Gabapentin Inhibits γ-Amino Butyric Acid Release in the Locus Coeruleus but Not in the Spinal Dorsal Horn after Peripheral Nerve Injury in Rats

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ABSTRACT

Background: Gabapentin reduces acute postoperative and chronic neuropathic pain, but its sites and mechanisms of action are unclear. Based on previous electrophysiologic studies, the authors tested whether gabapentin reduced γ-amino butyric acid (GABA) release in the locus coeruleus (LC), a major site of descending inhibition, rather than in the spinal cord.

Methods: Male Sprague-Dawley rats with or without L5–L6 spinal nerve ligation (SNL) were used. Immunostaining for glutamic acid decarboxylase and GABA release in synaptosomes and microdialyzates were examined in the LC and spinal dorsal horn.

Results: Basal GABA release and expression of glutamic acid decarboxylase increased in the LC but decreased in the spinal dorsal horn after SNL. In microdialyzates from the LC, intravenously administered gabapentin decreased extracellular GABA concentration in normal and SNL rats. In synaptosomes prepared from the LC, gabapentin and other α2δ ligands inhibited KCl-evoked GABA release in normal and SNL rats. In microdialyzates from the spinal dorsal horn, intravenous gabapentin did not alter GABA concentrations in normal rats but slightly increased them in SNL rats. In synaptosomes from the spinal dorsal horn, neither gabapentin nor other α2δ ligands affected KCl-evoked GABA release in normal and SNL rats.

Discussion: These results suggest that peripheral nerve injury induces plasticity of GABAergic neurons differently in the LC and spinal dorsal horn that gabapentin reduces presynaptic GABA release in the LC but not in the spinal dorsal horn. The current study supports the idea that gabapentin activates descending noradrenergic inhibition via disinhibition of LC neurons.

GABAPENTIN produces analgesia in a wide range of animal pain models1–5 and in patients with acute postoperative pain and chronic neuropathic pain.6,7 Despite this widespread use, the sites and mechanisms of analgesic action of gabapentin remain uncertain. Gabapentin binds with high affinity to the α2δ subunits of voltage-gated Ca2+ channels,8 and this molecular site is important to gabapentin-induced analgesia.9–11 Although acute inhibition of Ca2+ currents by gabapentin is either very minor or absent,12 gabapentin inhibits trafficking of voltage-gated Ca2+ channels to the cell membrane by binding to α2δ subunits,13–15 which are up-regulated in primary sensory afferents and the spinal cord in rats after peripheral nerve injury.11 Despite demonstration of α2δ subunits as molecular targets of gabapentin action, the identity of and neuronal circuits essential to gabapentin analgesia remain obscure.

Because spinal plasticity and sensitization play pivotal roles in neuropathic pain after peripheral nerve injury, most
laboratory studies have focused on actions of gabapentin in the spinal cord, where it reduces afferent traffic and responses of spinal projection neurons. However, it is unlikely that gabapentin relies exclusively on spinal actions for analgesia. We and others have demonstrated in rodents after paw incision and peripheral nerve injury and in humans with chronic pain that systemically administered gabapentin activates descending bulbospinal noradrenergic inhibition to produce analgesia, blocked by intrathecal α2-adrenoceptor antagonists. In addition, gabapentin, both systemically administered in vivo and locally applied to isolated brainstem slices, activates noradrenergic neurons in the locus coeruleus (LC). These results suggest that gabapentin acts on the local circuits within the brainstem to activate descending noradrenergic inhibition.

γ-Aminobutyric acid (GABA), the prototypical inhibitory neurotransmitter of the central nervous system, plays an important role in sensory processing, and analgesic effects of gabapentin may reflect modulation of GABA release. Gabapentin’s actions on GABA release are a matter of controversy and likely depend on the site in the central nervous system. For example, some studies show an increase in extracellular GABA concentration from gabapentin, whereas others show a direct reduction in GABA release when cortical synaptosomes are exposed to gabapentin. In the LC, gabapentin reduces GABA-mediated inhibitory postsynaptic currents and thereby disinhibits LC neurons, consistent with the activation of LC neurons by gabapentin. The effect of gabapentin on spinal GABA release in normal and neuropathic states has not been fully examined.

The current study tested whether gabapentin and other α2δ ligands inhibit GABA release in the LC and spinal cord, using intact (in vivo microdialysis) and isolated (in vitro synaptosomes) approaches to examine circuit and direct effects, respectively, in normal and neuropathic rats. We further tested whether peripheral nerve injury regulates extracellular GABA concentration and expression of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) in the LC and spinal cord.

Materials and Methods

Animals and Surgeries

Male Sprague-Dawley rats, weighing 180–250 g, from Harlan Industries (Indianapolis, IN), housed under a 12-h light–dark cycle with food and water ad libitum, were used. All experiments were approved by the Animal Care and Use Committee at Wake Forest University (Winston Salem, NC). L5–L6 spinal nerve ligation (SNL) was performed as described previously. Briefly, while the animal was anesthetized with isoflurane, 2%, in oxygen, the right L6 transverse process was removed, and the right L5 and L6 spinal nerves were tightly ligated using 5–0 silk suture. Seven to 10 days after SNL, some animals were anesthetized with isoflurane, 2%, and placed securely in a stereotaxic frame. A sterile steel guide cannula (CXG-8, EICOM, Kyoto, Japan) was implanted into the right LC as described previously. The coordinates for placement of the tip of the guide cannula were 9.8 mm posterior and 1.4 mm lateral to the bregma and 6.5 mm ventral from the surface of the dura mater, according to the rat brain atlas. Animals were allowed to recover for at least 5 days before the study.

Timing and Rationale of Experimental Manipulations

The primary purpose of the current study was to probe the effect and mechanisms of the action of gabapentin on GABA release in the LC. We therefore examined more concentrations of gabapentin as well as other α2δ ligands (L-isoleucine, pregabalin) in the LC than in the spinal cord.

Microdialysis for GABA Measurement

On the day of experiment, anesthesia was induced with isoflurane, 2%, and then maintained with isoflurane, 1.25–1.5%, during the study. A heating blanket was used to maintain rectal temperature 36.5 ± 0.5°C, and the right jugular vein was cannulated for saline infusion (1.2 ml·kg⁻¹·h⁻¹) and gabapentin (Toronto Research Chemicals, North York, Toronto, Canada) or pregabalin (gift from Pfizer, New York, NY) injection. For microdialysis in the spinal dorsal horn, the L3–L6 level of spinal cord was exposed by the T13-L1 laminectomy. A microdialysis probe (OD = 0.22 mm, ID = 0.20 mm, length = 1 mm, CX-I-8-01, EICOM) was inserted from just lateral to the right dorsal root 1 h before the study and perfused with Ringer’s solution (1.0 μl/min). Fractions were collected every 30 min for 2.5 h starting 1 h before drug treatment, and samples were kept at −80°C until assayed for GABA. For microdialysis in the LC, a probe was inserted through the guide cannula, and microdialysis was performed using the same protocol as described for the spinal cord. After the experiment, the probe was perfused for 10 min with methylene blue to stain the areas surrounding the active dialysate window in the brainstem. Rats were then killed by decapitation, and the brainstem was removed and postfixed with 8% buffered paraformaldehyde overnight. After sectioning, the placement of the probe was verified microscopically. Data were used only from animals with staining in the LC. GABA content in the microdialysates was measured by a high-pressure liquid chromatography system with electrochemical detection (HTEC-500, EICOM). GABA in samples were derivatized with 2-mercaptoethanol and o-phthalaldehyde (4 mM) in 0.1 M carbonate buffer (pH = 9.5). The o-phthalaldehyde derivatives were then separated on the column (3.0 mm × 150 mm, SC-5ODS, EICOM) at 30°C, using a mobile phase consisting of 50 mM phosphate buffer (pH = 2.8) and methanol (1:1 vol/vol) containing 5 mg/ml EDTA-2Na at a flow rate of 0.5 ml/min. The limit of detection of GABA assay in the current study was 1.5 pg per injection.
GABA Release from Synaptosomes

Under deep anesthesia with isoflurane, 5% animals were killed by decapitation, and the brainstem and spinal lumbar enlargement were quickly removed and placed in ice-cold sucrose (0.32 M)-HEPES (10 mM) buffer, pH = 7.4. Brainstem slices (2-mm thickness) containing the LC were obtained using a precision brain slicer (RBM-400OC, ASI Instruments, Warren, MI), and the region of the LC was carefully dissected under the surgical microscope and homogenized in ice-cold sucrose-HEPES buffer. Each brainstem synaptosome preparation contained the bilateral LC and adjacent tissue from four normal or four SNL rats. Because SNL surgery or gabapentin equally activates the LC bilaterally,18 we used bilateral LCs for the current study. The lumbar spinal dorsal horn tissue ipsilateral to SNL surgery was obtained from four SNL rats for each synaptosome preparation, as described previously.26 Control spinal cord tissue was obtained from four normal or four SNL rats. Because SNL surgery or gabapentin equally activates the LC bilaterally,18 we used bilateral LCs for the current study. The lumbar spinal dorsal horn tissue ipsilateral to SNL surgery was obtained from four SNL rats for each synaptosome preparation, as described previously.26 Control spinal cord tissue was obtained bilaterally from two normal rats. The initial homogenate was centrifuged at 1,000 g for 3 min, and the resulting supernatant was centrifuged again at 10,000 g for 13 min. The supernatant was discarded, and the pellet was resuspended in Krebs buffer (in mM: NaCl 124, KCl 3, MgSO4 2, CaCl2 2, NaH2PO4 1.25, NaHCO3 25, and glucose 10, saturated with 95% O2-5% CO2, pH = 7.4). [3H]-GABA release from synaptosomes was measured as described previously,22 with minor modifications. After incubation with [3H]-GABA and unlabeled GABA (final concentration of 0.25 μCi/ml and 1 μM, respectively) for 20 min at 37°C, the synaptosome-containing solution was centrifuged at 10,000 g for 5 min, and the pellet was resuspended in Krebs buffer. Each synaptosome preparation was divided into six equal aliquots and transferred to Whatman filters in temperature-controlled perfusion chambers (SF-12, Brandel, Gaithersburg, MD). Synaptosomes were perfused with Krebs buffer (0.8 ml/min) for 25 min to remove radioactivity, and then fractions were collected every 5 min for 20 min. After a 10-min baseline collection, synaptosomes were perfused with a test drug, either gabapentin, pregabalin, t-isoleucine (Sigma Chemical, St. Louis, MO), or D-isoleucine (Sigma Chemical), for 2 min and then stimulated with cold phosphate-buffered saline (pH 7.4) containing 1% sodium nitrite followed by 4% paraformaldehyde in phosphate-buffered saline. The spinal cord and brainstem from normal and SNL rats were postfixed with paraformaldehyde, 4%, for 3 h, cryoprotected with sucrose, 30%, for 48 h at 4°C, and sectioned on a cryostat at a 16-μm thickness. For staining in the spinal cord, after pretreatments with hydrogen peroxide, 0.3%, ethanol, 50%, and normal donkey serum, 1.5% (Jackson ImmunoResearch Laboratories, West Grove, PA), the sections were incubated with a mouse anti-GAD67 antibody (1:1000; Millipore, Billerica, MA) in normal donkey serum, 1.5%, for 24 h at 4°C. The sections were then incubated with biotinylated donkey antimagE IgG (1:200; Jackson ImmunoResearch Laboratories), processed using an Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA), and developed by the standard glucose oxidase-nickel method. For staining in the LC, the sections were incubated with a rabbit antityrosine hydroxylase antibody (1:500; Pel-Freez Biologicals, Rogers, AR) and a mouse anti-GAD67 antibody (1:500) followed by a Cy3-conjugated donkey antirabbit IgG (1:600; Jackson ImmunoResearch Laboratories), a biotinylated donkey antimagE IgG (1:200), and Cy2-conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories).

For quantification of GAD67 immunoreactivity, four to five brainstem and spinal cord sections were selected randomly from each rat. Images of dorsal horns and LCs from normal or SNL rats were captured using a digital charge-coupled device camera. The area of the LC was identified by a cluster of tyrosine hydroxylase immunoreactive cells. By using an image analysis software (SigmaScan; Systat Software, San Jose, CA), pixels of GAD67-immunoreactive objects within the area of the dorsal horn containing lamina I–IV or the LC were quantified based on a constant threshold of optical density. Data are expressed as a percentage of GAD67-immunoreactive pixels in total pixels of the quantified area. The person performing image analysis was blinded to treatment.

Statistical Analyses

Unless otherwise noted, data are presented as mean ± SE. Differences among groups for microdialysis experiments were determined using two-way repeated-measures analysis of variance (ANOVA), and other data were analyzed using one-way ANOVA. P < 0.05 was considered significant.

Results

Basal GABA Release and GAD Immunoreactivity in the LC and Spinal Dorsal Horn

In microdialysates from the LC, basal GABA concentrations were significantly greater from SNL rats than from normal ones (fig. 1). The opposite effect of SNL was observed in the spinal cord, where basal GABA concentrations in microdialysates were significantly less from SNL rats than from normal ones.
Figure 2 A through F depict the LC, identified by a cluster of tyrosine hydroxylase-immunoreactive cells, and GAD67 immunoreactivity within and adjacent to the LC in normal and SNL rats. GAD67 immunoreactivity within the LC was found mainly in axons, but a few GAD67-positive cells were found in both normal and SNL rats. Quantitatively, GAD67 immunoreactivity in the LC was significantly greater in tissue from SNL rats than from those of normal rats (2.94 ± 0.51% pixels above threshold vs. 1.03 ± 0.20%, respectively; n = 5 per group; P < 0.01). In the lumbar spinal dorsal horn, GAD67 immunoreactivity was found in axons and cells in both normal and SNL rats (fig. 2, G and H). As with basal GABA concentrations, the effect of SNL on GAD67 immunoreactivity in the spinal dorsal horn was opposite that observed in LC, with less immunoreactivity in SNL rats than in normal rats (5.21 ± 0.94% pixels above threshold vs. 8.04 ± 0.49%, respectively; n = 6 per group; P < 0.01).

Microdialysis Studies

In normal animals, intravenous injection of gabapentin (10 and 50 mg/kg) dose-dependently decreased GABA concentrations in microdialysates from the LC compared with saline (fig. 3A). Similarly, another gabapentin-related α2δ ligand pregabalin (30 mg/kg) significantly decreased GABA concentrations in microdialysates from the LC compared with saline in normal rats (fig. 3A). The peak effect of both drugs was observed at 30 min after injection. In SNL rats, only a high dose of gabapentin (50 mg/kg) significantly decreased GABA concentrations in microdialysates from the LC compared with saline (fig. 3B). Although the percentage decreases from the baseline by gabapentin (50 mg/kg) did not differ in normal and SNL rats (P = 0.127), the quantitative decrease in GABA concentration in the dialysates collected from 0 to 30 min after gabapentin injection compared with the baseline was significantly greater in SNL rats (0.18 ± 0.03 pmol/30 μL, n = 12) than in normal ones (0.10 ± 0.02 pmol/30 μL, n = 13, P < 0.05).

GABA Regulation by Gabapentin

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In the spinal dorsal horn, gabapentin (50 mg/kg) did not affect GABA concentrations in microdialysates compared with saline in normal rats (fig. 3C). In SNL rats, gabapentin (50 mg/kg) slightly but significantly increased GABA concentrations in microdialysates from the spinal dorsal horn compared with saline (fig. 3D).

Synaptosome Studies
In LC-containing synaptosomes, gabapentin reduced 25 mM KCl-evoked [3H]-GABA release in a concentration-dependent manner in both normal and SNL rats (fig. 4). Pregabalin (100 μM) or an endogenous α2δ subunit ligand L-isoleucine (10 μM) also significantly reduced KCl-evoked [3H]-GABA release from the LC synaptosomes in normal and SNL rats. In contrast, the inactive enantiomer of L-isoleucine, D-isoleucine (10 μM), did not affect KCl-evoked [3H]-GABA release from the LC synaptosomes in normal rats (fig. 4).

As shown in figure 5, neither gabapentin nor L-isoleucine affected KCl-evoked [3H]-GABA release from the lumbar spinal dorsal horn synaptosomes in either normal or SNL rats.

Discussion
Despite its name and structural similarity to GABA, gabapentin is thought to act primarily on subunits of Ca2+ channels without direct effects on GABA or its receptors. In addition, a focus on the spinal cord as a key site of sensory processing and plasticity after nerve injury has led to an assumption, supported by some electrophysiologic experiments, that gabapentin has a primary site of action in the spinal cord. Recently, we and others have challenged this dogma, demonstrating that noradrenergic neurons in the LC are excited by gabapentin, coincident with noradrenaline release in the spinal cord and behavioral antihypersensitivity,2,5,18 and a GABA-mediated response of gabapentin in the LC has been suggested in electrophysiologic experiments.23 The current study adds to these single-cell studies by demonstrating that gabapentin and other α2δ ligands inhibit GABA release in vivo, and that this effect is in part attributable to direct presynaptic inhibition, as demonstrated in vitro using synaptosomes. We report that the substrate for this effect of gabapentin is increased after the stress of peripheral nerve injury, confirming previous results with other non-painful stressors.27,28 The lack of α2δ ligand action on GABA release in the spinal dorsal horn suggests that supraspinal, rather than spinal, sites of action are important to gabapentin analgesia, at least as it relates to GABA-mediated mechanisms.

Basal GABA release and GAD immunoreactivity in the LC increased after nerve injury in the current study, consistent with previous observations of increased GAD expression...
in the LC under other forms of chronic physical and psychologic stress conditions in rats.27,28 Thus, one might expect that increased GABA tone in the LC after peripheral nerve injury would result in reduced activity of noradrenergic neurons and of descending inhibition. However, we and others have demonstrated just the opposite: peripheral nerve injury in rats increases noradrenergic cell activation in the LC, coincident with increased basal release of noradrenaline in the spinal dorsal horn.18 The reasons for this discrepancy are unclear. Other, nonpainful chronic stress states increase glutamate content in brain regions29,30 and down-regulate the expression of GABA-A receptors.31,32 Should similar effects occur in the LC, it is possible that nerve injury, despite increased basal GABA release, results in a net excitation by increasing glutamate release and reducing postsynaptic GABA receptor expression. We are testing this hypothesis.

Although the results are controversial, several studies have observed a reduction of GABA release in the spinal cord after nerve injury, presumably allowing hyperexcitation of spinal neurons and resulting in behavioral hypersensitivity.33 In the current study, basal GABA release and GAD immunoreactivity decreased in the spinal dorsal horn after nerve injury, consistent with previous observations in rats after peripheral nerve or spinal cord injury.34–36

The effect of gabapentin on GABA release varies widely and in different directions, depending on the brain region.22,37–39 Here and in other studies, gabapentin and other α2δ ligands decrease presynaptic GABA release in the LC,25 consistent with gabapentin-induced activation of noradrenergic neurons in the LC and noradrenaline release in the spinal dorsal horn.18 These results suggest that gabapentin reduces presynaptic GABA release to disinhibit LC neurons via α2δ interactions in normal and SNL animals. Interestingly, gabapentin does not affect the mechanical withdrawal threshold in normal rats.4,18 The reasons an increased withdrawal threshold by gabapentin is seen only after SNL, whereas similar effects on GABA release in the LC by gabapentin are observed in normal and SNL rats, are unknown. Perhaps the hypersensitive state of the spinal cord after nerve injury may be required to unmask a significant effect of descending noradrenergic tone. It is also conceivable that increased basal release of noradrenaline and the α2-adrenoceptor–mediated acetylcholine release that develop after injury3,18,26,40 are responsible. It should be noted that microdialysis experiments were performed in the presence of isoflurane, which could have reduced the dynamic range of GABA release observed.

In contrast to the LC, both gabapentin and L-isoleucine failed to affect KCl-evoked GABA release from spinal dorsal horn synaptosomes in normal and SNL animals, suggesting that expression or function of α2δ subunits on GABAergic terminals is either very low or absent in the spinal dorsal horn. On the other hand, systemically administered gabapentin induced a small but significant increase in spinal GABA release in SNL rats but not in normal rats. Because systemic administered gabapentin induces more spinal noradrenaline release in SNL rats than in normal ones18 and because noradrenaline activates some GABAergic neurons in the spinal dorsal horn via α1-adrenoceptors,41,42 gabapentin–induced spinal noradrenaline release may contribute to this small increase of spinal GABA release in the presence of nerve injury. The role of this mildly enhanced spinal GABA release for gabapentin analgesia is unclear, because GABA receptor type A and GABA receptor type B receptor antagonists did not affect analgesia from intrathecally injected gabapentin in SNL rats.43

In summary, peripheral nerve injury induces different GABAergic neuronal plasticity in the LC and spinal dorsal horn, increasing expression and release of GABA in the LC but decreasing it in the spinal cord. Gabapentin and other α2δ ligands inhibit presynaptic GABA release in the LC but not in the spinal dorsal horn. These results suggest that gabapentin reduces the influence of GABA in the LC as one mechanism by which it activates descending inhibition from this structure.

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