Chronic Morphine Induces Downregulation of Spinal Glutamate Transporters: Implications in Morphine Tolerance and Abnormal Pain Sensitivity

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Tolerance to the analgesic effects of an opioid occurs after its chronic administration, a pharmacological phenomenon that has been associated with the development of abnormal pain sensitivity such as hyperalgesia. In the present study, we examined the role of spinal glutamate transporters (GTs) in the development of both morphine tolerance and associated thermal hyperalgesia. Chronic morphine administered through either intrathecal boluses or continuous infusion induced a dose-dependent downregulation of GTs (EAAC1 and GLAST) in the rat's superficial spinal cord dorsal horn. This GT downregulation was mediated through opioid receptors because naloxone blocked such GT changes. Morphine-induced GT downregulation reduced the ability to maintain *in vivo* glutamate homeostasis at the spinal level, because the hyperalgesic response to exogenous glutamate was enhanced, including an increased magnitude and a prolonged time course, in morphine-treated rats with reduced spinal GTs. Moreover, the downregulation of spinal GTs exhibited a temporal correlation with the development of morphine tolerance and thermal hyperalgesia. Consistently, the GT inhibitor L-*trans*-pyrrolidine-2-4-dicarboxylate (PDC) potentiated, whereas the positive GT regulator riluzole reduced, the development of both morphine tolerance and thermal hyperalgesia. The effects from regulating spinal GT activity by PDC were at least in part mediated through activation of the NMDA receptor (NMDAR), because the noncompetitive NMDAR antagonist MK-801 blocked both morphine tolerance and thermal hyperalgesia that were potentiated by PDC. These results indicate that spinal GTs may contribute to the neural mechanisms of morphine tolerance and associated abnormal pain sensitivity by means of regulating regional glutamate homeostasis.

Key words: tolerance; opioid; glutamate transporter; NMDA; hyperalgesia; riluzole; PDC

Opioids are a class of the most effective analgesics for treating many forms of acute and chronic pain. Besides the known side effects, the clinical utility of opioid analgesics is often hampered by the development of analgesic tolerance that necessitates dose escalation regardless of the disease progression. The development of opioid tolerance also has been associated with enhanced pain sensitivity such as hyperalgesia (exacerbated pain in response to noxious stimulation) in both laboratory and clinical settings (Sjogren et al., 1993; Mao et al., 1994; Ossipov et al., 1995; Devulder, 1997; Wegert et al., 1997; Vanderah et al., 2000; Celerier et al., 2001). Several lines of recent research have shed light on the neurobiology of opioid tolerance. For instance, β-arrestin, a regulatory protein, has been shown to play an important role in the development of opioid tolerance (Bohn et al., 1999, 2000; Whistler and von Zastrow, 1999). More recently, the activity of μ-opioid receptor oligomerization and endocytosis has been suggested to be critical to the prevention of morphine tolerance (Finn and Whistler, 2001; He et al., 2002). As such, an opioid agonist that would facilitate the μ-opioid receptor endocytosis has been shown to reduce the development of morphine tolerance (He et al., 2002). Another interesting development is that activation of excitatory amino acid receptors such as the NMDA receptor (NMDAR) has been implicated in the mechanisms of opioid tolerance, particularly μ-opioid tolerance, and associated abnormal pain sensitivity (Marek et al. 1991a,b; Trujillo and Akil, 1991; Tiseo and Inturrisi, 1993; Elliott et al., 1994a,b; Mao et al., 1994, 1996; Manning et al., 1996). There is also emerging evidence suggesting that opioid tolerance and abnormal pain sensitivity may share common cellular mechanisms mediated in part through NMDARs (Mao et al., 1994, 1995b).

Despite a large number of studies over a decade that indicate the involvement of NMDARs in the development of μ-opioid tolerance and associated abnormal pain sensitivity, it remains unclear how activation of NMDARs could be initiated in response to opioids that are known to have overwhelmingly inhibitory effects. Opioids such as morphine do not have detectable binding affinity with NMDARs (Mao, 1999), making it unlikely that opioids directly interact with NMDARs at the receptor level. On the other hand, neither acute opioid analgesic effects nor the expression of opioid tolerance is affected by an NMDAR antagonist (Trujillo and Akil, 1991; Mao et al., 1994), indicating that the NMDAR itself does not directly modulate opioid receptor functions.

The homeostasis of the extracellular glutamate level, a primary endogenous ligand for the NMDAR, is actively and tightly regulated by the glutamate transporter (GT) system (Robinson and Dowd, 1997; Semba and Wakuta, 1998; Mennerick et al., 1999;

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9Jabaudon et al., 2000; Danbolt, 2001). There are at least five identified Na^+-dependent GT proteins, which are differentially expressed in specific cell types with EAAC1 primarily being in neurons and GLAST and GLT-1 in glial cells (Robinson and Dowd, 1997; Danbolt, 2001). A number of studies have shown that GTs play a critical role in the prevention of glutamate neurotoxicity under both physiological and pathological conditions (Mennerick et al., 1999; Lievens et al., 2000; Vorwerk et al., 2000; Trotti et al., 2001). In brain regions, changes in GLT-1 mRNAs have been observed after naloxone-precipitated morphine withdrawal (Ozawa et al., 2001), and morphine tolerance decreases after subcutaneous injection of a proposed glial GT activator, MS-135 (Nakagawa et al., 2001). Conceivably, activation of NMDARs would become possible, despite the overwhelmingly inhibitory opioid effects, if the GT function were reduced after chronic opioid administration with a resultant increase in synaptic glutamate availability. In this series of experiments, we examined the possibilities that chronic morphine administration downregulates both neuronal and glial GTs in the spinal cord, which in turn contributes to the neural mechanisms of morphine tolerance and abnormal pain sensitivity in part through the activation of NMDARs.

MATERIALS AND METHODS

Experimental animals and drugs

Adult male Sprague Dawley rats weighing 300–350 gm were used. Rats were housed in individual cages with water and food pellets available *ad libitum*. The animal room was artificially illuminated from 7:00 A.M. to 7:00 P.M.. The protocols were approved by our Institutional Animal Care and Use Committee. The following drugs were purchased from Sigma: MK-801, morphine, riluzole, glutamate, naloxone, and L-*trans*-pyrrolidine-2-4-dicarboxylate (PDC).

Intrathecal catheter and osmotic pump implantation

A polyethylene (PE)-10 intrathecal catheter was implanted in each rat (Yaksh and Rudy, 1976). Those animals that exhibited neurological deficits after intrathecal catheter implantation were excluded from the experiments. Drugs were delivered via an intrathecal catheter in a total volume of 10 μl followed by a saline flush. For continuous intrathecal infusion, osmotic minipumps (Alza, Mountain View, CA) were implanted as described previously (Granados-Soto et al., 2000; Vanderah et al., 2000). An osmotic pump was connected to an intrathecal catheter via a piece of PE-60 catheter. The filled minipumps were soaked in normal saline for 4 hr before the insertion to ensure an immediate drug delivery. The integrity of the pump delivery system was reexamined when the spinal cords were harvested for the immunocytochemical or Western blot analysis. The experiments including behavioral testing were conducted so that the experimenters were blinded to treatment conditions.

Induction of morphine tolerance

Tolerance to the antinociceptive effect of morphine was induced using two intrathecal treatment regimens: repeated boluses and continuous infusion. Morphine was given twice daily for 7 d in the repeated bolus regimen, whereas continuous morphine infusion was given for 7 d via an implanted osmotic pump system. Because the osmotic pump infusion began on day 1, day 8 was the last day of a full 7 d delivery using an osmotic pump. Differences in morphine antinociception among treatment groups were assessed on the test day using the tail-flick test at 30 min after a probe dose containing either 10 μg of morphine (intrathecal) for repeated bolus groups or 5 mg/kg of morphine (intraperitoneal) for continuous infusion groups.

Behavioral tests

The routine tail-flick test was made with baseline latencies of 4–6 sec and a cutoff time of 10 sec to assess the antinociceptive effects of morphine (D'Amour, Smith, 1941; Akil and Mayer, 1972). The percentage of maximal possible antinociceptive effect (%MPAE) was calculated by comparing the test latency before [baseline (BL)] and after a drug injection (TL) using the equation: $\%MPAE = [(TL - BL)/(cutoff - BL)] \times 100$. In dose–response experiments, the generation of cumulative dose–response curves, as described in the literature (Paronis and Holtzman, 1991; Elliott et al., 1994a,b; Mao et al. 1995a), was used to reduce the total number of rats used in experiments. To examine changes in baseline nociceptive responses before and after chronic administration of morphine or a GT regulator, the paw-withdrawal test with baseline latencies of 9–11 sec and a cutoff time of 22 sec was used as described previously (Hargreaves et al., 1988). The paw-withdrawal test was used because this test has been shown to be sensitive in detecting subtle changes of a baseline nociceptive response because of its slow-rising temperature change during the test (Mao et al., 1994). In this study, the development of morphine tolerance was assessed by the tail-flick test, whereas the development of thermal hyperalgesia was evaluated by the paw-withdrawal test.

Statistics for behavioral data

Data obtained from the tail-flick test were first calculated to yield mean %MPAE as shown previously (Mao et al., 1994). The data for both tail-flick and paw-withdrawal tests were then analyzed by using two-way ANOVA to detect overall differences among treatment groups. When significant main effects were observed, the Waller–Duncan K-ratio *t* tests were performed to determine sources of differences. For the dose–response data analysis, AD_{50} values and 95% confidence intervals (CIs) were computed using a computerized Litchfield and Wilcoxon calculation.

Immunocytochemistry and Western blotting

The routine immunostaining procedure was followed. Briefly, rats were perfused through the ascending aorta with saline followed by 4% paraformaldehyde. Spinal cord lumbar segments in which the intrathecal catheter was implanted were removed, postfixed for 2 hr, and kept overnight in 15% sucrose. Spinal cord samples from both experimental and control groups were mounted on the same block, and 10 μm transverse sections were cut together on a cryostat. These sections were then treated under the same condition during the immunocytochemical procedure to minimize the between-group variability. Sections were blocked with 1% goat serum in 0.3% Triton X-100 for 1 hr at room temperature and incubated overnight at 4°C with a primary antibody (EAAC1 or GLAST, 1:2000; Chemicon). The sections were then incubated for 1 hr at room temperature with the corresponding CY3-conjugated secondary antibody (1:300; Chemicon).

For Western blotting, rats were killed rapidly (<1 min) in a CO_2 chamber. The dorsal horn from the lumbar spinal cord, corresponding to the site where samples for immunocytochemistry were collected, was removed and homogenized in SDS sample buffer containing a mixture of proteinase inhibitors (Sigma). The spinal cord dorsal horn was sampled because the immunocytochemical staining showed that EAAC1 and GLAST, as well as their changes after morphine treatment, were presented primarily in this spinal region. Protein samples were separated on SDS-PAGE gel (4–15% gradient gel; Bio-Rad) and transferred to polyvinylidene difluoride filters (Millipore). The filters were blocked with 3% milk and incubated overnight at 4°C with a primary antibody (EAAC1, 1:2000; GLAST, 1:2000) and for 1 hr at room temperature with HRP-conjugated secondary antibody (Amersham; 1:10,000). The blots were then visualized in ECL solution (NEN) for 1 min and exposed onto hyperfilms (Amersham) for 1–10 min.

Image analysis

For the immunostaining analysis, six spinal sections were randomly selected and scanned using a Nikon fluorescence microscope. Images were then captured with a CCD Spot camera (Diagnostic Instruments, Inc.), and image densities were analyzed (Adobe PhotoShop) according to the division of spinal cord dorsal horn regions (Molander et al., 1984; Mao et al., 1992, 1993). Relative density of images was determined by subtracting the background density in each image. The percentage change of staining density in morphine-treated groups from the corresponding saline group was calculated by the following equation: (density of the saline group − density of the morphine group)/(density of the saline group) × 100. For Western blotting, the developed films were scanned, and the density of immunoreactive bands was measured and normalized with internal control bands (ERK2 as loading control). For both immunostaining and Western blotting, differences in image density were compared using the Student's *t* test (two groups) or ANOVA (multiple groups) followed by the Waller–Duncan K-ratio *t* tests.

Experimental design

Experiment 1: changes in spinal GTs after chronic morphine. A total of 10 groups of rats were used in this experiment. To investigate whether repeated exposure to morphine boluses would result in changes in spinal GTs, three groups of rats ($n = 5$) were given intrathecal 10 or 20 μg of morphine or saline twice daily for 7 d. In addition, three more groups of rats ($n = 5$) were infused, via an intrathecal osmotic pump for 7 d, with 10 or 20 nM $\cdot \mu l^{-1} \cdot hr^{-1}$ morphine or saline. The continuous infusion regimen was included because it has been suggested that the cellular mechanisms of opioid tolerance might differ between repeated boluses and continuous administration (Ibuki et al., 1997; Dunbar and Pulai, 1998). In either case, morphine doses were chosen on the basis of previous studies that showed the reliable development of morphine tolerance using these doses (Mao et al., 1994; Ibuki et al., 1997). Additional two groups of rats ($n = 4$) were included to examine whether the EAAC1 or GLAST protein content (Western blot) would be changed after a 7 d continuous intrathecal infusion with either saline or 20 nM $\cdot \mu l^{-1} \cdot hr^{-1}$ morphine.

To examine whether blockade of opioid receptors would prevent morphine-induced changes in spinal GTs, morphine (20 μg) was coadministered intrathecally (twice daily) with naloxone (10 μg, a generic opioid receptor antagonist) for 7 d ($n = 5$). As a control, naloxone (10 μg) also was given alone twice daily for 7 d ($n = 5$). In all groups, spinal cords were harvested after the final behavioral test on day 8 (see above for the time line for pump groups), and samples were prepared for either immunocytochemical or Western blot analysis.

Experiment 2: effect of spinal GT changes on the response to exogenous glutamate. To examine whether changes in spinal GTs would alter the baseline latency to noxious thermal stimulation (i.e., the development of thermal hyperalgesia) and the response to exogenous glutamate, baseline paw-withdrawal latencies were compared between day 0 and day 8 in four groups of rats ($n = 5$), each receiving intrathecal infusion of saline, 10 nM $\cdot \mu l^{-1} \cdot hr^{-1}$ morphine, or 20 nM $\cdot \mu l^{-1} \cdot hr^{-1}$ morphine (two groups). On day 8 after determining the baseline latency, glutamate (5 nM) was given intrathecally to the saline and morphine (20 nM $\cdot \mu l^{-1} \cdot hr^{-1}$) groups. Paw-withdrawal latencies to noxious thermal stimulation were again measured at 30, 60, 120, and 240 min after the glutamate administration. In the second 20 nM $\cdot \mu l^{-1} \cdot hr^{-1}$ morphine group, 20 μg of riluzole (a positive GT regulator) was given intrathecally at 30 min before the injection of 5 nM glutamate to determine whether increasing GT activity with acute riluzole treatment would affect either the response magnitude or the time course of exogenous glutamate. A control group of rats ($n = 4$) received a single injection of 20 μg of riluzole on day 8 to examine the effect of riluzole alone on the baseline nociceptive response.

Experiment 3: time course between GT changes, morphine tolerance, and thermal hyperalgesia. To examine the temporal relationship between changes in spinal GTs and the development of morphine tolerance and thermal hyperalgesia, eight groups ($n = 8$–9) of rats were used, each receiving 20 μg of either morphine or saline (twice daily), and their spinal cords were harvested for either immunocytochemistry or Western blotting on days 2, 4, 6, or 8 after the behavioral tests. The behavioral tests included the paw-withdrawal test to examine thermal hyperalgesia and the tail-flick test (cumulative dose–response) to examine the antinociceptive effect of intrathecal morphine.

Experiment 4: effect of regulating spinal GT activity and blocking NMDARs on morphine tolerance and thermal hyperalgesia. To investigate the role of spinal GTs in the development of morphine tolerance and associated thermal hyperalgesia, the GT inhibitor PDC (Lievens et al., 2000; Matthews et al., 2000) and the positive GT regulator riluzole (Azbill et al., 2000) were used to determine whether inhibiting and activating spinal GT activity would enhance and attenuate, respectively, morphine tolerance and thermal hyperalgesia. Ten groups of rats ($n = 5$) were used as follows: groups 1–3 (10 μg of morphine + 5, 10, or 20 μg of riluzole), group 4 (20 μg of riluzole alone), groups 5–7 (10 μg of morphine + 5, 10, or 20 μg of PDC), group 8 (20 μg of PDC alone), group 9 (10 μg of morphine + vehicle), and group 10 (vehicle alone). The drug combinations were given intrathecally twice daily for 7 d. The equivalent doses of PDC and riluzole have been demonstrated to be effective in regulating the extracellular glutamate concentration and NMDAR-mediated activities under both *in vivo* and *in vitro* experimental conditions (Semba and Wakuta, 1998; ; Azbill et al., 2000; Jabaudon et al., 2000; Lievens et al., 2000; Matthews et al., 2000). Additionally, two more groups of rats each received a 7 d intrathecal treatment (twice daily) with either 10 μg of morphine + 10 nM MK-801 + 20 μg of PDC ($n = 6$) or 10 nM MK-801 alone ($n = 4$). The MK-801 dose was selected on the basis of a previous study that showed its prevention of morphine tolerance and hyperalgesia (Mao et al., 1994). The data from these two groups were compared with the above morphine alone and morphine plus PDC group to examine whether the effect of PDC on the development of morphine tolerance and hyperalgesia is mediated through NMDARs.

RESULTS

Downregulation of spinal GTs after chronic morphine administration

In naïve rats there was a basal level of EAAC1 and GLAST immunoreactivity (ir) primarily within laminas I–II of the spinal cord dorsal horn. When examined on day 8, both EAAC1-ir and GLAST-ir in laminas I–II were significantly reduced in rats receiving a 7 d intrathecal morphine treatment given either as repeated boluses (10 or 20 μg, twice daily) or continuous infusion (10 or 20 nM $\cdot \mu l^{-1} \cdot hr^{-1}$), as compared with the corresponding saline group (Figs. 1*A*,*B*,*D*,*E*, 2*A*,*B*). There were no differences in the level of EAAC1-ir and GLAST-ir between saline-treated and naïve rats, indicating a specific effect of morphine on EAAC1-ir and GLAST-ir. A much lower basal level of EAAC1-ir and GLAST-ir was observed in laminas III–VI (Fig. 1*A*,*D*), as compared with that in laminas I–II, and the EAAC1 and GLAST immunostaining in laminas III–VI was not significantly changed after chronic morphine.

Quantitatively, chronic morphine administration resulted in a 30–40% reduction of EAAC1-ir and GLAST-ir in laminas I–II from that of saline-treated rats on day 8, and such reductions were morphine dose dependent (Fig. 2*A*,*B*). In addition, the level of reduction for both EAAC1-ir and GLAST-ir was comparable between two morphine treatment regimens (Fig. 2*A*,*B*). The downregulation of EAAC1 and GLAST expression also was revealed by the Western blot assay. There was a clear reduction of the EAAC1 and GLAST protein content in the corresponding Western blots after a 7 d infusion of 20 nM $\cdot \mu l^{-1} \cdot hr^{-1}$ morphine as compared with the saline control (Fig. 3). Thus, spinal EAAC1 and GLAST expression was downregulated dose dependently after two independent morphine treatment regimens.

The downregulation of EAAC1 and GLAST was prevented by coadministration of morphine (20 μg) with naloxone (10 μg) twice daily for 7 d (Figs. 1*C*,*F*, 2*C*), indicating that changes in spinal GTs were mediated through opioid receptors. The naloxone treatment alone did not affect the GT level (Fig. 2*C*). Naloxone (10 μg) also blocked the development of tolerance to morphine (20 μg) antinociception and thermal hyperalgesia in the same group of rats (Figs. 4*B*, 5*B*).

Temporal correlation between GT downregulation, morphine tolerance, and thermal hyperalgesia

Consistent with the downregulation of spinal GTs revealed by the immunocytochemical and Western blot assays, tolerance to the antinociceptive effects of morphine developed dose dependently in morphine- but not saline-treated rats (bolus or infusion) when tested on day 8 (Fig. 4*A*). In these same morphine-treated rats, the baseline paw-withdrawal latency to noxious radiant heat was dose-dependently reduced on day 8 as compared with the baseline latency before the morphine treatment (Fig. 5*A*), indicating the development of thermal hyperalgesia.

The time course between the GT downregulation and the development of morphine tolerance and thermal hyperalgesia was compared at days 2, 4, 6, and 8 of a repeated morphine (20 μg, twice daily) treatment regimen. Neither reduction of GTs

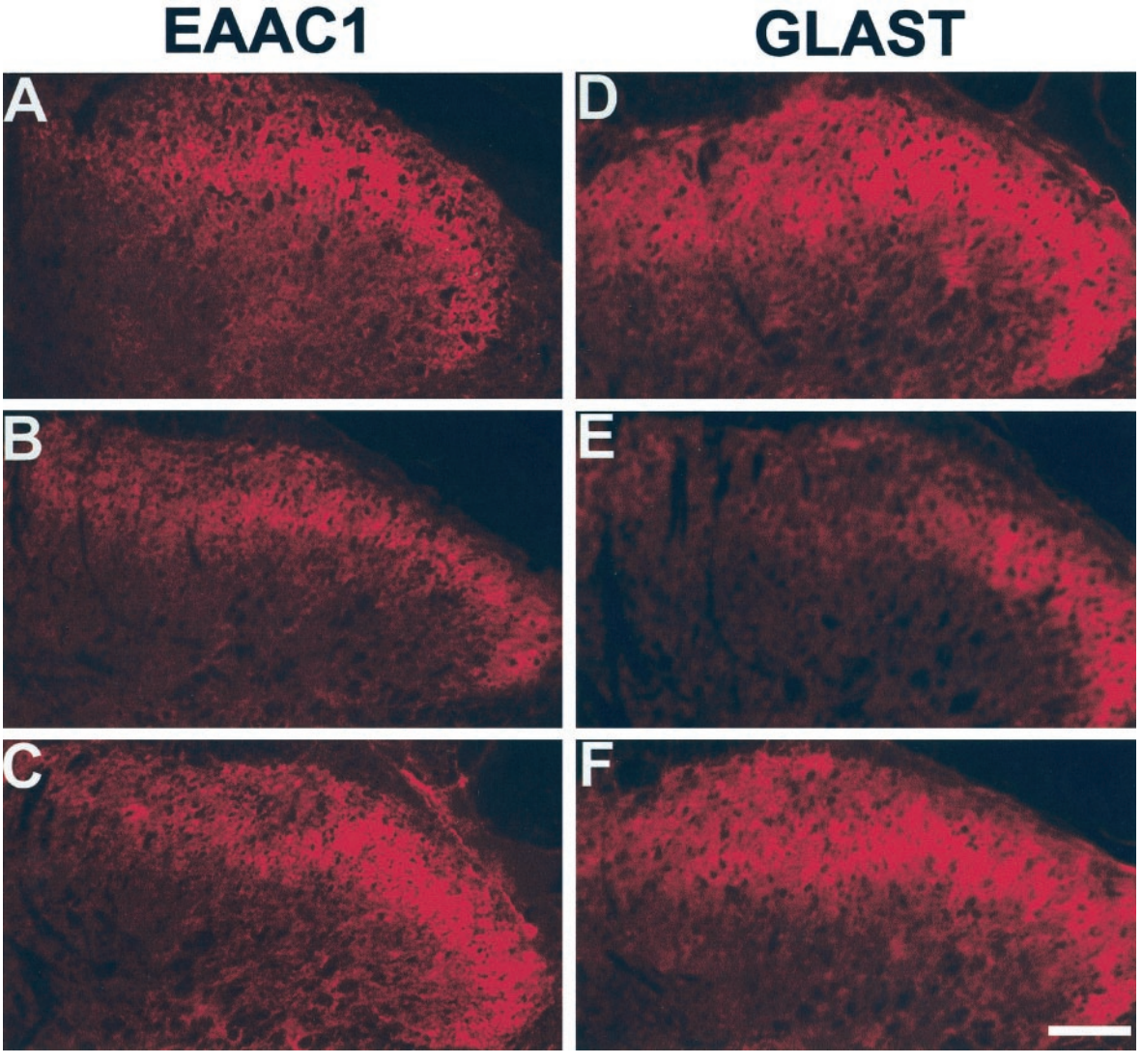

Figure 1. Downregulation of spinal GTs after chronic morphine. Both EAAC1-ir (*B*) and GLAST-ir (*E*) were reduced in rats receiving a 7 d intrathecal, twice daily treatment with 20 μg of morphine as compared with the corresponding saline control (*A, D*). Coadministration of morphine (20 μg) with naloxone (10 μg), twice daily for 7 d, blocked the reduction of both EAAC1-ir (*C*) and GLAST-ir (*F*). Scale bar, 50 μm.

(EAAC1 and GLAST) in Western blotting nor the development of morphine tolerance or thermal hyperalgesia was observed on days 2 and 4 (Fig. 6), and there was a transient increase in both EAAC1 and GLAST in Western blots at least on day 2 (Fig. 6). However, both EAAC1 and GLAST in Western blotting were clearly reduced on days 6 and 8 as compared with the saline group (Fig. 6). Consistent with the GT downregulation, the reduction of both morphine antinociception (tolerance) and baseline paw-withdrawal latency (hyperalgesia) also was present on days 6 and 8 (Fig. 7, Table 1). Together, these results indicate a temporal correlation between the GT downregulation and the development of morphine tolerance and associated thermal hyperalgesia after chronic morphine.

Exacerbation of the hyperalgesic response to exogenous glutamate after the GT downregulation

Thermal hyperalgesia in rats treated with morphine (20 $\text{nM} \cdot \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) was further exacerbated in response to exogenous glutamate (5 nM, i.t.), as compared with the corresponding saline group (Fig. 5*C*). The exacerbation of thermal hyperalgesia to exogenous glutamate was reflected by (1) an increased magnitude, i.e., a further reduction of the paw-withdrawal latency, and (2) a prolonged time course of hyperalgesia lasting at least 4 hr versus <2 hr in saline-treated rats (Fig. 5*C*). Moreover, riluzole (20 μg, a positive GT regulator), given at 30 min before administering 5 nM glutamate, attenuated the hyperalgesic response to exogenous glutamate (Fig. 5*C*). Riluzole (20 μg) alone in the absence of exogenous glutamate resulted in an increase in the baseline paw-withdrawal latency (10–12% from the baseline) in saline-treated rats (Fig. 5*C*). These results indicate that the downregulation of spinal GTs induced by chronic morphine has a functional impact on maintaining *in vivo* glutamate homeostasis within the spinal cord dorsal horn.

Potentiation of morphine tolerance and thermal hyperalgesia by the GT inhibitor PDC

The antinociceptive effect of morphine examined by the tail-flick test was significantly reduced beginning on day 4 in rats treated repeatedly with 10 μg of morphine plus 20 μg of PDC (Fig. 8*A*). In contrast, the antinociceptive effects of morphine were not

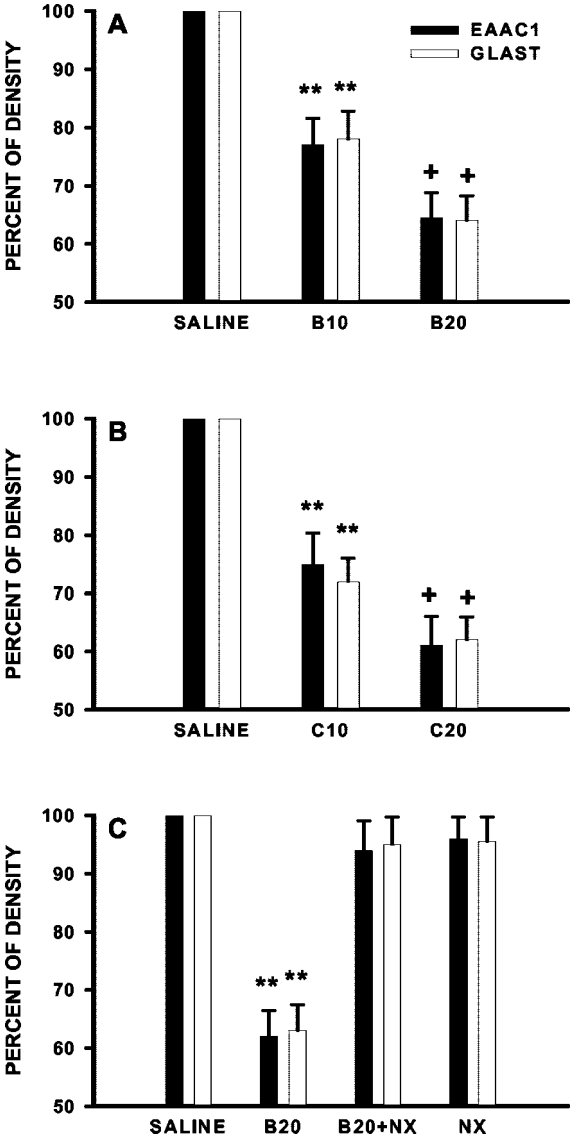

Figure 2. Quantification of the EAAC1-ir and GLAST-ir reduction. The relative density of immunostaining in laminas I–II was measured by subtracting the background density in each image. The percentage reduction of density from the corresponding saline group was calculated as described in Materials and Methods. *A*, *B10*, *B20*, 10 or 20 μg of morphine boluses; *B*, *C10*, *C20*, 10 or 20 nM · μl^{-1} · hr^{-1} morphine infusion; *C*, *B20*, 20 μg of morphine bolus alone; *B20+NX*, 20 μg of morphine plus 10 μg of naloxone boluses; *NX*, 10 μg of naloxone bolus alone. $^{**}p < 0.01$ as compared with the saline group and $^{+}p < 0.01$ as compared with the corresponding low morphine dose or saline groups.

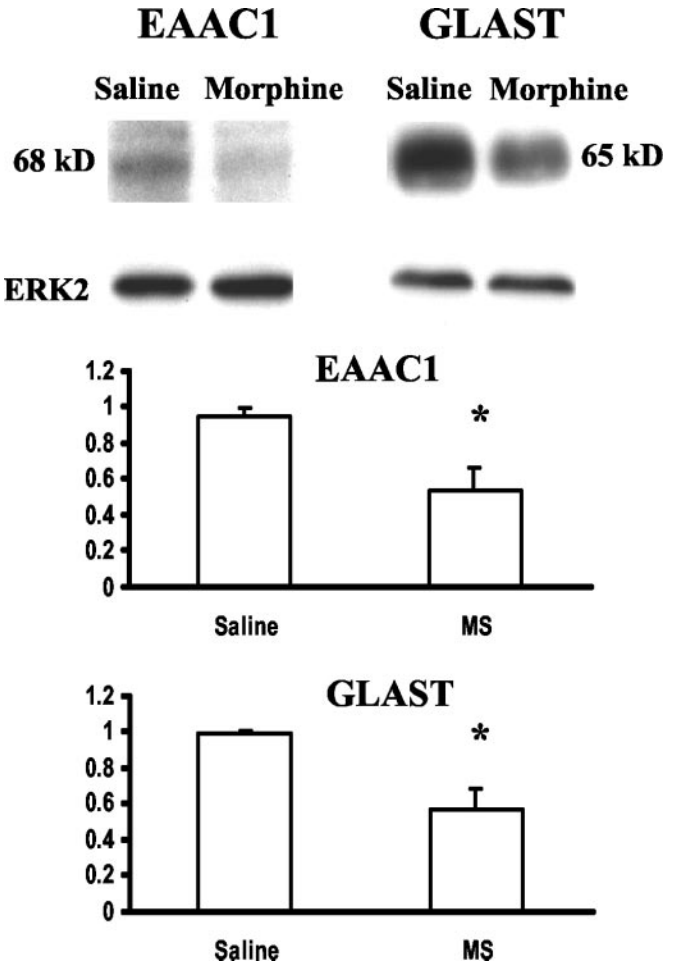

Figure 3. Morphine-induced EAAC1 and GLAST reduction in Western blotting. Both EAAC1 and GLAST protein contents were reduced in rats receiving a 7 d intrathecal infusion with 20 nM · μl^{-1} · hr^{-1} morphine as compared with the saline control. $^{*}p < 0.05$; two-tailed Student's *t* test. ERK2 is a loading control.

significantly reduced until day 6 in the morphine (10 μg) plus vehicle group (Fig. 8*A*). That is, the onset for the development of morphine tolerance was shortened in rats coadministered with morphine and PDC. The potentiation of morphine tolerance by PDC was further indicated by an increased rightward shift of the antinociceptive dose–response curve in the morphine plus PDC groups when tested on day 8 as compared with the morphine alone group (Fig. 8*B*, Table 2). Taken together, the GT inhibitor PDC potentiated the development of morphine tolerance by shortening the onset and enhancing the level of antinociceptive tolerance.

Although repeated intrathecal treatment with PDC (20 μg) alone for 7 d did not produce detectable changes in the baseline tail-flick latency, this treatment reduced the baseline paw-withdrawal latency on day 8 (Fig. 9*A*), indicating a role of PDC in regulating GT activity. However, there was a further reduction of the paw-withdrawal latency in the morphine (10 μg) plus PDC (20 μg) group, as compared with either the PDC or morphine alone group (Fig. 9*A*). Thus, PDC also potentiated the development of thermal hyperalgesia associated with morphine tolerance.

Reduction of morphine tolerance and thermal hyperalgesia by the positive GT regulator riluzole

There was a nearly fivefold rightward shift of the morphine antinociceptive dose–response curve in rats receiving morphine (10 μg) plus vehicle twice daily for 7 d. The positive GT regulator riluzole dose-dependently reduced the rightward shift of the dose–response curve (Fig. 8*C*, Table 2). Consistent with this observation, reduced antinociception as demonstrated in the morphine plus vehicle group on day 6 was absent in those rats receiving intrathecal coadministration of morphine (10 μg) with riluzole (20 μg) for 5 d (Fig. 8*A*), indicating that riluzole also prolonged the onset of the tolerance development. Moreover, although repeated intrathecal treatment with 20 μg of riluzole alone for 7 d had a negligent effect on the baseline tail-flick latency, it did moderately raise the paw-withdrawal latency on day

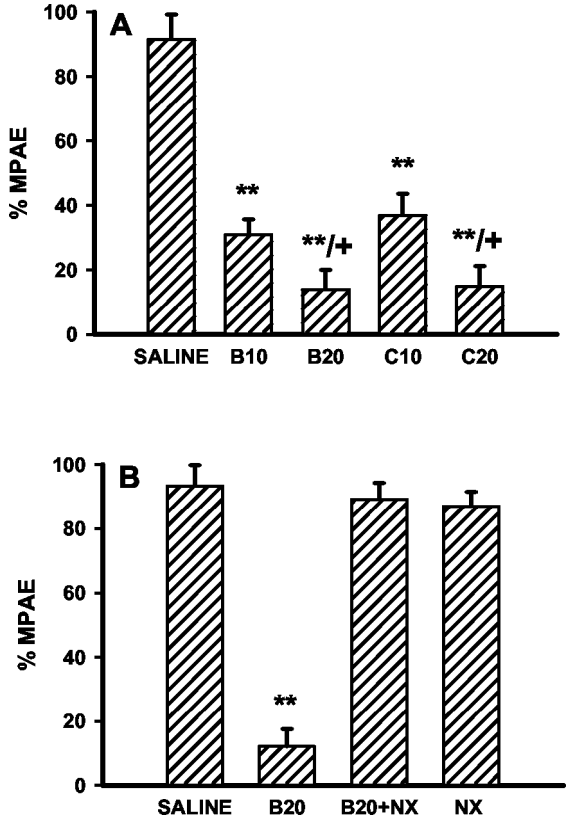

Figure 4. Development of morphine tolerance and its blockade by naloxone. *A*, Morphine antinociception was dose-dependently reduced on day 8 in rats receiving a 7 d intrathecal morphine treatment of either twice daily boluses or continuous infusion. *B*, Coadministration of morphine (20 μg) with naloxone (10 μg) for 7 d blocked the development of morphine tolerance (*B20+NX*), and a 7 d naloxone (10 μg; *NX*) treatment alone did not affect the morphine antinociception. See Figure 2 for the details of each group. $^{**}p < 0.01$ as compared with the saline group, and $^{+}p < 0.05$ as compared with the corresponding low morphine dose group.

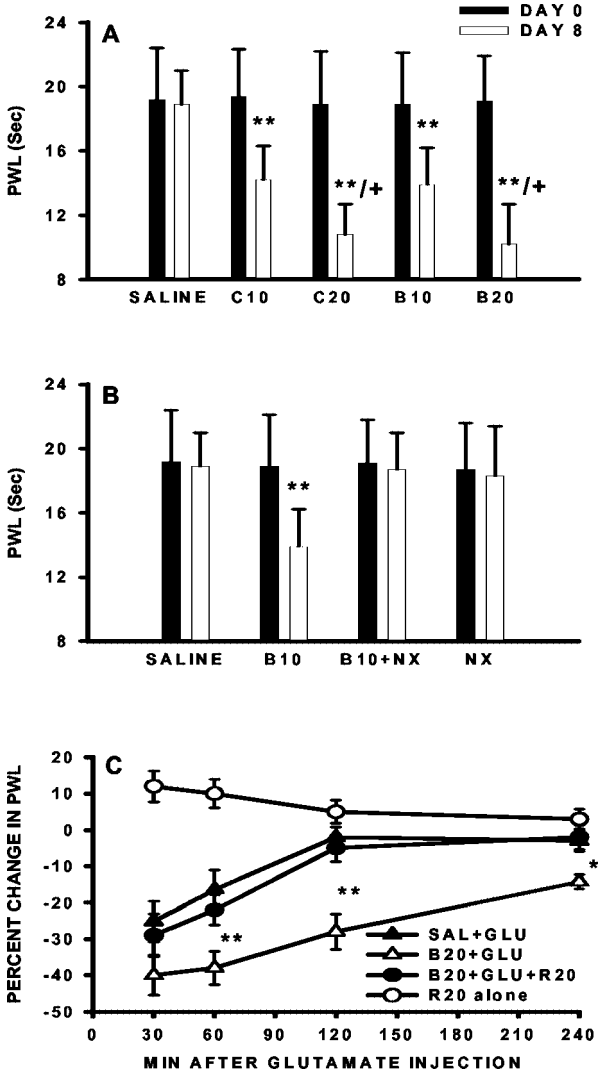

Figure 5. Development of thermal hyperalgesia and its reversal by riluzole. *A*, The paw-withdrawal latency (*PWL*) was reduced on day 8 in the absence of exogenous glutamate as compared with that on day 1 in rats receiving either 10 (*B10*) or 20 μg (*B20*) of morphine boluses or continuous infusion of 10 (*C10*) or 20 nM · μl^{-1} · hr^{-1} (*C20*) morphine for 7 d. *B*, Coadministration of morphine (20 μg) with naloxone (10 μg) for 7 d blocked the development of thermal hyperalgesia (*B20+NX*), and a 7 d naloxone (10 μg; *NX*) treatment alone did not affect baseline paw-withdrawal latency. *C*, The response to intrathecal 5 nM glutamate was exacerbated in morphine-infused rats (*B20+GLU*) as compared with saline-treated rats (*SAL+GLU*). A single intrathecal pretreatment with 20 μg of riluzole at 30 min before the glutamate injection attenuated the hyperalgesia (*B20+GLU+R20*). Riluzole alone (*R20 alone*) transiently increased the baseline paw-withdrawal latency. The data were presented as the percentage change of the paw-withdrawal latency from that of before the glutamate treatment on day 8 in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with the corresponding *SAL+GLU* group. The paw-withdrawal latency in the *R20 alone* group was compared before and after riluzole treatment, and its change did not reach statistical significance.

8 at the current dose (Fig. 9*A*). Similar to the effect of PDC on thermal hyperalgesia, coadministration of morphine (10 μg) and riluzole (20 μg) for 7 d significantly reduced the development of thermal hyperalgesia as compared with either the morphine or riluzole alone group (Fig. 9*A*).

Riluzole, however, did not reverse the behavioral manifestation of morphine tolerance once it had developed. Thus, an acute injection of riluzole (20 μg), given at 30 min before the behavioral test of morphine antinociception on day 8, failed to restore the antinociceptive effects of morphine in rats receiving repeated morphine (20 μg) treatment for 7 d (Fig. 9*B*). The results indicate that changes in spinal GT activity are contributory to the development of morphine tolerance.

Inhibition by MK-801 of morphine tolerance and thermal hyperalgesia potentiated by PDC

The noncompetitive NMDAR antagonist MK-801 (10 nM), given intrathecally with morphine (10 μg) or morphine (10 μg) plus PDC (20 μg) for 7 d, effectively blocked the development of morphine tolerance (Fig. 9*C*). The same treatment regimen also prevented the development of thermal hyperalgesia in the same rats when tested on day 8 (paw-withdrawal latencies: saline, 18.7 ± 2.1 sec; 10 μg of morphine, 13.1 ± 1.9 sec; 10 μg of morphine/20 μg of PDC, 7.2 ± 2.1 sec; 10 μg of morphine/20 μg of PDC/10 nM MK-801, 18.4 ± 2.0 sec; $p > 0.05$ as compared with the saline group). As a control, repeated intrathecal treatment with MK-801 (10 nM) alone for 7 d did not alter the baseline tail-flick or paw-withdrawal latency or the acute antinociceptive effects of morphine (Fig. 9*C*). The results indicated that NMDARs play a role in the development of morphine tolerance and thermal hyperalgesia that were potentiated by PDC.

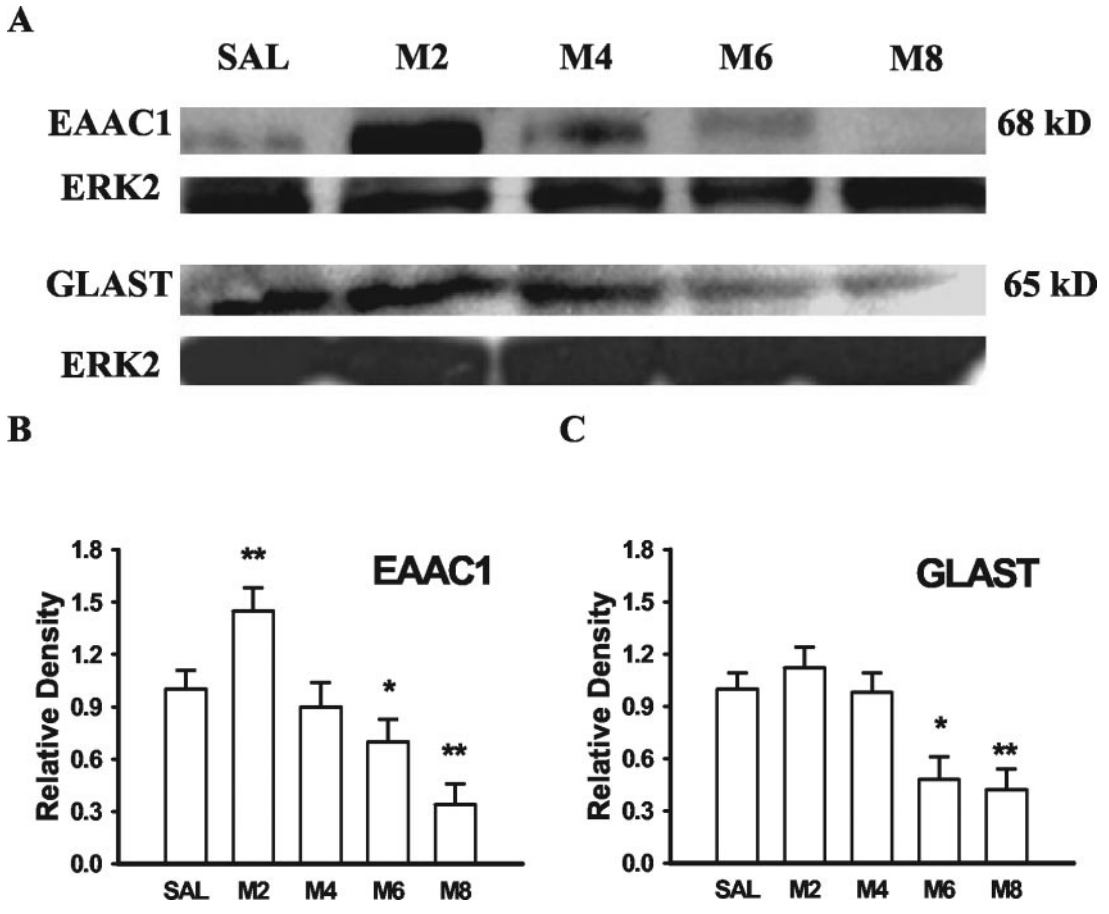

Figure 6. Time course of EAAC1 and GLAST changes after chronic morphine. Both EAAC1 and GLAST protein contents were progressively reduced after twice daily intrathecal treatment with 20 μg of morphine. The *M2* to *M8* groups stand for rats receiving 20 μg of morphine, and their spinal cords were harvested at day 2, 4, 6, or 8 of the treatment period. $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with the corresponding saline group. ERK2 is a loading control.

DISCUSSION

The present results indicate that chronic morphine induces downregulation of spinal GTs, which contributes to the development of morphine tolerance and associated thermal hyperalgesia. First, the time course of morphine-induced GT downregulation correlated with that of the behavioral manifestation of morphine tolerance and thermal hyperalgesia. Second, morphine-induced downregulation of spinal GTs reduced the ability to maintain *in vivo* regional glutamate homeostasis, as shown by the development of thermal hyperalgesia in morphine-tolerant rats in the absence of exogenous glutamate and the exacerbated thermal hyperalgesia in response to exogenous glutamate. Third, perturbation of spinal GT activity by PDC or riluzole modulated the development of both morphine tolerance and associated thermal hyperalgesia. Fourth, the noncompetitive NMDAR antagonist MK-801 prevented the development of morphine tolerance and thermal hyperalgesia potentiated by the GT inhibitor PDC, indicating that the role of spinal GTs in morphine tolerance and thermal hyperalgesia is mediated at least in part through NMDARs.

Overall consideration on data interpretation

This series of experiments extended previous *in vitro* findings of synaptic glutamate regulation by GTs to examine the relationship between changes in spinal GT expression and activity and the development of morphine tolerance and associated thermal hyperalgesia under an *in vivo* experimental condition. Several lines of evidence including the exacerbated hyperalgesia to exogenous glutamate in rats with reduced GTs and its reduction by riluzole support a functional link between changes in GT expression and activity and the regulation of *in vivo* glutamate homeostasis. This is consistent with the role of GTs in regulating glutamate uptake at the synaptic level in previous *in vitro* studies (Semba and Wakuta, 1998; Azbill et al., 2000; Jabaudon et al., 2000; Lievens et al., 2000; Matthews et al., 2000). A caveat is that although PDC has been used extensively as a GT inhibitor, riluzole as a specific GT activator was controversial (Cheramy et al., 1992; Martin et al., 1993; Doble, 1996). This agent has recently been shown to be a positive regulator of GT activity that increases glutamate uptake in synaptosomes when given under both *in vitro* and *in vivo* conditions (Azbill et al., 2000). However, neither PDC nor riluzole is selective for neuronal or glial GT activity. As such, the effects from PDC and riluzole should be considered to be on both neuronal and glial GTs, and other GTs such as GLT-1 not examined in this study may also play a role in morphine tolerance and thermal hyperalgesia.

An interesting observation in morphine-tolerant rats is the development of abnormal nociceptive sensitivity shown as thermal hyperalgesia that was regulated by spinal GT activity. Hyperalgesia is a known sign of naloxone-precipitated withdrawal indicative of physical dependence after chronic morphine treat-

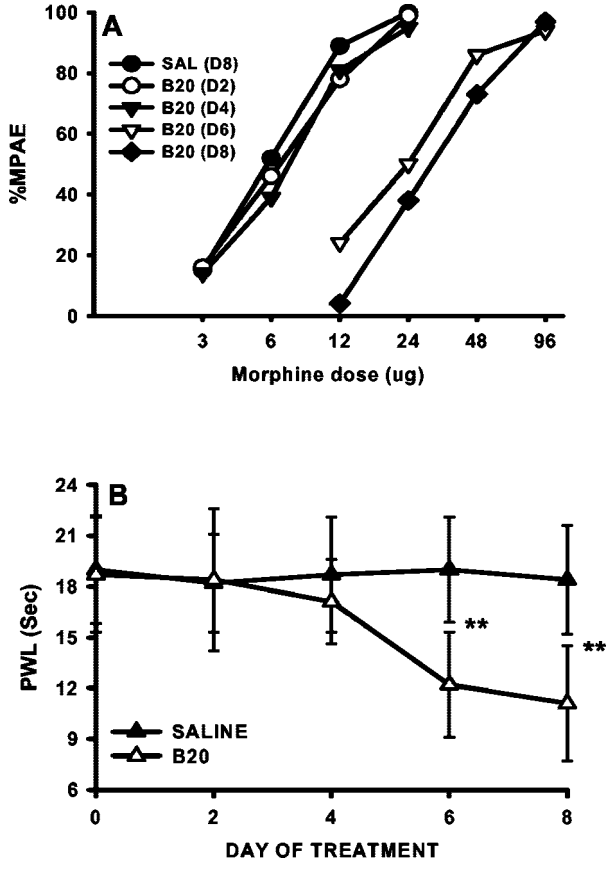

Figure 7. Time course of the development of morphine tolerance and thermal hyperalgesia after chronic morphine. Both morphine tolerance and thermal hyperalgesia developed on days 6 (*D6*) and 8 (*D8*) after twice daily intrathecal treatment with 20 μg of morphine. Note that the time course of behavioral changes correlated with that of EAAC1 and GLAST changes in Western blot analysis (Fig. 6). $^{**}p < 0.01$, as compared with the corresponding saline group.

Table 1. Time course of morphine tolerance

Group	AD_{50} (μg)	95% CI (μg)
Saline	5.1	1.6–7.9
M2	5.8	2.9–8.2
M4	8.1	3.5–15.2
M6	53.5	31.2–88.2
M8	67.1	37.7–101.4

The M2 to M8 groups stand for rats receiving 20 μg of morphine twice daily, and their spinal cords were harvested at day 2, 4, 6, or 8 of the treatment period. Note that both M6 and M8 groups were significantly different from the remaining groups based on the AD_{50} value and 95% CI.

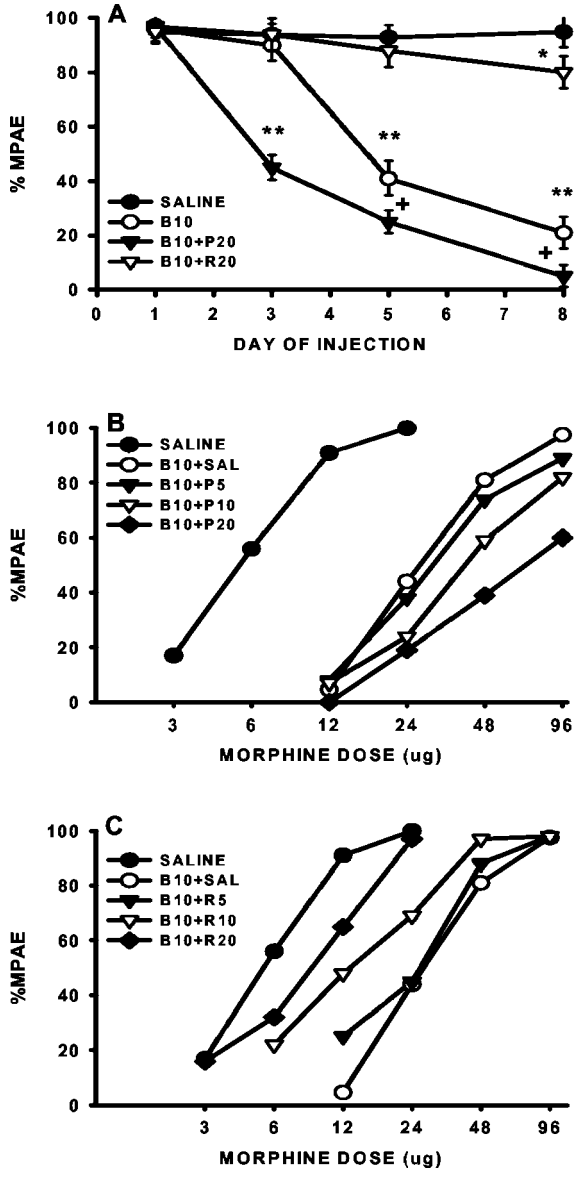

Figure 8. Regulation of morphine tolerance by the GT inhibitor PDC and activator riluzole. *A*, The onset for the development of morphine tolerance was shortened by coadministration of 10 μg of morphine (*B10*) with 20 μg of PDC (*B10+P20*) but prolonged by coadministration of 10 μg of morphine (*B10*) with 20 μg of riluzole (*B10+R20*). $^{**}p < 0.01$, as compared with the saline group, and $^{+}p < 0.05$, as compared with the morphine alone group. *B*, *C*, The cumulative dose–response curves were shifted dose dependently to the right in rats treated with 10 μg of morphine (*B10*) with 5, 10, or 20 μg of PDC (*B10+P5*, *B10+P10*, *B10+P20*), whereas the dose–response curves were shifted dose dependently to the left in rats treated with 10 μg of morphine with 5, 10, or 20 μg of riluzole (*B10+R5*, *B10+R10*, *B10+R20*), as compared with the rats receiving either saline alone or 10 μg of morphine plus saline (*B10+SAL*).

ment (Mao et al., 1994; Jhamandas et al., 1996). A group of recent studies have shown the development of thermal hyperalgesia in association with the development of morphine tolerance in the absence of naloxone-precipitated withdrawal (Mao et al., 1994; Ossipov et al., 1995; Wegert et al., 1997; Vanderah et al., 2000). Although there is a possibility that thermal hyperalgesia after opioid boluses could result from mini-withdrawals (Jhamandas et al., 1996), thermal hyperalgesia also has been observed in rats infused continuously with morphine via an osmotic pump at the time of the hyperalgesia test (Vanderah et al., 2000). This is also observed in the present study, showing a comparable degree of thermal hyperalgesia on day 8 between rats receiving either morphine boluses or continuous infusion via an implanted osmotic pump, and the pump infusion was not disconnected during the hyperalgesic test on day 8.

Of interest to note is that PDC or riluzole treatment alone for 7 d changed the baseline paw-withdrawal latency in morphine-naïve rats, albeit less profoundly as compared with that of morphine-treated rats, although changes in the tail-flick latency in

Table 2. Effect of PDC and riluzole on morphine tolerance

Group	AD_{50} (μg)	95% CI (μg)
Saline	4.8	1.3–8.2
B10+SAL	22.6	6.9–38.2
B10+P5	24.4	3.5–45.2
B10+P10	30.5	5.6–66.8
B10+P20	68.1	27.7–108.5
B10+R5	19.5	14.4–53.4
B10+R10	10.5	4.8–25.7
B10+R20	6.1	1.4–16.3

See the Figure 8 legend for the details for each group. Note that both B10+P20 and B10+R20 groups were significantly different from the B10+saline group based on the AD_{50} value and 95% CI.

these same rats were not detected. This difference is likely attributable to the fast-rising temperature in the tail-flick test as compared with that in the paw-withdrawal test (Mao et al., 1994). Thus, the data suggest that spinal GT activity, in addition to the GT expression, could contribute to the behavioral manifestation of thermal hyperalgesia in morphine-treated rats. Indeed, a single pretreatment with riluzole attenuated thermal hyperalgesia to exogenous glutamate in morphine-treated rats, indicating that enhancing the activity of existing GTs was able to compensate for, at least in part, the GT downregulation resulting from chronic morphine administration.

Relation to mechanisms of morphine tolerance and associated thermal hyperalgesia

To date, several intriguing hypotheses have been proposed concerning the cellular and molecular mechanisms of opioid tolerance, including recent findings of the role of β-arrestin, excitatory amino acid receptors including NMDARs, and μ-opioid receptor oligomerization/endocytosis (Guitart and Nestler, 1989; Marek et al. 1991a; Trujillo and Akil, 1991; Nestler, 1992; Bohn et al., 1999, 2000; Whistler and von Zastrow, 1999; Finn and Whistler, 2001; He et al., 2002; Kieffer and Evans, 2002). With regard to the role of NMDARs, previous *in vitro* studies have suggested that NMDARs may be primed (i.e., increased excitability) after exposure to morphine via activation of intracellular protein kinase C (PKC) (Chen and Huang, 1991; Mao et al. 1995c). PKC may directly or indirectly modulate NMDARs by removing the Mg^{++} blockade from the NMDAR-Ca^{2+} channel site (Chen and Huang, 1992) and regulating NMDAR trafficking and gating (Xiong et al., 1998; Lan et al., 2001). The present findings of morphine-induced GT downregulation and its relation to the regulation of morphine tolerance and associated hyperalgesia that were preventable by the NMDAR inhibition provides additional evidence for the NMDAR involvement in this process.

There is a basal level of extracellular glutamate (Jhamandas et al., 1996) that is actively and tightly regulated by GTs (Robinson and Dowd, 1997; Danbolt, 2001). Chronic morphine induces downregulation of spinal GTs leading to the reduced ability to maintain regional glutamate homeostasis as indicated in the present *in vivo* study. That is, morphine-induced GT downregulation would increase the availability of extracellular glutamate, although such changes may not necessarily be seen as a gross increase in the regional glutamate level (Jhamandas et al., 1996). Increased glutamate availability at the extracellular level would increase the probability of excitatory amino acid receptor activation including NMDARs. Conceivably, activation of NMDARs under such circumstances could make contributions to the previously proposed intracellular mechanisms of morphine tolerance that involve PKC, cAMP, and nitric oxide (Kolesnikov et al., 1993; Elliott et al., 1994a,b; Mao et al. 1995c; Mayer et al., 1995; Bilsky et al., 1996; Narita et al., 1996, 2001; Ma et al., 2001; Zeitz et al., 2002).

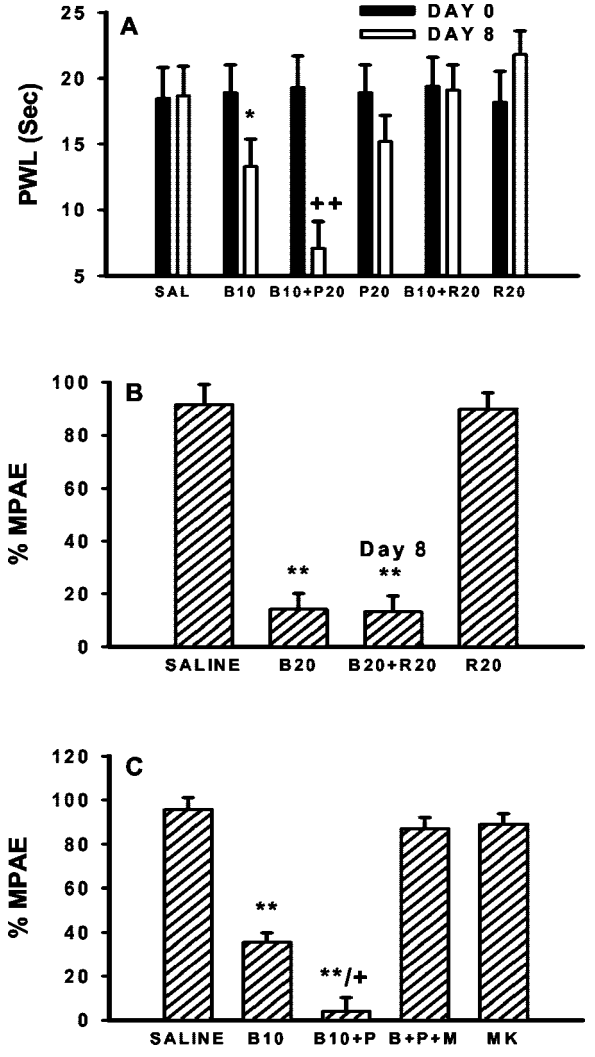

Figure 9. Inhibition by MK-801 of morphine tolerance and thermal hyperalgesia potentiated by PDC. *A*, The development of thermal hyperalgesia was potentiated in rats treated with 10 μg of morphine plus 20 μg of PDC (*B10+P20*) but prevented in rats receiving 10 μg of morphine plus 20 μg of riluzole (*B10+R20*). PDC or riluzole alone changed baseline paw-withdrawal latencies (*PWL*) on day 8 but did not reach the statistical significance at the current dose. *B*, The morphine antinociception was dose-dependently reduced on day 8 in rats receiving 7 d intrathecal 20 μg of morphine boluses (*B20*). The GT activator riluzole (20 μg), given intrathecally at 30 min before the morphine antinociceptive test on day 8 (*B20+R20*; *Day 8*), did not reverse the behavioral manifestation of morphine tolerance. *C*, The development of morphine tolerance was potentiated by intrathecal coadministration of 10 μg of morphine with 20 μg of PDC (*B10+P*) twice daily for 7 d. This potentiation was blocked by adding 10 nM MK-801 into this combination (*B+P+M*). Treatment with 10 nM MK-801 alone (*MK*) for 7 d did not change the antinociceptive effects of morphine. $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with the corresponding saline group, and $^{+}p < 0.05$, $^{++}p < 0.01$, as compared with the corresponding morphine alone group.

Several issues are noteworthy with regard to contributions of morphine-induced GT changes to the neural mechanisms of morphine tolerance and associated hyperalgesia. First, changes in

regional glutamate availability caused by the GT downregulation would modulate the activity of excitatory amino acid receptors including NMDARs at both presynaptic and postsynaptic sites, considering that NMDARs as well as μ-opioid receptors are located both presynaptically and postsynaptically (Yaksh, 1986; Liu et al., 1994). This extends previous views of NMDA and μ-opioid receptor interactions that have focused on the postsynaptic site (Mao et al. 1995b). Second, besides the interaction between NMDA and μ-opioid receptors within a single cell as demonstrated by *in vitro* studies (Chen and Huang, 1991), such an interaction could also occur involving neural circuits (Mao et al., 1994; Zeitz et al., 2002). Changes in regional glutamate availability from the downregulation of both glial and neuronal GTs would support such mechanisms. Third, both basic and clinical research have demonstrated the association between chronic opioid treatment and the development of abnormal pain sensitivity, and NMDAR activation is contributory to such an association (Sjogren et al., 1993; Mao et al., 1994; Ossipov et al., 1995; Devulder, 1997; Wegert et al., 1997; Vanderah et al., 2000; Celerier et al., 2001). Morphine-induced GT downregulation would play an important role in the association between morphine tolerance and hyperalgesia, because both tolerance and hyperalgesia are preventable by blocking NMDARs (Mao et al. 1995b). Fourth, the cellular and molecular mechanisms of opioid tolerance are complex, and multiple mechanisms are likely to be involved depending on opioid receptor agonists, route of treatment, assay methods, and clinical relevance (Kieffer and Evans, 2002). Of significance is that morphine-induced GT downregulation and its functional role may help explain the interaction between two opioid-related clinical observations: analgesic tolerance and associated abnormal pain sensitivity. It remains to be seen how morphine-induced GT downregulation would interact with other proposed cellular mechanisms of opioid tolerance.

Potential mechanisms of morphine-induced GT downregulation

The cellular mechanisms of morphine-induced downregulation of spinal GTs remain to be investigated. There are at least two possibilities of GT regulation by chronic morphine. Spinal GT expression could be regulated by extracellular glutamate (Danbolt, 2001). This is suggested by the observations that downregulation of GLT-1 and GLAST occurs in the rat's brain regions after an impaired cortical glutamatergic connection (Ginsberg et al., 1995), and conversely, that an increase in extracellular glutamate upregulates GLT-1 in astroglial cultures (Thorlin et al., 1998). If this is the case, a decreased level of extracellular glutamate resulting from the inhibitory effect of morphine on neurotransmitter (e.g., glutamate) release (Yaksh, 1986) could lead to a simultaneous downregulation of both EAAC1 and GLAST to maintain regional glutamate homeostasis. The present data showing a time course of progressive GT downregulation after chronic morphine would lend some support to this possibility. However, this regulation would be difficult to explain a transient increase in GT expression after morphine treatment as seen in the present study.

Another possibility is that morphine could regulate GTs via opioid receptor-mediated intracellular changes such as cAMP (She et al., 2000; Wang and Sadee, 2000), because cAMP has been shown to regulate the expression of GLT-1 and GLAST in cell cultures (Swanson et al., 1997; Schlag et al., 1998). This possibility is supported by the reduced GLT-1 mRNAs in response to a δ-opioid agonist acting directly on cultured glial cells (Thorlin et al., 1998). In addition, previous studies have shown the colocalization of μ-opioid receptors and glial cells at both developmental and adult stages (Ruzicka et al., 1995; Ruzicka and Akil, 1997; Stiene-Martin et al., 1998, 2001; Thorlin et al., 1999; Tryoen-Toth et al., 2000). Nonetheless, glutamate regulation and opioid receptor-mediated intracellular changes could each play a role in morphine-induced GT changes, and both possibilities merit future investigation.

Clinical implications

The present findings indicate a functional role of spinal GTs in the development of morphine tolerance and associated thermal hyperalgesia and suggest a new strategy for preventing opioid tolerance and the associated abnormal pain sensitivity by regulating regional glutamate homeostasis using a GT regulator such as riluzole. Furthermore, the present study may provide some insights into the neural mechanisms of substance abuse. Activation of NMDARs has been shown to play a role in the neural mechanisms of many forms of substance abuse (De Montis et al., 1992; Churchill et al., 1999; Huber et al., 2001). Thus, a corollary of the present data is that the involvement of NMDARs in substance abuse could be related to changes of brain GTs after exposure to a substance of abuse. This may be particularly relevant to the mechanisms of heroin addiction, because heroin metabolites (6-monoacetylmorphine or morphine) do indeed interact with opioid receptors (Sim-Selley et al., 2000; Kreek, 2001).

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