Immunoneutralization of Agmatine Sensitizes Mice to μ -Opioid Receptor Tolerance

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ABSTRACT

Systemically or centrally administered agmatine (decarboxylated arginine) prevents, moderates, or reverses opioid-induced tolerance and self-administration, inflammatory and neuropathic pain, and sequelae associated with ischemia and spinal cord injury in rodents. These behavioral models invoke the *N*-methyl-D-aspartate (NMDA) receptor/nitric-oxide synthase cascade. Agmatine (AG) antagonizes the NMDA receptor and inhibits nitric-oxide synthase in vitro and in vivo, which may explain its effect in models of neural plasticity. Agmatine has been detected biochemically and immunohistochemically in the central nervous system. Consequently, it is conceivable that agmatine operates in an anti-glutamatergic manner in vivo; the role of endogenous agmatine in the central nervous system remains minimally defined. The current study used an immunoneutralization strategy to evaluate the effect of sequestration of endogenous agmatine in acute opioid analgesic tolerance in

mice. First, intrathecal pretreatment with an anti-AG IgG (but not normal IgG) reversed an established pharmacological effect of intrathecal agmatine: antagonism of NMDA-evoked behavior. This result justified the use of anti-AG IgG to sequester endogenous agmatine in vivo. Second, intrathecal pretreatment with the anti-AG IgG sensitized mice to induction of acute spinal tolerance of two μ -opioid receptor-selective agonists, [D-Ala², *N*-Me-Phe⁴, Gly⁵-ol]-enkephalin and endomorphin-2. A lower dose of either agonist that, under normal conditions, produces moderate or no tolerance was tolerance-inducing after intrathecal pretreatment of anti-AG IgG (but not normal IgG). The effect of the anti-AG IgG lasted for at least 24 h in both NMDA-evoked behavior and the acute opioid tolerance. These results suggest that endogenous spinal agmatine may moderate glutamate-dependent neuroplasticity.

Decarboxylated arginine (agmatine) has been identified in the mammalian central nervous system (CNS) both biochemically (Li et al., 1994; Fairbanks et al., 2000) and neuroanatomically (Fairbanks et al., 2000; Goracke-Postle et al., 2006). Agmatine was discovered as a clonidine-displacing substance (Li et al., 1994) that bound but did not activate or inhibit α -2 adrenergic receptors (Pinthong et al., 1995). However, agmatine also acts as an *N*-methyl-D-aspartate (NMDA)

receptor antagonist at polyamine site ($K_i = 15 \mu\text{M}$) (Gibson et al., 2002) and the MK-801 binding site (Reynolds, 1990). Agmatine also inhibits (Galea et al., 1996) or inactivates (Demady et al., 2001) nitric-oxide synthase. Electrophysiological (Yang and Reis, 1999) and pharmacological (Fairbanks et al., 2000; Roberts et al., 2005) evidence supports agmatine antagonism/inhibition at both NMDA receptor and nitric-oxide synthase, proteins known as essential components of glutamatergic neurotransmission. This dual activity raises the question as to whether agmatine functions endogenously as an anti-glutamatergic neuromodulator. This proposed role for agmatine is also suggested from reports describing agmatine-mediated inhibition of opioid tolerance (Kolesnikov et al., 1996; Fairbanks and Wilcox, 1997), opioid self-administration (Morgan et al., 2002; Wade et al., 2008), inflammation- and neuropathy-induced hyperalgesia (Fair-

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ABBREVIATIONS: CNS, central nervous system; NMDA, *N*-methyl-D-aspartate; MK-801, 5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine (dizocilpine maleate); AG, agmatine; Endo-2, endomorphin-2; %MPE, percentage maximal possible effect; CI, confidence intervals; GP, guinea pig; ELISA, enzyme-linked immunosorbent assay; IR, immunoreactivity; NeuN, neuronal nuclei; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride.

banks et al., 2000), behavioral sequelae after spinal cord injury (Fairbanks et al., 2000; Gilad and Gilad, 2000; Yu et al., 2000) and evoked seizure (Feng et al., 2005).

It has been suggested (Reis and Regunathan, 1998) that agmatine meets several criteria characteristic of an endogenous neuromodulator, including synthesis of agmatine in the brain (Reis and Regunathan, 1998), localization to neurons and synaptic vesicles (Otake et al., 1998; Goracke-Postle et al., 2006), transport into nerve terminals (Sastre et al., 1997; Goracke-Postle et al., 2006, 2007a,b), release by depolarization (Reis and Regunathan, 1998; Goracke-Postle et al., 2006, 2007b), transport into astrocytes (Regunathan et al., 1995), and enzymatic degradation by CNS agmatinase (Sastre et al., 1996). An important criterion yet to be tested includes a demonstration that endogenous agmatine performs the same physiological function as does exogenously administered agmatine. This criterion has been tested for a variety of neurotransmitters and neuropeptides, including β -endorphin (Guerrero-Munoz et al., 1979), endomorphin (Zadina et al., 1997), anandamide (Devane et al., 1992), substance P (Share and Rackham, 1981), calcitonin gene-related peptide (CGRP) (Tan et al., 1994), Met- and Leu-enkephalin (Mulder et al., 1984; Hardy and Haigler, 1985), and small molecules such as norepinephrine (Hardy and Haigler, 1985). In view of the ability of exogenously administered agmatine to prevent opioid analgesic tolerance (Nguyen et al., 2003), we hypothesized that endogenous agmatine participates in control of opioidergic processes. Previous studies of potential antinociceptive endogenous compounds have inferred the activity of these neuromodulators through the use of pharmacological antagonists *in vivo*. However, agmatine is itself a receptor antagonist and an enzyme inhibitor, rendering such a strategy inappropriate. We applied an immunoneutralization strategy using scavenger antisera to evaluate the physiological role of agmatine, a method previously used for other pharmacological antagonists (Vanderah et al., 1994; Tseng et al., 2000; Ohsawa et al., 2001). We hypothesized that sequestration of endogenous agmatine using a structure-specific anti-agmatine (AG) IgG antibody would invoke the induction of acute μ -opioid receptor tolerance at doses that normally are not tolerance-inducing. Our objective was to assess the impact of spinal delivery of anti-AG IgG on acute homologous tolerance induced by both [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO) and endomorphin-2 (Endo-2) to determine whether diminished availability of endogenous agmatine affected that process.

We show that exogenously applied agmatine prevents the induction of DAMGO- and Endo-2-induced acute spinal tolerance, as has been shown previously for morphine. The present study demonstrates that pretreatment with anti-AG IgG (but not normal IgG) increases DAMGO- and Endo-2-induced acute spinal tolerance, supporting the proposal that endogenous agmatine exerts a modifying affect on μ -opioid receptor acute tolerance and providing a mirror image parallel to the studies using exogenous agmatine in the same paradigm.

Materials and Methods

Animals

Experimental subjects were Institute of Cancer Research male mice (21–30 g; Harlan Teklad, Madison, WI). Subjects were housed

in groups of eight in a temperature- and humidity-controlled environment and maintained on a 12-h light/dark cycle with free access to food and water. These experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee.

Chemicals

Agmatine sulfate, aminoguanidine, L-arginine, D-arginine, and NMDA were purchased from Sigma-Aldrich (St. Louis, MO). MK-801 was a gift from Merck (Rahway, NJ). Endo-2 (YPFF) was synthesized by the University of Minnesota's Microchemical Facility (Minneapolis, MN). DAMGO (mol. wt., 513.7) was purchased from Tocris Bioscience (Ellisville, MO). All drugs were dissolved in 0.9% saline.

Intrathecal Injection

Agmatine was administered intrathecally in conscious mice according to the method of Hylden and Wilcox (1980). In brief, the pelvic girdle (ileac crest) of the mouse is gripped firmly by the thumb and forefinger of the injector's nondominant hand. The skin above the ileac crest is pulled tautly to create a horizontal plane where the needle is inserted. The needle is a 30-gauge, 0.5-inch sterile disposable needle connected to a 50- μ l Luer-hub Hamilton syringe (Hamilton Co., Reno, NV). All injections were delivered in 5- μ l volume.

NMDA-Induced Nociceptive Test

Nociceptive responsiveness was tested in the NMDA nociceptive test. A constant dose of NMDA (0.3 nmol) was injected intrathecally to produce approximately 40 to 60 behaviors (scratches and bites directed to the hindlimbs) in the 1st min after injection. Coadministration of agmatine dose-dependently inhibits those behaviors (Fairbanks et al., 2000). In these experiments, anti-AG IgG, preimmune serum, normal guinea pig IgG, or saline was administered as pretreatments before agmatine and NMDA injection.

L-Arginine-Induced Nociceptive Behavioral Responses

Biting and scratching responses were induced by a single intrathecal injection (0.3 nmol) of L-arginine. The animal's scratching and biting responses were counted for 90 s after injection.

Antinociception. In the opioid tolerance studies, thermal nociceptive responsiveness in the opioid tolerance studies was assessed using the warm water (52.5°C) tail-immersion assay. In brief, mice were gently wrapped in a soft cloth such that their tails were exposed, and three quarters of the length of the tail was dipped into the warm water. Tail-flick latencies were obtained before drug application to establish a baseline response. To test for analgesia, opioid agonists (DAMGO and endomorphin-2) were injected intrathecally as 2.5-min pretreatments, respectively. A maximal cut-off of 12 s was set to avoid tissue damage. The results were then expressed as a percentage maximum possible effect (%MPE) according to the equation (postdrug latency – predrug latency)/(cut-off – predrug latency) $\times$ 100.

Tolerance Induction. Tolerance was induced in mice using the following protocol: Mice were injected intrathecally with a high dose of either DAMGO (0.6 pmol) or Endo-2 (30 nmol) (Stone et al., 1997) or saline, respectively, to induce tolerance (or 0.06 pmol or 10 nmol for doses that do not induce tolerance). Thirty minutes after the tolerance-inducing injection, the tail-flick latencies of the mice had returned to baseline. At that point, mice were injected with either DAMGO or Endo-2 at varying doses to create an analgesic dose-response curve.

Dose-Response Analysis

The ED₅₀ values and 95% confidence intervals (CI) of drugs were calculated using the graded dose-response curve method of Tallarida and Murray (1987). A minimum of three doses were used for each drug. When evaluating the extent of a potency shift

between treatment groups, a potency ratio was calculated. These calculations were performed using the pharmacological statistics software FlashCalc, version 4.3.2 (Michael Ossipov, University of Arizona, Tucson, AZ).

Anti-Agmatine Antisera Generation

Agmatine sulfate was coupled to bovine thyroglobulin (BTG) with glutaraldehyde. The conjugate was dialyzed to remove excess glutaraldehyde. The conjugate was frozen in aliquots of 1 mg/ml concentration and stored at -20°C for use in immunizations. Preimmune serum was collected from four female guinea pigs (Duncan Hartley) before the first intradermal immunization with AG-BTG conjugate, which was mixed as an emulsion in a 1:1 ratio with Complete Freund's adjuvant. Subsequent immunizations were administered every 2 weeks using incomplete Freund's adjuvant in a 1:1 ratio with the AG-BTG conjugate. After 6 weeks, microliter quantities of sera were collected biweekly (alternating with the immunoboosts) and screened for immunoreactivity. After 11 weeks, the guinea pigs were anesthetized and exsanguinated by cardiac puncture. Blood was centrifuged, and serum was collected, measured in aliquots (1 ml), and stored at -80°C . Initial screening of the antisera by immunohistochemistry from three of the four guinea pigs (GP1, 3, and 4) showed similar patterns of immunofluorescence with some variation in background and in-

tensity. Sera from the second guinea pig did not seem immunoreactive. Samples of the stored serum were subsequently thawed and subjected to protein A column purification to reduce the samples to the IgG fraction, measured in aliquots, and stored at -20°C . There is added value in using protein A-purified IgG (rather than serum) for immunoneutralization studies, particularly in neuroscience, because both glutamate and glycine (both ligands of the NMDA receptor) are present in serum. In addition, antisera for all four guinea pigs were screened in the bioassay described in Fig. 1 representing agmatine inhibition of NMDA-elicited behavioral responses; the IgG from GP1, 3, and 4 reversed agmatine-induced inhibition of NMDA responses, whereas IgG from GP2 did not (consistent with the evaluation by immunohistochemistry). In the current study, the data presented were obtained from the third guinea pig (GP3), which seemed to have the best response in both the immunoassay and behavioral bioassay. A sample of the protein A-purified IgG from GP3 was further purified by affinity purification using the ImmunoPure Immobilized Protein A column (Pierce Chemical, Rockford, IL) according to the manufacturer's protocol. Samples of the eluents from both the protein A purification and the affinity purifications were confirmed to contain IgG through ELISA identification on protein A-preloaded ELISA plates purchased from Pierce Chemical.

Fig. 1. In vivo behavioral specificity of anti-AG IgG for agmatine. A, B, C, and D illustrate the impact of anti-AG IgG on modulation of NMDA-evoked behavior by agmatine, aminoguanidine, and MK-801, as well as on similar behaviors evoked by L-arginine in mouse spinal cord. In A, B, and C, NMDA (0.3 nmol i.t.) produces scratching and biting behaviors (first bars of A, B, and C). Agmatine (60 nmol i.t.), aminoguanidine (1 nmol i.t.), and MK-801 (1 nmol i.t.), all equieffectively block NMDA-evoked behavior (second bars of A, B, and C). Five-minute pretreatment with anti-AG IgG dose-dependently reverses agmatine (A) and aminoguanidine (B) but not MK-801 (C) inhibition of NMDA-evoked behavior. D shows that L-arginine (but not D-arginine) produces scratching and biting behaviors similar to NMDA (first and second bars of D). Five-minute pretreatment with anti-AG IgG does not impact L-arginine-induced scratching and biting behaviors even at twice the dose effective for reversal of aminoguanidine and agmatine effects. *, $p < 0.05$, as evaluated by ANOVA followed by Dunnett's post hoc test for multiple comparisons to a control: $F_{(4,35)} = 27$ (A); $F_{(4,35)} = 12$ (B); $F_{(5,42)} = 76$ (C); and $F_{(5,42)} = 18$ (D).

Immunohistochemistry

Male rats (Sprague-Dawley, 120–150 g; Harlan Teklad) were deeply anesthetized (75 mg/kg ketamine, 5 mg/kg xylazine, and 1 mg/kg acepromazine i.m.) and fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate-buffered saline, pH 6.9, by vascular perfusion. Spinal cords were removed and placed in 10% sucrose in phosphate-buffered saline overnight at 4°C. Spinal segments were frozen, and thaw-mounted cryostat sections (14 μ m) were prepared for indirect immunofluorescence histochemistry. The sections were preincubated for 1 h at room temperature in diluent containing 1% normal donkey serum, 0.3% Triton X-100, 0.01% sodium azide, and 1% bovine serum albumin. Sections were then incubated overnight in a humid chamber with primary antisera and rinsed several times with phosphate-buffered saline. Sections were then incubated with secondary antisera for 1 h at room temperature, rinsed, and coverslipped in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). Other primary antisera used were rabbit-derived anti-AG (dilution 1:50; a gift from Dr. Donald Reis, Cornell University, Ithaca, NY), rabbit-derived anti-glial fibrillary acidic protein (1:50; MP Biomedicals, Irvine, CA), and mouse-derived anti-neuronal nuclei (NeuN) (1:500; Millipore Bioscience Research Reagents, Temecula, CA). Preparations were visualized with RedX rhodamine-conjugated secondary antisera (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and examined with an MRC-1000 confocal imaging system (Bio-Rad Microscience Division, Cambridge, MA). Micrographs were assembled using Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Experimental Standards

All experiments were repeated at least once by blinded investigators and showed consistent results.

Results

Anti-Agmatine Immunoreactivity. To develop an anti-serum to agmatine, guinea pigs were immunized with a conjugate of agmatine and BTG, and polyvinylidene fluoride transfer membrane was spotted with agmatine sulfate-BTG conjugate. Dot blots were probed with the protein A-purified anti-AG IgG in the presence or absence of varying concentrations (10, 1.0, and 0.1 mM) of agmatine and its precursor L-arginine. The antibody binding to the polyvinylidene fluoride membrane was diminished by agmatine, but not L-arginine, in a concentration-dependent manner, indicating the specificity of agmatine for the IgG (data not shown).

We detected agmatine immunoreactivity (AG-IR) in rat spinal cord with guinea pig-derived anti-AG IgG that colabeled spinal structures with the rabbit-derived anti-AG antiserum used in previous reports (Otake et al., 1998; Fairbanks et al., 2000). The pattern of AG-IR included labeling of structures surrounding the perikarya of anti-NeuN-identified spinal and glial fibers, consistent with previous reports that agmatine may be present in glia (Regunathan et al., 1995). AG-IR was not observed in tissue treated with preimmune serum (data not shown), and the AG-IR observed in spinal structures was concentration-dependently diminished after preincubation of antiserum with free agmatine sulfate, but not L-arginine (data not shown). Immunoreactivity to other antisera (NeuN and glial fibrillary acidic protein) was not changed by preincubation with agmatine, a result that ensures that the observed agmatine absorption control was not due to a nonspecific impact of agmatine sulfate on other IgG immunoreactivity (data not shown).

In Vivo Specificity of Anti-AG IgG for Agmatine. We conducted a series of studies to examine the in vivo effects of the anti-AG IgG upon delivery of either NMDA alone or NMDA + AG in an established model of NMDA-evoked biting and scratching behaviors (Fairbanks et al., 2000). Consistent with previous reports (Fairbanks et al., 2000), agmatine (60 nmol i.t.) significantly reduced NMDA-evoked biting and scratching behavior (Fig. 1, A–C, bars 1 and 2). In contrast, pretreatment with the anti-AG IgG showed a reversal of the agmatine-mediated inhibition of NMDA-evoked behavior, supporting the concept of IgG-mediated agmatine sequestration (Fig. 1A). Heating the anti-AG IgG to 100°C for 5 min before intrathecal administration abolished this effect (data not shown), indicating that this is dependent upon the intact anti-AG IgG protein structure. In the absence of agmatine, NMDA responses did not differ in subjects pretreated with either preimmune serum or anti-AG IgG (data not shown). The anti-AG IgG also showed a dose dependence, with the most effective dose being 150 ng (Fig. 1A, bars 3–5). To determine what part of the agmatine molecule may bind to the IgG, the same experiment was conducted using aminoguanidine (Fig. 1B), the chemical structure of which is a guanidino group, a constituent of the agmatine molecule. Aminoguanidine (10 nmol i.t.) significantly reduced NMDA-evoked behavior. As in the agmatine experiment, pretreatment with the anti-AG IgG showed a dose-dependent reversal of aminoguanidine-mediated inhibition of NMDA behavior, suggesting that the guanidino group may be important in the binding of agmatine to both the NMDA receptor and the IgG (Fig. 1B). To control for a potential nonspecific IgG effect on the NMDA receptor or downstream signaling that governs that behavioral response, the same experiment was conducted with MK-801 (Fig. 1C), an NMDA receptor antagonist with a chemical structure unrelated to that of agmatine. MK-801 (1 nmol i.t.) significantly reduced NMDA-evoked biting and scratching behavior. Pretreatment with the anti-AG IgG showed no effect upon MK-801-mediated inhibition of NMDA behavior, arguing against a nonspecific effect of the IgG on the NMDA receptor or downstream mediators. Finally, we evaluated the possibility that the IgG could bind to L-arginine, which also contains a guanidino group (Fig. 1D). Intrathecal injection of L-arginine (600 nmol) elicits a scratching and biting behavior similar to NMDA. Pretreatment of the anti-AG IgG showed no effect on L-arginine-evoked behavior up to 300 ng. Taken collectively, these in vivo bioassays (Fig. 1, A–D) indicate the in vivo specificity of the anti-AG IgG for the target molecule agmatine.

We next studied the duration of action for the anti-AG IgG. Either anti-AG IgG or saline were given as 1-min, 24-h, or 48-h pretreatments before coadministration of the NMDA and agmatine (Fig. 2). As expected, agmatine effectively inhibited the responses in mice that had been pretreated with saline at 1 min, 24 h, and 48 h (light gray bars). However, agmatine did not inhibit NMDA-evoked responses in mice pretreated with anti-AG IgG at 1 min or at 24 h (Fig. 2, first and second dark bars). This reversal was no longer observed at the 48-h pretreatment time point. Therefore, the anti-AG IgG pretreatment seemed to reverse the effect of agmatine for at least 24 h.

Agmatinergic Modulation of DAMGO- and Endo-2-Induced Acute Tolerance. Figure 1 shows that anti-AG IgG reversed exogenous agmatine-induced (but not MK-801-

Fig. 2. Duration of anti-AG IgG effect on the agmatine inhibition of NMDA-evoked responses. Anti-AG IgG effectively reverses agmatine inhibition of NMDA-evoked behavior when given as a 1-min and 24-h (but not 48-h) pretreatment. First bar represents NMDA (0.3-nmol) injection to establish the baseline number of behaviors. Grey bars represent a saline injection given at various pretreatment times, followed by an NMDA + agmatine (60 nmol i.t.) coinjection. Black bars represent an anti-AG IgG injection (150 ng) given at various pretreatment times followed by an NMDA + agmatine (60 nmol i.t.) coinjection. *, $p < 0.05$, as evaluated by ANOVA followed by Dunnett's post hoc test for multiple comparisons with a control: $F_{(6,49)} = 86$.

induced) inhibition of NMDA-evoked behavior in mice, suggesting specificity of the anti-AG IgG for the target molecule. This provided the rationale to test the anti-AG IgG in a model of opioid receptor agonist-induced plasticity. It had been shown previously that acute analgesic tolerance develops to supramaximal doses of intrathecally administered morphine in an NMDA-receptor/nitric-oxide synthase-dependent manner (Fairbanks and Wilcox, 1997). In that study, it was also shown that intrathecally administered agmatine prevented acute spinal morphine tolerance (Fairbanks and Wilcox, 1997). We hypothesized that sequestration of endogenous agmatine by intrathecal administration of anti-AG IgG should reduce inhibitory tone upon development of acute opioid tolerance, sensitizing subjects to induction of acute tolerance by lower doses of opioid that normally induce moderate amounts of tolerance.

We tested agmatine for blockade of acutely induced tolerance to intrathecal DAMGO (Fig. 3A) and Endo-2 (Fig. 3B). To characterize acute spinal DAMGO and Endo-2 tolerance, we determined the antinociceptive dose-response curves in saline-, DAMGO (0.6 pmol i.t.)-, and Endo-2 (30 nmol i.t.)-pretreated mice. Although the μ -opioid agonists produced dose-dependent antinociception in saline-pretreated mice, pretreatment with either DAMGO (Fig. 3A) or Endo-2 (Fig. 3B) significantly reduced the potency of the respective agonists at all doses tested, confirming induction of acute tolerance. When agmatine (4 nmol i.t.) was administered as a copretreatment with the same tolerance-inducing doses of the agonists acute tolerance was prevented (Fig. 3, A and B). The probe agonist dose-response curves in DAMGO-AG or Endo-2-AG-pretreated subjects resulted in an ED_{50} value comparable with that of the saline pretreatment group and significantly different from the agonist pretreatment group (Fig. 3, A and B; Tables 1 and 2). This result demonstrates that agmatine robustly attenuates acutely induced tolerance to the spinally administered μ -opioid agonists DAMGO or Endo-2.

The doses of DAMGO and Endo-2 used to induce analgesic tolerance were 0.6 pmol and 30 nmol, respectively. Having

Fig. 3. Agmatinergetic effects on acute DAMGO and Endo-2 analgesic tolerance. A, pretreatment with DAMGO (0.6 pmol i.t., inverted triangles) decreased DAMGO potency and efficacy compared with saline-treated controls (open circles), indicating induction of acute opioid analgesic tolerance. Coadministration (triangles) of agmatine (4 nmol i.t.) with this same tolerance-inducing dose of DAMGO (0.6 pmol) prevented the induction of DAMGO acute tolerance. B, pretreatment with Endo-2 (30 nmol i.t., inverted triangles) decreased Endo-2 potency compared with saline-treated controls (open circles), indicating induction of acute opioid analgesic tolerance. Coadministration of agmatine (4 nmol i.t.) with this same tolerance-inducing dose of Endo-2 (30 nmol) prevented induction of acute Endo-2 tolerance (triangles). C, response to DAMGO in mice copretreated with normal guinea pig IgG + low dose DAMGO (0.06 pmol, diamonds) was comparable with that of mice pretreated with saline (circles) indicating lack of induction of acute tolerance. In contrast, the DAMGO analgesic response in mice that received a copretreatment with anti-AG IgG + DAMGO (0.06 pmol, inverted triangles) was of significantly lower efficacy. D, response to Endo-2 in mice copretreated with normal guinea pig IgG + low dose Endo-2 (10 nmol, diamonds) was comparable with that of mice pretreated with saline (circles) indicating lack of induction of acute tolerance. In contrast, the Endo-2 response in mice that received a copretreatment with anti-AG IgG + Endo-2 (10 nmol, inverted triangles) was of significantly lower potency.

observed a pharmacological effect for exogenous agmatine, we next evaluated the impact of pretreatment with the anti-AG IgG with administration of lower doses of DAMGO (0.06 pmol) and Endo-2 (10 nmol) that do not evoke analgesic tolerance (Fig. 3, C and D). To characterize the impact of anti-AG IgG pretreatment on acute spinal opioid-induced tolerance, we determined the antinociceptive dose-response curves in mice pretreated with the μ -opioid agonists or copretreated with the μ -opioid agonists and either normal guinea pig IgG or anti-AG IgG. When normal guinea pig IgG is administered before the DAMGO (0.06 pmol) or Endo-2 (10 nmol) low-dose pretreatment, the probe agonist dose-response curves are comparable with that of the saline-treated mice, indicating that normal IgG has no effect on the analgesic dose-response curves of DAMGO or Endo-2. However, when anti-AG IgG is administered before either DAMGO or Endo-2 low-dose pretreatment, the subsequent DAMGO and Endo-2 probe analgesic potency is lower than the saline or

TABLE 1

ED₅₀ values for Fig. 3, A and C, pretreatment group

Pretreatment Group	Probe DAMGO ED ₅₀ (95% CI)	Relative Potency (95% CI) (Compared with Saline)	Pretreatment Group	Probe DAMGO ED ₅₀ (95% CI)	Relative Potency (95% CI) (Compared with Saline Pretreatment)
	<i>pmol i.t.</i>			<i>pmol i.t.</i>	
Saline	51 (34–75)	1	Saline	52 (39–68)	1
DAMGO (0.6 pmol)	Not calculable	Not calculable	Normal IgG + DAMGO (0.06 pmol)	50 (32–77)	0.9 (0.58–1.6)
DAMGO (0.6 pmol) + agmatine (4 nmol)	75 (39–140)	1.5 (0.69–3.1)	Anti-AG IgG + DAMGO (0.06 pmol)	Not calculable	Not calculable

TABLE 2

ED₅₀ values for Fig. 3, B and D, pretreatment groups

Pretreatment Group	Probe ENDO-2 ED ₅₀ (95% CI)	Relative Potency (95% CI) (Compared with Saline)	Pretreatment Group	Probe ENDO-2 ED ₅₀ (95% CI)	Relative Potency (Compared with Saline)
	<i>nmol i.t.</i>			<i>nmol i.t.</i>	
Saline	5.8 (4.3–8)	1	Saline	3.0 (2.3–4.0)	1
Endo-2 (30 nmol)	22 (18–27)	3.7 (2.5–7.9)*	Normal IgG + Endo-2 (10 nmol)	2.8 (1.7–4.8)	0.9 (0.51–1.7)
Endo-2 (30 nmol) + agmatine (4 nmol)	8.2 (5.9–12)	1.4 (0.9–2.2)	Anti-AG IgG + Endo-2 (10 nmol)	14 (12–16)	4.6 (3.3–6.4) ^a

^a Significant difference relative to the saline pretreatment group.

normal IgG pretreatment groups (Table 2). These decreases in potency are significant and suggest that anti-AG IgG sensitized the mice to μ -opioid agonist-evoked spinal tolerance. Therefore, the anti-AG IgG sensitization of mice to acutely induced DAMGO tolerance is not a nonspecific effect of pretreatment with IgG.

The data presented in Fig. 3, B and D, show that pretreatment with the anti-AG IgG causes the lower dose of agonist to induce tolerance; at this dose, tolerance is not observed with control pretreatments of either saline or normal guinea pig IgG. This result supports the concept that reduction of endogenous agmatine by anti-AG IgG sensitizes subjects to induction of spinal opioid tolerance peptides.

As a control for potential anti-AG IgG effects on acute Endo-2 antinociception, the anti-AG IgG was administered in one treatment group after the Endo-2 pretreatment. Thus, it was given as a cotreatment with the Endo-2 probe dose (10 nmol). The resulting antinociceptive response (%MPE = 71 ± 9.1 ; $n = 7$) was comparable with responses observed from the administration of probe doses of Endo-2 (10 nmol) to the groups pretreated with saline (%MPE = 88 ± 5.5 ; $n = 8$) or 10 nmol of Endo-2 (%MPE = 74 ± 8.8 ; $n = 8$) but different from the group pretreated with anti-AG IgG + 10 nmol of Endo-2 (%MPE = 27 ± 9.8 ; $n = 8$). These results demonstrate that the sensitizing effect of the anti-AG IgG, is on the Endo-2 pretreatment induced tolerance component of the experiment rather than a putative antagonizing effect at the time of the ENDO-2 probe antinociception.

It was of interest to determine the duration of the effect of the anti-AG IgG administration in the opioid tolerance assay. To assess that, anti-AG IgG was delivered as a cotreatment or as 1-min, 24-h, or 48-h pretreatments before induction of Endo-2 tolerance (Fig. 4). The first bar shows that pretreatment with normal guinea pig serum (150 ng) and the same dose of Endo-2 (10 nmol) does not alter the analgesic response of Endo-2 given 30 min later. This response gives an approximate 70% MPE analgesic response, which is similar to the response seen with saline or Endo-2 (10 nmol) pretreatment (data not shown). However, consistent with the

Fig. 4. Duration of agmatineric effects on acute Endo-2 analgesic tolerance. Anti-AG IgG effectively sensitizes mice to acute Endo-2 analgesic tolerance when given as a 1-min, 24-h, or 48-h pretreatment. When normal guinea pig serum is given with the pretreatment of Endo-2 (10 nmol), there is no impact on the level of analgesia (first bar). However, when anti-AG IgG is given as a cotreatment (second bar) or as a pretreatment (third, fourth, and fifth bars) to Endo-2, antinociception is significantly diminished relative to the normal guinea pig IgG-pretreated control. *, $p < 0.05$, significant difference from the Endo-2 + normal GP serum pretreatment group; both measures were evaluated by ANOVA (Dunnett's post hoc test for multiple comparisons with a control). $F_{(4,45)} = 3.4$.

data profiled in Fig. 3B, a coadministration of anti-AG IgG (150 ng) given with the 10-nmol dose of Endo-2 results in a significantly diminished analgesic response to the probe dose of Endo-2 (second bar), presumably sensitizing the subjects to opioid-induced tolerance. Furthermore, the third, fourth, and fifth bars, respectively, show that, when the anti-AG IgG pretreatment is administered to the mice 15 min, 24 h, and 48 h before administration of the Endo-2 pretreatment, the anti-AG IgG still invokes sensitization to the development of acute opioid tolerance represented by an apparent analgesic tolerance to the low dose of Endo-2 (10 nmol). Therefore, the

anti-AG pretreatment seemed to sensitize the mice to opioid tolerance for up to 48 h.

Discussion

The current study examines the effect of endogenous agmatine in a model of acute opioid tolerance. It has been shown by a number of research groups that exogenously administered agmatine prevents opioid induced analgesic tolerance (for review, see Nguyen et al., 2003). Such evidence suggests that endogenous agmatine could moderate the development of opioid induced analgesic tolerance. It was observed that pretreatment with anti-agmatine IgG allowed lower doses of intrathecal opioid to evoke acute spinal analgesic tolerance. This provides proof of concept for the endogenous role of agmatine as modulator of spinal neural plasticity.

Other research groups have shown that antisera to endogenous compounds can be used to interfere with the actions of endogenous compounds in *in vivo* models of opioid tolerance and analgesia, including neuropeptide FF (Lake et al., 1991), Leu- and Met-enkephalin (Vanderah et al., 1994; Tseng et al., 2000; Ohsawa et al., 2001), β -endorphin (Tseng et al., 2000; Ohsawa et al., 2001), and dynorphin (Ossipov et al., 1996; Tseng et al., 2000; Ohsawa et al., 2001).

The present study demonstrates that intrathecal pretreatment with protein A-purified agmatine IgG (e.g., antiserum purified to the IgG fraction) dose-dependently and specifically interferes with agmatine-induced inhibition of NMDA-evoked behavior. The anti-AG IgG dose-dependently reversed the ability of aminoguanidine to inhibit NMDA-evoked behavior, which is significant because aminoguanidine is in effect a guanidine group and a chemical constituent of the agmatine molecule; and as such, suggests an epitope for the antibody. The guanidino moiety is also represented in the structure of the agmatine precursor L-arginine. Consequently, the IgG protein was evaluated for cross-reactivity to L-arginine in this bioassay. However, pretreatment with the anti-AG IgG did not affect L-arginine evoked behaviors even at twice the dose effective for reversal of the agmatine response. Therefore, it seems unlikely that the anti-AG IgG binds to L-arginine. Furthermore, previously generated and studied anti-AG antisera raised in rabbit did not show L-arginine cross-reactivity in ELISA binding assays (Otake et al., 1998). Having documented proof-of-concept that agmatine can be selectively immunoneutralized *in vivo*, we next evaluated the effects of intrathecally injected antiserum in a model of acute spinal opioid tolerance using the opioid peptides DAMGO and Endo-2 (YPFF).

Anti-Agmatine IgG Effect on Spinal μ -Opioid Receptor Tolerance. We showed that homologous tolerance induced by each of those two agents resulted in an approximately 10-fold decrease in analgesic potency of each agent compared with saline-pretreated controls. Coadministration of agmatine with the tolerance-inducing opioid prevented induction of opioid tolerance, consistent with previous reports (for review, see Nguyen et al., 2003). Conversely, cotreatment with anti-AG IgG (but not normal IgG) permitted lower doses of DAMGO or Endo-2 to become tolerance-inducing. This effect was not observed in animals that received a pretreatment of IgG from nonimmunized guinea pigs (nor-

mal IgG). We interpret these results to mean that sequestration of endogenous agmatine by agmatine-selective IgG increases susceptibility of mice to tolerance induced by these opioids. These data suggest that, under normal conditions, endogenous agmatinergetic tone may control or dampen the development of μ -opioid receptor tolerance.

Several inhibitory neurotransmission systems (enkephalins, GABA, norepinephrine, and endocannabinoids) are known to exert control over excitatory neurotransmission in CNS. Each of these agonists has a G_i -coupled seven transmembrane receptor and, as such, has been readily characterized through neuropharmacological studies using selective antagonists such as naloxone, phaclofen, yohimbine, and SR 141716A. Prior reports that exogenous agmatine prevented glutamate-dependent behavioral events formed the rationale for the proposal that endogenous agmatine could exert some anti-glutamatergic control. The current study extends that result to include a parallel observation: that possible reduction of freely available agmatine could sensitize subjects to glutamatergic events. As a putative inhibitor of glutamate neurotransmission, agmatine is distinct in that it may exert dual activity upon the NMDA receptor and/or its downstream signal transduction mediator, nitric-oxide synthase. Furthermore, agmatine is reported to have an uptake mechanism into purified nerve terminals (Sastre et al., 1997; Goracke-Postle et al., 2006, 2007a) and is also released from these structures by either K^+ or evoked depolarization (Reis and Regunathan, 1998; Goracke-Postle et al., 2006, 2007b) or capsaicin exposure (Goracke-Postle et al. 2007b). Consequently, the agmatinergetic modulation of glutamatergic neuromodulation may be of neuronal origin. However, because agmatine has also been shown to be synthesized and stored in astrocytes (Regunathan et al., 1995), the site(s) of cellular synthesis, uptake, and release of agmatine remains under investigation. Identification of such agmatinergetic neurodynamics may be key to defining its role in chronic neuroplastic processes. The scope of the present study is limited to the action of the anti-agmatine IgG in an acute model of spinal opioid tolerance. We and others have reported that exogenous agmatine inhibits neuroplastic behavior accompanying opioid tolerance and addiction, chronic pain, and spinal cord injury (Nguyen et al., 2003). It is possible that endogenous agmatine plays a similar role in control of those processes. Evidence supports the role of glia in neuroplasticity; the role of glia as a contributor to neuroplasticity is increasingly appreciated, and the source of endogenous agmatine regulation may involve glia as well as neurons. The complexity of the system permits the participation of agmatine as an intensity control affecting neuroplastic events in the CNS.

Increasing evidence suggests that decarboxylated arginine, agmatine, operates as a novel neurotransmitter and/or neuromodulator in mammals (Reis and Regunathan, 1998; Goracke-Postle et al., 2006, 2007a,b). Asserting that claim requires, in part, testing the hypothesis that, when administered exogenously, agmatine produces pharmacological effects comparable with the proposed physiological effects of the putative endogenous molecule. Since its discovery in the CNS (Li et al., 1994), there have been numerous studies evaluating a variety of physiological effects produced by exogenous administration of agmatine. To our knowledge, the current study is the first to conduct a direct side-by-side comparison with exogenous administration of agmatine and

a tool intended to neutralize endogenous agmatine. Additional studies are needed that modulate endogenous agmatine levels in in vivo models to further define agmatinergic CNS function.

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