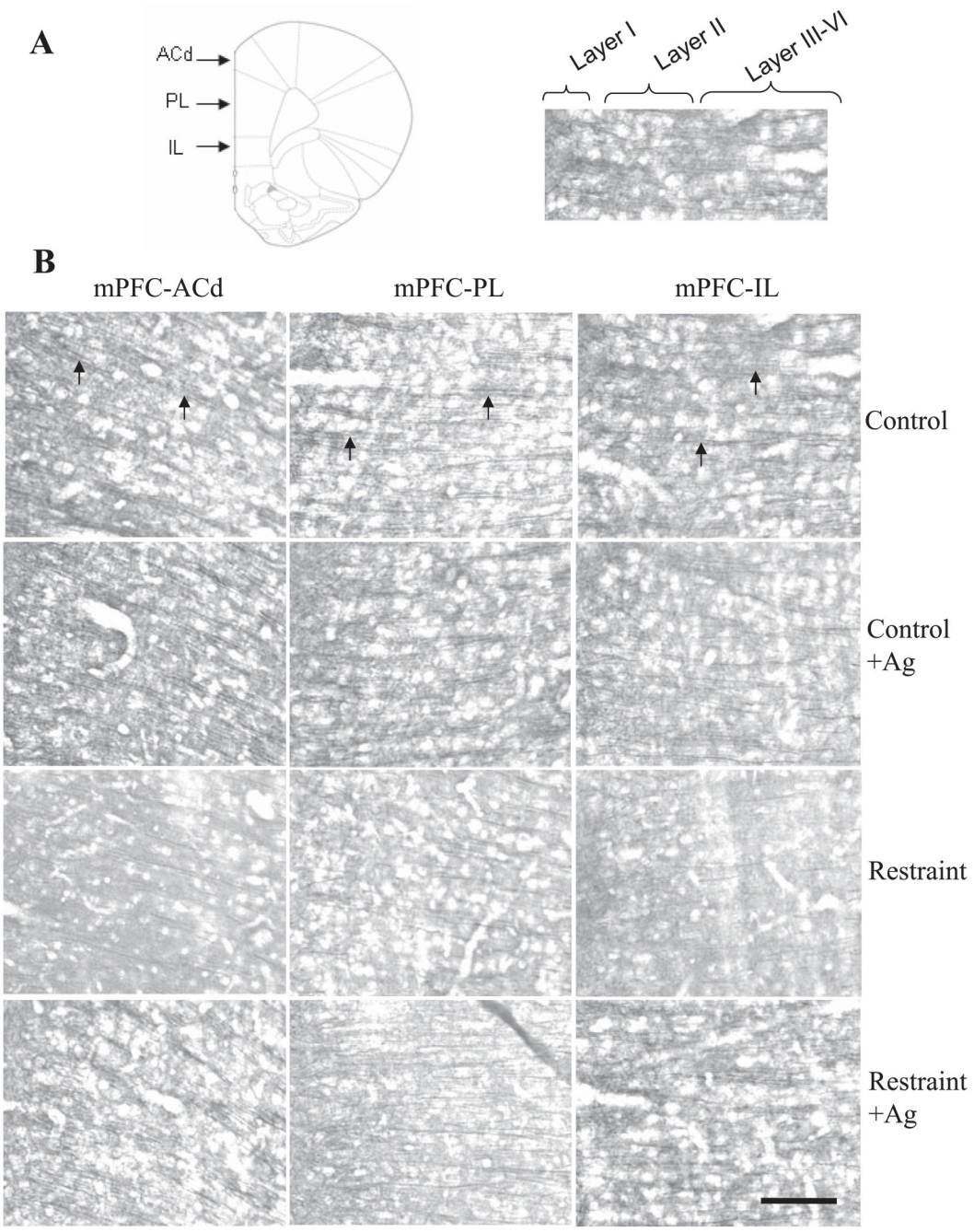


**FIG. 3.**

(A) Left: Schematic representation of the division of the mPFC in the coronal plane (approximately Bregma +3.24 mm; Paxinos & Watson, 2005). Right: The same image of mPFC-IL showing boundaries between layers. (B) Photomicrographs showing the ACd, PL and IL in the rat mPFC immunostained with βT-III antibody after injection of saline (Control), agmatine alone (50 mg/kg/day, i.p., Control + Ag), repeated restraint stress plus saline (Restraint) and repeated restraint stress plus agmatine (50 mg/kg/day, i.p., Restraint + Ag) for 21 days ($n = 6$ for each group). Arrows indicate apical dendrites immunostained for β-III. Scale bar, 100 μm for all images.

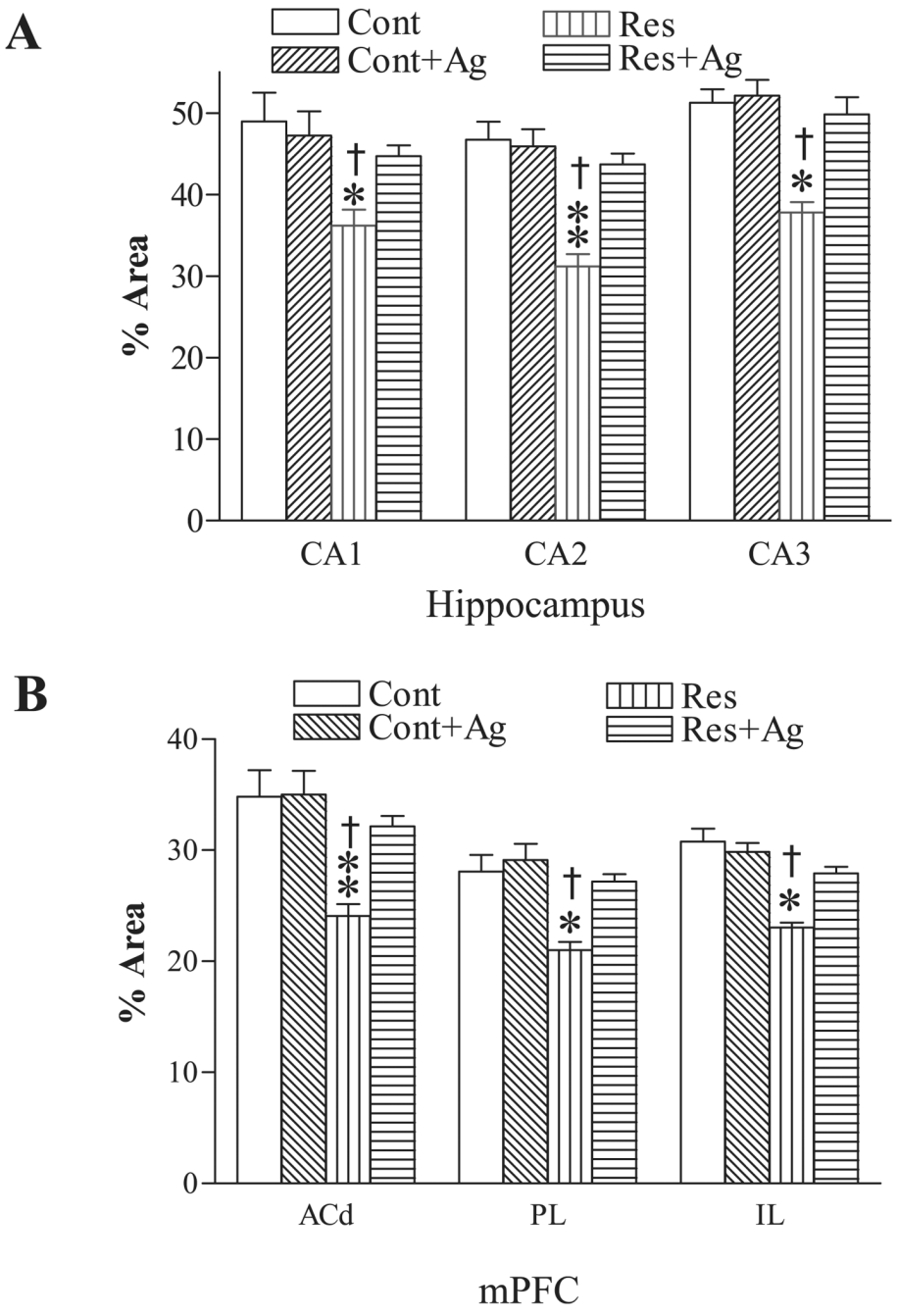


**FIG. 4.**

Graphs showing semiquantitative changes of β-III-immunoreactive intensities in the hippocampus (A) and mPFC (B) among the control (Cont), control plus agmatine (Cont + Ag), restraint (Res) and restraint plus agmatine (Res + Ag) groups by the analysis using ImageJ image software ($n = 6$ for each group). *$P < 0.05$, **$P < 0.01$, compared with the control (Cont). $^{\dagger}P < 0.05$, compared with the restraint plus agmatine (Res + Ag) group.

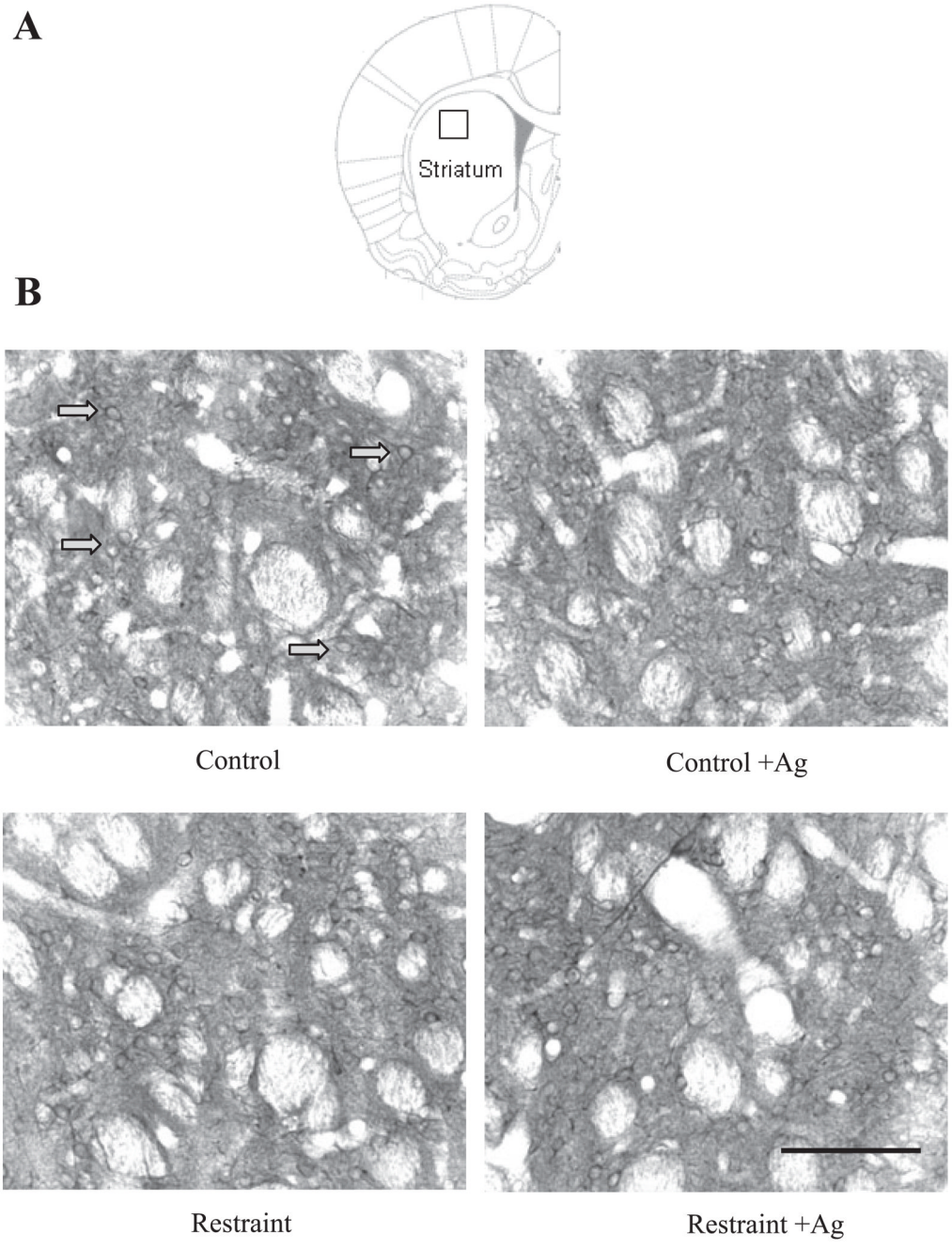


**FIG. 5.**

(A) Schematic representation of the striatum in the coronal plane (approximately Bregma 1.08 mm; Paxinos & Watson, 2005). (B) Photomicrographs show the area (boxed zone in A) in the rat striatum immunostained with βT-III after the injection of saline (Control), agmatine alone (50 mg/kg/day, i.p., Control + Ag), repeated restraint plus saline (Restraint) and repeated restraint plus agmatine (50 mg/kg/day, i.p., Restraint + Ag) for 21 days ($n$ = 6 for each group). Open arrows point to neuronal cells that are clustered around striatal fiber bundles. Scale bars, 50 μm.

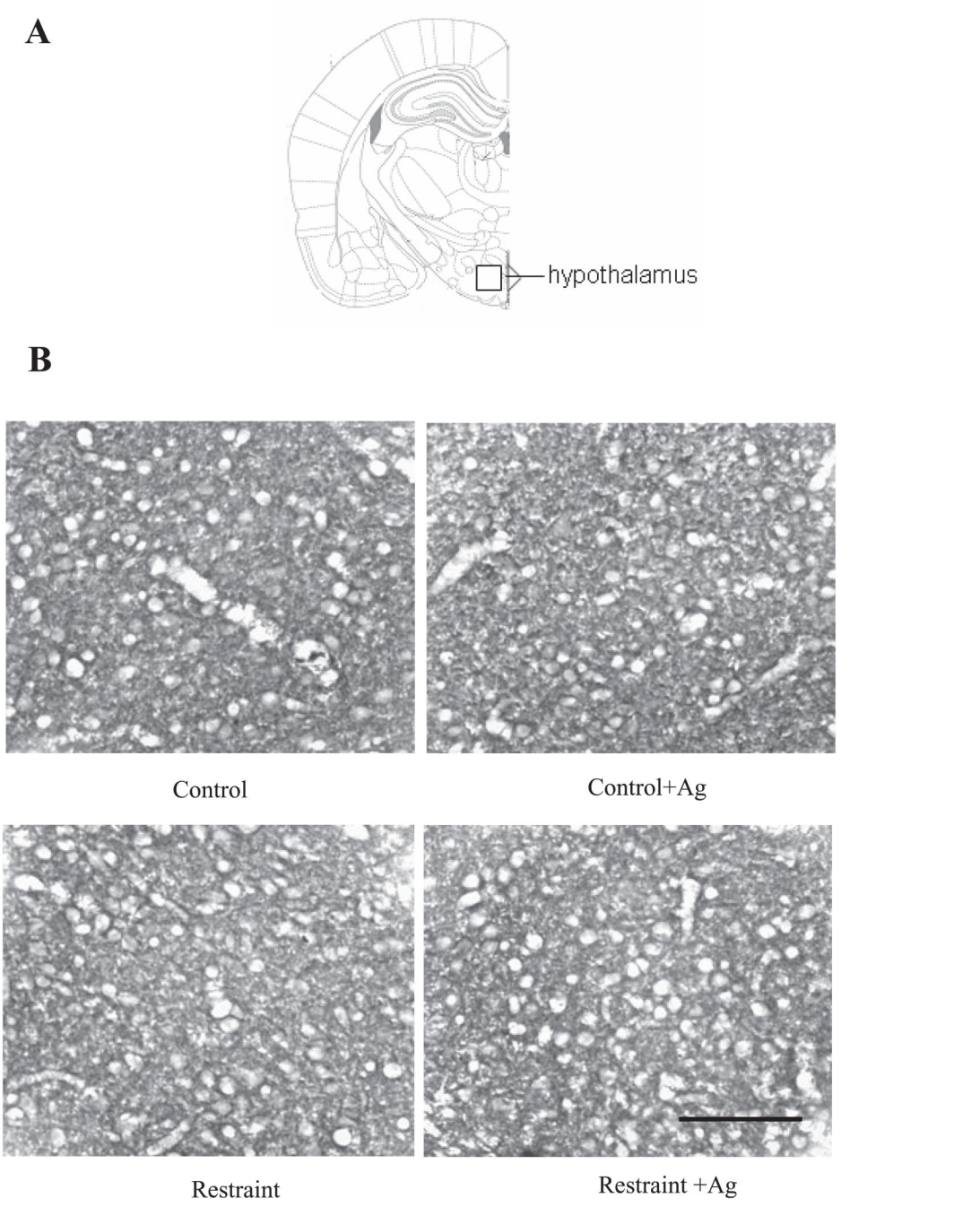


**FIG. 6.**

(A) Schematic representation of the hypothalamus in the coronal plane (approximately Bregma −3.12 mm; Paxinos &Watson, 2005). (B) Photomicrographs show the area (boxed zone in A) in the rat hypothalamus immunostained with βT-III after the injection of saline (Control), agmatine alone (50 mg/kg/day, i.p., Control + Ag), repeated restraint plus saline (Restraint) and repeated restraint plus agmatine (50 mg/kg/day, i.p., Restraint + Ag) for 21 days (*n* = 6 for each group). Scale bar, 50 μm.

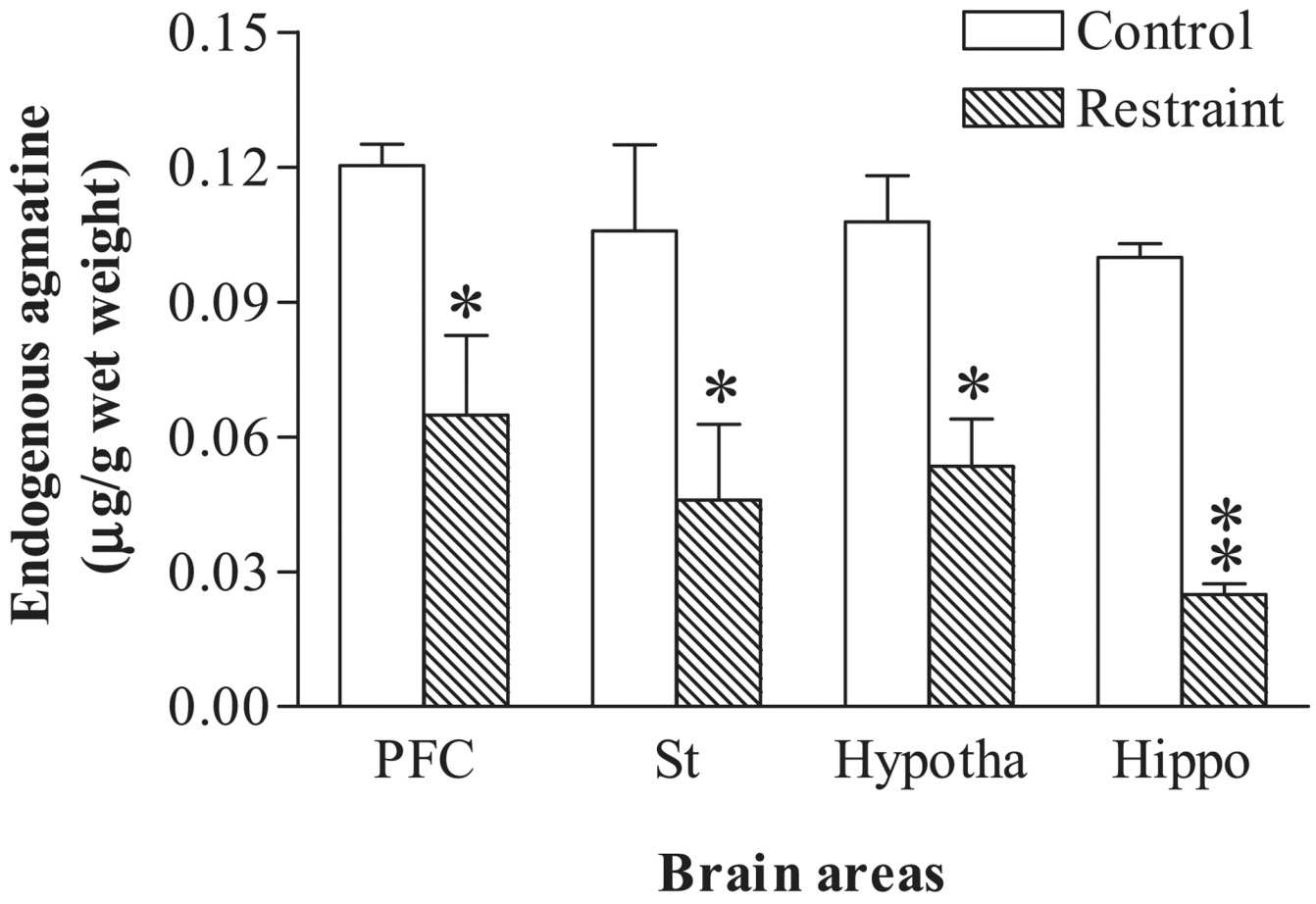


**FIG. 7.**

Endogenous agmatine levels were measured by HPLC in the prefrontal cortex (PFC), striatum (St), hypothalamus (Hypotha) and hippocampus (Hippo) in control and repeated restraint rats ($n$ = 6). Results are the mean ± SEM. *$P$ < 0.05, **$P$ < 0.01, compared with the control.

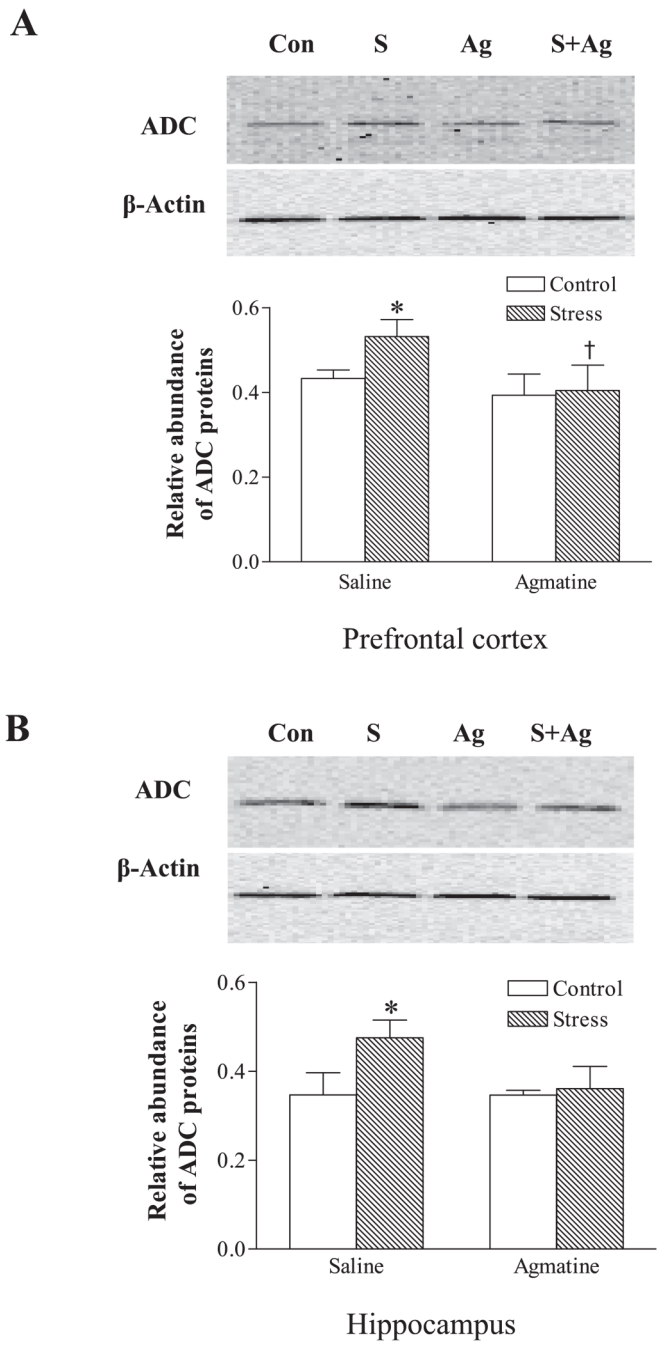


**FIG. 8.**

Upper panel: Photograph of immunoblotted ADC in the prefrontal cortex (A) and hippocampus (B) in control and repeated restraint rats that were injected with saline or agmatine, respectively ($n$ = 6). Middle panel: The same membrane was again probed with anti-β-actin antibody (after stripping) as an internal control. Lower panel: Semiquantitative analysis by densitometry of bands after western blotting. Results are the mean ± SEM ($n$ = ¼ 6). *$P$ < 0.05, compared with control; $^{\dagger}P$ < 0.05, compared with the stress plus saline group. Con, control plus saline; S, stress plus saline; Ag, injection with agmatine; S+Ag, stress plus injection with agmatine.

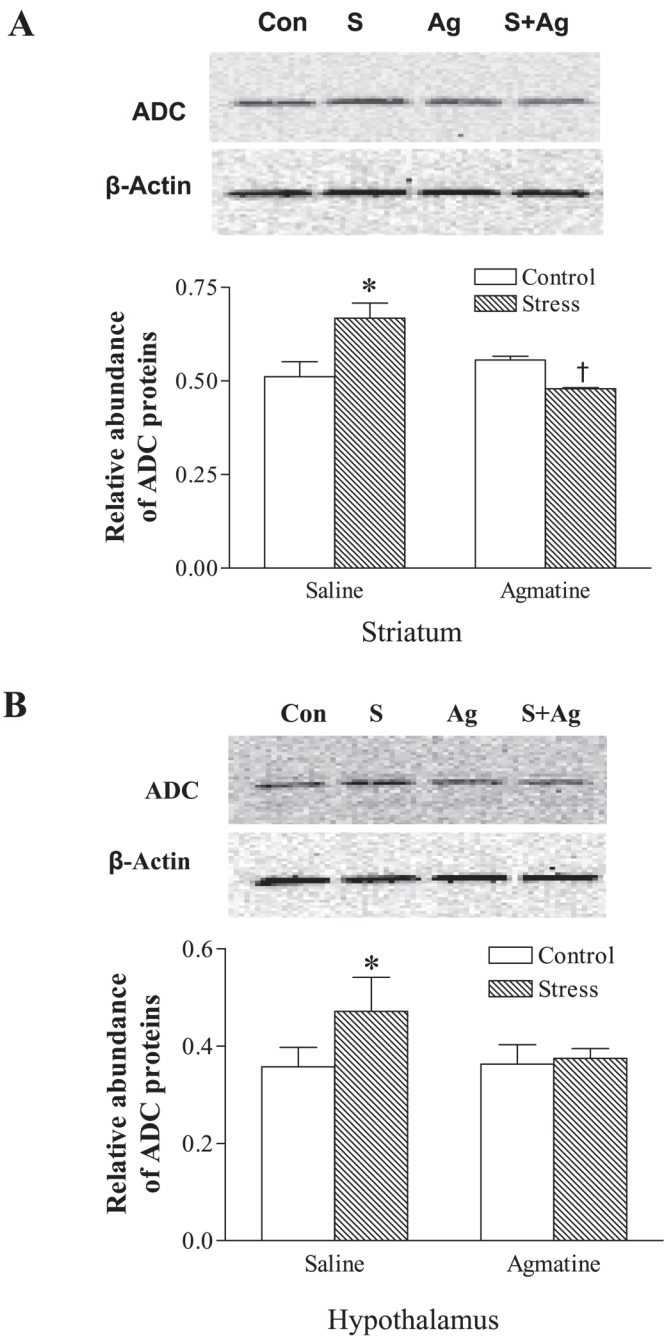


**FIG. 9.**

Upper panel: Photograph of immunoblotted ADC in the striatum (A) and hypothalamus (B) in control and repeated restraint rats that were, respectively, injected with saline or agmatine ($n = 6$). Middle panel: The same membrane was again probed with anti-β-actin antibody (after stripping) as an internal control. Lower panel: Semiquantitative analysis by densitometry of bands after western blotting. Results are the mean ± SEM ($n = 6$). *$P < 0.05$, compared with control. $^{\dagger}P < 0.01$, compared with the group of stress plus saline. Con, control plus saline; S, stress plus saline; Ag, injection with agmatine; S+Ag, stress plus injection with agmatine.