

Role of Adenosine Receptors in Spinal G-Protein Activation after Peripheral Nerve Injury

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Background: Spinally injected adenosine induces antinociception in animal models of neuropathic but not acute pain. The reasons for this discrepancy remain unclear. Adenosine receptors are coupled to G proteins, and increased efficiency of adenosine-induced G-protein activity in neuropathic pain could contribute to the antinociceptive effect of adenosine. In this study the authors used [³⁵S]guanosine-5'-O-(3-thiotriphosphate) ([³⁵S]GTPγS) autoradiography in rat spinal cord sections to test this possibility.

Methods: The spinal cords of normal animals and those that underwent left L5 and L6 spinal nerve ligation (SNL) were removed and immediately frozen. Horizontal spinal cord sections were cut and mounted on chrom-alum gelatin-subbed slides. Sections were incubated with guanosine diphosphate, [³⁵S]GTPγS, the adenosine A1 agonist R-N6-phenylisopropyladenosine, and various other drugs, apposed to films, and analyzed.

Results: Baseline and R-N6-phenylisopropyladenosine-stimulated [³⁵S]GTPγS binding was predominantly localized to the superficial dorsal horns of both normal and SNL animals. This binding was significantly increased in SNL compared with normal animals. In contrast, no difference in R-N6-phenylisopropyladenosine-stimulated [³⁵S]GTPγS binding was observed between SNL and normal animals. Blockade of adenosine A1 receptors by 1,3-dipropyl-8-cyclopentylxanthine, or adenosine destruction by added adenosine deaminase, reduced the increased basal activity in SNL to baseline levels of normal dorsal horns, whereas atropine and naloxone had no effect.

Conclusion: This study shows an increased basal G-protein activity in lumbar spinal cords during conditions of SNL. The data suggest that increased adenosine release during conditions of SNL results in an increased basal activity of G proteins in the spinal cord during neuropathic pain.

RECENT clinical and preclinical studies strongly indicate the value of spinal application of the endogenous nucleoside adenosine in the treatment of chronic pain. Spinally administered adenosine appears to be particularly effective in central sensitivity evoked states in animals modeling neuropathic pain, which are characterized by the appearance of allodynia and hyperalgesia. During such conditions, adenosine shows a pronounced and long-lasting (> 24 h) antihypersensitivity effect.^{1,2}

Adenosine acts by binding to four types of specific adenosine receptors, including A1, A2A, A2B, and A3. Although the A1 receptor type is predominantly inhibitory, hence contributing to the spinal antinociceptive properties of adenosine, A2 receptors are considered excitatory, and the role of A3 receptors in pain processing in the spinal cord remains unclear.^{3,4} Furthermore, there is an increasing body of evidence suggesting a direct interaction of A1 and A2 receptors, with each type inhibiting the action of the other.^{5,6} All adenosine receptors belong to the superfamily of G-protein coupled receptors.⁷ G proteins are intracellular membrane-bound heterotrimeric guanosine triphosphate (GTP)-binding proteins that are activated when receptor agonists increase affinities of G-protein α subunits for GTP. After activation, G proteins regulate the activities of effector enzymes such as adenylyl cyclase and ion channels. They therefore mediate intracellular signal transduction systems induced by the binding of an agonist such as adenosine to its receptor. A1 adenosine receptors couple primarily to the Gi/o class of G proteins, which mediate intracellular effects by decreasing cyclic adenosine monophosphate, increasing K⁺ conductance and decreasing Ca²⁺ conductance.⁷⁻⁹

Receptor activation of G proteins can be measured by agonist-stimulated binding of the hydrolysis-resistant GTP analog, [³⁵S]guanosine-5'-O-(3-thiotriphosphate) ([³⁵S]GTPγS), to α subunits of G proteins in membranes.^{10,11} The development of [³⁵S]GTPγS autoradiography in brain sections extended this technique to provide a neuroanatomic localization of receptor-activated G proteins in different brain regions.¹² Recently, [³⁵S]GTPγS autoradiography was extended to spinal cord, where stimulation of [³⁵S]GTPγS binding by various receptor agonists (including adenosine A1) was localized to superficial laminae of dorsal horns.¹³

The effect of adenosine on hypersensitivity states is in stark contrast to its lack of effect on acute nociceptive stimuli.¹⁴ The reason for these divergent effects on different pain states remains unclear. One explanation could be that the pathophysiology of neuropathic pain leads to alterations of G-protein activities and consequently changes in intracellular signal transduction cascades. In its simplest form, one might expect increased spinal G-protein activation by adenosine agonists after spinal nerve ligation as the explanation for the increased efficacy and potency of adenosine. Therefore, this study was conducted to determine the activity of G proteins and the role of adenosine receptor activation, using [³⁵S]GTPγS binding autoradiog-

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raphy, in lumbar spinal cords of rats with peripheral nerve injury-induced hypersensitivity.

Materials and Methods

After obtaining approval from the Animal Care and Use Committee of Wake Forest University (Winston-Salem, NC), male rats (Harlan Sprague-Dawley), weighing 200–250 g at the time of surgery, were studied. Animals were housed at 22°C with a 12–12 h light–dark cycle, with free access to food and water.

Spinal Nerve Ligation

Spinal nerve ligation (SNL) was performed as previously described.¹⁵ In brief, during halothane anesthesia, the left L5 and L6 spinal nerves were isolated adjacent to the vertebral column in both the SNL and sham groups. In the SNL group, L5 and L6 spinal nerves were further tightly ligated with 6–0 silk sutures distal to the dorsal root ganglion. After a recovery period of 13 days, left paw tactile hypersensitivity was confirmed by measuring hind-paw withdrawal threshold to von Frey filaments, using an up–down method previously described.¹⁶ Only rats with a withdrawal threshold less than 4 g and without signs of neurologic impairment were included in the study.

[³⁵S]Guanosine-5'-O-(3-thiotriphosphate)

Autoradiography

[³⁵S]guanosine-5'-O-(3-thiotriphosphate) autoradiography was performed as previously described.¹² Briefly, six normal and six SNL animals were killed by decapitation during halothane anesthesia, and spinal cords were removed and immediately immersed in isopentane at

–30°C until frozen, then stored at –80°C until use. Horizontal spinal cord sections (20 μm) were cut on a cryostat, thaw-mounted onto chrom-alum gelatin-subbed slides, desiccated under a vacuum at 5°C overnight, and stored at –80°C until use. Sections were preincubated in TME buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4) for 10 min at 25°C, then in TME with 2 mM guanosine diphosphate at 25°C for 15 min. Sections were then incubated at 25°C for 90 min with 2 mM guanosine diphosphate and 0.04 nM [³⁵S]GTPγS (New England Nuclear Corp., Boston, MA) in TME buffer in combination with the various agonists and antagonists. Other sections were incubated in the absence of endogenous ligands to determine basal G-protein activity as reflected by [³⁵S]GTPγS binding. Adenosine A1 receptor-stimulated [³⁵S]GTPγS binding was determined with 1 μM of the A1 adenosine receptor-specific agonist R-N6-phenylisopropyladenosine (R-PIA). Effects of receptor antagonists on basal [³⁵S]GTPγS binding included the A1 antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 1–100 nM), the muscarinic cholinergic antagonist atropine (100 nM), and the μ-opioid antagonist naloxone (100 nM). To determine the role of A2 adenosine receptors on basal [³⁵S]GTPγS binding in the absence of exogenous agonist, sections were incubated with the A2A receptor-selective agonist 2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS21680). To further examine the role of endogenous adenosine in [³⁵S]GTPγS binding in the absence of agonist, endogenous adenosine in some spinal cord sections was metabolized by addition of 100 mU adenosine deaminase in the preincubation and incubation media.¹⁷ After incubation, sections were rinsed twice in 50 mM Tris-HCl buffer (pH 7.4) for 2 min at 5°C

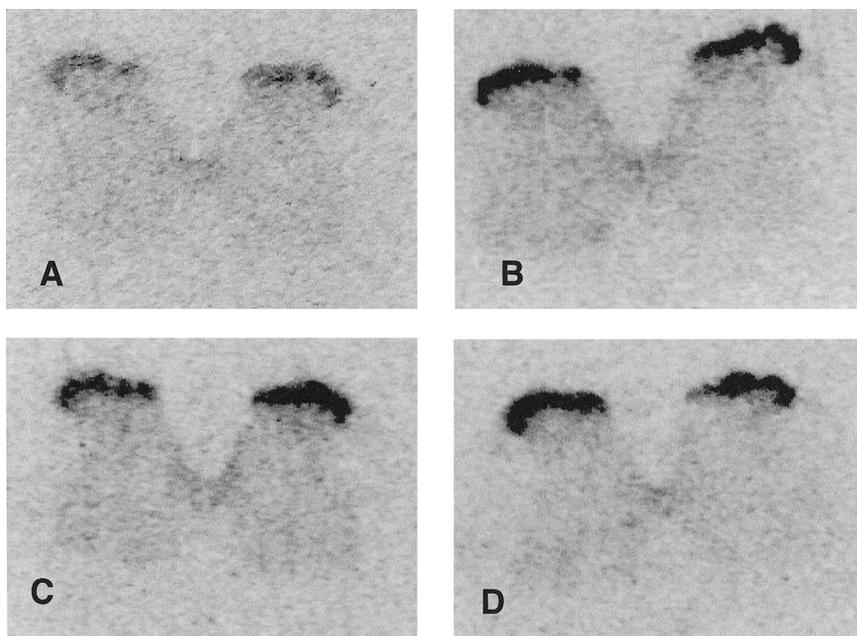


Fig. 1. Autoradiograms of baseline [³⁵S]GTPγS binding in lumbar spinal cords. Basal binding was determined in normal animals (A) and in those that underwent spinal nerve ligation (B). Adenosine A1-stimulated binding was determined with 1 μM R-N6-phenylisopropyladenosine in normal animals (C) and in those that underwent spinal nerve ligation (D).

and finally once in deionized distilled H₂O for 1 min at 5°C. Slides were dried overnight and then, along with [¹⁴C] standards, apposed to x-ray film for approximately 48 h. Films were digitized and analyzed using NIH IMAGE 1.62C for Macintosh computers (Scion Corp., Frederick, MD). Measurements of quantitative densitometry were referenced to [¹⁴C] standards and converted to nanocuries per gram [³⁵S], and data were presented as mean ± SEM of percent increase from normal baseline. Statistical significance was determined by one-way analysis of variance and Student-Newman-Keuls *post hoc* test. *P* < 0.05 was considered significant.

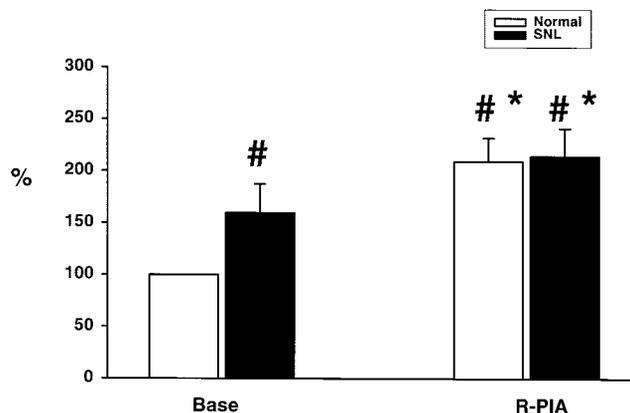
Results

Data obtained from spinal cord sections of sham-operated animals showed no change in basal [³⁵S]GTPγS binding compared with normal animals, with 6% difference ipsilateral and 1% contralateral to nerve injury. R-PIA stimulation increased [³⁵S]GTPγS binding to the same extent in sections from normal and sham-operated animals, with no significant difference between the groups. Compared with normal baseline, the increase was significant in both groups, with 121% ipsilateral and 107% contralateral in normal as well as 93% ipsilateral and 91% contralateral in sham-operated animals. Because these experiments showed no difference between sham-operated and normal animals, we used naive animals as controls in the current study. These findings are in agreement with the SNL literature, which show no difference between normal and sham-operated animals in behavioral, electrophysiological, anatomic, or biochemical endpoints.^{15,18-20,21}

Figure 1 shows autoradiograms of basal and R-PIA-stimulated [³⁵S]GTPγS binding sections from control and SNL animals. A number of G-protein-coupled receptors, including adenosine A1, μ-opioid, and muscarinic cholinergic receptors, are predominantly localized to the superficial laminae of dorsal horns.²²⁻²⁴ This localization contributes to a relatively high level of receptor-activated and basal [³⁵S]GTPγS binding in the same area, which is seen in the autoradiograms of the current study. In both groups, location of basal and R-PIA-stimulated [³⁵S]GTPγS binding was the same, with high levels observed in superficial dorsal horns. The spinal cord sections from SNL animals (fig. 1B) demonstrated an increase in basal [³⁵S]GTPγS binding in the dorsal horns compared with those obtained from normal animals (fig. 1A). R-PIA stimulation resulted in [³⁵S]GTPγS binding to approximately the same degree in the dorsal horns of SNL and normal animals (figs. 1C and D).

To quantify this increase in basal binding, [³⁵S]GTPγS autoradiographic images from control and SNL animals were analyzed by densitometry, comparing data both ipsilateral and contralateral to the spinal nerve ligation.

ipsilateral



contralateral

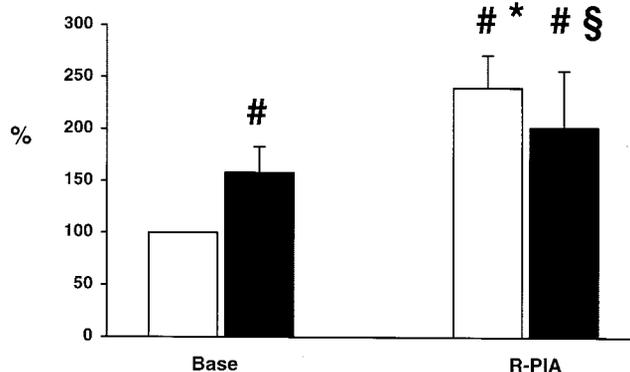


Fig. 2. Results of quantitative densitometry of [³⁵S]GTPγS binding shown as mean values of percent change from normal spinal cord baseline ± SEM. (Top) Ipsilateral baseline [³⁵S]GTPγS binding in normal (0.68 ± 0.07 nCi/g) and spinal nerve-ligated (SNL; 1.01 ± 0.11 nCi/g) dorsal horns and binding after stimulation with 1 μM R-N6-phenylisopropyladenosine (R-PIA) of normal and SNL animals. (Bottom) Contralateral baseline [³⁵S]GTPγS binding in normal (0.63 ± 0.08 nCi/g) and SNL (0.91 ± 0.07 nCi/g) dorsal horns and binding after stimulation with 1 μM R-PIA of normal and SNL animals. #*P* < 0.05 compared with normal baseline; **P* < 0.05 compared with SNL baseline; §*P* < 0.05 between R-PIA-stimulated normal and SNL animals.

Results of basal [³⁵S]GTPγS binding showed significantly increased activity in SNL compared with normal animals. This increase was 60% (*P* < 0.05) ipsilateral and 58% (*P* < 0.05) contralateral to nerve injury compared with uninjured animals.

R-N6-phenylisopropyladenosine stimulated [³⁵S]GTPγS binding significantly in both normal and SNL animals (fig. 2). Maximum stimulation from R-PIA did not differ between normal and SNL animals.

In brain sections, areas with high concentrations of adenosine A1 receptors show relatively high basal [³⁵S]GTPγS binding, produced by activation from endogenous adenosine.¹⁷ Therefore, it is possible that the increased basal [³⁵S]GTPγS binding in spinal cord sec-

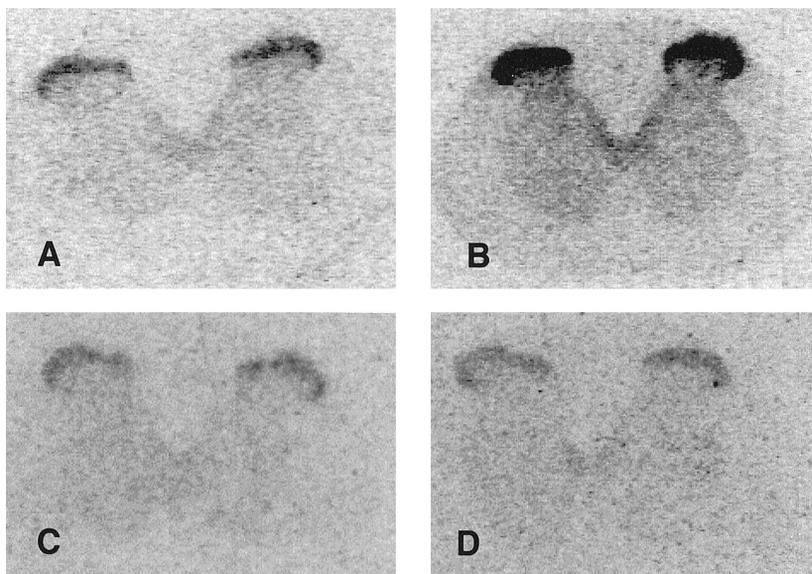


Fig. 3. Lumbar spinal cord autoradiograms of basal [^{35}S]GTP γ S binding (A) in normal animals (B) and in those that underwent spinal nerve ligation, and binding after incubation with 100 nM 1,3-dipropyl-8-cyclopentylxanthine in normal animals (C) and in those that underwent spinal nerve ligation (D).

tions of SNL animals was caused by an increased release of endogenous adenosine. To test this possibility, spinal cord sections of normal and SNL animals were incubated with the specific adenosine A1 antagonist DPCPX. Figure 3 shows the autoradiograms of basal [^{35}S]GTP γ S binding. These images show that increased SNL basal binding (fig. 3B) is eliminated in sections incubated with 100 nM DPCPX (fig. 3D). Quantitative densitometry of basal [^{35}S]GTP γ S autoradiograms was performed after incubating with several concentrations of DPCPX in sections of normal and SNL animals (fig. 4). The basal [^{35}S]GTP γ S binding of SNL animals was significantly increased compared with normal baseline (ipsilateral 128% and contralateral 83%; $P < 0.05$). Although ineffective in altering the basal [^{35}S]GTP γ S binding in spinal cord sections of normal animals, addition of DPCPX reduced the increase in basal binding in SNL animals in a concentration-dependent manner on both sides of the spinal cord of SNL animals, completely eliminating the difference between control and SNL animals. This effect of DPCPX was mimicked by treatment of sections with the adenosine metabolizing enzyme adenosine deaminase, thus confirming the role of endogenous adenosine in contributing to the increase in basal [^{35}S]GTP γ S binding in SNL animals.

Finally, we determined whether the increased basal G-protein activity in SNL compared with normal dorsal horns was exclusively caused by an A1 receptor-mediated stimulation of G proteins. For these studies, sections were incubated with either DPCPX, the muscarinic cholinergic antagonist atropine, or the μ -opioid antagonist naloxone. To determine whether A2A receptor activation diminished the elevated basal [^{35}S]GTP γ S binding in SNL animals because of crosstalk between A1 and A2A receptors, some sections were incubated with the A2A receptor agonist CGS21680. These data showed an increase of 185% in basal [^{35}S]GTP γ S binding in SNL

compared with control dorsal horn (fig. 5). Although addition of DPCPX eliminated this increase and CGS21680 reduced it to 31%, neither atropine nor naloxone had any significant effect, with G-protein activity still at 58% (atropine) and 78% (naloxone) greater than normal control values. Membrane binding data indicated that the effect of CGS21680 was more likely caused by a nonspecific inhibitory effect on A1 receptors rather than a reflection of an A1-A2A receptor crosstalk (data not shown).

Discussion

This study clearly demonstrates for the first time a bilaterally increased activity of G proteins in spinal cord after nerve injury, an unexpected finding. The cause of this increase in this nerve injury model of chronic allodynia, increased adenosine receptor activation, is also surprising.

Increase in Spinal G-protein Activity after Nerve Injury

Because of their large preponderance in nervous tissue, the method of [^{35}S]GTP γ S binding predominantly detects G proteins belonging to the Gi/o subfamily.¹³ An increased activity of these Gi/o proteins, which are associated with inhibitory mechanisms, could reflect an elevated inhibitory tone in response to the ongoing excitatory activity in the spinal cord after nerve injury, as recently suggested from studies of spinal γ -aminobutyric acid-mediated neurons.²⁵

Increased spinal G-protein activity in animals after SNL could reflect up-regulation of G-protein α subunits or an increase in receptor-G-protein coupling. In each case, one would expect that addition of an exogenous agonist would enhance [^{35}S]GTP γ S binding because it would

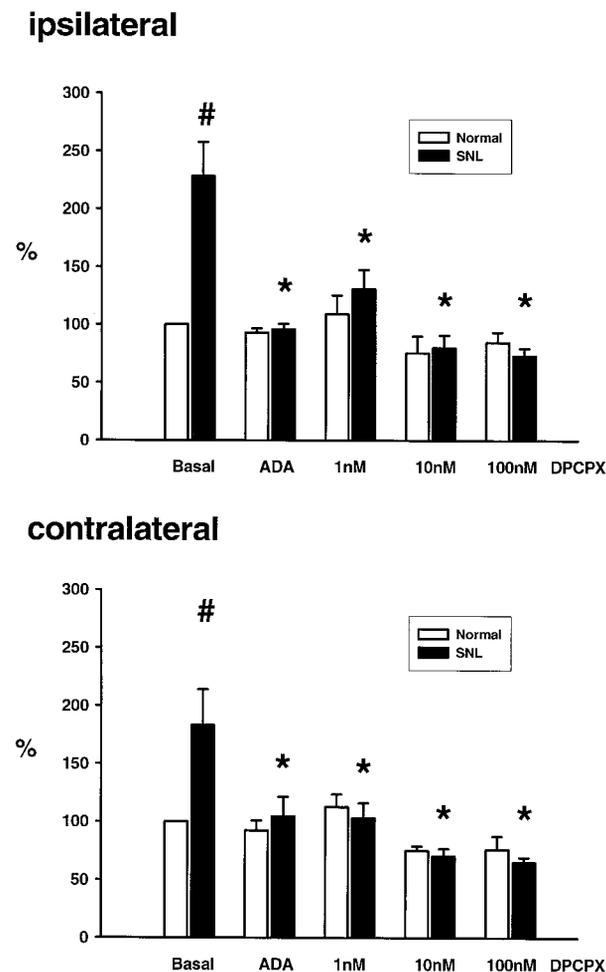


Fig. 4. Results of quantitative densitometry of [³⁵S]GTPγS binding shown as mean values of percent change from normal spinal cord baseline ± SEM. (Top) Ipsilateral baseline [³⁵S]GTPγS binding in normal (1.22 ± 0.13 nCi/g) and spinal cord-ligated (SNL; 2.74 ± 0.42 nCi/g) dorsal horns and binding after incubation with 1–100 nM 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 100 mU adenosine deaminase (ADA) in normal and SNL animals. (Bottom) Contralateral baseline [³⁵S]GTPγS binding in normal (1.29 ± 0.08 nCi/g) and SNL (2.27 ± 0.36 nCi/g) dorsal horns and binding after incubation with 1–100 nM DPCPX and 100 mU ADA in normal and SNL animals. #P < 0.05 compared with normal baseline; *P < 0.05 compared with SNL baseline.

either interact with more G proteins if their number were increased or in a more efficient manner if coupling were increased. However, the A1 agonist R-PIA, at a maximally effective concentration of 1 μM, stimulated [³⁵S]GTPγS binding to the same degree in normal and SNL dorsal horns. Consequently, these mechanisms seem unlikely to explain the elevated activity in dorsal horns after nerve injury.

Another unanticipated result of the current study was that the increase in G-protein activity was not restricted to the side ipsilateral to the nerve injury but also extended to the contralateral side. However, an expansion of pathophysiologic changes contralateral to spinal nerve ligation has been observed in behavioral studies in

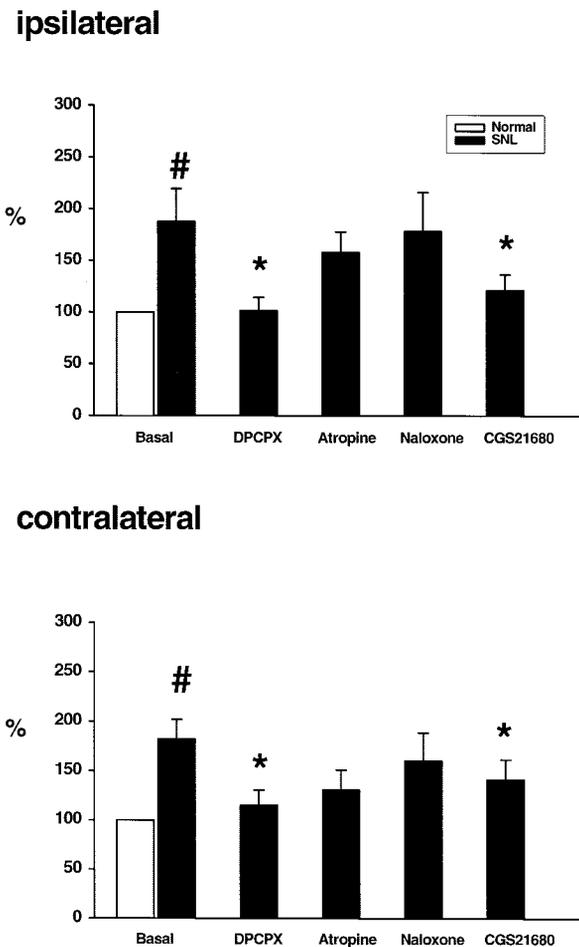


Fig. 5. Results of quantitative densitometry of [³⁵S]GTPγS binding shown as mean value of percent change from normal spinal cord baseline ± SEM. (Top) Ipsilateral baseline [³⁵S]GTPγS binding in normal (0.77 ± 0.09 nCi/g) and spinal cord-ligated (SNL; 1.35 ± 0.12 nCi/g) dorsal horns and the effects of 100 nM 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 100 nM atropine, 100 nM naloxone, and 30 nM CGS21680 on [³⁵S]GTPγS binding in normal and SNL animals. (Bottom) Contralateral baseline [³⁵S]GTPγS binding in normal (0.74 ± 0.08 nCi/g) and SNL (1.28 ± 0.09 nCi/g) dorsal horns and the effects of 100 nM DPCPX, 100 nM atropine, 100 nM naloxone, and 30 nM CGS21680 on [³⁵S]GTPγS binding in normal and SNL animals. #P < 0.05 compared with normal baseline; *P < 0.05 compared with SNL baseline.

rats, where SNL induced a bilateral development of hypersensitivity.²⁶ It is believed that hypersensitivity is induced by alterations in the central nervous system, such as sprouting of Aβ fibers, change in neurotransmitter release, or signal transduction cascades.^{27,28} These changes often extend beyond the original involved spinal cord areas and are referred as “plasticity.”²⁹ It might be possible that increased G-protein activity after SNL reflects these plastic changes.

Adenosine as the Cause of Increased Spinal G-protein Activity

The current study suggests that adenosine, rather than other Gi/o coupled receptor systems, including musca-

rinic or cholinergic or opioid, is responsible for increased spinal G-protein activity after SNL, because the A1 receptor antagonist DPCPX, but not atropine or naloxone, returned this activity to normal. Moreover, the effective concentrations of DPCPX were consistent with specific blockade of adenosine A1 receptor-coupled G proteins and could be mimicked by metabolism of endogenous adenosine with adenosine deaminase.¹⁷ These data strongly suggest that the observed phenomenon of elevated G-protein activities in dorsal horns of SNL animals is almost exclusively mediated by A1 adenosine receptors. The idea of endogenous adenosine being responsible for the increased G-protein activities is further supported by the unique ability of adenosine to bind very tightly to its receptors.^{30,31} Therefore, even small and temporary changes in spinal cord extracellular adenosine concentration could contribute to an enhanced inhibitory tone by increasing the activation of A1 receptors.

The reason for the increased A1 receptor stimulation in the spinal cord sections from SNL animals is uncertain. It is possible that alterations in A1 receptor activation properties could account for this effect. This possibility is not excluded but is unlikely given our recent observations of no change in receptor number and affinity between SNL and normal spinal dorsal horns.³² Another cause might be increased release of adenosine after SNL, perhaps because of cell injury, increased adenosine triphosphate degradation, or altered purine (adenosine) metabolism and transport. However, previous work suggested a decrease rather than an increase in extracellular adenosine in the spinal cord after nerve injury.³³

Regardless of the cause, we propose that increased spinal adenosine-mediated G-protein activation after SNL reflects a primed system, amplifying the effect of exogenous adenosine or adenosine ligands. Further work examining these and other hypotheses will clarify the role of this large, A1 adenosine receptor-mediated increase in G-protein activity after peripheral nerve injury in the generation and maintenance of hypersensitivity.

Role of Spinal Adenosine in Nerve Injury-induced Hypersensitivity

The mechanisms by which intrathecally administered adenosine relieves allodynia after nerve injury, whereas it is without any effect in acute pain, remain unclear; therefore, the current study was conducted to test the involvement of G proteins in this phenomenon. We recently excluded prolonged residence in cerebrospinal fluid or increased spinal cord adenosine A1 receptor number as causes.³² We reasoned that, although the number of receptors was unchanged after nerve injury, increased G-protein coupling efficiency by A1 adenosine receptor activation could explain the enhanced activity of intrathecal adenosine to treat allodynia. Our current results suggest that this is unlikely, because [³⁵S]GTPγS

binding from R-PIA exposure was not increased in spinal cords from nerve-injured animals. If anything, one could say that the effect was decreased, in that the difference between unstimulated and R-PIA-induced binding was reduced after nerve injury. This paradox of adenosine having a pronounced antinociceptive effect in the behaving SNL animal *in vivo* but showing a reduced net G-protein activity in the spinal cord dorsal horns *in vitro* could be explained by several possible mechanisms.

First, adenosine may be acting by a non-G-protein mechanism. For example, A1 receptor activation could increase norepinephrine release in the spinal cord by direct interactions with protein kinase C rather than G proteins.³⁴ Second, adenosine is acting on a small but strategically important subpopulation of G-protein-coupled receptors that are altered after nerve injury, not measurable against the large pool of total A1 receptors. Third, for adenosine to be capable of exerting antinociceptive properties, it must exceed some extracellular (threshold) concentration. Because of the usual rapid reuptake of adenosine once released, this specific concentration might never be reached during normal conditions unless the metabolism of adenosine is being blocked. Indeed, during such circumstances, *i.e.*, inhibition of adenosine kinase, adenosine is capable of being antinociceptive even during conditions of acute pain.³⁵ Because of an altered spinal physiology after nerve injury, there might be an increased concentration of endogenous extracellular adenosine that nearly reaches the threshold for antinociception. Further administered exogenous adenosine might then be able to exceed the threshold, hence inducing antinociception.

In summary, this is the first study to show increased basal G-protein activity in lumbar spinal cords after SNL by measuring [³⁵S]GTPγS binding. The data further indicate that this elevated activity is caused by an increased amount of extracellular adenosine acting on A1 receptors.

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