

# Functional $\mu$ Opioid Receptors Are Reduced in the Spinal Cord Dorsal Horn of Diabetic Rats

Shao-Rui Chen, M.D.,\* Kristi L. Sweigart, B.S.,† Joan M. Lakoski, Ph.D.,‡ Hui-Lin Pan, M.D., Ph.D.§

**Background:** The mechanisms of decreased spinal analgesic potency of morphine in neuropathic pain are not fully known. Agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S receptor autoradiography has been used to measure receptor activation of G proteins *in vitro*. Using this technique, we determined changes in the functional  $\mu$  opioid receptors in the spinal dorsal horn in diabetic rats.

**Methods:** Rats were rendered diabetic with an intraperitoneal injection of streptozotocin. The lumbar spinal cord was obtained from age-matched normal and diabetic rats 4 weeks after streptozotocin treatment. [D-Ala<sup>2</sup>,N-MePhe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin (DAMGO, 10  $\mu$ M)-stimulated [ $^{35}$ S]GTP $\gamma$ S binding was performed in both tissue sections and isolated membranes.

**Results:** The DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the spinal dorsal horn was significantly reduced (approximately 37%) in diabetic rats compared with normal rats. However, [ $^{35}$ S]GTP $\gamma$ S bindings in the spinal dorsal horn stimulated by other G protein-coupled receptor agonists, including [D-Pen<sup>2</sup>,D-Pen<sup>5</sup>]-enkephalin, R(-)-N<sup>6</sup>-(2-phenylisopropyl)-adenosine, and WIN-55212, were not significantly altered in diabetic rats. The basal [ $^{35}$ S]GTP $\gamma$ S binding in the spinal dorsal horn was slightly (approximately 13%) but significantly increased in diabetic rats. Western blot analysis revealed no significant difference in the expression of the  $\alpha$  subunits of G<sub>i</sub> and G<sub>o</sub> proteins in the dorsal spinal cord between normal and diabetic rats.

**Conclusions:** These data suggest that the functional  $\mu$  opioid receptors in the spinal cord dorsal horn of diabetic rats are reduced. The impaired functional  $\mu$  opioid receptors in the spinal cord may constitute one of the mechanisms underlying the reduced spinal analgesic effect of  $\mu$  opioids in diabetic neuropathic pain.

DIABETIC neuropathy is a common late complication of diabetes and is frequently painful, with the pain involving predominantly the distal extremities.<sup>1-3</sup> Pain associated with diabetic neuropathy can occur either spontaneously or as a result of exposure to only mildly painful stimuli (hyperalgesia) or to stimuli not normally perceived as painful (allodynia).<sup>1,3</sup> Opioids are widely used to treat patients with acute and chronic pain. However, diabetic neuropathic pain is often resistant to opioid analgesics.<sup>3,4</sup> For instance, many animal and human studies have shown a decreased analgesic potency of  $\mu$  opioid agonists in diabetic neuropathic pain.<sup>5-8</sup> A previous study suggests that there is no significant difference

in the  $\mu$  opioid receptor binding in the whole brain homogenate between normal and diabetic rats and that the decreased potency of systemic morphine may be related to altered distribution and clearance kinetics in diabetic rats.<sup>9</sup> We recently found that the inhibitory effect of morphine on spinothalamic tract neurons is substantially reduced in diabetic rats,<sup>10</sup> suggesting inadequate presence or dysfunction of  $\mu$  opioid receptors in the spinal cord dorsal horn in diabetes. Although some studies have shown a reduction in spinal  $\mu$  opioid receptors following peripheral nerve ligation,<sup>11,12</sup> changes in functional  $\mu$  opioid receptors in the spinal cord in diabetic neuropathy have not been studied previously.

The  $\mu$  opioid receptor signal transduction system requires activation of pertussis toxin-sensitive G<sub>i</sub> and G<sub>o</sub> proteins.<sup>13,14</sup> After coupling to the inhibitory G proteins,  $\mu$  opioid agonists elicit biologic actions through decreased production of cAMP, opening of G protein-gated, inwardly rectifying potassium channels, and closing of voltage-gated calcium channels.<sup>15,16</sup> The development of [ $^{35}$ S]GTP $\gamma$ S autoradiography provides an important means for anatomic localization of receptor-activated G proteins *in vitro*.<sup>17</sup> The agonist-induced stimulation of [ $^{35}$ S]GTP $\gamma$ S binding to the  $\alpha$  subunit of G proteins is based on the G protein activation cycle. Unlike the traditional receptor autoradiograph binding, the [ $^{35}$ S]GTP $\gamma$ S binding is a functional assay of receptors since it measures an intracellular signal transduction system coupled to a membrane-bound receptor. Using this technique, it has been shown that the functional  $\mu$  opioid receptors are highly concentrated in the superficial dorsal horn of the spinal cord.<sup>18</sup> In the present study, we tested a hypothesis that diabetic neuropathy is associated with a reduction in functional  $\mu$  opioid receptors in the spinal cord dorsal horn. Also, since alterations of inhibitory G protein concentrations may account for the change of functional  $\mu$  opioid receptors in diabetes, the expression of  $\alpha$  subunits of G<sub>i</sub>- and G<sub>o</sub>-type G proteins that mediate the signaling of activation of  $\mu$  opioid receptors was measured in the dorsal spinal cord of normal and diabetic rats.

\* Research Associate, § Associate Professor and Director of Basic Research in Anesthesiology, Department of Anesthesiology, † Graduate Student, ‡ Professor of Pharmacology and Anesthesiology, Department of Pharmacology.

Received from the Departments of Anesthesiology and Pharmacology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania. Submitted for publication December 6, 2001. Accepted for publication July 23, 2002. Supported by grant Nos. GM64830, HL04199, and NS41178 from the National Institutes of Health, Bethesda, Maryland, and by the Juvenile Diabetes Foundation International, New York, New York.

Address reprint requests to Dr. Pan: Department of Anesthesiology, H187, Penn State University College of Medicine, 500 University Drive, Hershey, Pennsylvania 17033-0850. Address electronic mail to: hpan@psu.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

## Materials and Methods

### Induction of Diabetes

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) initially weighing 220–250 g were used in this study. The experimental protocols were approved by the Animal Care and Use Committee of the Penn State University College of Medicine. Diabetes was induced by a

single intraperitoneal injection of 50 mg/kg of streptozotocin (Sigma, St. Louis, MO) freshly dissolved in 0.9% sterile saline.<sup>10,19</sup> One week later, diabetes was confirmed in streptozotocin-injected rats by measuring plasma glucose concentrations (>350 mg/dl) in blood samples obtained from the tail vein. The glucose concentration was assayed enzymatically using Sigma diagnostic glucose reagents, and the colorimetric absorbance readings were performed at 450 nm using a microplate spectrophotometer (SPECTRAMax Plus, Molecular Devices Co., Sunnyvale, CA). This experimental model of diabetes (Type I diabetes) has been used as a relevant model of chronic neuropathic pain, and it shows a poor response to opioids administered systemically or intrathecally.<sup>6,8,20,21</sup> Previous studies have demonstrated that this method induces reproducible and reliable mechanical hyperalgesia and allodynia within 3 weeks, which last for at least 6 weeks after streptozotocin injection.<sup>6,8,20,21</sup> Age-matched normal (controls) and diabetic rats 4 weeks after streptozotocin treatment were used in this study. Mechanical allodynia was confirmed in all diabetic rats using calibrated von Frey filaments.<sup>10,19</sup>

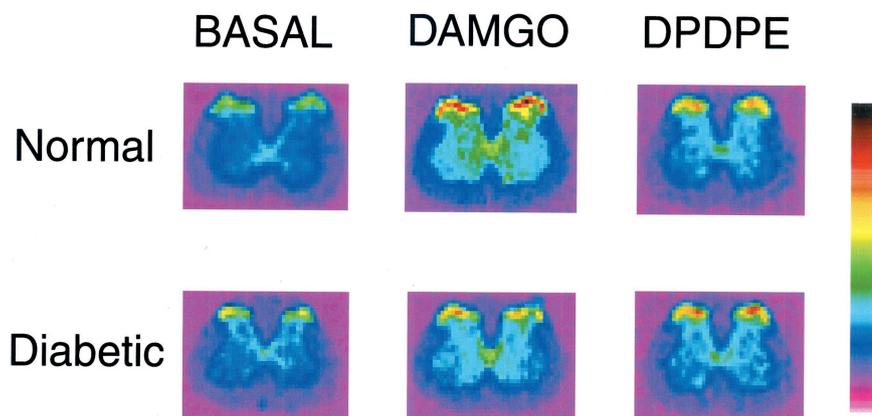
#### *Autoradiography of Agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S Binding in Spinal Cord Sections*

The lumbar spinal cord was rapidly removed from rats through limited laminectomy using halothane anesthesia. The tissue was frozen in a mixture of isopentane and dry ice, and kept at  $-80^{\circ}\text{C}$ . Coronal sections (20  $\mu\text{m}$ ) of the lumbar spinal cord were cut on a cryostat at  $-20^{\circ}\text{C}$  (OTF5000, Bright Instrument, Cambridgeshire, England). The sections were mounted on gelatin-subbed slides, rinsed in assay buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) at  $25^{\circ}\text{C}$  for 10 min, followed by a 15 min preincubation in assay buffer containing 2 mM GDP and 10 mU/ml adenosine deaminase at  $25^{\circ}\text{C}$ . The sections were then incubated in assay buffer with 2 mM GDP, 10 mU/ml adenosine deaminase and 0.04 nM [<sup>35</sup>S]GTP $\gamma$ S (PerkinElmer Life Sciences Inc., Boston, MA), with (stimulated) or without (basal) agonists at  $25^{\circ}\text{C}$  for 2 h. As previously described,<sup>18</sup> agonists were used at maximally-effective concentrations, which include [D-Ala,<sup>2</sup>N-MePhe,<sup>4</sup>Gly<sup>5</sup>-ol]-enkephalin (DAMGO, 10  $\mu\text{M}$ , a  $\mu$  opioid receptor agonist), [D-Pen,<sup>2</sup>D-Pen<sup>5</sup>]-enkephalin (DPDPE, 3  $\mu\text{M}$ , a  $\delta$  opioid receptor agonist), *R*(-)-N<sup>6</sup>-(2-phenylisopropyl)-adenosine (PIA, 1  $\mu\text{M}$ , an adenosine A<sub>1</sub> agonist), and WIN-55212 (10  $\mu\text{M}$ , a cannabinoid CB<sub>1</sub> agonist). Higher concentrations of receptor agonists were used because a higher concentration of GDP was required to reduce the basal [<sup>35</sup>S]GTP $\gamma$ S binding using this technique. All the receptor agonists used in this study were obtained from RBI-Sigma (St. Louis, MO). After incubation, slides were rinsed twice in cold Tris buffer (50 mM Tris-HCl, pH 7.0) for 2 min and once in deionized water for 30 s. The

sections were dried at room temperature overnight and exposed to the Kodak MR-1 film for 72–96 h in cassettes containing [<sup>14</sup>C] standards (Amersham, Piscataway, NJ) for densitometric analysis. After the films were developed and digitized into a computer, the regions of interest in the dorsal spinal cord were analyzed densitometrically using an imaging analysis program (AIS, Imaging Research Inc., St. Catharines, Ontario, Canada). The optical density in the sections was analyzed by drawing regional boundaries to select the appropriate area of interest (laminae I-III). The area of increased binding was first visualized and defined in agonist-stimulated normal spinal cord section, and then it was applied to the corresponding nonstimulated sections and sections from the diabetic rats. Values were corrected for [<sup>35</sup>S] based on incorporation of [<sup>35</sup>S] into sections of frozen brain paste, and correction factors were used to convert [<sup>14</sup>C] values to [<sup>35</sup>S] data.<sup>17,18</sup> The net agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding was calculated by subtracting the basal binding from the agonist-stimulated binding. Data were expressed as nCi [<sup>35</sup>S]GTP $\gamma$ S/g tissue.

#### *DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S Binding in Spinal Cord Membranes*

To further confirm the finding from the autoradiographic experiment that spinal functional  $\mu$  opioid receptors were reduced in diabetic rats, the DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding was also performed using isolated membranes. The dorsal spinal cord tissue was homogenized in cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, pH 7.4) and centrifuged at  $48,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Pellets were resuspended in the membrane buffer and centrifuged again under identical conditions. After centrifugation, pellets were homogenized in assay buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 100 mM NaCl, pH 7.7) and measured for protein content. Concentration-effect curves of the DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding included 0.1–10  $\mu\text{M}$  DAMGO with and without 1  $\mu\text{M}$  D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub> (CTAP, a  $\mu$  opioid receptor antagonist), 30 PM GDP, 0.05 nM [<sup>35</sup>S]GTP $\gamma$ S, 4 mU/ml adenosine deaminase, 20  $\mu\text{g}$  protein, and assay buffer in a final volume of 1 ml.<sup>17,18</sup> The basal binding was determined in the presence of GDP and absence of agonists, and the nonspecific binding was assessed in the presence of 10  $\mu\text{M}$  GTP $\gamma$ S. After incubation for 1 h at  $30^{\circ}\text{C}$ , reactions were terminated by rapid filtration (Brandel, Gaithersburg, MD) through Whatman GF/B glass fiber filters followed by three washes with 3 ml cold Tris buffer (50 mM Tris-HCl, pH 7.7). Bound radioactivity was determined by the liquid scintillation counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA) after overnight extraction of the filter in 4 ml scintillation fluid.



**Fig. 1.** Representative autoradiograms of the basal and DAMGO ([D-Ala,<sup>2</sup>N-MePhe,<sup>4</sup>Gly<sup>5</sup>-ol]-enkephalin, 10  $\mu$ M)- or DPDPE ([D-Pen,<sup>2</sup>D-Pen<sup>5</sup>]-enkephalin, 3  $\mu$ M)-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in the lumbar spinal cord from a normal and a diabetic rat. Note that the tissue sections were presented with the pseudocolor scale for better visualization of the density changes in the spinal dorsal horn. The density indication bar is shown on the right side.

#### Western Immunoblot of G protein $\alpha$ Subunits in the Dorsal Spinal Cord

To further determine if the inhibitory G protein concentrations in the dorsal spinal cord are altered in diabetic rats, expression of  $\alpha$  subunits of G<sub>i</sub> and G<sub>o</sub> proteins was assessed in the dorsal spinal cord obtained from control and diabetic rats ( $n = 6$  in each group) 4 weeks after streptozotocin injection. The dorsal spinal cord was rapidly removed from rats through laminectomy using halothane anesthesia. Protein concentrations of homogenized tissues from each animal were measured, and SDS/polyacrylamide gel electrophoresis (12% running, 6% stacking gel) was performed using 60  $\mu$ g of protein per lane. Each run included molecular weight markers and tissues from a pair of diabetic and age-matched control animals. The proteins were electrophoretically transferred onto nitrocellular membranes, as we described previously.<sup>22</sup> Immunoblots were performed using the specific rabbit polyclonal antisera against the  $\alpha$  subunits of G<sub>i1</sub> (G<sub>i $\alpha$ 1</sub>/G<sub>i $\alpha$ 2</sub>, 1:1000), G<sub>i3</sub> (G<sub>i $\alpha$ 3</sub>/G<sub>o $\alpha$</sub> , 1:1000), and G<sub>o</sub> (G<sub>o1</sub>/G<sub>o $\alpha$</sub> , 1:1000) (PerkinElmer Life Sciences Inc., Boston, MA).<sup>23,24</sup> The membranes were washed twice with the PBS buffer containing 0.05% Tween 20 and then treated with alkaline phosphatase-conjugated goat antirabbit antibody (1:2000, Jackson ImmunoResearch, West Grove, PA). The protein bands were developed with BCIP/NBT and analyzed densitometrically using an imaging analysis program (AIS, Imaging Research Inc., St. Catharine, Ontario, Canada). To minimize interrun variability, the pixel density of bands from diabetic animals were expressed as the percentage difference compared with age-matched controls run on the same gel using the formula: [(diabetic/control) - 1]  $\times$  100.

Data are presented in mean  $\pm$  SEM of at least four separate experiments, each performed in 15–20 sections (autoradiography) or triplicate (membrane bindings). Percent stimulation was calculated as: (net stimulated binding/basal binding)  $\times$  100%. Differences in the [<sup>35</sup>S]GTP $\gamma$ S binding and G protein expression in the spinal cord between the normal and diabetic rats were compared by either Student *t* test or repeat measures

analysis of variance followed by Tukey *post hoc* test (Prism, GraphPad Software, San Diego, CA).  $P < 0.05$  was considered statistically significant.

## Results

The diabetic rats developed hyperglycemia within 1 week after streptozotocin injection; these rats displayed polyuria, a reduced growth rate, and a marked increase in food and water intake. The paw-withdrawal threshold in response to von Frey filaments was  $26.5 \pm 0.9$  g before streptozotocin treatment in all diabetic rats used for this study. The mechanical threshold decreased significantly ( $4.4 \pm 0.5$  g,  $P < 0.05$ ) 4 weeks after streptozotocin injection.

#### Autoradiography of Agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S Binding in Spinal Cord Sections

In normal rats, the [<sup>35</sup>S]GTP $\gamma$ S binding stimulated by the agonists used, including DAMGO and DPDPE, was highly concentrated in the superficial dorsal horn of the spinal cord (fig. 1), similar to findings reported recently.<sup>18</sup> Densitometric measurement of the autoradiograms revealed that the basal [<sup>35</sup>S]GTP $\gamma$ S binding was slightly (approximately 13%) but significantly increased in the spinal dorsal horn of diabetic rats compared to that in normal rats (figs. 1 and 2;  $n = 5$  in each group). The net DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in the spinal cord dorsal horn was reduced by 37% in diabetic rats, which was significantly different from that in normal rats (figs. 1 and 2). By contrast, the [<sup>35</sup>S]GTP $\gamma$ S bindings stimulated by DPDPE, PIA, and WIN-55212 in the spinal dorsal horn of diabetic rats did not differ significantly from those in normal rats (fig. 2).

#### DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S Binding in Spinal Cord Membranes

To further confirm the changes of functional  $\mu$  opioid receptors in the spinal cord caused by diabetes, the [<sup>35</sup>S]GTP $\gamma$ S binding stimulated by DAMGO was also performed using isolated membranes from lumbar spinal

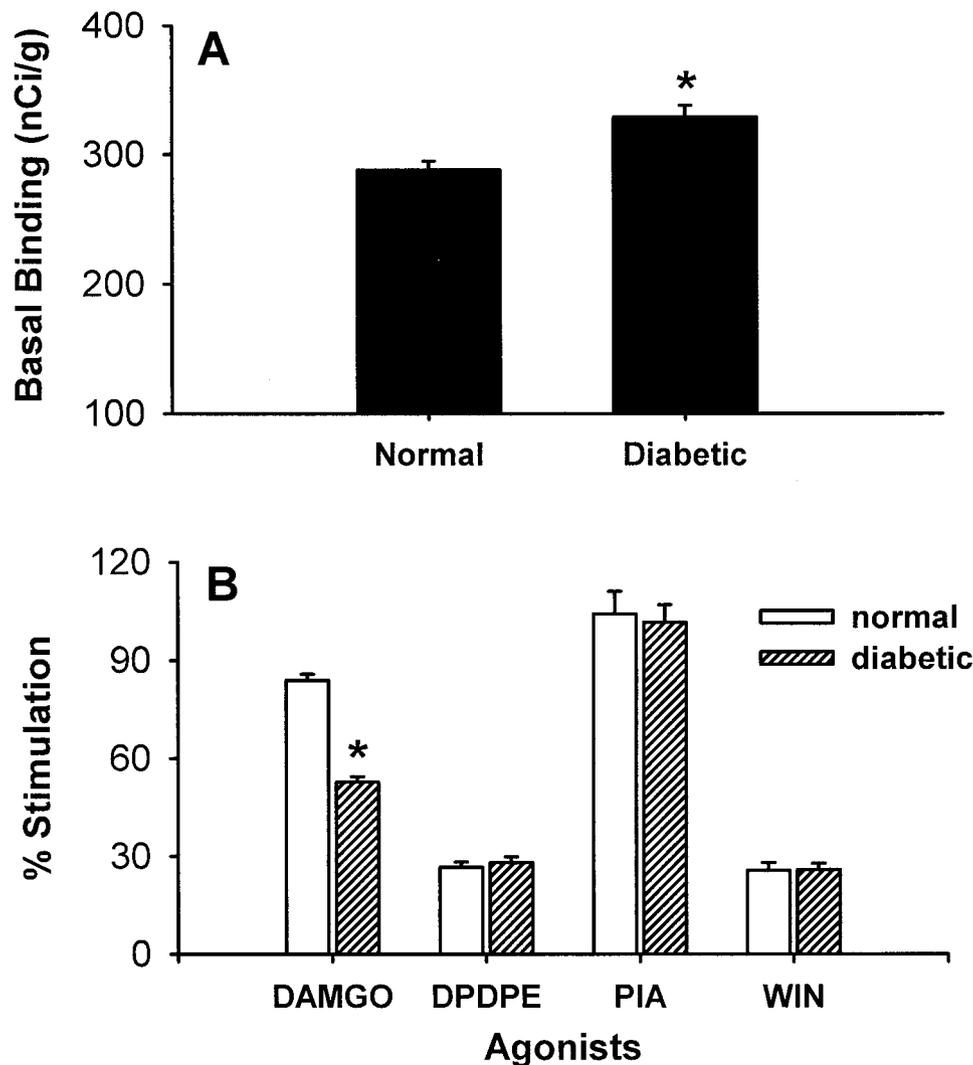


Fig. 2. Changes in basal (A) and agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S bindings (B) in the dorsal horn of spinal cord sections from normal and diabetic rats (n = 5 in each group). The data (mean  $\pm$  SEM) in B are expressed as the percentage of basal [ $^{35}$ S]GTP $\gamma$ S binding, as determined from densitometric analysis of autoradiograms. DAMGO: [D-Ala, $^2$ N-MePhe, $^4$ Gly $^5$ -ol]-enkephalin, 10  $\mu$ M; DPDPE: [D-Pen, $^2$ D-Pen $^5$ ]-enkephalin, 3  $\mu$ M; PIA: *R*(-) $N^6$ -(2-phenylisopropyl)-adenosine, 1  $\mu$ M; WIN: WIN-55212, 10  $\mu$ M. \* $P$  < 0.05 compared to the corresponding normal group.

cords of normal and diabetic rats (n = 4 in each group). The concentration-effect curve for the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in spinal cord membranes is shown in figure 3. The DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding was completely blocked by 1  $\mu$ M CTAP (data not shown). Compared to that in normal rats, the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding was significantly attenuated in diabetic rats at concentrations greater than 0.5  $\mu$ M (fig. 3). The basal [ $^{35}$ S]GTP $\gamma$ S binding was also significantly higher in diabetic rats than normal rats (56.3  $\pm$  3.0 vs. 49.5  $\pm$  2.3 fmol/mg protein,  $P$  < 0.05).

#### Expression of G protein $\alpha$ Subunits in the Dorsal Spinal Cord

The representative immunoblots for the  $\alpha$  subunits of  $G_{11}$ ,  $G_{13}$ , and  $G_o$  proteins were shown in figure 4. Compared to that in normal rats, the density of the protein

band for the  $\alpha$  subunits of  $G_{11}$  (-5.1  $\pm$  0.7%),  $G_{13}$  (6.9  $\pm$  0.6%), and  $G_o$  (-4.6  $\pm$  0.6%) in the dorsal spinal cord of diabetic rats was not significantly altered (n = 6 in each group).

#### Discussion

In the current study, we determined the potential changes of functional  $\mu$  opioid receptors in the spinal cord dorsal horn in a rat model of diabetic neuropathic pain. For quantitative assessment of functional  $\mu$  opioid receptors, the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S bindings in both isolated membranes and tissue sections were studied. We observed that the basal [ $^{35}$ S]GTP $\gamma$ S binding was slightly increased in the dorsal spinal cord in diabetic rats. The important finding from the present study

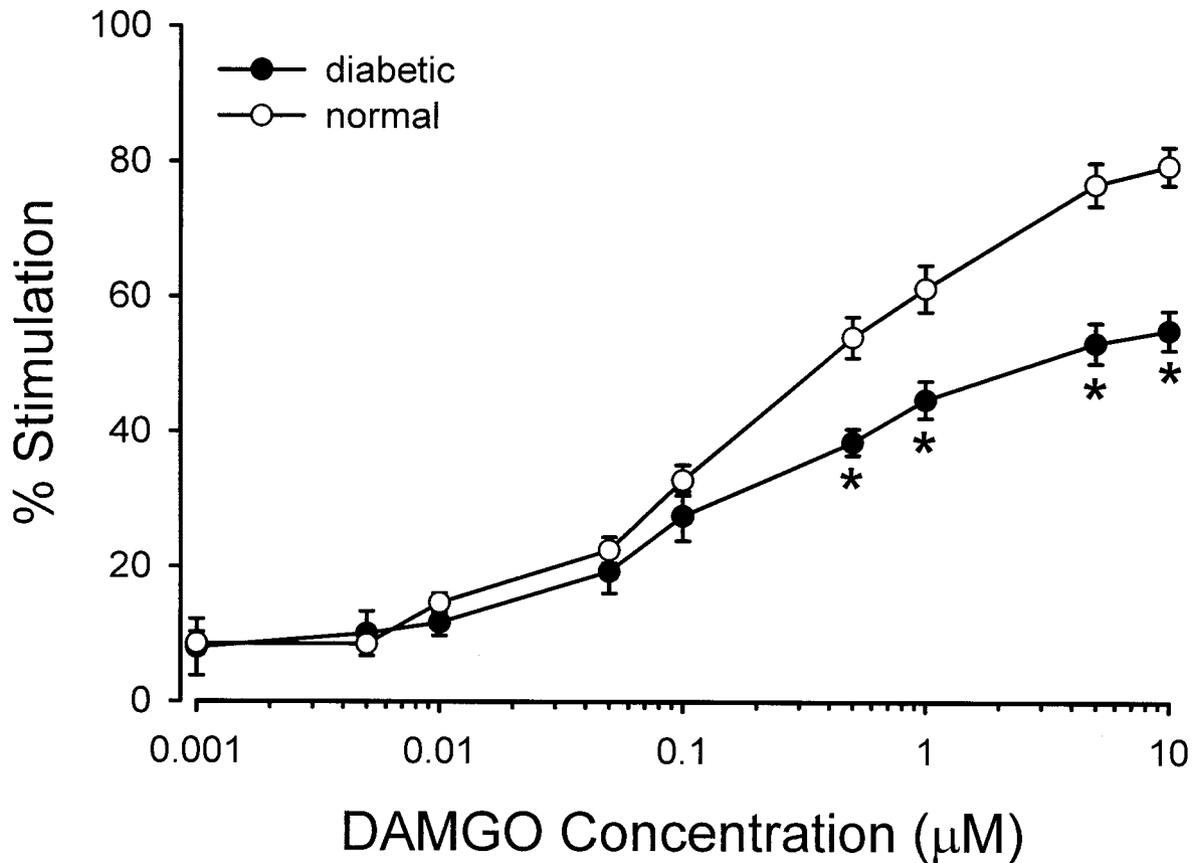


Fig. 3. Concentration-effect curve for the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the spinal cord membrane obtained from normal and diabetic rats 4 weeks after streptozotocin injection. The data are expressed as the percentage of basal [ $^{35}$ S]GTP $\gamma$ S binding (mean  $\pm$  SEM,  $n = 4$  in each group). \* $P < 0.05$  compared with the value in the normal group at the same concentration of DAMGO. The basal [ $^{35}$ S]GTP $\gamma$ S binding was significantly higher in diabetic rats than normal rats ( $56.3 \pm 3.0$  vs.  $49.5 \pm 2.3$  fmol/mg protein).

was that the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding was significantly reduced in the spinal dorsal horn of diabetic compared with normal rats. However, the expression level of  $G_i$ - and  $G_o$ -type G proteins was not significantly altered in the dorsal spinal cord of diabetic rats. Therefore, these data support the hypothesis that the functional  $\mu$  opioid receptors in the spinal dorsal horn are reduced in diabetes.

The spinal cord is an important site for modulation of nociception by  $\mu$  opioid receptors. Previous studies have shown that the analgesic potency of morphine and fentanyl are reduced in diabetic animals and patients.<sup>5,7,8,20</sup> In a recent study, we found that the inhibitory effect of intravenous morphine on the evoked responses of spinal dorsal horn neurons to noxious stimuli was largely diminished in diabetic animals.<sup>10</sup> Thus, the

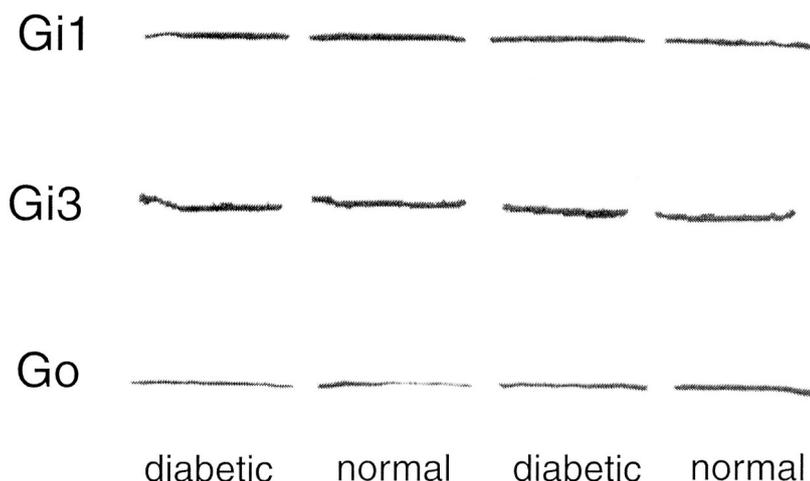


Fig. 4. Representative Western blots showing similar expression levels of  $\alpha$  subunits of  $G_{i1}$  (M.W. = 41 kd),  $G_{i3}$  (M.W. = 41 kd), and  $G_o$  (M.W. = 39 kd) proteins in the dorsal spinal cord from one normal and one diabetic rat.

attenuated inhibitory effect of morphine on dorsal horn neurons in diabetes may be due to altered  $\mu$  opioid receptors or their signal transduction system in the spinal cord. However, alteration of G protein activation by  $\mu$  opioid receptors in the spinal cord in the animal model of diabetes has not previously been investigated. The agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S binding was used in this study since activation of G proteins by G protein-coupled receptors in discrete areas can be precisely localized and measured with this technique. The development of an assay for agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in tissue sections allows the visualization of receptor-activated G proteins in specific tissue regions.<sup>17,18</sup> The [ $^{35}$ S]GTP $\gamma$ S assay is based on the principle that the inactive state of the G protein  $\alpha$  subunit has a relatively high affinity for GDP over GTP, whereas activation of a receptor by its agonist shifts the  $\alpha$  subunit into a higher affinity for GTP *versus* GDP. The [ $^{35}$ S]GTP $\gamma$ S is a hydrolysis-resistant form of GTP, and the degree to which an agonist stimulates the [ $^{35}$ S]GTP $\gamma$ S binding can be measured in tissue sections or membranes.<sup>17,25</sup> A number of G protein-coupled receptors have been localized in the spinal cord by the agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S autoradiography, including  $\mu$  and  $\delta$  opioid, adenosine A<sub>1</sub>, and cannabinoid CB<sub>1</sub> receptors.<sup>18</sup> These data complement findings from traditional receptor autoradiography because they provide information not only about the location of the receptor, but also its activation of G proteins. In this study, we observed a small but consistent increase in the basal [ $^{35}$ S]GTP $\gamma$ S binding in the spinal dorsal horn in diabetic rats. It is uncertain what causes the increased basal [ $^{35}$ S]GTP $\gamma$ S binding in the dorsal spinal cord in diabetes. Since the [ $^{35}$ S]GTP $\gamma$ S binding assay primarily measures receptors coupled to G<sub>i</sub> and G<sub>o</sub>,<sup>17,18</sup> we also examined the potential changes in the  $\alpha$  subunits of G<sub>i</sub> and G<sub>o</sub> proteins in the dorsal spinal cord. We found that the expression level of  $\alpha$  subunits of inhibitory G proteins (G<sub>i</sub> and G<sub>o</sub>) in the lumbar spinal cord was similar in normal and diabetic rats. Thus, it is less likely that the increased basal [ $^{35}$ S]GTP $\gamma$ S binding is due to an increase in the inhibitory G protein expression in the spinal cord in diabetes. Further studies are warranted to determine if changes in the affinity of G proteins for GTP and GDP contribute to the increased basal [ $^{35}$ S]GTP $\gamma$ S binding in the spinal dorsal horn in diabetes.

In the current study, we observed a significant reduction (37%) in the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the spinal cord dorsal horn in diabetic rats, which may partially account for the decreased analgesic action of morphine on diabetic neuropathic pain. Consistent with the autoradiographic data, the membrane binding experiments also showed a similar magnitude of reduction in the maximal DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the dorsal spinal cord in diabetic rats compared with normal rats. We found that attenuation of the DAMGO-

stimulated [ $^{35}$ S]GTP $\gamma$ S binding was especially prominent at higher DAMGO concentrations. These data suggest that the functional  $\mu$  opioid receptors in the spinal dorsal horn may be decreased in rats with diabetic neuropathy. Importantly, the [ $^{35}$ S]GTP $\gamma$ S bindings in the spinal cord dorsal horn stimulated by other G protein-coupled receptor agonists, including DPDPE, PIA, and WIN55212, were not significantly altered in diabetic rats. With the diabetics having a slightly higher basal binding than normal controls, a portion of the decreased DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S activity could be accounted for by the increased basal [ $^{35}$ S]GTP $\gamma$ S binding. However, the significant decrease (37%) in the net DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the spinal cord dorsal horn in diabetic rats could not be fully explained by a small increase (13%) in the basal [ $^{35}$ S]GTP $\gamma$ S binding in the spinal cord of diabetic rats. The  $\mu$  opioid receptor is coupled to G<sub>i</sub> and G<sub>o</sub>-type G proteins.<sup>13,14</sup> Using the Western blot analysis of the  $\alpha$  subunit of G<sub>i</sub> and G<sub>o</sub>, we found no significant difference in the expression of the types of inhibitory G proteins that preferentially couple to the opioid receptor in the dorsal spinal cord between normal and diabetic rats. As a result, it is unlikely that attenuation in the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the spinal cord dorsal horn in diabetic rats is due to a decrease in the expression of inhibitory G proteins.

The observed selective reduction in functional  $\mu$ , but not  $\delta$ , opioid receptors in the spinal dorsal horn in diabetes is supported by the behavioral studies showing that morphine analgesia is reduced, while DPDPE-produced analgesia is not altered in diabetic animals.<sup>8,21,26</sup> Our conclusion that the functional  $\mu$  opioid receptor is reduced in the spinal cord dorsal horn in diabetes is solely based on the significant decrease in the DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the spinal cord of diabetic rats. It is important to acknowledge that the decreased [ $^{35}$ S]GTP $\gamma$ S binding stimulated by DAMGO in diabetes could be due to impairment of  $\mu$  receptor-G protein coupling and/or alteration of  $\mu$  opioid receptor density and affinity. Future studies are required to delineate the precise causes for the reduced DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding in the spinal cord dorsal horn in diabetes. Furthermore, Zurek *et al.* have shown a complete resistance to fentanyl when the drug is restricted to act only at the spinal cord level in diabetes.<sup>8</sup> Thus, partial loss of functional  $\mu$  opioid receptors may not account fully for the diminished analgesic effect of  $\mu$  opioids. Other mechanisms, such as impaired regulation of cytosolic calcium also may be involved in the loss of the action of  $\mu$  opioids in diabetes.<sup>27</sup> In addition, a recent study suggests that insulin can affect phosphorylation of  $\mu$  opioid receptors and G protein coupling efficacy.<sup>28</sup> In this regard, alteration of tyrosine phosphorylation state of  $\mu$  opioid receptors due to insulin defi-

ciency in diabetes could constitute an additional mechanism of the reduced analgesic efficacy of  $\mu$  opioids.

In summary, we found in the present study that the [ $^{35}$ S]GTP $\gamma$ S binding stimulated by a  $\mu$  opioid agonist, DAMGO, was significantly reduced in the spinal cord dorsal horn of diabetic rats. However, the [ $^{35}$ S]GTP $\gamma$ S binding stimulated by other G protein-coupled receptor agonists and the expression of G $_i$  and G $_o$  proteins in the dorsal spinal cord were not significantly altered in diabetic rats. Thus, these results suggest that the functional  $\mu$  opioid receptors are reduced in the spinal dorsal horn in diabetes. Data from the present study provide new information that the impaired functional  $\mu$  opioid receptors in the spinal cord in diabetes likely contributes, at least in part, to the reduced analgesic action of spinally administered  $\mu$  opioid agonists in diabetic neuropathic pain.

## References

- Brown MJ, Asbury AK: Diabetic neuropathy. *Ann Neurol* 1984; 15:2-12
- Clark CM, Jr, Lee DA: Prevention and treatment of the complications of diabetes mellitus. *N Engl J Med* 1995; 332:1210-7
- Boulton AJ, Gries FA, Jervell JA: Guidelines for the diagnosis and outpatient management of diabetic peripheral neuropathy. *Diabet Med* 1998; 15:508-14
- Wright JM: Review of the symptomatic treatment of diabetic neuropathy. *Pharmacotherapy* 1994; 14:689-97
- Arner S, Meyerson BA: Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. *Pain* 1988; 33:11-23
- Courteix C, Eschali r A, Lavarenne J: Streptozotocin-induced diabetic rats: behavioural evidence for a model of chronic pain. *Pain* 1993; 53:81-8
- Courteix C, Bardin M, Chantelauze C, Lavarenne J, Eschali r A: Study of the sensitivity of the diabetes-induced pain model in rats to a range of analgesics. *Pain* 1994; 57:153-60
- Zurek JR, Nadeson R, Goodchild CS: Spinal and supraspinal components of opioid antinociception in streptozotocin induced diabetic neuropathy in rats. *Pain* 2001; 90:57-63
- Courteix C, Bourget P, Caussade F, Bardin M, Coudore F, Fialip J, Eschali r A: Is the reduced efficacy of morphine in diabetic rats caused by alterations of opiate receptors or of morphine pharmacokinetics? *J Pharmacol Exp Ther* 1998; 285:63-70
- Chen SR, Pan HL: Hypersensitivity of spinothalamic tract neurons associated with diabetic neuropathic pain in rats. *J Neurophysiol* 2002; 87:2726-2733
- Zhang X, Bao L, Shi TJ, Ju G, Elde R, Hokfelt T: Down-regulation of mu-opioid receptors in rat and monkey dorsal root ganglion neurons and spinal cord after peripheral axotomy. *Neuroscience* 1998; 82:223-40
- Porreca F, Tang QB, Bian D, Riedl M, Elde R, Lai J: Spinal opioid mu receptor expression in lumbar spinal cord of rats following nerve injury. *Brain Res* 1998; 795:197-203
- Laugwitz KL, Offermanns S, Spicher K, Schultz G: mu and delta opioid receptors differentially couple to G protein subtypes in membranes of human neuroblastoma SH-SY5Y cells. *Neuron* 1993; 10:233-42
- Offermanns S, Schultz G, Rosenthal W: Evidence for opioid receptor-mediated activation of the G-proteins, Go and Gi2, in membranes of neuroblastoma x glioma (NG108-15) hybrid cells. *J Biol Chem* 1991; 266:3365-8
- Schneider SP, Eckert WA, 3rd, Light AR: Opioid-activated postsynaptic, inward rectifying potassium currents in whole cell recordings in substantia gelatinosa neurons. *J Neurophysiol* 1998; 80:2954-62
- Wilding TJ, Womack MD, McCleskey EW: Fast, local signal transduction between the mu opioid receptor and Ca $^{2+}$  channels. *J Neurosci* 1995; 15:4124-32
- Sim IJ, Selley DE, Childers SR: In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5'-[gamma- $^{35}$ S]thio]-triphosphate binding. *Proc Natl Acad Sci U S A* 1995; 92:7242-6
- Maher CE, Eisenach JC, Pan HL, Xiao R, Childers SR: Chronic intrathecal morphine administration produces homologous mu receptor/G-protein desensitization specifically in spinal cord. *Brain Res* 2001; 895:1-8
- Chen SR, Khan GM, Pan HL: Antiallodynic effect of intrathecal neostigmine is mediated by spinal nitric oxide in a rat model of diabetic neuropathic pain. *ANESTHESIOLOGY* 2001; 95:1007-12
- Calcutt NA, Chaplan SR: Spinal pharmacology of tactile allodynia in diabetic rats. *Br J Pharmacol* 1997; 122:1478-82
- Malcangio M, Tomlinson DR: A pharmacologic analysis of mechanical hyperalgesia in streptozotocin/diabetic rats. *Pain* 1998; 76:151-7
- Zhao Z, Chen SR, Eisenach JC, Busija DW, Pan HL: Spinal cyclooxygenase-2 is involved in development of allodynia after nerve injury in rats. *Neuroscience* 2000; 97:743-8
- Lin S, Kajimura M, Takeuchi K, Kodaira M, Hanai H, Nishimura M, Kaneko E: Alterations of GTP-binding proteins (G $\alpha$  and G $\beta$ /11 $\alpha$ ) in gastric smooth muscle cells from streptozotocin-induced and WBN/Kob diabetic rats. *Dig Dis Sci* 2000; 45:1517-24
- Hall KE, Liu J, Sima AA, Wiley JW: Impaired inhibitory G-protein function contributes to increased calcium currents in rats with diabetic neuropathy. *J Neurophysiol* 2001; 86:760-70
- Traynor JR, Nahorski SR: Modulation by mu-opioid agonists of guanosine 5'-O-(3-[ $^{35}$ S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* 1995; 47:848-54
- Kamei J, Ohhashi Y, Aoki T, Kawasima N, Kasuya Y: Streptozotocin-induced diabetes selectively alters the potency of analgesia produced by mu-opioid agonists, but not by delta- and kappa-opioid agonists. *Brain Res* 1992; 571:199-203
- Hall KE, Sima AA, Wiley JW: Opiate-mediated inhibition of calcium signaling is decreased in dorsal root ganglion neurons from the diabetic BB/W rat. *J Clin Invest* 1996; 97:1165-72
- McLaughlin JP, Chavkin C: Tyrosine phosphorylation of the mu-opioid receptor regulates agonist intrinsic efficacy. *Mol Pharmacol* 2001; 59:1360-8