Agmatine reverses pain induced by inflammation, neuropathy, and spinal cord injury

Carolyn A. Fairbanks*[†], Kristin L. Schreiber[†], Kori L. Brewer[‡], Chen-Guang Yu[§], Laura S. Stone[†], Kelley F. Kitto*[†], H. Oanh Nguyen*, Brent M. Grocholski*, Don W. Shoeman*, Lois J. Kehl[¶], Soundararajan Regunathan[∥], Donald J. Reis[∥], Robert P. Yezierski[§], and George L. Wilcox*[†]**

Departments of *Pharmacology and [†]Neuroscience and [¶]Oral Science, University of Minnesota, Minneapolis, MN 55455; [§]University of Miami, The Miami Project, Miami, FL 33136; [∥]Department of Neurology and Neuroscience, Weill–Cornell University Medical College, New York, NY 10021; and [‡]East Carolina University School of Medicine, Department of Emergency Medicine, Greenville, NC 27858

Antagonists of glutamate receptors of the *N*-methyl-D-aspartate subclass (NMDAR) or inhibitors of nitric oxide synthase (NOS) prevent nervous system plasticity. Inflammatory and neuropathic pain rely on plasticity, presenting a clinical opportunity for the use of NMDAR antagonists and NOS inhibitors in chronic pain. Agmatine (AG), an endogenous neuromodulator present in brain and spinal cord, has both NMDAR antagonist and NOS inhibitor activities. We report here that AG, exogenously administered to rodents, decreased hyperalgesia accompanying inflammation, normalized the mechanical hypersensitivity (allodynia/hyperalgesia) produced by chemical or mechanical nerve injury, and reduced autotomy-like behavior and lesion size after excitotoxic spinal cord injury. AG produced these effects in the absence of antinociceptive effects in acute pain tests. Endogenous AG also was detected in rodent lumbosacral spinal cord in concentrations similar to those previously detected in brain. The evidence suggests a unique antiplasticity and neuroprotective role for AG in processes underlying persistent pain and neuronal injury.

Agmatine (AG) is formed by the enzymatic decarboxylation of L-arginine (1). It has been discovered recently in mammals (2, 3), where it is expressed in the central nervous system. In brain, AG meets most of the criteria of a neurotransmitter/neuromodulator (4): it is synthesized, stored, and released from specific networks of neurons (5, 6), is inactivated by energy-dependent reuptake mechanisms (7), is degraded enzymatically (8), and binds with high affinity to α_2-adrenergic and imidazoline (I$_1$) receptors (2, 9). In addition, AG antagonizes *N*-methyl-D-aspartate receptors (NMDAR) (10) and inhibits all isoforms of nitric oxide synthase (NOS) (11, 12). NMDAR antagonists and NOS inhibitors prevent adaptive changes in neuronal function, including opioid tolerance (13, 14), persistent pain (15–17), and spinal cord injury (SCI) (18–21). Therefore, AG, which antagonizes/inhibits both NMDAR and NOS, should moderate chronic pain accompanying inflammation, neuropathy or SCI. We report here that AG, when exogenously administered, selectively relieves allodynic, hyperalgesic, and autotomy-like states accompanying spinal nerve injury, peripheral inflammation, and excitotoxic SCI, respectively. Moreover, as in brain (5, 6), we have detected AG in spinal cord, indicating that AG may be an endogenous modulator of pain pathways.

Methods

Animals. Institute of Cancer Research mice (25–30 g, Harlan, Teklad, Madison, WI), Sprague–Dawley rats [125 g, Harlan Teklad (Fig. 1*D*; 400–500 g, Harlan Teklad (Fig. 5*C*); 200–250 g, Charles River Breeding Laboratories (Figs. 3 and 4)]. All experiments were approved by the Institutional Animal Care and Use Committees. Each group had at least five animals; each animal was used only once.

Chemicals. The following chemicals were used: MK801 (Merck); LY235959 (Lilly Research Laboratories, Indianapolis); carra-

geenan (CARRA), ketamine, dextromethorphan, ifenprodil, aminoguanidine, N$^\omega$-nitro-L-arginine methyl ester (L-NAME), AG, NMDA, substance P (SP), memantine, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/metabotropic agonist quisqualate (QUIS; Sigma); dynorphin (DYN; National Institute on Drug Abuse), SK&F 86466 (SmithKline Beecham), efaxoran (Research Biochemicals), and moxonidine (Solvay Pharma). SP and moxonidine were dissolved in acidified saline; CARRA was dissolved in PBS; and all the other drugs were dissolved in 0.9% normal saline.

Mechanical Sensitivity Testing. Application of von Frey (vF) filaments 2.44 (0.4 mN, innocuous) and 3.61 (3.3 mN, noxious) cause mice to lick, withdraw, and/or shake the paw, actions representing a behavioral endpoint. In Figs. 1 and 2, nociception was tested by multiple vF applications to the plantar (Figs. 1*A* and 2 *C–F*, 10 applications) or dorsal (Fig. 2 *A* and *B*, 6 applications) hindpaw surfaces.

CARRA-Evoked Mechanical Hyperalgesia. Intraplantar injections of CARRA (4%/50 μl), or PBS (50 μl) were given to halothane-anesthetized mice. After confirmation of hyperalgesia at 3 h postinjection, AG or SAL was delivered intrathecally (i.t.), followed by continued testing for mechanical sensitivity.

CARRA-Evoked Muscle Hyperalgesia. Intramuscular (triceps) injections of CARRA (4 mg/75 μl), but not PBS (75 μl), in halothane-anesthetized rats produced hyperalgesia, which was indicated by a decrease in forelimb grip tensile force (22) at 12 h after CARRA. AG or SAL then was delivered i.t., followed by continued testing. The data are pooled from three experiments conducted by a blinded observer.

Dynorphin-Induced Allodynia. Intrathecal injections of DYN (3 nmol) or SAL (5 μl) were given to awake mice. After confirmation of allodynia at 1 day postinjection, AG or SAL was delivered i.t., followed by continued testing for mechanical sensitivity by a blinded observer.

Nerve Injury-Induced Hypersensitivity. Spinal nerve ligation induces mechanical hypersensitivity of the ipsilateral hindpaw in mice

(24, 25). In halothane-anesthetized mice, the left paraspinal muscle (at the level of the lumbrosacral spinal nerves L4–S2) and the L6 transverse process were removed. The L5 spinal nerve was ligated (6-0 silk thread) distal to the dorsal root ganglion and proximal to the L4-L5 spinal nerve confluence. Control groups included naïve or sham-operated (no nerve ligation) mice. After induction of allodynia/hyperalgesia was confirmed, AG or SAL was injected followed by continued testing for mechanical sensitivity by blinded observers.

SCI. QUIS (125 nmol) or QUIS + AG (1, 5, and 10 nmol) was injected (1 μl) in anesthetized rats 900 μm below the dorsal surface of the spinal cord in laminae IV–VI.

Histology. After 3 days, rats were perfused transcardially (saline, then 10% formalin) and cords were removed for histology. Spinal cord cross-sections (75 μm) were examined with light microscopy, and reconstructions of the lesion were made with an overhead projector and camera lucida. Areas of tissue damage eight sections rostral and eight sections caudal (1,275 μm total length) to the lesion epicenter were measured by using a computer-aided imaging system (26) (IMAGE 1; Universal Imaging, Media, PA). Comparing the area of normal gray matter between each 75-μm section and the corresponding segmental level of uninjured control cords yielded a longitudinal profile of the percentage of gray matter damage in the injured cord. To obtain an overall descriptor of the amount of tissue damage, the total lesion volume was calculated by subtracting the volume of gray matter remaining in injured animals from the normal control volume. AG was given either (*i*) intraspinally at the time of QUIS administration (Fig. 3) or (*ii*) systemically (100 and 150 mg/kg, i.p.) 30 min after QUIS administration and once daily thereafter for 3 days (Fig. 4).

Behavior. The area of skin with evidence of excessive grooming was measured and compared between QUIS- and QUIS + AG-treated groups (Student's *t* test.). In these studies, AG was delivered systemically (100 mg/kg, i.p.) either (*i*) 30 min after QUIS and once daily thereafter for 14 days or (*ii*) 14 days after QUIS (after onset of the behavior) and once daily thereafter for 14 days.

Tail Flick Tests. *Antinociception.* Antinociception was tested by tail immersion in warm water (52.5°C), and the latency to rapid tail flick was measured (3.61 ± 0.17 s, *n* = 36). For this test, percent antinociception was calculated as percent maximum possible effect by the formula: (postdrug latency − predrug latency)/(12 − predrug latency) × 100%.

NMDA-evoked thermal hyperalgesia (NMDA hyperalgesia). Tail flick latency to radiant heat was measured before and 1, 3, and 10 min after NMDA (0.15 nmol, i.t.) (27). Data are expressed as the average percent inhibition ± SEM during this time period by the formula: [(ControlΔ − ExperimentalΔ)/ControlΔ] × 100%. ED$_{50}$ values were calculated by the method of Tallarida and Murray (28).

NMDA and SP Tests. Transmitter-induced behavior tests compare the number of hindlimb-directed behaviors elicited within 1 (mouse) or 1.5 (rat) min after i.t. delivery of SP (29) or NMDA (30). SP (15 ng i.t.) produces biting and scratching behavior (SP behavior) in mice (*n* = 12, 68 ± 9.0 behaviors). NMDA (0.3 nmol) elicits biting behavior in mice (*n* = 18, 56 ± 4.3) and nociceptive behaviors (spontaneous tail flicks and rapid circling) in rat (*n* = 10, 33.5 ± 5.4). AG was tested for inhibition of behaviors elicited by both SP (mice) and NMDA (mice, rats). MK801, ketamine, dextromethorphan, ifenprodil, memantine aminoguanidine, LY235959, and L-NAME also were tested for inhibition of NMDA behavior (mice). For these tests, percent antinociception was calculated as percent inhibition ± SEM by the formula [(Control − Experimental)/Control] × 100%.

Electrophysiology. Spinal L3-L5 laminectomy permitted extracellular single-neuron recordings in urethane-anaesthetized (1.2 g/kg i.p.) male rats (450 g, 38°C) via the center barrel (2 MΩ carbon) of seven-barreled glass microiontophoretic electrodes: 50 mM NMDA (pH 8.0) and 15 mM AG (pH 6.5), both in 150 mM NaCl, administered with automatic current balancing. Recordings were made from dorsal horn neurons responding to noxious or to both innocuous (brush) and noxious (pinch, squeeze) stimuli applied to the ipsilateral hindpaw receptive fields. Peristimulus time histograms of action potentials evoked by iontophoretic application of NMDA (20 neurons) or natural stimulation (vide infra) of the cutaneous receptive field involved detection (window discriminator) and accumulation (computer). Receptive fields of three spinal neurons were activated by cutaneous stimulation (vF 5.18, 140 mN, 0.5–1 Hz or no. 1 paintbrush, 1–5 min on, 1–5 min off, repeatedly for 15–30 min).

Rotarod Assay. After two training sessions, mice walked for 300 s on an accelerating (4–40 rpm) rotarod (Ugo Basile, Varese, Italy). We compared the latency to fall before (291 ± 5.4 s, *n* = 48) and after delivery (i.t.) of saline or drug (MK801, L-NAME, AG) by the formula: % motor impairment = (predrug latency − postdrug latency)/(predrug latency) × 100%. Mice that walked for 300 s were scored 100%.

HPLC. The HPLC method (31) used was modified from two reports (3, 32). Tissue extracts (32) were concentrated under vacuum and suspended in 75 μl of borate buffer (pH 9.4) containing 2-*p*-toluyl ethyl amine (internal standard). NaCN (25 μl, 10 mM) was added, followed by naphthalene dicarboxaldehyde (NDA; 200 μl, 1 mM) in MeOH for derivatization (at room temperature for 4 min). Three to 50 μl of the reaction mixture was injected onto a 250 × 4.6-mm Alltech Associates Nucleosil C8 10-μm HPLC cartridge and eluted (flow rate: 1.5 ml/min) with 55% acetonitrile in a buffer (3.42 g KH$_2$PO$_4$ and 4.32 g K$_2$HPO$_4$ in 1 liter of HPLC-grade H$_2$O, pH 6.81).

Immunocytochemistry. Naïve rats or mice were perfused (4% paraformaldehyde/0.2% picric acid/0.1 M PBS, pH 6.9), and brains or spinal cords were removed for histology (33). Primary antisera or preimmune serum was incubated overnight (4°C) on 14-μm brain or lumbar spinal cord cross-sections at the following dilutions: rabbit anti-AG, 1:50 (D.J.R.); mouse anti-NeuN (antineuronal nuclear protein), 1:500; and mouse antidopamine-β-hydroxylase, 1:500 (Chemicon). Double-labeled slides were visualized with a mixture of fluorescein isothiocyanate and lissamine-rhodamine-conjugated secondary antisera 1:200 (Jackson ImmunoResearch). Spinal cord sections (*n* ≥ 3 animals per experiment) were examined with a MRC-1000 Confocal Imaging System (Bio-Rad). Fields were selected to represent the pattern of AG-LI when single-labeled or in relation to NeuN or dopamine β-hydroxylase (DβH).

Results

Inflammatory and Neuropathic Pain Models. To test the effect of AG on persistent pain, AG was delivered after inflammation and nerve injury. Intrathecal AG (60 nmol) reversed preestablished CARRA mechanical (mice, Fig. 1*A* and *B*) and muscle (rats, Fig. 1 *C* and *D*) hyperalgesia. AG did not affect responses of PBS-treated rats, which were comparable to PBS-SAL controls.

We also tested AG's impact on neuropathic pain elicited by dynorphin or ligation of a peripheral nerve. A single injection of AG (0.3 nmol, i.t.) reversed dynorphin allodynia (23) for at least 28 days (Fig. 2 *A* and *B*, 1–21 days). The α_2/I_1 agonist moxonidine only transiently inhibited (2 h) allodynia (data not shown), suggesting that the persistent AG effect does not likely involve α_2/I_1 receptors.

AG reversal of allodynia was observed in another mouse model of neuropathic pain [Chung Model (24)]. L5 spinal nerve injury induced hypersensitivity of the ipsilateral hindpaw (Fig. 2
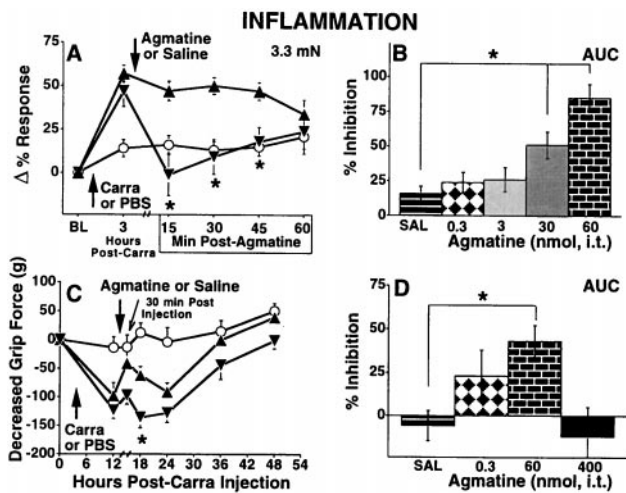
INFLAMMATION

Fig. 1. Inflammation. (*A* and *C*) Time course of AG or SAL treatment (i.t.) after inflammation-induced hyperalgesia. The duration of hypersensitivity was compared among CARRA + SAL, PBS + SAL, and CARRA + AG. (*B* and *D*) Area under the curve (AUC; average across time) showing dose-related inhibition of hyperalgesia by AG. Included data coincide with times when preliminary studies had revealed significant differences between CARRA- and PBS-treated groups. The percent inhibition was calculated from the equation [(Control − Experimental)/Control] × 100%, and * indicates a statistically significant difference (ANOVA with Bonferroni) between the SAL-injected control and AG-treated groups (doses at and below the most effective inhibitory dose). These experiments were replicated and produced comparable results. (*A*) Mechanical hyperalgesia. Three hours after CARRA injection into the hindpaw, mice showed increased responses (▲) compared with PBS-injected controls (figchr;○) ($P \leq 0.001$, Student's *t* test). AG (60 nmol, i.t.) (figchr;▼) injected 3 h after CARRA reduced hyperalgesia. The * indicates a statistically significant difference (ANOVA-repeated measures with Bonferroni). (*B*) AUC (15, 30, and 45 min) after AG injection. (*C*) Muscle hyperalgesia. Twelve hours after CARRA injection into the triceps, rats showed decreased grip force (▼) compared with PBS-injected controls (○) ($P \leq 0.001$, Student's *t* test); AG (60 nmol, i.t.) (▲) injected 12.5 h after CARRA reduced hyperalgesia. (*D*) AUC (12.5, 18, and 24 h after CARRA injection).

C–F). A single injection of AG (0.3 nmol, i.t.) reversed this allodynia (Fig. 2*C*, 2 weeks) and hyperalgesia (Fig. 2*E*, 3 weeks). Responses did not increase throughout the test period in naïve or sham-operated mice (AG-treated or SAL-treated, data not shown). Again, in contrast to AG, moxonidine only transiently (2 h) inhibited hyperalgesia (25). These results demonstrate that AG (i.t.) decreases established hypersensitivity induced by inflammatory, chemical, and mechanical insults in rodents.

SCI. To determine the effects of AG on neuronal damage, AG was given either concurrently or after intraspinal QUIS injection. QUIS (Fig. 3*A*) produced excitotoxic injury similar to that associated with ischemic and traumatic SCI (34, 35). Intraspinally administered AG (1–10 nmol, Fig. 3 *B–E*) injected with QUIS reduced the lesion. Pathological effects of intraspinal AG alone were not evident at these doses (data not shown). AG administered systemically (100, 150 mg/kg, i.p.) 30 min after QUIS (Fig. 4 *A–C*) significantly reduced the lesion (Fig. 4*D*). QUIS also causes delayed (≈2 weeks) excessive grooming behavior directed to specific measurable areas of dermatomes corresponding to the lesion. AG (100 mg/kg, i.p. 30 min post-QUIS) significantly reduced the grooming area (QUIS, 9.75 ± 1.9; QUIS + AG, 0.98 ± 0.7 cm², Student's *t* test). AG (100 mg/kg i.p., once daily for 14 days beginning after establishment of the grooming behavior, ≈2 weeks post-QUIS) also reduced the grooming area (QUIS, 7.9 ± 1.2; QUIS + AG, 1.3 ± 0.45 cm², Student's *t* test).
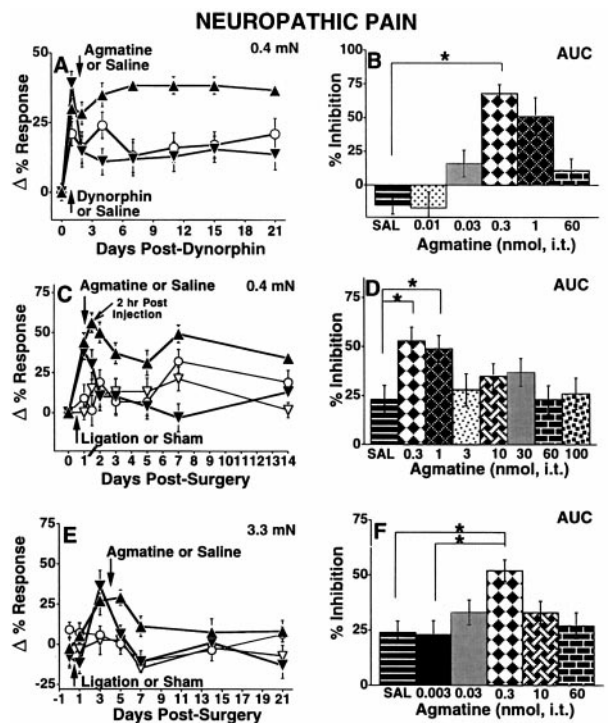
NEUROPATHIC PAIN

Fig. 2. Neuropathic pain models. (*A*, *C*, and *E*) Time course of AG (▼) or SAL (▲) treatment (i.t.) after induction of hyperalgesia representing neuropathic pain. The duration of hypersensitivity was compared among injured (▲), vehicle-treated or sham-operated (○), or naïve (▽) mice. (*B*, *D*, and *F*) AUC showing dose-related inhibition of hyperalgesia by AG. Data analysis is identical to that described for Fig. 1 *B* and *D*. These experiments were replicated and produced comparable results. (*A*) Dynorphin allodynia. DYN-treated mice show increased responsiveness (▲) compared with SAL-injected controls (○) ($P \leq 0.0001$, Student's *t* test). AG (0.3 nmol) (▼) injected 1 day after DYN reduced allodynia. (*B*) AUC (days 2, 3, and 7 after DYN injection). Spinal nerve injury. (*C*) Allodynia. Nerve-injured mice showed increased responses (▲) compared with sham-operated (○) or naïve (▽) controls (ANOVA, $P \leq 0.001$). AG (0.3 nmol) (▼) injected 1 day after surgery reduced allodynia. (*D*) AUC (2, 3, 5, 7, and 14 days after ligation). (*E*) Hyperalgesia. Three days after surgery, nerve-ligated mice showed increased responses (▲) compared with sham-operated (○) or naïve (▽) controls; AG (0.3 nmol) (▼) injected 1 day after surgery reduced hyperalgesia. (*F*) AUC (5, 7, 14, and 21 days after ligation).

Antinociception. To determine whether AG is antinociceptive, we tested for inhibition of responses to noxious thermal and chemical stimuli. AG (i.t.) neither prolonged tail flick latencies nor inhibited SP behavior (Fig. 5*A*). These results do not support an antinociceptive action for AG.
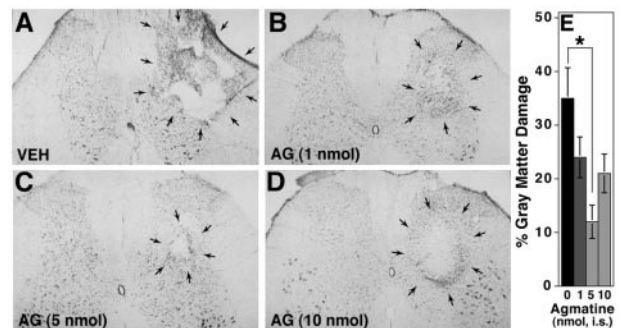

Fig. 3. (*A–E*) Concurrent intraspinal AG reduces QUIS-induced SCI. (*A*) QUIS (125 nmol). (*B*) QUIS (125 nmol) + AG (1 nmol). (*C*) QUIS (125 nmol) + AG (5 nmol). (*D*) QUIS (125 nmol) + AG (10 nmol). (*E*) AG-treated spinal cords (5 nmol) showed significantly less damage than those exposed to QUIS alone (ANOVA, $P < 0.05$).
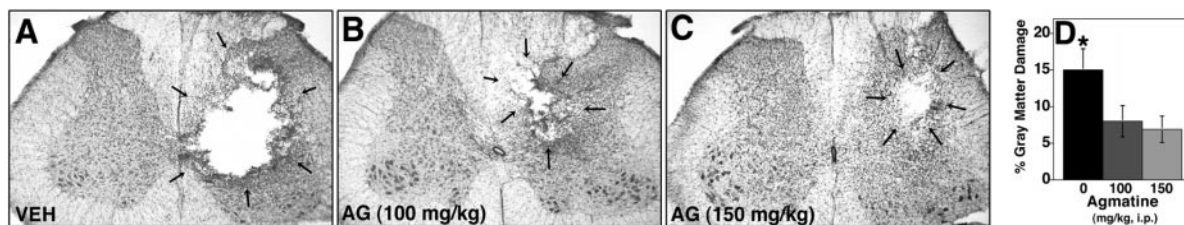
Fig. 4. (A–D) Systemic AG reduces QUIS-induced SCI. (A) QUIS (125 nmol). (B) QUIS (125 nmol) + AG (100 mg/kg). (C) QUIS (125 nmol) + AG (150 mg/kg). (D) AG-treated (30 min after QUIS) spinal cords showed significantly less damage than those exposed to QUIS alone (ANOVA, $P < 0.05$).

NMDA Behavior. To test for AG-mediated NMDAR blockade *in vivo*, we coadministered AG with NMDA (i.t.). AG antagonized NMDA behavior in mouse ($ED_{50} = 30$, 18–50 nmol, Fig. 5A) and rat ($ED_{50} = 19$, 4–85 nmol, data not shown), but not AMPA-evoked behavior in mice (0.3 nmol, data not shown). Other NMDAR antagonists (ED_{50}: ketamine, 0.1, 0.48–0.21; memantine, 0.16, 0.085–0.31; dextromethorphan, 0.6, 0.37–1.0; MK801, 0.92, 0.5–1.7; ifenprodil, 0.17, 0.07–0.39 nmol i.t.; LY235959, 0.06, 0.02–0.16 pmol i.t.) inhibited NMDA behavior with significantly greater potency than AG. Whereas the nonselective NOS inhibitor L-NAME did not inhibit NMDA behavior (0.1–10 nmol i.t., data not shown), the selective iNOS inhibitor aminoguanidine did (ED_{50}: 0.3, 0.14–0.62 nmol i.t.), suggesting that, like AG, aminoguanidine may antagonize NMDAR. Because AG binds to α_2-adrenergic receptors (ARs) (36) and I_1 receptors (2), we also tested antagonists selective for α_2-AR (SK&F 86466) and α_2/I_1 (efaroxan) for antagonism of AG-induced inhibition of NMDA behavior. Neither antagonist interfered with the action of AG in this test, indicating that α_2/I_1 receptors are not involved.

NMDA Hyperalgesia. To test AG for inhibition of NMDA hyperalgesia, we compared AG with MK801 and L-NAME (27). NMDA decreased ($P < 0.0001$, Student's t test) tail flick latencies (3.1 ± 0.1 s, $n = 9$) relative to SAL controls (4.4 ± 0.15 s, $n = 9$) (27). MK801 inhibited this hyperalgesia with a potency (ED_{50}: 2.6 nmol, 0.96–4.8) comparable to that of its inhibition of the corresponding nociceptive behavior (ED_{50}: 1.6 nmol, 0.92–2.7) measured in the same mice. This result confirms that both of MK801's behavioral actions rely on NMDAR activation. Coadministration of L-NAME (550 nmol, i.t.) with NMDA fully prevents NMDA hyperalgesia (27) with only partial (43%) inhibition of NMDA behavior (present study). This result indicates that NOS may contribute more to thermal hyperalgesia than to nociceptive behavior. Interestingly, AG inhibits the thermal hyperalgesia at significantly lower doses (ED_{50}: 0.45 nmol, 0.089–2.2) than those required

to inhibit the corresponding nociceptive behavior (ED_{50}: 53 nmol, 25–112, Fig. 5B). This discrepancy supports the proposal that, at the lower doses, AG may act at another effector (e.g., NOS) to prevent the hyperalgesia.

NMDA-Evoked Firing. Consistent with these behavioral studies, iontophoretically (37) applied AG inhibited NMDA-evoked firing in 7 of 20 rat spinal neurons studied (Fig. 5C) but did not change activity evoked by physiological stimulation of cutaneous receptive fields, a result consistent with lack of AG-induced inhibition of spinal nociceptive reflexes (38). Failure of AG to inhibit physiologically evoked responses is in agreement with its apparently selective action at NMDAR and minimal inhibition of AMPA-evoked current (10) and behavior (data not shown). Presumably, activity evoked synaptically from glutamatergic afferent axons activates secondary sensory neurons by using both receptor subtypes.

Rotarod Test. Acute delivery of MK801 impaired motor function with an ED_{50} (11 nmol, 6.3–18) 4-fold higher than the antihyperalgesic ED_{50} (2.6 nmol, 0.96–4.8). Motor function was not affected by L-NAME (550 nmol, i.t.) or AG (0.3–240 nmol, i.t.), suggesting that (using this test) the therapeutic index of spinally administered AG is substantially higher than that of MK801.

Endogenous AG in Rodent Spinal Cord. AG levels in naïve and saline-treated (5 μl, i.t.) mouse lumbosacral spinal cord were 0.96 ± 0.14 and 0.56 ± 0.2 μg/g wet weight ($n = 4$–5), respectively, values similar to those reported in mammalian brain (2, 32). Intrathecally administered AG (60 but not 0.3 nmol) significantly increased spinal AG levels (2.6 ± 0.65 μg/g wet weight, $n = 6$, ANOVA, $P < 0.05$).

Spinal AG was immunocytochemically localized with AG antiserum. Preincubation of the antiserum with free AG (but not structurally related compounds arginine, spermidine, spermine, ornithine, putrescine, or citrulline) inhibited binding to conju-
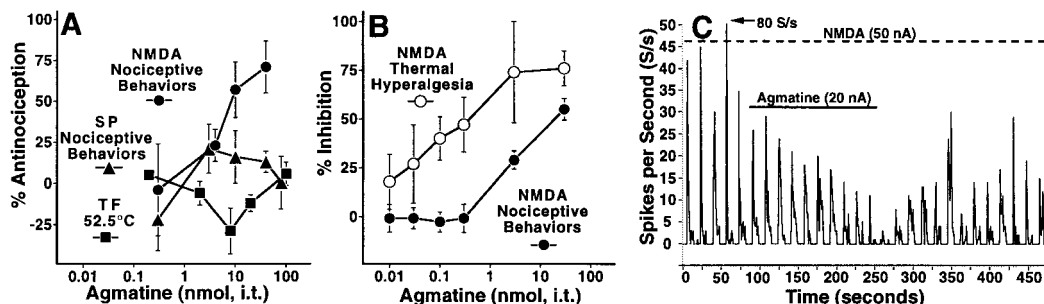

Fig. 5. (A–C) Acute nociceptive tests. (A) AG was inactive in the tail flick (10-min pretreatment) and SP (coadministered) behavioral tests. AG inhibited NMDA behaviors (ED_{50}: 30 nmol, 18–50). (B) NMDA hyperalgesia and nociceptive behavior. AG inhibited NMDA hyperalgesia with greater potency (ED_{50}: 0.45 nmol, 0.089–2.2) than inhibition of NMDA behavior in the same animals (ED_{50}: 53 nmol, 25–112). (C) NMDA-evoked firing. Iontophoretically applied AG inhibited NMDA-evoked firing in 7 of 20 NMDA-responsive dorsal horn neurons tested.
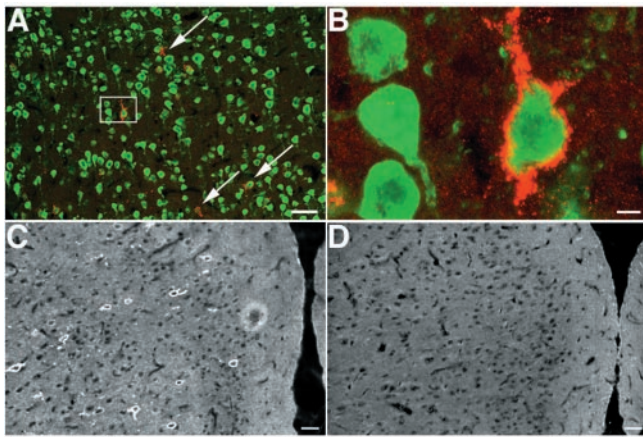
NEUROBIOLOGY

Fig. 6. AG-LI in rat cortex. Single, 14-μm coronal sections were single- or double-labeled with AG (red) and NeuN (green) antisera. (*A*) AG-LI associated with NeuN-positive cells (arrows). (Bar = 200 μm.) (*B*) The boxed portion of *A* at higher magnification projected from eight optical sections acquired at 1-μm intervals by using a $\times$60 objective. (Bar = 5 μm.) (*C*) AG-LI in cingulate cortex. (Bar = 50 μm.) (*D*) Absence of AG-LI in cingulate cortex after preabsorption with free AG (100 mM). (Bar = 50 μm.)

Fig. 7. AG-LI in mouse spinal cord. Single, 14-μm cross-sections were single- or double-labeled by using antisera to NeuN (green) or DβH (green, noradrenergic) and AG (red). Double-labeled micrographs represent projected images from multiple optical sections (o.s.) acquired at 1-μm intervals using a $\times$60 objective and confocal zoom. (*A*) AG-LI in dorsal horn is in a few fibers; the midline is on the left. (Bar = 200 μm.) (*B*) Area dorsolateral to central canal: AG and NeuN (5 o.s.). (Bar = 5 μm.) (*C*) Lumbar dorsal horn: AG and DβH (6 o.s.). (Bar = 5 μm.)

gated agmatine (5, 39). The antiserum specificity was confirmed for the conditions used in these studies. Neurons were identified by using neuronal marker NeuN (40) (Fig. 6 *A* and *B*). AG-like immunoreactivity (AG-LI) was associated with cingulate cortical neurons (Fig. 6*C*) as shown previously (5), was not observed in tissue incubated with preimmune serum (data not shown), and was concentration-dependently decreased after preincubation of antiserum with free AG (0.1, 1, and 10 mM, data not shown; 100 mM, Fig. 6*D*). AG-LI (fibers and/or puncta) was sparsely but consistently observed in all areas of the mouse spinal cord gray matter (Fig. 7*A*); fibers also were present in the surrounding white matter. At higher magnification, AG-LI was observed surrounding NeuN immunoreactivity lateral to the central canal in sacral spinal cord sections (Fig. 7*B*). AG's association with α_2AR and I$_1$ receptors implied a common source with norepinephrine; therefore, we compared AG-LI with that of DβH, a marker for noradrenergic terminals. High magnification showed that AG-LI fibers were distinct from DβH-containing processes (Fig. 7*C*), suggesting independent sources for AG and norepinephrine.

Discussion

The localization of AG and its synthetic and degradative enzymes in mammalian brain (2) established a potentially novel neurotransmitter (4). The characterization of its dual-activity profile, NMDAR antagonism (10) with NOS inhibition (11, 12), may have equal impact in several areas of neuroscience relating to glutamatergic neurotransmission and synaptic plasticity. This report shows modulation by exogenous AG of spinally mediated pain states that depend on glutamate receptors and neuronal plasticity.

The present experiments reveal the ability of AG to restore injured, hypersensitive mice to normal levels of sensation. Intrathecally administered AG reversed CARRA mechanical and muscle inflammatory hyperalgesia. These actions were dose-dependent, transient (1–6 h, consistent with ref. 41), and comparable in efficacy to levorphanol, dexamethasone, and indomethacin (22). A single posttreatment of AG (0.3 nmol, i.t.) persistently reversed long-lasting hypersensitivity induced by two models of neuropathic pain: dynorphin, i.t. (23, 42), or nerve injury (Chung Model; ref. 24). AG efficacy in the Chung Model is notable because it is a widely accepted neuropathic pain
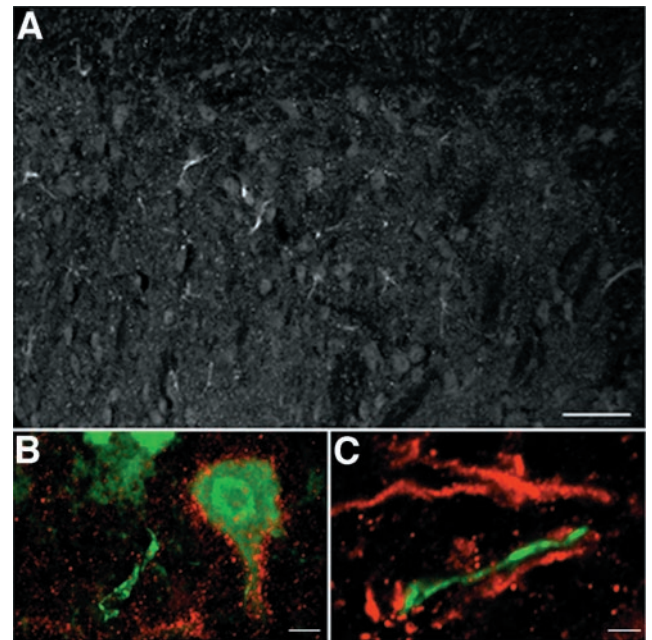
model, corresponding well with clinical observations (43). AG's moderation of neuropathic pain is consistent with the antihyperalgesic activity of an analog of histogranin, another endogenous NMDAR antagonist (16). However, AG's reversal is persistent and possibly reliant on an additional action other than NMDAR antagonism.

Traumatic SCI also induces chronic pain (44) and may invoke common, secondary pathological cascades, including activation of NMDAR (26, 45), AMPA/kainate receptors (46), and NOS (44). AG reduced QUIS-induced spinal gray matter injury when administered intraspinally (34) or systemically (i.p.) 30 min after QUIS. Systemic, daily AG treatment also reduced autotomy-like behavior whether started 30 min or 14 days after QUIS (after establishment of the behavior). These data concur with evidence showing that AG (i.p.) prevented ischemia-induced neuronal loss (47). AG's reduction of hypersensitivity and SCI demonstrates a postinjury therapeutic potential in multiple preclinical models of persistent pain.

The effects of AG in these neuropathic pain models are distinctly antiallodynic and antihyperalgesic. Agents such as morphine and clonidine, which transiently relieve allodynia/hyperalgesia, are also analgesic in normal subjects; in contrast, AG did not modify nociceptive responses to acute thermal (48), chemical, or mechanical (data not shown) stimuli. These results agree with evidence that NMDAR antagonists and NOS inhibitors are not antinociceptive (49) and distinguish AG from conventional analgesics.

AG inhibits NMDA-evoked currents in cultured rodent hippocampal neurons by channel blockade (10). The present report extends this finding to show that AG blocks spinal NMDARs by inhibition of both NMDA behavior and firing in spinal neurons. Consequently, AG may reverse spinal hypersensitivity through NMDAR antagonism, in agreement with evidence that NMDAR antagonist pretreatment prevents the induction of CARRA-

induced (50, 51), DYN-induced (23, 42), and nerve injury-induced (15) hypersensitivity. The doses necessary for temporary reversal of inflammation-induced hyperalgesia (60 nmol, i.t.) and for protection from excitotoxic injury (5 nmol, intraspinally) are comparable to the doses (30–60 nmol, i.t.) required to inhibit NMDA behavior. This correspondence suggests that these three effects of AG require NMDAR antagonism.

That the doses necessary for reversal of neuropathic pain (0.3, 1 nmol, i.t.) and NMDA hyperalgesia (0.4 nmol, i.t.) are 100-times lower than those required to antagonize NMDAR-mediated behavioral actions (30–60 nmol, i.t.) suggests that the mechanism for AG-mediated recovery from neuropathic pain requires activity other than NMDAR blockade. NMDA hyperalgesia relies on NOS activation (27), and AG inhibits all isoforms of NOS (12). We speculate that NOS inhibition, concerted action at both NMDAR and NOS, or some unknown action mediates these high-potency actions. AG's low potency against NMDA behavior, 30- to 500,000-times lower than those of clinically (ketamine, memantine, dextromethorphan) and experimentally (MK801, aminoguanidine, ifenprodil, LY235959) used NMDAR antagonists, may be predictive of an improved therapeutic potential for AG relative to previously used agents (14, 52, 53).

AG's exogenous profile suggests that endogenous AG could play a similar role. Endogenous control of spinal plasticity by AG would require localization in spinal tissue. Immunocytochemistry (5), electron microscopy (5), and HPLC (2, 3) previously have identified AG in brain. AG-LI has been observed in association with small synaptic vesicles in axons and axon terminals (6) in hippocampus, suggesting a neuronal source of AG in the central nervous system. We report AG levels in spinal cord consistent with previously reported values in brain. Additionally, the localization of AG-LI in spinal cord is suggestive of a neuromodulatory role for AG. Further studies are warranted to determine whether endogenous AG participates in the spinal processing of plasticity associated with persistent pain syndromes.

Exogenous administration of AG hours to days after injury significantly reduces pain induced by inflammation, neuropathy, and SCI, suggesting a new therapeutic direction for plasticity-mediated neurodysfunction. Several reports describe the phenomena that exogenous AG pretreatment reduces the development of opioid tolerance (48, 54), inflammation-induced thermal hyperalgesia (41), and ischemia-induced neuronal lesion (47). The present study demonstrates an endogenous location and potential mechanisms for AG-mediated antiplasticity action in spinal cord postinjury. The apparently low toxicity and selective antihyperalgesic (nonanalgesic, nonsedating) profile of AG make the compound a novel and potentially advantageous therapeutic agent for treatment of chronic pain and SCI.

1. Tabor, C. W. & Tabor, H. (1984) *Annu. Rev. Biochem.* **53,** 749–790.
2. Li, G., Regunathan, S., Barrow, C. J., Eshraghi, J., Cooper, R. & Reis, D. J. (1994) *Science* **263,** 966–969.
3. Raasch, W., Regunathan, S., Li, G. & Reis, D. J. (1995) *Life Sci.* **56,** 2319–2330.
4. Reis, D. J. & Regunathan, S. (1999) *Ann. N. Y. Acad. Sci.* **881,** 65–80.
5. Otake, K., Ruggiero, D. A., Regunathan, S., Wang, H., Milner, T. A. & Reis, D. J. (1998) *Brain Res.* **787,** 1–14.
6. Reis, D. J., Yang, X. C. & Milner, T. A. (1998) *Neurosci. Lett.* **250,** 185–188.
7. Sastre, M., Regunathan, S. & Reis, D. J. (1997) *J. Neurochem.* **68,** 2421–2426.
8. Sastre, M., Regunathan, S., Galea, E. & Reis, D. J. (1996) *J. Neurochem.* **67,** 1761–1765.
9. Piletz, J. E., Chikkala, D. N. & Ernsberger, P. (1995) *J. Pharmacol. Exp. Ther.* **272,** 581–587.
10. Yang, X. C. & Reis, D. L. (1999) *J. Pharmacol. Exp. Ther.* **288,** 544–549.
11. Auguet, M., Viossat, I., Marin, J. G. & Chabrier, P. E. (1995) *Jpn. J. Pharmacol.* **69,** 285–287.
12. Galea, E., Regunathan, S., Eliopoulos, V., Feinstein, D. L. & Reis, D. J. (1996) *Biochem. J.* **316,** 247–249.
13. Trujillo, K. A. & Akil, H. (1991) *Science* **251,** 85–87.
14. Elliott, K., Kest, B., Man, A., Kao, B. & Inturrisi, C. E. (1995) *Neuropsychopharmacology* **13,** 347–356.
15. Chaplan, S. R., Malmberg, A. B. & Yaksh, T. L. (1997) *J. Pharmacol. Exp. Ther.* **280,** 829–838.
16. Siegan, J. B., Hama, A. T. & Sagen, J. (1997) *Brain Res.* **755,** 331–334.
17. Yoon, Y. W., Sung, B. & Chung, J. M. (1998) *NeuroReport* **9,** 367–372.
18. Wu, W., Han, K., Li, L. & Schinco, F. P. (1994) *Exp. Neurol.* **129,** 335–339.
19. Wu, W. & Li, L. (1993) *Neurosci. Lett.* **153,** 121–124.
20. Faden, A. I., Demediuk, P., Panter, S. S. & Vink, R. (1989) *Science* **244,** 798–800.
21. Faden, A. I. & Simon, R. P. (1988) *Ann. Neurol.* **23,** 623–626.
22. Kehl, L. J., Trempe, T. M. & Hargreaves, K. M. (2000) *Pain* **85,** 333–343.
23. Laughlin, T. M., Vanderah, T., Lashbrook, J. M., Nichols, M. L., Ossipov, M. H., Porecca, F. & Wilcox, G. L. (1997) *Pain* **72,** 253–260.
24. Mogil, J. S., Wilson, S. G., Bon, K., Lee, S. E., Chung, K., Raber, P., Pieper, J. O., Hain, H. S., Belknap, J. K., Hubert, L., *et al.* (1999) *Pain* **80,** 67–82.
25. Fairbanks, C. A., Nguyen, H. O., Grocholski, B. M. & Wilcox, G. L. (2000) *Anesthesiology* **93,** 765–773.
26. Liu, S., Ruenes, G. L. & Yezierski, R. P. (1997) *Brain Res.* **756,** 160–167.
27. Kitto, K. F., Haley, J. E. & Wilcox, G. L. (1992) *Neurosci. Lett.* **148,** 1–5.
28. Tallarida, R. J. & Murray, R. B. (1987) *Manual of Pharmacological Calculations with Computer Programs* (Springer, New York), pp. 26–31.
29. Hylden, J. L. K. & Wilcox, G. L. (1981) *Brain Res.* **217,** 212–215.
30. Aanonsen, L. M. & Wilcox, G. L. (1987) *J. Pharmacol. Exp. Ther.* **243,** 9–19.
31. deMontigny, P., Stobach, J. F., Givens, R. S., Carlson, R. G., Srinivasachar, K., Sternson, L. A. & Higuchi, T. (1987) *Anal. Chem.* **59,** 1096–1101.
32. Feng, Y., Halaris, A. E. & Piletz, J. E. (1997) *J. Chromatograph. Biomed. Appl.* **691,** 277–286.
33. Wessendorf, M. W. & Elde, R. P. (1985) *J. Histochem. Cytochem.* **33,** 984–994.
34. Yezierski, R. P., Santana, M., Park, S. H. & Madsen, P. W. (1993) *J. Neurotrauma* **10,** 445–456.
35. Yezierski, R. P., Liu, S., Ruenes, G. L., Kajander, K. J. & Brewer, K. L. (1998) *Pain* **75,** 141–155.
36. Pinthong, D., Wright, I. K., Hanmer, C., Millns, P., Mason, R., Kendall, D. A. & Wilson, V. G. (1995) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **351,** 10–16.
37. Budai, D., Wilcox, G. L. & Larson, A. A. (1995) *Eur. J. Pharmacol.* **278,** 39–47.
38. Bradley, K. J. & Headley, P. M. (1997) *Eur. J. Pharmacol.* **331,** 133–138.
39. Wang, H., Regunathan, S., Youngson, C., Bramwell, S. & Reis, D. J. (1995) *Neurosci. Lett.* **183,** 17–21.
40. Mullen, R. J., Buck, C. R. & Smith, A. M. (1992) *Development* **116,** 201–211.
41. Horvath, G., Kekesi, G., Dobos, I., Szikszay, M., Klimscha, W. & Benedek, G. (1999) *Eur. J. Pharmacol.* **368,** 197–204.
42. Vanderah, T., Laughlin, T., Lashbrook, J., Nichols, M., Wilcox, G., Ossipov, M., Malan, T. & Porreca, F. (1996) *Pain* **68,** 275–281.
43. Kim, S. H. & Chung, J. M. (1992) *Pain* **50,** 355–363.
44. Hamada, Y., Ikata, T., Katoh, S., Tsuchiya, K., Niwa, M., Tsutsumishita, Y. & Fukuzawa, K. (1996) *Free Radical Biol. Med.* **20,** 1–9.
45. Haghighi, S. S., Johnson, G. C., de Vergel, C. F. & Vergel Rivas, B. J. (1996) *Neurol. Res.* **18,** 509–515.
46. Grossman, S. D., Wolfe, B. B., Yasuda, R. P. & Wrathall, J. R. (1999) *J. Neurosci.* **19,** 5711–5720.
47. Gilad, G. M., Salame, K., Rabey, J. M. & Gilad, V. H. (1996) *Life Sci.* **58,** PL 41–46.
48. Kolesnikov, Y., Jain, S. & Pasternak, G. W. (1996) *Eur. J. Pharmacol.* **296,** 17–22.
49. Nishiyama, T., Yaksh, T. L. & Weber, E. (1998) *Anesthesiology* **89,** 715–722.
50. Ren, K., Hylden, J. L., Williams, G. M., Ruda, M. A. & Dubner, R. (1992) *Pain* **50,** 331–344.
51. Ren, K., Williams, G. M., Hylden, J. L., Ruda, M. A. & Dubner, R. (1992) *Eur. J. Pharmacol.* **219,** 235–243.
52. Porter, R. H. & Greenamyre, J. T. (1995) *J. Neurochem.* **64,** 614–623.
53. Nelson, K. A., Park, K. M., Robinovitz, E., Tsigos, C. & Max, M. B. (1997) *Neurology* **48,** 1212–1218.
54. Fairbanks, C. A. & Wilcox, G. L. (1997) *J. Pharmacol. Exp. Ther.* **282,** 1408–1417.

NEUROBIOLOGY