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Expression of arginine decarboxylase in brain regions and neuronal cells

Abiye H. Iyo, **Meng-Yang Zhu**, **Gregory A. Ordway**, and **Soundar Regunathan**
Division of Neurobiology and Behavior Research, Department of Psychiatry and Human Behavior and Center for Psychiatric Neuroscience, University of Mississippi Medical Center, Jackson, Mississippi, USA

Abstract

After our initial report of a mammalian gene for arginine decarboxylase, an enzyme for the synthesis of agmatine from arginine, we have determined the regional expression of ADC in rat. We have analyzed the expression of ADC in rat brain regions by activity, protein and mRNA levels, and the regulation of expression in neuronal cells by RNA interference. In rat brain, ADC was widely expressed in major brain regions, with a substantial amount in hypothalamus, followed by cortex, and with least amounts in locus coeruleus and medulla. ADC mRNA was detected in primary astrocytes and C6 glioma cells. While no ADC message was detected in fresh neurons (3 days old), significant message appeared in differentiated neurons (3 weeks old). PC12 cells, treated with nerve growth factor, had higher ADC mRNA compared with naive cells. The siRNA mixture directed towards the N-terminal regions of ADC cDNA down-regulated the levels of mRNA and protein in cultured neurons/C6 glioma cells and these cells produced lower agmatine. Thus, this study demonstrates that ADC message is expressed in rat brain regions, that it is regulated in neuronal cells and that the down-regulation of ADC activity by specific siRNA leads to lower agmatine production.

Keywords

agmatine; arginine; arginine decarboxylase; astrocytes; neurons; siRNA

After the discovery of agmatine and arginine decarboxylase (ADC) in rat and bovine brains (Li *et al.* 1994), subsequent studies have demonstrated their presence in many other tissues and cell types (Li *et al.* 1994, 1995; Raasch *et al*. 1995; Regunathan *et al.* 1995; Lortie *et al.* 1996; Regunathan *et al.* 1996; Sastre *et al*. 1998). The findings that agmatine is synthesized and stored in neuronal cells (Feng *et al.* 1997) suggested that the amine may have physiological functions as a neuromodulator (Reis and Regunathan 1998). Agmatine binds to imidazoline, α2-adrenergic and NMDA receptors and is proposed as an endogenous ligand for imidazoline receptors (Li *et al.* 1994; Piletz *et al.* 1995). Agmatine has been shown to modulate transmitter/hormone release, including norepinephrine, vasopressin and glutamate probably acting at voltage-gated calcium channels (Li *et al.* 1994; Kalra *et al.* 1995; Wang *et al.* 2002). The pharmacological actions of agmatine include the potentiation of morphine analgesia and anti-nociceptive effects in various pain models (Kolesnikov *et al.* 1996; Fairbanks *et al.* 2000; Gilad and Gilad 2000; Yu *et al.* 2000; Onal and Soykan 2001), anti-inflammatory effects (Reis and Regunathan 2000; Regunathan and Piletz 2003),

Address correspondence and reprint requests to Dr Soundar Regunathan, PhD, Division of Neurobiology and Behavior Research, Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA. sregunathan@psychiatry.umsmed.edu.

protection against ischemic neuronal injury (Fairbanks *et al.* 2000; Gilad and Gilad 2000; Yu *et al.* 2000; Zhu *et al.* 2003), anti-seizure activity (Aricioglu *et al.* 2003; Su *et al.* 2004; Feng *et al.* 2005) and reduction of tolerance and withdrawal symptoms to morphine (Kolesnikov *et al.* 1996; Li *et al.* 1999; Aricioglu *et al.* 2004). While the physiological role of endogenous agmatine in these actions is not clear, it is evident that ADC may play a crucial role in determining the availability of agmatine in neuronal cells.

We have recently reported the identification of a mammalian cDNA and amino acid sequence that encodes for ADC (Zhu *et al.* 2004). Our findings provided the first molecular evidence that mammalian ADC is a unique enzyme, distinct but related to ornithine decarboyxlase (ODC) and different from mammalian basic amino acid decarboxylases and ADC of bacteria and plants. While the localization of agmatine in brain has been reported (Otake *et al.* 1998), little is known about the regional localization of ADC and its regulation in neuronal cells. Moreover, there appears to be some controversy regarding the function of ADC. While one earlier report questioned the production of agmatine *in vivo* (Coleman *et al.* 2004), another recent report confirmed the decarboxylation of arginine to produce agmatine in rat liver (Horyn *et al.* 2005). Therefore, in this study, we addressed three important issues related to the neurobiology of the ADC/agmatine system in brain. Our objectives were: (i) to understand the regional expression of ADC in rat brain by measuring the activity, protein and mRNA levels; (ii) to determine whether ADC is expressed in cultured neurons/PC12 cells and whether the expression is regulated by the differentiation state of the cells; (iii) to demonstrate that the down-regulation of the ADC gene results in reduced expression of the enzyme and lower production of agmatine using RNA interference (siRNA) technique in cultured neuronal cells.

Material and methods

Culturing of neurons, astrocytes, PC12 cells and C6 glioma cells

Primary neuronal cultures were prepared from rat embryos (E18) as described earlier (Zhu *et al.* 2003). Briefly, cortex was dissected out from the brains of 18-day fetuses of Long–Evans rats and placed in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (Gibco BRL, Grand Island, NY, USA) containing 1 mM sodium pyruvate and 10 mM HEPES. After dissociating in Hanks' balanced salt solution containing 0.125% trypsin solution and 0.1 mg/mL deoxyribonuclease for 15 min at 37°C, the 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 1000 *g* for 90 s. The pellet was re-suspended in a neuron-defined culture medium, serum-free neurobasal medium (Gibco BRL), supplement with B-27, 0.5 mM L-glutamine, 100 IU/mL penicillin, 100 mg/mL stresptomycin and 25 μM glutamate. Trypan blue-excluding cells were counted. Then, the cells were plated onto 6-well plates coated with poly D-lysine (100 μg/mL; BD Biosciences, Bedford, MA, USA) at 2.5–3 × 10^5 cells/well. Cell cultures were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For immunocytochemistry, cells were plated in glass coverslips coated with poly D-lysine (100 μg/mL) that were placed in the middle of wells in 12-well plates. Half of the medium was replaced with fresh medium without glutamate every 3–4 days. Under these culture conditions, 95% of the cells were as determined by GFAP and neuron-specific enolase immunostaining.

Primary cultures of glial cells were prepared from neocortex of rat pups (post-natal day 1) as described earlier (Regunathan *et al.* 1995). Briefly, tissue was dissociated with repeated passage through hypodermic needles (1.7 and 1.0 mm). Cells were plated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and media changed every 3 days. After 14 days, the confluent cell cultures contained only glial cells and the purity of the cultures was verified by immunostaining with glial-specific marker, GFAP. PC12 cells

were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and were maintained in DMEM medium (Cellgro, Mediatech, Herndon, VA, USA) supplemented with 5% heat-inactivated fetal calf serum, 10% horse serum (Summit Biotechnology, Fort Collins, CO, USA), penicillin (100 U/mL), streptomycin (100 mg/mL) and L-glutamine (2 mM). Cells were grown at 37°C in 95% humidified air with 5% CO_2 in plates coated with poly L-lysine. For nerve growth factor (NGF) treatment, semiconfluent (50%) PC12 cells were placed in serum-free RPMI medium supplemented with glutamine and containing NGF (100 ng/mL) or in medium without NGF. C6 glioma cells and COS-7 cells were obtained from ATCC and cultured as described earlier in DMEM with 10% fetal calf serum (Regunathan *et al.* 1995; Zhu *et al.* 2004). Semi-confluent COS-7 cells were transfected with ADC cDNA as described in our earlier publication (Zhu *et al.* 2004).

ADC activity measurements

ADC activity was measured by the release of $^{14}CO_2$ from 1^{14}C-arginine as described previously (Regunathan and Reis 2000). Brains were excised quickly on an ice-cold Petri dish and regions including the hypothalamus, hippocampus, frontal cortex, locus coeruleus (LC), striatum and medulla were dissected out as previously described (Palkovits and Brownstein 1998). For cultured cells, harvested cells were sonicated in Tris-HCl buffer (pH 7.5) and centrifuged to obtain the whole membrane fraction. The activity of ADC was measured in membrane fractions prepared from each brain region or cultured cells. For regions such as LC, and hypothalamus, tissues from three rat brains were combined. Briefly, the membrane pellet was washed once by sonication and re-centrifugation in Tris-HCl buffer (pH 8.25) and re-suspended in the incubation buffer. The membrane suspension (250 μL) was incubated for 1 h at 25°C in 20 mM Tris-HCl buffer (pH 8.25), containing 1 mM $MgSO_4$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 0.1 mM L-arginine and 7.28 μM L[1–^{14}C] arginine. Release of $^{14}CO_2$ from 1 to ^{14}C-arginine was measured by trapping the $^{14}CO_2$ in filter paper wicks saturated with benzethonium hydroxide. The incubation mixture also contained 100 μM of difluromethyl ornithine to inhibit any ODC activity that might contribute to CO_2 production. The reaction was stopped by the injection of 40% Trichloroacetic acid (TCA) into the reaction chamber, the filters transferred to minivials containing 5 mL CytoScint cocktail (ICN Biomedicals Aurora, OH, USA), and counted for radioactivity by liquid scintillation spectrometry (Beckman model LS 5801). Protein concentrations were determined using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as standard.

ADC protein expression in rat brain and cultured cells

Based on the amino acid sequence of ADC (Zhu *et al.* 2004), we have identified an 18-amino acid peptide sequence in the N-terminal region that is unique for ADC. This peptide peptide (STRDLLKELTLGASQATC) was synthesized and polyclonal antibodies were produced in rabbit against this peptide by a commercial vendor (Sigma–Genosys Woodlam, TX, USA). These antibodies were initially verified by immunoblot analysis of membranes of COS-7 cells transfected with ADC cDNA that showed a single band at about 42 kDa (Fig. 3). These antibodies were used for immunoblot analysis of rat brain regions, human brain and cultured cells. The IgG fraction of the antiserum was purified using protein A column (Pierce, Rockford, IL, USA) and used for immunocytochemistry of cultured neurons. The purified IgG was characterized for its specificity by pre-absorption with the antigen peptide. The IgG fraction was incubated overnight at room temperature (30°C) with and without the antigen peptide (50 μg) and used for the immunoblot analysis at 1/3000 dilution (Fig. 4).

For immunoblot analysis, rat brain regions were dissected out as described above and homogenized in HEPES buffer (pH 7.4). After centrifugation at 1000 *g*, the supernatant is centrifuged at 30 000 *g* for 20 min and the resulting pellet was used for ADC immunoblot

analysis. The human brain frontal cortex samples were also homogenized in HEPES buffer and membrane and cytosol fractions were prepared after centiguation at 30 000 *g* for 20 min. The membrane pellet was solubilized in sodium dodecyl sulfate sample buffer for polyacrylamide gel electrophoresis. The amount of protein in each sample was determined before electrophoresis and the volume was adjusted to have same amount of amount of protein loaded (about 1 μg/well) for each sample. The separated protein was transferred to polyvinylidene difluoride membrane and exposed to polyclonal antibody to ADC at 1/3000 dilution. The membranes were processed for immunoblot analysis using the enhanced chemiluniscence method. The immunoreactive band was visualized using Kodak Imagestation and quantitated using the imaging software.

Freshly cultured neurons (2 days) or matured neurons (3 weeks old) were immunostained using IgG fraction of the antiserum to ADC by the immunoperoxidase method (Regunathan *et al.* 1996). Briefly, cultures were fixed with 4% paraformaldehyde and blocks prepared with 0.5% BSA in 0.1 M Tris-saline. Cultures were incubated overnight with IgG fraction of the antiserum to ADC or pre-immune control serum (1/2000 dilution) in 0.1% BSA containing Tris-saline at 4°C. After removing the primary antiserum and washing, the immunoreactivity was visualized by ABC kit (Vector Laboratories, Burlingame, CA, USA) using goat anti-rabbit IgG and 3,3′-diaminobenzidine/hydrogen peroxide. Triton X-100 (0.1–0.3%) was included in all steps to permeabilize the cells and improve antibody penetration. The slides were dehydrated, mounted in DPX mounting medium and photographed using a Nikon digital microscope system.

RT–PCR analysis of ADC mRNA rat brain and neuronal cells

The levels of mRNA encoding the previously cloned ADC gene (Zhu *et al.* 2004) were assessed by semiquantitative RT–PCR. Forward and reverse primers were designed at the 5′ end of the cDNA that is unique for the ADC gene, yielding about 300-bp PCR product. Total RNA was isolated from tissues and cells using trizol reagent from Gibco BRL according to the manufacturer's instructions. Briefly, tissues were homogenized in trizol reagent using a Teflon homogenizer for approximately 30 s and re-suspended in the same reagent. Quality and quantity of total RNA were detected spectrophotometrically using a Bio-Rad DU 650 spectrophotometer at 230/260/320 nm. First-strand cDNA synthesis was carried out using the Promega ImProm-II Reverse Transcription System (Madison, WI, USA) using random primers. Each reaction was transcribed from 1 μg total RNA following an initial annealing at 25°C for 5 min and a further incubation at 42°C for 1 h. Reactions were terminated by heating at 70°C for 15 min to inactivate the reverse transcriptase. For PCR, 5 μL of cDNA reaction products was used as template in a 50-μL reaction buffer using the following primer pairs: ADC forward ATGGCTGGCTACCTGAGTGAA, reverse GACCAACTCCATCTCTGCCTTGT; β-actin forward GCTCGTCGTCGACAACGGCTC, reverse CAAACATGATCTGGGTCATCTTCTC. Amplification of ADC was carried out for 35 cycles, consisting of an initial 2 min at 94°C followed by 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C. Following this step, a final extension was carried out at 72°C for 10 min. For β-actin, the conditions were essentially the same except that the number of cycles was 27. The different cycles were optimized for amplification in the exponential phase of PCR. Reaction mixture without template cDNA was used as negative control. PCR products were visualized in tris-acetate-EDTA buffered 1% agarose gels with incorporated ethidium bromide. When appropriate, quantitative evaluation of RT–PCR signals was carried out by densitometry using the Kodak ID Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA). Values of target PCR product were normalized to those of β-actin.

ADC down-regulation by siRNA

The down-regulation of ADC expression was accomplished using RNA interference technique that is based on the selective degradation of mRNA by small 21–23-bp RNA species (siRNA). RNA interference proceeds via a two-step mechanism. In the first step, long dsRNA are recognized by the Dicer enzyme, which cleaves the double-stranded RNA (dsRNA) into siRNA. These 21–23-bp siRNA are then incorporated into the RNA-induced silencing complex, a multi-enzyme complex responsible for specific cleavage and destruction of target mRNA. Selective small interfering RNAs (siRNA) were generated using the BLOCK-iT RNAi TOPO transcription kit from Invitrogen (Carlsberg, CA, USA). Briefly, ssRNA were obtained from PCR-derived templates, which contained T7 promoter sites engineered by TOPO linking. The following primers were used in PCR reaction to generate templates: N-terminal forward 5′-ATGGCTGGCTACCTGAGTGAA, reverse 5′-GACCAACTCCATCTCTGCCTTGT; C-terminal forward 5′-CAGGGAGGAGGAAAATGGTT, reverse 5′-TCACATGATGCTCGCTGG. Transcription reactions to produce single-stranded RNA (ssRNA) were carried out following manufacturers' instructions. Equal amounts of ssRNA were annealed by incubation in boiling water and cooled gradually to room temperature. One hundred micrograms of dsRNA was digested using 60 units of BLOCK-iT dicer (enzyme) at 37°C for 18 h. Following this digestion step, siRNAs present in the samples were purified using the BLOCK-iT RNAi purification kit, quantified and stored at −80°C until further use.

Tissue culture and transfection conditions

Transfection of cells with purified siRNA was carried out using lipofectamine 2000 (Life Technologies) in six-well culture plates according to manufacturer's instructions. Briefly, 250 ng of purified siRNAs and 5 μL of lipofectamine were diluted separately in 250 μL of Opti-MEM 1 medium and incubated for 5 min at room temperature. Lipofectamine and siRNA mixtures were then combined and incubated further for 20 min at room temperature. The DNA/lipofectamine complex was then added to cultured cells grown to about 50% confluency (C6 glioma cells) or 3 weeks after culture (neurons). Cells were incubated at 37°C for 24–48 h and used for the determination of ADC gene knock-down by the measurement of enzyme activity, mRNA levels by RT–PCR, protein levels by western blot and cellular agmatine levels by HPLC.

Measurement of agmatine by HPLC

The amount of agmatine in control and transfected cells was measured by HPLC as described extensively in earlier papers (Raasch *et al.* 1995; Feng *et al.* 1997). Cells were harvested 48 h after transfection and re-suspended in phosphate buffer (pH 5.7) containing amino picollinate as internal standard. The cell suspension was then homogenized in 10% TCA, centrifuged at 10 000 *g* for 10 min and the supernatant used for HPLC analysis. Samples were derivatized with *o*-pthaladehyde and injected into a reverse-phase column (5 μm) with fluorescence detector. Recovery of agmatine was calculated from the added external standard and expressed as ng/mg protein.

Animals and materials

Adult male Sprague–Dawley rats were obtained from the Jackson laboratory and housed under constant conditions of light, temperature and humidity. Animals were kept under these conditions for at least for 4 days before killing for brain dissection. Monoclonal ODC antiserum was obtained from Sigma (St Louis, MO, USA). NGF was purchased from Invitrogen and total RNA of human brain regions (nucleus accumbens, hippocampus and cerebral cortex) were obtained from Clontech (Carlsbad, CA, USA). Human brain tissue for immunoblot analysis was from the departmental brain collection (UMC IRB protocol no.

1999–1002). Human brain tissue was collected at autopsy at the Cuyahoga County Coroner's Office in Cleveland, Ohio. Informed written consent was received from the next of kin and tissue was collected in accordance with an approved Institutional Review Board Protocol (Klimek *et al.* 1999). Prefrontal cortex (Brodmann's area 10) was collected from two subjects who died of natural causes related to the cardiovascular system. Based on a retrospective review of medical records and a structured clinical interview with the next of kin, there was no lifetime or current history of a psychiatric disorder in either subject. Blood and urine samples from both subjects were examined by the coroner's office for psychotropic medications and substances of abuse, and no such drugs were detected

Results

Regional expression of ADC activity and protein

ADC activity was observed in six regions, although with regional differences, whereas cerebellum had no activity. The highest activity was observed in hypothalamus followed by frontal cortex, striatum; with LC, medulla and hippocampus had the lowest activity (Fig. 1). To determine whether ADC activity is associated with corresponding protein expression, the level of ADC protein was measured by immunoblot analysis using a polyclonal antibody produced against a unique ADC peptide. The ADC peptide antiserum recognized a major protein band of about 43-kDa size in membrane fractions, whereas pre-immune control serum from the same rabbit showed no immunoreactive band (Fig. 2a). We also detected a significant immunoreactive band in human cortex membrane with very little protein in the cytosol fraction (Fig. 2b). In all rat brain regions tested, the ADC immunoreactive band roughly correlated with the activity. For example, the highest protein band intensity was observed in hypothalamus which had the highest activity. In contrast, LC had the lowest protein band and lowest enzyme activity. The protein expression and activity in other regions ranged between the two. The pretreatment of the IgG fraction of this antiserum with peptide antigen largely reduced the immunoreactive band in rat brain cortex and hippocampus, indicating the specificity of the antiserum (Fig. 3). Because of the sequence similarity between ADC and ODC, it was essential to test whether our ADC antiserum recognizes ODC protein. Therefore, we compared the immunoreactivity of ADC antiserum with commercially available ODC antiserum in ADC cDNA-transfected COS-7 cells and PC12 cells. Two sets of tissue samples were separated in the same gel, transferred to membranes and then cut into two parts; one used for ADC antiserum and the other for ODC antiserum. The ADC antiserum recognized a strong protein band of about 43 kDa in COS-7 cells transfected with ADC cDNA, similar to brain ADC protein, with very little expression in control cells (Fig. 4). With ODC antiserum, we observed a protein band of 55 kDa that was present equally in control and transfected COS-7 cells. In PC12 cells, ODC antiserum recognized several protein bands with a major 55-kDa protein, whereas ADC antiserum recognized a single 43-kDa protein band. These results clearly indicate that the ADC antiserum does not recognize ODC and that ADC and ODC are distinct protein species.

Expression of ADC mRNA in rat and human brain

The RT–PCR amplification of total RNA from rat brain, using specific primers flanking a unique coding region of ADC, yielded a single product of 297 bp which matched with the ADC cDNA sequence as confirmed by DNA sequencing. The direct PCR amplification of ADC cDNA and cDNA obtained from total RNA of ADC cDNA transfected COS-7 cells also produced the same size PCR product, thus confirming the identity of the 297-bp product. Using this primer pair, we were able to demonstrate the expression of the ADC gene in different regions of the rat and human brain. In rat brain, the highest level of expression was noted in hypothalamus, followed by striatum, frontal cortex and hippocampus, with lower expression levels in LC and medulla (Fig. 5a). This pattern of

mRNA expression is consistent with ADC enzyme activity and protein levels in rat brain regions. In three human brain regions tested, we have observed significant expression of ADC mRNA in frontal cortex, hippocampus and nucleus accumbens (Fig. 5b).

ADC expression in cultured neuronal cells

The regulation of ADC is critical in determining the availability of agmatine in cells. We showed earlier that ADC activity is higher in confluent C6 glioma cells compared with dividing cells (Regunathan and Reis 2000). We have also observed that ADC activity is expressed in cultured glial cells, but not in neurons, which led us to hypothesize that agmatine is primarily synthesized in glial cells (Regunathan *et al.* 1995). Therefore, in the next experiment, we sought to analyze the expression of ADC gene in cultured cells including glial cells, C6 cells, neurons and PC12 cells to ascertain whether the mRNA level is regulated by the stages of differentiation of neurons and PC12 cells. The RT–PCR analysis confirmed moderate expression of ADC transcript in glial cells, C6 glioma cells and PC12 cells (Fig. 5c). While 3-day-old neurons failed to demonstrate any positive amplification product, there was an induction of ADC gene transcript after 3 weeks of culturing, as evidenced by the positive PCR product (Fig. 5c). Although naive PC12 cells (no NGF treatment) expressed some ADC message, gene expression was increased to about fourfold when the cells were treated with NGF for 3 days. To determine whether these changes in mRNA levels are reflected in ADC protein, we measured the expression of ADC by immunoblot and immuncytochemistry in cultured neurons that are freshly cultured (2 days) and mature (3 weeks). As shown in Fig. 6, the freshly cultured neurons showed very little immunostaining, whereas neurons in culture for 3 weeks showed several immunopositive cells. No immunostaining was observed with pre-immune control serum.

Effect of siRNA on ADC mRNA

To assess the effectiveness of RNAi on endogenous expression of ADC in cell culture, the cells were exposed to siRNA specific for the ADC gene. Two siRNA species, targeted to a highly conserved 285-bp N-terminal region and a less conserved 475-bp C-terminal region, were made. When these two siRNAs were used to transfect cultured C6 glioma cells, the N-terminal siRNA almost completely reduced the levels of ADC mRNA (Fig. 7a). The effect was much smaller with the C-terminal duplex (Fig. 7a). N-terminal specific siRNA was also effective in reducing the levels of ADC mRNA at two different concentrations in cultured neurons (Fig. 7b). Therefore, we used N-terminal specific siRNA in subsequent experiments to evaluate the effects on enzyme activity measurements and agmatine production. In both C6 glioma cells and neurons, the mRNA for β-actin was not altered by siRNA transfection, indicating that the effect is specific for ADC mRNA (Fig. 6).

Effect of siRNA on ADC protein expression

To verify the levels of ADC protein after siRNA transfection, we used immunoblot analysis using specific antibodies to ADC peptide. As shown in Fig. 2, the antiserum, but not the control pre-immune serum (not shown), produced against a unique ADC peptide, recognizes the ADC protein of 43 kDa in rat brain regions, cultured neurons, glial cells and C6 glioma cells. When siRNA-transfected C6 glioma cells were tested using this antiserum, these cells had significantly less ADC protein compared with non-transfected cells (Fig. 7c). While both C-terminal- and N-terminal-specific siRNA reduced the amount of ADC, N-terminal-specific siRNA was more effective and the effect was maximal at 24 h after transfection. The reduction in protein synthesis was approximately 95% in the siRNA-transfected cells compared with control cells at 24 h. The control β-actin expression was not affected by the transfection (Fig. 7c), indicating the specificity of the siRNA.

Effect of siRNA on enzyme activity and agmatine production

Arginine decarboxylase activity and agmatine levels were measured in cultured rat cortical neurons and C6 glioma cells transfected with N-terminal-specific siRNA for 24 h. As shown in Table 1, the ADC activity was significantly lower in C6 cells and neurons that were transfected with N-terminal-specific siRNA. The amount of agmatine in these cells, as measured by the HPLC method, was also lower when transfected with siRNA to ADC. Although the decrease in ADC activity in neurons was more pronounced than in C6 cells, the decrease in agmatine levels was higher in C6 cells.

Discussion

In this study, we report the expression of ADC as measured by activity, protein and mRNA levels in rat brain regions and the ability of siRNAs to down-regulate the expression in cultured neuronal cells. While ADC activity in whole brain has been reported earlier, the regional differences in the expression of ADC are not known. ADC activity was observed in all regions, although with large regional differences. The highest activity was observed in hypothalamus, the region which also has extensive agmatine immunoreactive cells (Otake *et al.* 1998; Gorbatyuk *et al.* 2002). Other regions that showed measurable ADC activity include frontal cortex, striatum, medulla, hippocampus and LC (Fig. 1). Using a polyclonal antibody produced against the unique ADC peptide, we were able to identify the immunoreactive band in rat brain regions. Moreover, this antibody recognizes specifically ADC of rat and human brain of about 43 kDa, but not the ODC protein of 55 kDa. The immunoblot data from rat brain regions roughly correlates with the activity data. For example, the highest ADC activity was detected in hypothalamus which also had the highest protein levels. This was followed by frontal cortex, striatum and hippocampus, whereas the lowest activity and protein levels were detected in LC and medulla. The levels of ADC mRNA as measured by RT–PCR also showed a similar pattern of expression with highest expression in hypothalamus and lowest in brainstem. These findings also parallel our previous report on the distribution of agmatine immunoreactivity in rat brain. For example, extensive agmatine immunoreactivity was observed in several nuclei of hypothalamus (Gorbatyuk *et al.* 2002) and neurons in frontal cortex and hippocampus (Otake *et al.* 1998). These observations also support the reported functional data on the effects of agmatine on hypothalamic/neurohypophysis peptide release (Wang *et al.* 2002), as well as the regulation of NMDA channels in hippocampus (Yang and Reis 1999).

The levels of ADC mRNA in cultured neurons and PC12 cells revealed a specific pattern of expression that is associated with the differentiated state of these cells. Compared with freshly cultured cortical neurons, the mature neurons after 3 weeks in culture show extensive dendritic processes making contacts with other neurons. A similar neurite outgrowth of PC12 occurs after treatment with NGF compared with non-treated cells. The levels of ADC mRNA was not detectable (in fresh neurons) or lower (in naive PC12 cells) but appeared in mature neurons and higher in differentiated PC12 cells. These results indicate that the biosynthesis of agmatine probably occurs only in differentiated neurons. ADC mRNA was also detected in cultured glial cells and C6 glioma cells, confirming our earlier observation by enzyme activity measurements (Regunathan *et al.* 1995). However, in contrast to our earlier findings in freshly cultured neurons, we detected ADC mRNA in neurons after prolonged culture. Thus, it appears that both glia and mature differentiated neurons express ADC activity in brain.

One controversial issue since the publication of our earlier paper (Zhu *et al.* 2004) is whether this ADC gene is responsible for the biosynthesis of agmatine in mammalian tissues. While one study questioned this contention (Coleman *et al.* 2004), another recent study, using ^{15}N-labeled arginine, verified that agmatine can be produced by the

decarboxylation of arginine in rat liver mitochondria (Horyn *et al.* 2005). However, it was important to resolve whether this ADCgene is responsible for the production of agmatine in brain We have previously shown the presence of a mammalian cDNA encoding for ADC and that COS-7 cells, transfected with a plasmid containing the ADC cDNA, expressed ADC activity and produced agmatine (Zhu *et al.* 2004). Here, we demonstrate that specific siRNA fragments generated from the N-terminal region of ADC cause a selective suppression of endogenous expression of ADC in cultured cells, resulting in reduced enzyme activity and agmatine production. The effect was much smaller with the C-terminal duplex. With successful RNAi experiments to down-regulate the expression of ADC message, we sought to find out what effect, if any, this had on agmatine biosynthesis and ADC activity. Analysis of siRNA transfected and naive cells indicated that naive cells had a higher level of endogenous agmatine compared with transfected cells for both C6 and neurons. The reduction in the level of agmatine was associated with a parallel decrease in ADC activity in neurons. However, the decrease in the steady-state levels agmatine in C6 glioma cells was larger than that in neurons, although the suppression of ADC message was more prominent in neurons. Such a lack of correlation could be as a result of the differential expression of the degradative enzyme, agmatinase (Sastre *et al.* 1996), as well as the ability of neurons to store agmatine in vesicles (Reis *et al.* 1998; Wang *et al.* 2002) which could be lacking in C6 cells. Thus, it may take a higher amount and longer time of exposure to siRNA in neurons to completely reduce the levels of agmatine.

Findings from this study have several important implications in understanding the neurobiology of the agmatine/ADC system. The production and characterization of specific antibodies to ADC will be a valuable tool in determining the cellular and subcellular distribution of this protein in brain. The RT–PCR method to identify and quantitate the expression of ADC mRNA levels will be a valuable tool in evaluating the regulation of the synthesis of agmatine in brain. As enzyme activity will be difficult to measure in small regions or specific nucleus of rat brain without pooling from several animals, RT–PCR will be able to detect the changes in ADC mRNA levels from very small amounts of tissue. The analysis of mRNA and protein will be especially useful in investigating the changes in human brain samples, as ADC enzyme activity is extremely labile (Regunathan and Reis 2000), whereas mRNA/protein appears to be more stable to post-mortem delay. The siRNA results confirm that this ADC gene is responsible for the production of agmatine in brain and also provide an experimental tool for further studies to understand the function/neurobiology of the agmatine/ADC system in brain by selectively down-regulating the expression of ADC.

Abbreviations used

ADC	arginine decarboxylase
BSA	bovine serum albumin
DMEM	Dulbecco’s modified Eagle’s medium
dsRNA	double-stranded RNA
LC	locus coeruleus
ODC	ornithine decarboyxlase
siRNA	small interfering RNA
ssRNA	single-stranded RNA
RPMI	Roswell Park Memorial Institute

TCA Trichloroacetic acid

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References

Aricioglu F, Kan B, Yillar O, Korcegez E, Berkman K. Effect of agmatine on electrically and chemically induced seizures in mice. Ann. NY Acad. Sci. 2003; 1009:141–146. [PubMed: 15028579]

Aricioglu F, Means A, Regunathan S. Effect of agmatine on the development of morphine dependence in rats: potential role of cAMP system. Eur. J. Pharmacol. 2004; 504:191–197. [PubMed: 15541421]

Coleman CS, Hu G, Pegg AE. Putrescine biosynthesis in mammalian tissues. Biochem. J. 2004; 379:849–855. [PubMed: 14763899]

Fairbanks CA, Schreiber KL, Brewer KL, et al. Agmatine reverses pain induced by inflammation, neuropathy, and spinal cord injury. Proc. Natl Acad. Sci. USA. 2000; 97:10 584–10 589.

Feng Y, Halaris AE, Piletz JE. Determination of agmatine in brain and plasma using high-performance liquid chromatography with flourescence detection. J. Chromatogr. 1997; 691:277–286.

Feng Y, Leblanc MH, Regunathan S. Agmatine reduces extracellular glutamate during pentylenetetrazole-induced seizures in rat brain: a potential mechanism for the anticonvulsive effects. Neurosci. Lett. 2005; 390:129–133. [PubMed: 16125317]

Gilad GM, Gilad VH. Accelerated functional recovery and neuroprotection by agmatine after spinal cord ischemia in rats. Neurosci. Lett. 2000; 296:97–100. [PubMed: 11108990]

Gorbatyuk OS, Milner TA, Wang G, Reis DJ, Regunathan S. Localization of agmatine in vasopressin and oxytocin neurons of the rat hypothalamic paraventricular and supraoptic nuclei. Exp. Neurol. 2002; 171:235–245. [PubMed: 11573976]

Horyn O, Luhovvy B, Lazarow A, Daikhin Y, Nissim I, Yudkoff M, Nissim I. Biosynthesis of agmatine in isolated mitochondria and perfused rat liver: studies with ^{15}N-labeled arginine. Biochem. J. 2005; 388:419–425. [PubMed: 15656789]

Kalra SP, Pearson E, Sahu A, Kalra PS. Agmatine, a novel hypothalamic amine, stimulates pituitary luteinizing hormone release *in vivo* and hypothalamic luteinizing hormone-releasing hormone release *in vitro*. Neurosci. Lett. 1995; 194:165–168. [PubMed: 7478229]

Klimek V, Rajkowska G, Luker SN, Dilley G, Meltzer HY, Overholser JC, Stockmeier CA, Ordway GA. Brain noradrenergic receptors in major depression and schizophrenia. Neuropsychopharmacology. 1999; 21:69–81. [PubMed: 10379521]

Kolesnikov Y, Jain S, Pasternak GW. Modulation of opioid analgesia by agmatine. Eur. J. Pharmacol. 1996; 296:17–22. [PubMed: 8720472]

Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ. Agmatine: an endogenous clonidine-displacing substance in the brain. Science. 1994; 263:966–969. [PubMed: 7906055]

Li G, Regunathan S, Reis DJ. Agmatine is synthesized by a mitochondrial arginine decarboxylase in rat brain. Ann. NY Acad. Sci. 1995; 763:325–329. [PubMed: 7677342]

Li J, Li X, Pei G, Qin BY. Correlation between inhibitions of morphine withdrawal and nitric-oxide synthase by agmatine. Acta Pharmacologica Sinica. 1999; 20:375–380. [PubMed: 10452129]

Lortie MJ, Novotny WF, Peterson OW, Vallon V, Malvey K, Mendonca M, Satriano J, Insel P, Thomson SC, Blantz RC. Agmatine, a bioactive metabolite of arginine. J. Clin. Invest. 1996; 97:413–420. [PubMed: 8567962]

Yu CG, Marcillo AE, Fairbanks CA, Wilcox GL, Yezierski RP. Agmatine improves locomotor function and reduces tissue damage following spinal cord injury. Neuroreport. 2000; 11:3203–3207. [PubMed: 11043549]

Onal A, Soykan N. Agmatine produces anti-nociception in tonic pain in mice. Pharmacol. Biochem. Behav. 2001; 69:93–97. [PubMed: 11420073]

Otake K, Ruggiero DA, Regunathan S, Wang H, Milner TA, Reis DJ. Regional loclalization of agmatine in the rat brain: an immunocytochemical study. Brain Res. 1998; 787:1–14. [PubMed: 9518530]

Palkovits, M.; Brownstein, MJ. Maps and Guide to Micro-dissection of the Rat Brain. New York: Elsevier; 1998.

Piletz JE, Chikkala DN, Ernsberger P. Comparison of the properties of agmatine and endogenous clonidine-displacing substance at imidazoline and α-2 adrenergic receptors. J. Pharmacol. Exp. Ther. 1995; 272:581–587. [PubMed: 7853171]

Raasch W, Regunathan S, Li G, Reis DJ. Agmatine, the bacterial amine, is widely distributed in mammalian tissues. Life Sci. 1995; 56:2319–2330. [PubMed: 7791519]

Regunathan S, Piletz JE. Regulation of inducible nitric oxide synthase and agmatine synthesis in macrophages and astrocytes. Ann. NY Acad. Sci. 2003; 1009:20–29. [PubMed: 15028566]

Regunathan S, Reis DJ. Characterization of arginine decarboxylase in rat brain and liver: distinction from ornithine decarboxylase. J. Neurochem. 2000; 74:2201–2208. [PubMed: 10800966]

Regunathan S, Feinstein DL, Raasch W, Reis DJ. Agmatine, the decarboxylated arginine is localized and synthesized in glial cells. Neuroreport. 1995; 6:1897–1900. [PubMed: 8547593]

Regunathan S, Youngson C, Raasch W, Wang H, Reis DJ. Imidazoline receptors and agmatine in blood vessels: a novel system inhibiting vascular smooth muscle proliferation. J. Pharmacol. Exp. Ther. 1996; 276:1272–1282. [PubMed: 8786560]

Reis DJ, Regunathan S. Agmatine: a novel neurotransmitter? Adv Pharmacol. 1998; 42:645–649. [PubMed: 9327985]

Reis DJ, Regunathan S. Is agmatine a novel neurotransmitter in brain? Trends Pharmacol. Sci. 2000; 21:187–193. [PubMed: 10785653]

Reis DJ, Yang X-C, Milner TA. Agmatine containing axon terminals in rat hippocampus form synapses on pyramidal cells. Neurosci. Lett. 1998; 250:185–188. [PubMed: 9708863]

Sastre M, Regunathan S, Reis DJ. Agmatinase activity in rat brain: a metabolic pathway for the degradation of agmatine. J. Neurochem. 1996; 67:1761–1765. [PubMed: 8858963]

Sastre M, Galea E, Reis DJ, Regunathan S. Metabolism of agmatine in macrophages: modulation by lipopolysacharides and inhibitory cytokines. Biochem. J. 1998; 330:1405–1409. [PubMed: 9494113]

Su RB, Wei XL, Zheng JQ, Liu Y, Lu XQ, Li J. Anticonvulsive effect of agmatine in mice. Pharmacol. Biochem. Behav. 2004; 77:345–349. [PubMed: 14751463]

Wang G, Gorbatyuk O, Dayanithi G, Ouyang W, Wang J, Milner TA, Regunathan S, Reis DJ. Evidence for endogenous agmatine in hypothalamo-neurohypophysial tract and its modulation on vasopressin release and Ca2+ channels. Brain Res. 2002; 932:25–36. [PubMed: 11911858]

Yang X-C, Reis DJ. Agmatine selectively blocks the NMDA subclass of glutamate receptor channels in cultured mouse hippocampal neurons. J. Pharmacol. Exp. Ther. 1999; 288:544–549. [PubMed: 9918557]

Zhu MY, Piletz JE, Halaris A, Regunathan S. Effect of agmatine against cell death induced by NMDA and glutamate in neurons and PC12 cells. Cell Mol. Neurobiol. 2003; 23:865–872. [PubMed: 14514037]

Zhu MY, Iyo A, Piletz JE, Regunathan S. Expression of human arginine decarboxylase, the biosynthetic enzyme for agmatine. Biochem. Biophys. Acta. 2004; 1670:156–164. [PubMed: 14738999]

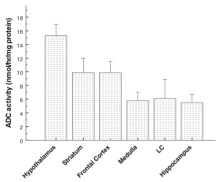

Fig. 1.

Activity of ADC in rat brain regions. Rats brain regions were dissected out and membrane fractions were used for ADC measurements. Values are mean ± SD from a total of 12 rats, with regions from three rat brains pooled for LC, hypothalamus and striatum.

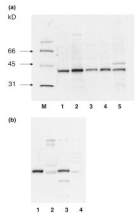

Fig. 2.

The expression of ADC protein in rat (a) and human brain (b). Rat brain regions were dissected out for ADC immunoblot analysis using ADC peptide antibodies. Normal human brain frontal cortex samples were obtained from departmental brain collection. Immunoblot analysis was performed in four rat brain and two human brain samples and a representative immnoblot is shown. (a) Lanes: M, marker; 1, rat frontal cortex; 2, hypothalamus; 3, LC; 4, striatum; 5, hippocampus; (b) 1, 3, human frontal cortex membrane; 2,4, human brain cortex cytosol.

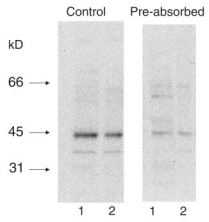

Fig. 3.

The effect of pre-absorption of ADC antiserum with peptide antigen. The IgG fraction of the antiserum was incubated with and without ADC peptide antigen (50 μg) for 16 h at room temperature and used for the immunoblot analysis (1/3000 dilution) of rat frontal cortex (lane 1) and hippocampus samples (lane 2).

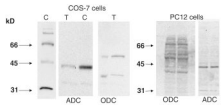

Fig. 4.

Immunoblot analysis of COS-7 and PC12 cells using ADC and ODC antibodies. The cell extracts, prepared from control COS-7 cells (C), ADC cDNA transfected COS-7 cells (T) and PC12 cells, were used for immunoblot analysis using ADC and ODC antisera. Two set of samples were separated in the same gel, transferred to membrane and then cut into two, one probed with ODC antiserum and the other with ADC antiserum.

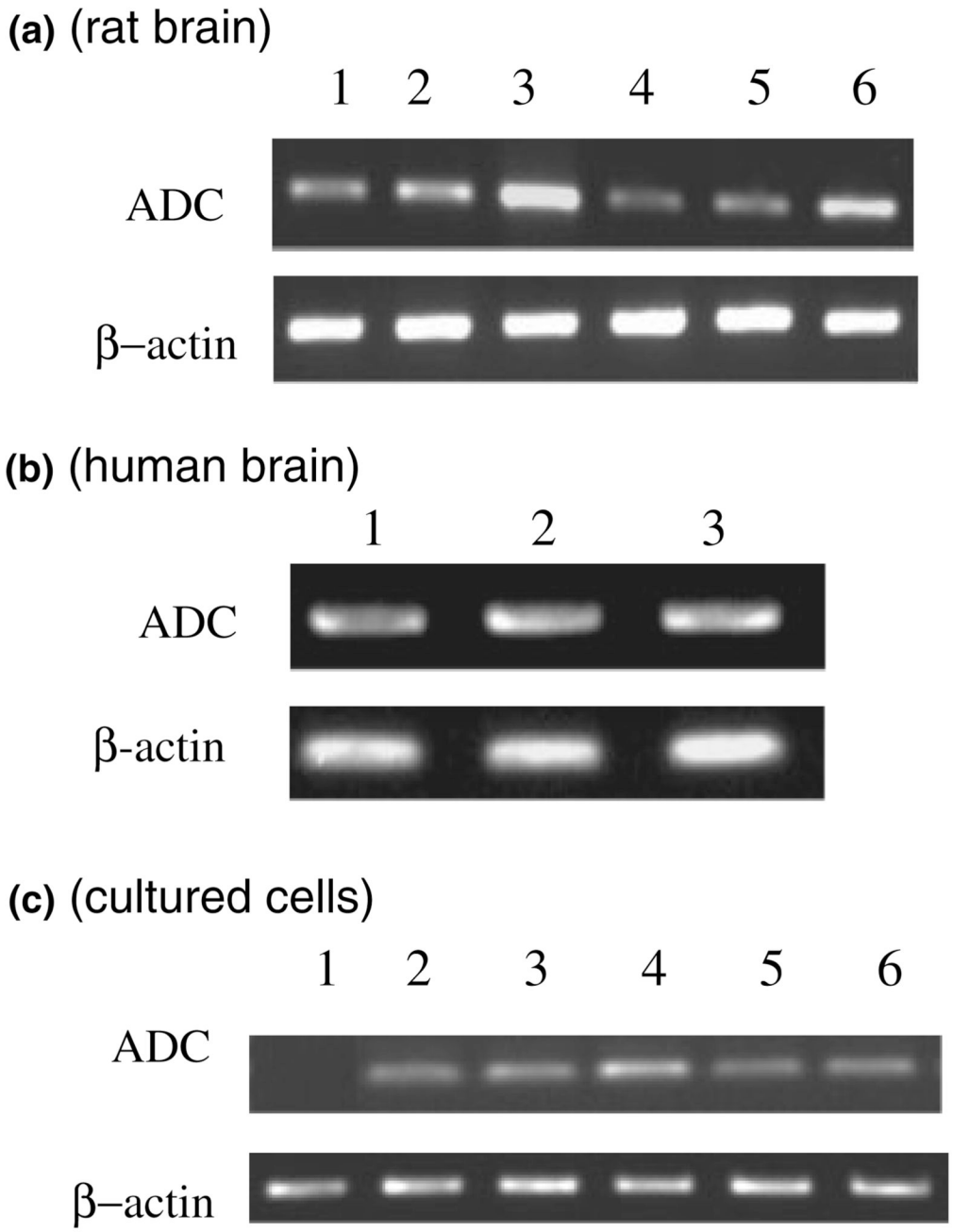

Fig. 5.

Expression of ADC mRNA in rat brain regions (a), human brain (b) and cultured neuronal cells (c). Total RNA, isolated from four rat brain regions, cultured cells or obtained from Clontech (human brain regions), was used for cDNA synthesis using reverse transcriptase. PCR was performed for cDNA from each region using specific primers yielding 297-bp PCR product and an example of the PCR product obtained in each region is shown. (a) Lanes: 1, frontal cortex; 2, hippocampus; 3, hypothalamus; 4, LC; 5, medulla; 6, striatum. (b) Lanes: 1, frontal cortex; 2, hippocampus; 3, nucleus accumbens. (c) Lanes: 1, neurons (3 days old); 2, neurons (4 weeks old); 3, PC12 cells (naive); 4, PC12 cells (NGF treated); 5,

astrocytes; 6, C6 glioma cells. The simultaneous PCR was performed using primers for β-actin in these cDNA samples to normalize the changes.

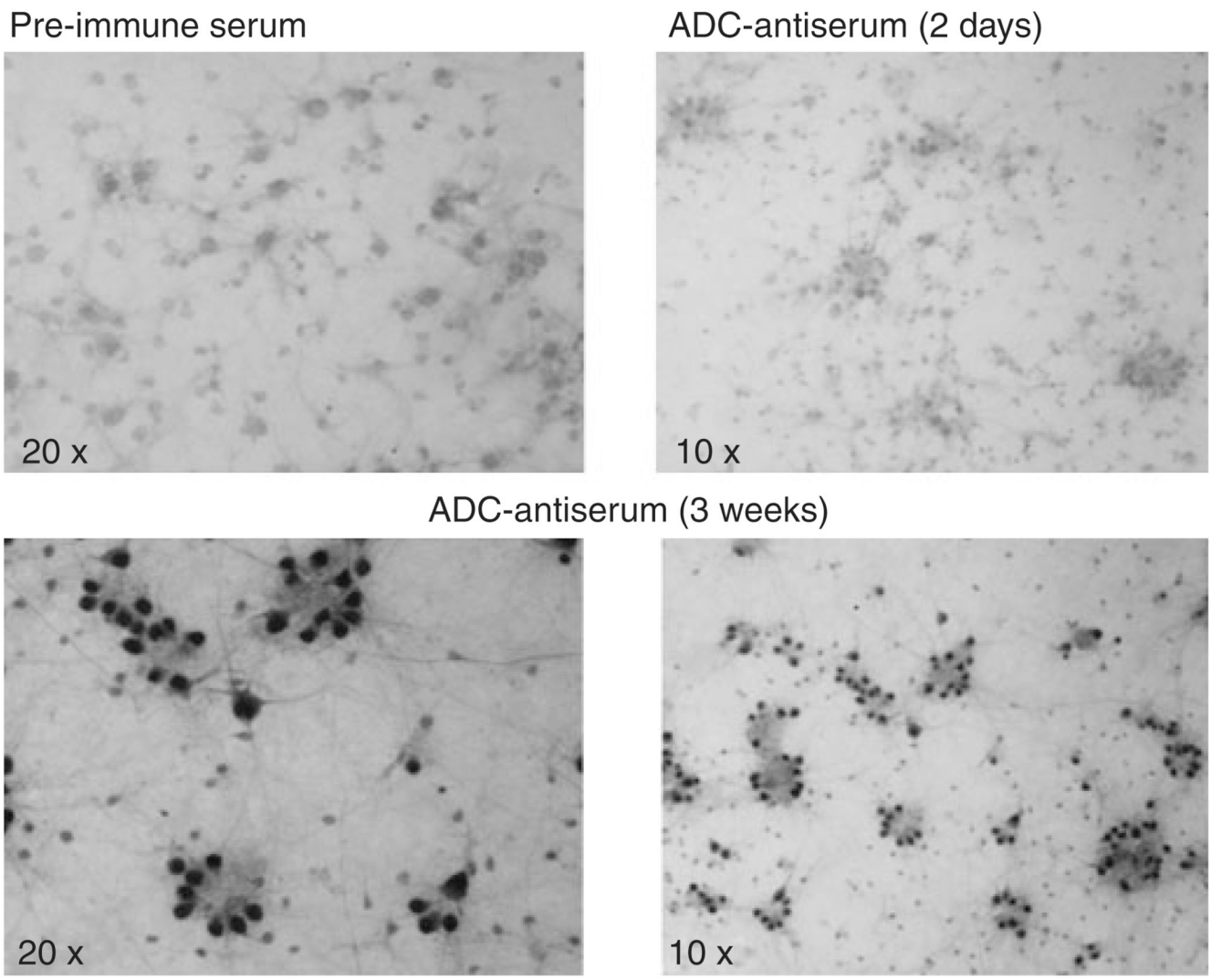

Fig. 6.

The immunocytochemical localization of ADC in cultured rat cortical neurons. Neurons were prepared from E15 rat embryo and immunostained with IgG fraction of specific ADC peptide antiserum (1/1000 dilution) or pre-immune control serum. This experiment was repeated in three different preparation of neurons with similar findings.

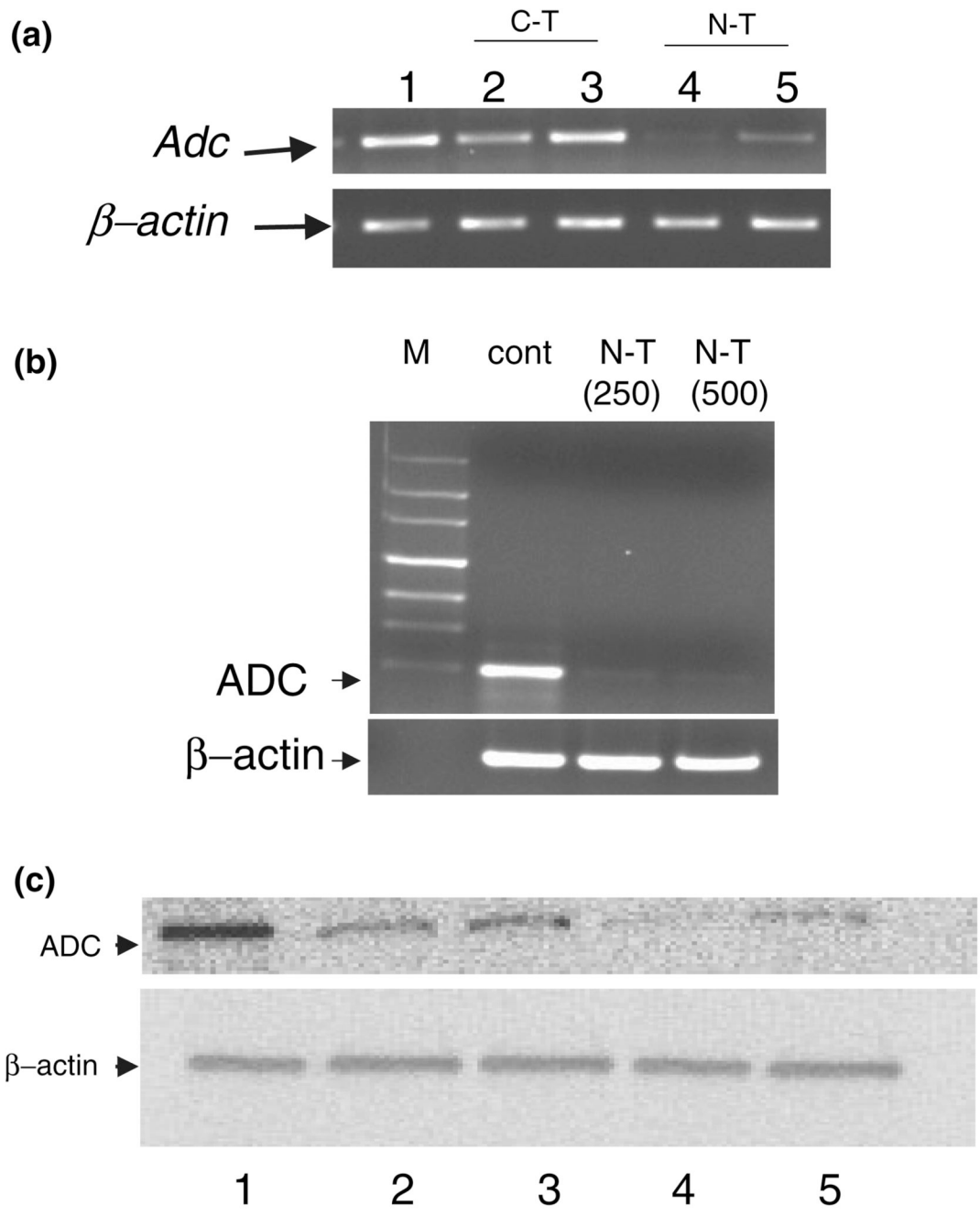

Fig. 7.

The expression of ADC in cells transfected with siRNA specific for ADC gene. Reverse transcripatase PCR showing the silencing of endogenous ADC message in cultured C6 glioma cells (a) and neurons (b). C6 glioma cells were transfected with either N-terminal- or C-terminal-specific siRNAs and ADC mRNA measured by RT–PCR after 24 h (lanes 2 and 4) and 48 h (lanes 3 and 5). Neurons were transfected with N-terminal siRNAs (250 and 500 ng) and ADC message was measured after 24 h. The β-actin mRNA was measured to control for the non-specific effects of siRNA. The expression of ADC protein by western blot analysis of membrane fractions prepared from siRNA-transfected C6 glioma cells using ADC peptide antiserum (c). Lanes: 1, control cells; 2, C-terminal (24 h), 3, C-terminal (48

h), 4, N-terminal (24 h), 5, N-terminal (48 h). The expression of β-actin was measured to detect any non-specific effects.

Table 1

Effect of siRNA on ADC activity and agmatine levels in cultured neuronal cells

	ADC activity (nmol/h/mg protein)	Agmatine (ng/mg protein)
Control C6 cells	15.4 ± 4.5	1.52 ± 0.45
N-T transfected C6 cells	6.5 ± 2.8 *	0.49 ± 0.23 *
Control neurons	38 ± 8.5	0.68 ± 0.11
N-T transfected neurons	5.3 ± 1.1 *	0.48 ± 0.1 *

ADC activity was measured in membrane fraction prepared from control or N-terminal-specific siRNA transfected cells. The amount of agmatine in control or siRNA-transfected cells was determined by HPLC. The cells were harvested 24 h after siRNA transfection and the cytosolic and membrane fractions were prepared for agmatine and ADC measurements, respectively. Values are mean ± SEM from three separate experiments, each performed in triplicate.

* $p < 0.001$ compared with control cells.