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Neuroprotective Effects of Agmatine Against Cell Damage Caused by Glucocorticoids in Cultured Rat Hippocampal Neurons

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Abstract

In the present study the neuroprotective effects of agmatine against neuronal damage caused by glucocorticoids were examined in cultured rat hippocampal neurons. Spectrophotometric measurements of lactate dehydrogenase activities, β -tubulin III immunocytochemical staining, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling assay (TUNEL) labeling and caspase-3 assays were carried out to detect cell damage or possible involved mechanisms. Our results show that dexamethasone and corticosterone produced a concentration-dependent increase of lactate dehydrogenase release in 12-day hippocampal cultures. Addition of 100 μ M agmatine into media prevented the glucocorticoid-induced increase of lactate dehydrogenase release, an effect also shared with the specific *N*-methyl-D-aspartate receptor antagonist MK801 and glucocorticoid receptor antagonists mifepristone and spironolactone. Arcaine, an analog of agmatine with similar structure as agmatine, also blocked glucocorticoid-induced increase of lactate dehydrogenase release. Spermine and putrescine, the endogenous polyamine and metabolic products of agmatine without the guanidino moiety of agmatine, have no appreciable effect on glucocorticoid-induced injuries, indicating a structural relevance for this neuroprotection. Immunocytochemical staining with β -tubulin III confirmed the substantial neuronal injuries caused by glucocorticoids and the neuroprotective effects of agmatine against these neuronal injuries. TUNEL labeling demonstrated that agmatine significantly reduced TUNEL-positive cell numbers induced by exposure of cultured neurons to dexamethasone. Moreover, exposure of hippocampal neurons to dexamethasone significantly increased caspase-3 activity, which was inhibited by co-treatment with agmatine. Taken together, these results demonstrate that agmatine can protect cultured hippocampal neurons from glucocorticoid-induced neurotoxicity, through a possible blockade of the *N*-methyl-D-aspartate receptor channels or a potential anti-apoptotic property.

Keywords

agmatine; hippocampus; glucocorticoids; apoptosis; lactate dehydrogenase; β -tubulin III

Agmatine is an endogenous polyamine derived from enzymatic decarboxylation of L-arginine (Tabor and Tabor, 1984) and is present in the brain and other tissues of mammals (Li et al., 1994; Lortie et al., 1996). In the past decade, accumulating pharmacological and physiological evidence has adumbrated a major role for agmatine in the CNS. In neuronal tissues, agmatine is present in axon terminals where it is associated with synaptic vesicles

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(Reis et al., 1998) and can be transported into synaptosomes via a Na⁺-independent system (Sastre et al., 1997). Agmatine has been reported to act as a ligand of the imidazoline receptor (Li et al., 1994). It inhibits all isoforms of nitric oxide synthase (NOS) (Galea et al., 1996), and blocks nicotinic receptors (Loring, 1990), voltage-gated Ca²⁺ channels (Weng et al., 2003; Zheng et al., 2004) and *N*-methyl-D-aspartate (NMDA) receptor channels (Yang and Reis, 1999). These functional characteristics suggest that agmatine may play a role as a neurotransmitter or neuromodulator in the brain.

Agmatine has previously been showed to exert neuroprotective actions by reducing the size of ischemic infarcts or the loss of cerebellar neurons after focal or global ischemia *in vivo* (Gilad et al., 1996; Kim et al., 2004). Agmatine has also been reported to attenuate the extent of neuronal loss following excitotoxic spinal cord injury (Fairbanks et al., 2000) and to prevent neurotoxicity produced by glutamate and NMDA in PC12 cells (Zhu et al., 2003) and the neuronal cultures of the rat cortex (Zhu et al., 2003) and cerebellums (Olmos et al., 1999). However, more compelling evidence is needed for elucidating its neuroprotective role on different brain neurons.

The hippocampus plays a vital role in learning and memory (Eichenbaum et al., 1992). It also influences autonomic and vegetative functions (Jacobson and Sapolsky, 1991). Many studies have demonstrated that the hippocampus is one of the most vulnerable brain regions to various neurobiological insults. Prolonged stress has long been considered as one of the primary insults to the hippocampus. During stress, enhanced activity of the adrenocortical axis results in elevated circulating glucocorticoid concentrations (Dallman et al., 1987). Prolonged and excessive glucocorticoid secretion can result in damaging effects on the structural integrity of brain neurons. The hippocampus is rich in glucocorticoid receptors (GR) (Joels, 2001) and has been recognized as a primary target for brain effects of glucocorticoids (McEwen et al., 1986). Indeed, chronic secretion of glucocorticoids induced by stress increases the incidence of hippocampal neuron loss in both rats and primates (Sapolsky et al., 1985, 1990). Additionally, exposure of the hippocampus to elevated glucocorticoid concentrations exacerbates the toxicity of excitotoxic seizures, hypoxic-ischemia, hypoglycemia, antimetabolites, and oxygen radical generators (Sapolsky, 1990). Given that glucocorticoid-induced neuronal death in the hippocampus has been implicated in mood disorders and neurodegenerative diseases, it would have significance in clinical practice to find a molecular agent which has ability to protect the hippocampal neurons from glucocorticoid-induced neuronal damage.

As an endogenous polyamine, agmatine is abundant in the hippocampus (Feng et al., 1997; Reis et al., 1998), and neuroprotective effects of agmatine have been demonstrated in the hippocampal neurons (Yang and Reis, 1999). On the other hand, glucocorticoid-induced hippocampal damage has been reported to be related to NMDA receptors (Armanini et al., 1990) and calcium channels (Elliott and Sapolsky, 1993; Fuller et al., 1997). Given the ability of agmatine to block NMDA receptor channels (Yang and Reis 1999) and calcium channels (Weng et al., 2003), therefore, agmatine may be able to reduce the neurotoxicity of glucocorticoids in hippocampal neurons. Likewise, glucocorticoids have recently been shown to induce apoptosis in hippocampal neurons (Lu et al., 2003). It would be interesting to find whether agmatine protects these neurons by preventing apoptosis.

In this study, we investigated the neuroprotective effect of agmatine against neuronal damage produced by glucocorticoids in hippocampal cultures and potential involved mechanisms. We hypothesized that exposure of neuronal cultures of rat hippocampus to glucocorticoids will result in neuronal damage, which can be prevented by agmatine. In addition, since apoptosis has been indicated to be involved in the glucocorticoid-induced neuronal damage, we hypothesized that an antiapoptotic characteristic may contribute to

agmatine's neuroprotective role. Further elucidation of the neuroprotective effects of agmatine on hippocampal neurons may lead to novel therapeutic strategies for some diseases such as depression, seizure and neurodegenerative illnesses.

Experimental Procedures

Chemicals and reagents

Agmatine sulfate salt, dexamethasone-water soluble, corticosterone (HBC complex), dizocilpine hydrogen maleate ((+)-MK801), mifepristone, arcaine sulfate salt, spermine dehydrochloride and putrescine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Spironolactone was purchased from Aldrich Chem. Co. (Milwaukee, WI, USA).

Culture of hippocampal neurons

The experimental procedure was approved by the Institutional Animal Use and Care Committee at the University of Mississippi Medical Center and was in accordance with the guidelines of the National Institutes of Health on the care and use of animals. Every effort was made to minimize the number of animals used and their suffering. Hippocampi were dissected from 18-day fetuses of Long Evans rats and placed in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco BRL, Grand Island, NY, USA) containing 1 mM sodium pyruvate and 10 mM HEPES. Then the hippocampal tissues were dissociated in HBSS solution containing 0.125% trypsin solution and 0.1 mg/ml deoxyribonuclease for 15 min at 37 °C. Subsequently, tissues were triturated by repeated passage through a constricted Pasteur pipette. The dispersed tissues were allowed to settle for 3 min. The supernatant was transferred to a fresh tube and centrifuged at 2000 r.p.m. for 90 s. The pellet was resuspended in a neuron-defined culture medium, serum-free neurobasal medium (Gibco BRL), supplemented with B-27, 0.5 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 25 μM glutamate. Trypan Blue-excluding cells were counted and cells were then plated onto six-well plates coated with poly-D-lysine (100 $\mu\text{g}/\text{ml}$; BD Biosciences, Bedford, MA, USA) at $2.5\text{--}3\times 10^5$ per well. Cell cultures were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Half of the medium was replaced with fresh one without glutamate every 3–4 days so as to leave a portion of “conditioned” medium in the culture to facilitate growth. For immunocytochemical staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling assay (TUNEL) labeling, a 13-mm glass coverslip coated with poly-D-lysine (100 $\mu\text{g}/\text{ml}$) was placed in the middle of each well in 12-well plates which were not coated. 1×10^5 Cells (1×10^5) were plated onto each well. Cells adhered to and grew on the coverslips. The medium was changed as previously done for the six-well plates.

Drug exposures

As 10–12 day-old hippocampal cultures have been considered as the mature cultures (Kimonides et al., 1999; Roy and Sapolsky, 2003), 12-day primary hippocampal cultures were used in all experiments of this study for drug exposure. The neuronal cultures were washed twice with Mg^{2+} -free, HEPES-buffered saline (HBS, 146 mM NaCl, 10 mM HEPES, 2 mM CaCl_2 , 5 mM KCl, 10 mM D-glucose, pH 7.4) and incubated in the same solution in the absence (control) or presence of different concentrations of corticosterone or dexamethasone, alone or in combination with agmatine, MK801, arcaine, spermine, putrescine, mifepristone or spironolactone for 1 h. After two washes with fresh HBS solution, cells were subsequently re-incubated in culture media lacking any drugs for an additional period of 23 h. At the end of exposure, cultured neurons were harvested and released LDH in the medium and cells measured. Cultured neurons used for immunocytochemical staining or TUNEL labeling were treated the same way. The

coverslips, where neurons were growing, were washed twice with Dulbecco's Phosphate-Saline (PBS) and fixed in 4% paraformaldehyde. Immunocytochemical staining or TUNEL labeling was performed immediately on the fixed cultures. Our previous experiment demonstrated that 10 μ M MK801 completely prevented glutamate- or NMDA-induced hippocampal neurons death (Wang et al., 2006). Therefore, 10 μ M MK801 was still used in this study. Moreover, in the same study, 100 μ M arcaine did, but 100 μ M spermine or putrescine failed to prevent NMDA- or glutamate-induced hippocampal neuronal damage (Wang et al., 2006). In order to keep consistent with our previous study, 100 μ M concentration of these reagents was chosen in the present experiments.

Lactate dehydrogenase (LDH) assay

The release of LDH, a widely used index of cellular injury (Koh and Choi, 1987), was measured using a cytotoxicity detection kit (LDH) (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to manufacturer's instructions. Briefly, culture medium was collected and immediately centrifuged to remove any cells and debris. Cells were harvested into microfuge tubes by adding 1 ml PBS and homogenized by ultra-sonication twice for 25 s each, followed by centrifuging at 14,000 r.p.m. for 4 min to remove cellular debris. Released LDH in the medium and cell homogenates were measured based on the protocol of kit using Microplate Reader (Bio-Rad, Hercules, CA, USA). Results are expressed as percentage of LDH released into the medium compared with total LDH (medium+cells).

Immunocytochemical staining for β -tubulin III (β T-III)—Immunocytochemical staining was performed using a monoclonal antibody to β T-III raised from mice (Chemicon, Temecula, CA, USA). After fixing with 4% paraformaldehyde, coverslips bearing neuronal cultures were preincubated in 5% bovine serum in PBS supplemented with 0.2% Triton-X 100 for 1 h at room temperature, followed by incubation in primary antibody (1:500 dilution, in PBS containing 0.2% Triton-X 100) overnight at 4 °C. The following day, binding of β T-III antibody was detected with a biotinylated secondary antibody using ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the instructions of the manufacturer. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was used as the substrate. Staining for β T-III was then visualized and analyzed microscopically. To verify the concurrent presence of glial cells in the culture, double immunocytochemical staining for β T-III and glial fibrillary acidic protein (GFAP) was performed on some coverslips bearing neuronal cultures using a protocol of multiple antigen labeling (Vector Laboratories).

TUNEL labeling (*in situ* labeling of nuclear DNA fragments)

DNA damage in dying cells was identified using ApopTag Peroxidase *In situ* Apoptosis Detection kits (Chemicon). Briefly, coverslips bearing neuronal cultures were incubated in an equilibration buffer (30 min) followed by addition of terminal deoxynucleotidyl transferase (TdT) and digoxigenin–deoxyuridine triphosphate (dUTP) reaction buffer (60 min) at 37 °C. Coverslips were then washed in stop/wash buffer for 10 min at 37 °C, followed by incubation in antidigoxigenin antibody conjugated to peroxidase for 30 min at room temperature. The digoxigenin–dUTP–peroxidase complex was visualized by reacting with DAB to generate a brown reaction product. Negative controls were performed by substituting distilled water for TdT in the working solution. TUNEL labeling was then analyzed microscopically by a technician unaware of the study group. Using a 10 $\times$ 10 reticule and 20 $\times$ objective, the total number of cell nuclei and the number of TUNEL-positive cell nuclei were counted in five randomly chosen fields on each treated or control coverslip. The numbers of TUNEL-positive nuclei in representative samples were expressed as a percentage of the total numbers of cell nuclei from five to six coverslips which respectively came from separate cultures.

Cellular caspase-3 activity determination

Cultured neurons used for caspase-3 assay were treated the same way as described above. A caspase-3 immunoassay/activity kit from Calbiochem (La Jolla, CA, USA) was used to measure the cellular caspase-3 activity. The assay utilizes a caspases-3 polyclonal antibody to capture activated caspase-3 from cell lysates. Briefly, following phosphate-buffered saline washing, cell lysate was prepared according to the manufacturer's instructions. The level of caspase-3 enzymatic activity on the cell lysate is directly proportional to the cleavage of 7-amino-4-trifluoromethylcoumarin-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (DEVD-AFC), a substrate of caspase-3, generating free AFC which is then analyzed fluorometrically using a fluorescence plate reader (Wallac Victor Fluorescein 1420 Multilabel Counter, Beckman Coulter, Inc., Fullerton, CA, USA) at excitation 425 and emission 525 nm. Data were corrected for background (no substrate or no cell lysate) and caspase-3 activities (unit) were calculated by comparison with the values of the standard recombinant caspase-3.

Statistics

Data are presented as means $\pm$ S.E.M. values and analyzed by analysis of variance (ANOVA) using single-factor ANOVA (SigmaStat, SYSTAT Software, Inc, Richmond, CA, USA). In the presence of significant *F* values, individual comparisons between means were made using the Student-Newman-Keuls test.

Results

Neuroprotective effects of agmatine on dexamethasone-induced cell damage in cultured hippocampal neurons

It was reported that neurobasal medium and B-27 supplement represent an optimized medium for sustaining the long-term survival of hippocampal neurons and glial growth in this medium is less than 0.5% of the nearly pure neuronal population (Brewer et al., 1993). The double immunocytochemical staining with β T-III (to display putative neurons) and GFAP (for astrocytes) in the present study revealed an almost pure population of neuronal cells with very little GFAP staining (less than 1%; data not shown), which is consistent with the report by Brewer et al. (1993).

We first examined the effect of dexamethasone on cultured hippocampal neurons. As shown in Fig. 1A, there was a significant effect of exposure of cultured hippocampal neurons to dexamethasone ($F_{3,20}=15.43$, $P<0.001$). The Student-Newman-Keuls test revealed that 0.5 and 5 μ M dexamethasone significantly increased LDH release by 23.8% ($P<0.05$) and 54.2% ($P<0.01$), respectively, whereas 0.05 μ M of that was without effect, indicating a concentration-dependent neurotoxicity of dexamethasone on these neurons. Based on these results, 5 μ M dexamethasone was chosen for later experiments in this study. Next, we examined possible neuroprotective effects of agmatine on dexamethasone-induced neuronal damage, and the results showed a significant influence of agmatine on such neuronal damage ($F_{4,25}=12.12$, $P<0.05$, Fig. 1B). While addition of 10 μ M agmatine into exposure buffers did not show significant effect on the increased LDH release produced by 5 μ M dexamethasone, 100 μ M agmatine prevented this increased LDH release, in which group the released LDH was significantly lower than the cultures exposed to 5 μ M dexamethasone alone ($P<0.05$); 100 μ M agmatine alone did not produce a significant change in the LDH release, but it was also significantly lower than that of cultures exposed to 5 μ M dexamethasone ($P<0.05$).

Comparison of effects of agmatine, MK801, GR antagonist and the analog of agmatine on dexamethasone-induced increase of LDH release

We further compared the effects of agmatine with MK801, a non-competitive NMDA receptor blocker, two polyamines arcaine and spermine, as well as mifepristone, an antagonist of GR. As illustrated in the Fig. 2, there were significant effects of these compounds on LDH release ($F_{7,65}=10.05$, $P<0.01$). The Student-Newman-Keuls test further revealed that 100 μM agmatine prevented the increase of LDH release produced by 5 μM dexamethasone ($P<0.05$). Similarly, 10 μM MK801 fully abolished the dexamethasone-induced increase of LDH release, as did 20 μM mifepristone (both $P<0.05$). At a concentration of 100 μM arcaine, a synthetic analog of agmatine with two terminal guanidino groups, had the same effect as agmatine in preventing the increase of LDH release ($P<0.05$). However, spermine, an endogenous polyamine without terminal guanidino groups, failed to prevent the increase of LDH release resulting from exposure of cultures to dexamethasone.

Neuroprotective effects of agmatine on corticosterone-induced cell damage in cultured hippocampal neurons

In order to verify whether natural glucocorticoids had similar effects as dexamethasone on hippocampal neurons, we tested the neurotoxic effect of corticosterone on these cultures. Exposure of hippocampal neurons to corticosterone caused a concentration-dependent neurotoxicity, as indexed by a significant increase of LDH release ($F_{4,28}=4.37$, $P<0.05$, Fig. 3A). The Student-Newman-Keuls test then indicated that exposure to 0.5 and 1 μM corticosterone increase LDH release by 26.9% ($P<0.05$) and 34.1% ($P<0.01$), respectively, whereas exposure of neurons to 0.1 μM corticosterone also increased LDH release by 20.3% but did not reach statistical significance. This corticosterone-induced increase of LDH release was abolished by addition of 100 μM agmatine to the exposure buffer, whereas 10 and 50 μM agmatine did not show this neuroprotective effect (Fig. 3B).

The neuroprotective effect of agmatine was also compared with that of antagonists of NMDA and GRs, as well as the analog of agmatine. Likewise, these agents produced significant effects on LDH release induced by corticosterone ($F_{8,45}=7.76$, $P<0.01$; Fig. 4). The Student-Newman-Keuls test further disclosed that 10 μM MK801 and 40 μM spironolactone, the specific antagonist of mineralocorticoid receptors (MR), completely abolished the corticosterone-induced increase of LDH release ($P<0.01$). Arcaine also prevented the increased LDH release induced by corticosterone ($P<0.05$). A similar effect, however, was not observed with spermine and putrescine, a metabolic product of agmatine without the guanidino moiety. We did not test mifepristone again in this experiment using corticosterone, because the involvement of GR had been demonstrated in the above experiment with dexamethasone.

Immunocytochemical study

The neuroprotective effects of agmatine were further studied using immunocytochemical staining with $\beta\text{T-III}$ in cultured hippocampal neurons treated as described in the experiments for LDH measurements. Control cultures exhibited $\beta\text{T-III}$ -positive cells with a homogeneous and compact morphology (Fig. 5A). Exposure of cultures to 5 μM dexamethasone for 1 h resulted in a significant loss of $\beta\text{T-III}$ -positive neurons (Fig. 5B), with the disappearance of neurites and appearance of disrupted membranes, distorted somata and condensed nuclei. This neuronal loss was prevented by the addition of 100 μM agmatine (Fig. 5C) or 10 μM MK801 (Fig. 5D) to the cultures. Agmatine alone did not markedly affect the morphology of $\beta\text{T-III}$ -positive neurons compared with those exposed to dexamethasone (data not shown).

TUNEL labeling study

The TUNEL labeling was performed on cell cultures treated as before. Sparse numbers of TUNEL labeled cells that exhibited inter-nucleosomal DNA fragmentation were found in the normal hippocampal cultures (Fig. 6A). In contrast, large numbers of TUNEL-positive cells were observed in the dexamethasone-treated cultures (Fig. 6B). After co-incubation of dexamethasone with agmatine or MK801, TUNEL labeled cells were markedly reduced (Fig. 6C and 6D). ANOVA analysis showed that the difference was very significant between these treatments ($F_{3,23} = 25.19$, $P < 0.01$; Fig. 6E).

Caspase-3 measurement

We also examined caspase-3 activity as a specific apoptotic marker in hippocampal neuronal cultures exposed to dexamethasone alone or in combination with agmatine. There was a significant effect for dexamethasone treatment on caspase-3 activity ($F_{2,15} = 8.55$, $P < 0.01$). The Student-Newman-Keuls test revealed that 5 μ M dexamethasone increased caspase-3 activity by 46% in comparison with control values ($P < 0.05$; Fig. 7). In the cultured hippocampal neurons exposed to 5 μ M dexamethasone plus 100 μ M agmatine, caspase-3 activity was similar to that of control.

Discussion

The hippocampal neuronal damage caused by glucocorticoids has been well documented *in vivo* and *in vitro* (Woolley et al., 1990; Virgin et al., 1991; Lu et al., 2003; Crochemore et al., 2005). Several putative mechanisms have been implicated in this damage. One is the glucocorticoid-induced increase in the extracellular glutamate (Sapolsky, 2000). Thus, glucocorticoid-induced hippocampus damage can be prevented by blocking NMDA receptors (Armanini et al., 1990). Consistent with those reports, our present study demonstrated that agmatine, which carries the guanidino group for blocking heteromeric NMDA receptor channels (Yang and Reis, 1999), prevented glucocorticoid-induced increases of LDH release and neuronal damage as indexed by β T-III immunocytochemical staining. This neuroprotective effect was shared by the NMDA receptor antagonist, MK801, and arcaine, a synthetic analog of agmatine that also carries two active moieties of guanidino for blocking NMDA receptor channels (Yang and Reis, 1999). In contrast, spermine and putrescine, two endogenous polyamines without the guanidino group, did not show the neuroprotective effect of agmatine. Therefore, the ability of agmatine to block NMDA receptor channels may contribute to its neuroprotective effect against glucocorticoid-induced neuronal damage in cultured hippocampal neurons, as indicated in our previous observations that agmatine can completely prevent NMDA- and glutamate-induced neuronal damage in neuronal cultures of the rat cortex (Zhu et al., 2003) and hippocampus (Wang et al., 2006).

Numerous studies have demonstrated that increases in intracellular calcium levels are involved in neuronal toxicity (Reagan and McEwen, 1997). Therefore, modulation of voltage-gated calcium channels (VGCC) has been considered as another possible mechanism responsible for glucocorticoid-induced tissue damage. For example, glucocorticoids were reported to significantly increase peak VGCC currents (Fuller et al., 1997), basal cytosolic free calcium concentrations (Elliott and Sapolsky, 1993) and mRNA levels of VGCC (Fomina et al., 1996) in cultured hippocampal neurons, which could be blocked by GR antagonists. Interestingly, 100 μ M agmatine has been reported to block VGCC with a high potency in cultured rat hippocampal neurons (Weng et al., 2003; Zheng et al., 2004). In this regard, this property of agmatine might also contribute to its neuroprotective effect observed in the present study. Further studies are warranted to elucidate this hypothesis.

As a comparison to agmatine, we investigated the possible effect of mifepristone and spironolactone, the antagonists for GR and MR, on glucocorticoid-induced cell damage. Our results showed both antagonists prevented the increase of released LDH release after co-incubation with glucocorticoids, indicating that glucocorticoid-induced neural damage in cultured hippocampal neurons may also be mediated by either class of GRs, in addition to glucocorticoid-induced elevations in glutamate and activation in VGCC. However, previous observations suggested that GR was a preferred one responsible for the “endangering” effects of glucocorticoids on hippocampal neurons (Sousa et al., 1999; Haynes et al., 2001; Lu et al., 2003; Mulholland et al., 2005). Whether MR plays a role during this “endangering” is still a matter of debate. Recent report showed that MR activation even reversed dexamethasone-induced apoptosis (Crochemore et al., 2005). It is likely contrary to our current observation. Different indexes of neuronal damage used to examine the role of MR in our (LDH measurement) and their studies (apoptosis) (Crochemore et al., 2005) may account for such discrepancy, as the involved mechanisms for LDH release and apoptosis could be different. In addition, it was reported that slightly elevated corticosterone protected against neurotoxic injury but high concentration of corticosterone increased the vulnerability of cultured cells to neurotoxic insults (Abraham et al., 2000). Given relatively high concentrations of corticosterone were used in our study, blocking MR might prevent the potential effects of corticosterone. Nevertheless, the precise role of MR in the glucocorticoid-induced “endangering” needs to be clarified by further study.

Perhaps the most significant finding of the present study was that both the increased TUNEL-positive cell numbers and the increased caspase-3 activity produced by exposure to dexamethasone were counteracted by addition of 100 μ M agmatine into the medium, suggesting that agmatine may exert a neuroprotective effect by blocking apoptosis. To our knowledge, this is the first report regarding the potential anti-apoptotic characteristics of agmatine. The precise mechanism by which agmatine blocks apoptosis is not known. One possibility may also be related to agmatine's ability to block NMDA receptor channels, as illustrated by the significant attenuation of TUNEL-positive cell numbers by MK801 (Fig. 6D and 6E). In fact, the antiapoptotic effects of MK801 have been well documented in cultured hippocampal neurons (Ogden et al., 1998; Lee et al., 2003; Lu et al., 2003; Mikati et al., 2003). However, as electron microscopic analysis remains the gold standard for detecting apoptotic damage, ultrastructural analysis is required to elucidate the anti-apoptotic ability of agmatine.

In the present study, exposure of cultured hippocampal neurons to 1 μ M corticosterone resulted in a significant neuronal damage (Fig. 3). This result appears to disagree with the previous reports that this concentration of corticosterone does not cause cell damage (Sapolsky et al., 1988; Roy and Sapolsky, 2003). One possibility for this disparity could be different experimental conditions between these studies. For example, the previous studies used growth media which facilitate the growth of glia (Almeida et al., 2000), and produced hippocampal cultures with a mixture of 30–40% neurons and largely GFAP positive glia (Sapolsky et al., 1988; Roy and Sapolsky, 2003). The present study used the neurobasal medium with a supplement of B-27, which produced an almost pure neuronal culture with less 1% glia. Cultured neurons are more vulnerable to the potential neurotoxicity of corticosterone when few glial cells are present in this culture (Dugan et al., 1995; Lewis et al., 1998). For example, when neuronal cells isolated from newborn rat were maintained in culture as enriched neuronal populations, 80% of the cells were killed by application of excitotoxic concentrations of glutamate and this neuronal death was not observed in mixed neuron/glial cultures (Heidinger et al., 1999).

Conclusion

In summary, results here demonstrated that agmatine protects cultured rat hippocampal neurons against cell damage produced by dexamethasone and corticosterone, as assessed by LDH measurements and immunocytochemical staining with β T-III. This effect is most likely related to the ability of agmatine to block NMDA receptor channels, as demonstrated by the similar protection provided by agmatine, NMDA receptor antagonist MK801, and its structural analog, arcaine. Our results also demonstrated that the neuroprotective effect of agmatine is possibly associated with antiapoptotic effects, as illustrated by the reduction in TUNEL-positive cell numbers and prevention of caspase-3 activation after co-incubation of agmatine with dexamethasone. Taken together, these data support a neuromodulatory role for agmatine in hippocampal neurons.

Acknowledgments

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Abbreviations

ANOVA	analysis of variance
DAB	3,3'-diaminobenzidine tetrahydrochloride
dUTP	digoxigenin–deoxyuridine triphosphate
GFAP	glial fibrillary acidic protein
GR	glucocorticoid receptor
HBBS	Hanks' balanced salt solution
HBS	HEPES-buffered saline
LDH	lactate dehydrogenase
MR	mineralocorticoid receptor
NMDA	<i>N</i> -methyl-D-aspartate
PBS	Dulbecco's Phosphate-Saline
TdT	terminal deoxynucleotidyl transferase
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling assay
βT-III	β -tubulin III
VGCC	voltage-gated calcium channel

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Fig. 1.

The LDH release in primary cultures of hippocampal neurons illustrating the neurotoxic effect of dexamethasone (A) and neuroprotective effect of agmatine (B). Data are shown as mean $\pm$ S.E.M. of six measurements. * $P<0.05$; ** $P<0.01$, compared with control (0); † $P<0.05$, compared with 5 μM dexamethasone.

Fig. 2.

The comparative neuroprotective effects of agmatine, MK801, mifepristone, arcaine and spermine against neuronal damage caused by exposure of neuronal cultures to dexamethasone. Data are shown as mean±S.E.M. of six to eight measurements. * $P<0.01$, compared with control (0); † $P<0.05$, ‡ $P<0.01$, compared with 5 μ M dexamethasone.

Fig. 3.

The LDH release in primary cultures of hippocampal neurons illustrating the neurotoxic effect of corticosterone (A) and neuroprotective effect of agmatine (B). Data are shown as mean $\pm$ S.E.M. of six measurements. * $P<0.05$; ** $P<0.01$, compared with control (0); † $P<0.01$, compared with 1 μ M corticosterone.

Fig. 4.

The comparative neuroprotective effects of agmatine, MK801, spironolactone, arcaine, spermine and putrescine against neuronal damage caused by exposure of neuronal cultures to corticosterone. Data are shown as mean±S.E.M. of five to nine measurements. * $P<0.05$; ** $P<0.01$, compared with control (0); † $P<0.05$, ‡ $P<0.01$, compared with 1 μM corticosterone.

Fig. 5. Agmatine reduces cell death in dexamethasone-treated rat hippocampal cultures. Hippocampal neurons (12 days) were exposed to vehicle (A), 5 μ M dexamethasone either alone (B) or in the presence of agmatine (100 μ M) (C) or MK801 (10 μ M) (D). The coverslips with neuronal cultures were then immunohistochemically stained with antibody to β T-III and microscopically analyzed. Scale bar=100 μ m for all pictures.

Fig. 6. TUNEL labeling in cultured hippocampal neurons (12 days) exposed to vehicle (A), 5 μ M dexamethasone either alone (B) or in combination with 100 μ M agmatine (C) or 10 μ M MK801 (D). These coverslips were performed TUNEL labeling and microscopically analyzed. TUNEL labeling shows the presence of DNA fragmentation in the nuclei of cultured hippocampal neurons as indicated by dark spots. Scale bar=100 μ m for all pictures. Above TUNEL labelings were quantitatively analyzed (E). Data were expressed as percentage of TUNEL positive cells to total cell numbers in five to six coverslips from separate cultures. * $P < 0.01$ versus the control; [†] $P < 0.01$ versus dexamethasone exposure.

Fig. 7. Agmatine blocks dexamethasone-induced activation of caspase-3 in cultured hippocampal neurons. Data expressed as percent of activity in control cultures are the mean $\pm$ S.E.M. of six measurements. * $P<0.01$, compared with control group. † $P<0.01$, compared with 5 μ M dexamethasone.