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Purinergic P2X Receptor Regulates *N*-Methyl-D-aspartate Receptor Expression and Synaptic Excitatory Amino Acid Concentration in Morphine-tolerant Rats

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ABSTRACT

Background: The present study examined the effect of P2X receptor antagonist 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) on morphine tolerance in rats.

Methods: Male Wistar rats were implanted with two intrathecal catheters with or without a microdialysis probe, then received a continuous intrathecal infusion of saline (control) or morphine (tolerance induction) for 5 days.

Results: Long-term morphine infusion induced antinociceptive tolerance and up-regulated *N*-methyl-D-aspartate receptor subunits NR1 and NR2B expression in both total lysate and synaptosome fraction of the spinal cord dorsal horn. TNP-ATP (50 μg) treatment potentiated the antinociceptive effect of morphine, with a 5.5-fold leftward shift of the morphine dose–response curve in morphine-tolerant rats, and this was associated with reversal of the up-regulated NR1 and NR2B subunits in the synaptosome fraction. NR1/NR2B–specific antagonist ifenprodil treatment produced a similar effect as TNP-ATP; it also potentiated the antinociceptive effect of morphine. On day 5, morphine challenge resulted in a significant increase in aspartate and glutamate concentration in the cerebrospinal fluid dialysates of morphine-tolerant rats, and this effect was reversed by TNP-ATP treatment. Moreover, the amount of immunoprecipitated postsynaptic density-95/NR1/NR2B complex was increased in morphine-tolerant rats, and this was prevented by the TNP-ATP treatment.

Conclusions: The findings suggest that attenuation of morphine tolerance by TNP-ATP is attributed to down-regulation of *N*-methyl-D-aspartate receptor subunits NR1 and NR2B expression in the synaptosomal membrane and inhibition of excitatory amino acids release in morphine-tolerant rats. The TNP-ATP regulation on the *N*-methyl-D-aspartate receptor expression may be involved in a loss of scaffolding proteins postsynaptic density-95.

What We Already Know about This Topic

- P2X receptors, a family of ion channels activated by extracellular adenosine 5′-triphosphate, may participate in neuropathic and inflammatory pain and present targets for analgesia.

What This Article Tells Us That Is New

- Treatment of rodents with a P2X receptor antagonist diminished opioid tolerance in part by down-regulating glutamate receptors and inhibiting excitatory amino acid release.

OPIOIDS, such as morphine, are a class of powerful analgesics used for treating moderate to severe pain in the clinic. However, long-term administration induces tolerance, which hampers their clinical use.[1] Morphine tolerance is a complex physiologic response; in addition to opioid receptor uncoupling and endocytosis/desensitization,[2,3] glutamatergic receptor activation and neuroinflammation has been demonstrated by ourselves and others.[4–7]

The excitatory amino acids (EAAs), glutamate and aspartate, are the principal excitatory neurotransmitters in the central nervous system and have a variety of functions, including nociceptive transmission and modification.[8] The glutamatergic receptor system, especially the *N*-methyl-D-aspartate (NMDA) receptor, plays an important role in synaptic plasticity and chronic pain formation.[9] NMDA receptors are tetrameric hetero-oligomers consisting of the essential NR1 subunit and one or more modulatory

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NR2A-D and NR3 subunits. Activation of spinal NMDA receptors plays a crucial role in the development of morphine tolerance.[4,10] Pharmacological blockade of NMDA receptors or disruption of the NR1 subunit gene significantly attenuates morphine tolerance,[11,12] suggesting an involvement of NMDA receptors in morphine tolerance.

P2X receptors are a family of ligand-gated ion channels activated by extracellular adenosine 5′-triphosphate (ATP) that are involved in pain mechanisms.[13] The $P2X_3$ and $P2X_{2/3}$ receptors located on primary afferent nerve terminals in the inner lamina II of the spinal cord play a significant role in neuropathic and inflammatory pain.[14,15] A number of studies have demonstrated the therapeutic potential of modulating P2X receptors in treating neuropathic pain.[16] Intrathecal administration of ATP produces long lasting allodynia, probably through $P2X_{2/3}$ receptors.[17] Studies using gene knockout, antisense oligonucleotides, or the selective $P2X_3$ antagonist A-317491 indicate that ATP and $P2X_3$ receptors are involved in chronic pain, particularly chronic inflammatory and neuropathic pain.[15,18–20] McGaraughty *et al.*[21] reported that antagonism of $P2X_3$ and $P2X_{2/3}$ receptors reduces inflammatory hyperalgesia and chemogenic nociception, possibly through the spinal opioid receptor system. Mao *et al.*[22] suggested that neuropathic pain and morphine tolerance share common mechanisms of nociception sensitization and morphine resistance. The present study examined the effect of the P2X receptor antagonist 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) on morphine tolerance and its possible mechanism.

Materials and Methods

Animal Preparation and Intrathecal Drug Delivery

All experiments conformed to the Guiding Principles in the Care and Use of Animals of the American Physiology Society and were approved by the National Defense Medical Center Animal Care and Use Committee (National Defense Medical Center, Taipei, Taiwan). Intrathecal catheters and microdialysis probe implantation were performed as described previously.[7] In brief, male Wistar rats (350–400 g) were anesthetized with phenobarbital (60 mg/kg, intraperitoneally) and implanted with two intrathecal catheters (8.5 cm) with or without a microdialysis loop probe *via* the atlantooccipital membrane down to the lumbar enlargement L1–L2 of the spinal bony structure. The levels of L1–L2 spinal bony structure correspond to the spinal cord segments of L5, L6, and S1–S3, which are responsible for the tail-flick reflex.[23] One intrathecal catheter was connected to a mini-osmotic pump for infusion of saline (1 μl/h) (Sal rats) or morphine (15 μg/h) (MO rats) for 5 days, whereas the other was used for the subsequent injection of saline (Sal/Sal or MO/Sal rats) or TNP-ATP (Sal/TNP-ATP or MO/TNP-ATP rats) or ifenprodil (Sal/IFE or MO/IFE rats). On day 5 after development of morphine tolerance, the rats were injected with either TNP-ATP (50 μg or 12.5–50 μg as indicated) or saline (as control) or ifenprodil (10 μg/5 μl, intrathecally), then 30 min later, a single dose of morphine (15 μg/5 μl, intrathecally) was injected and its antinociceptive effect measured. All rats were maintained on a 12-h light/dark cycle with food and water freely available. Rats with neurologic deficits were excluded from the study. All drugs were purchased from Sigma (St. Louis, MO). Preliminary results showed no abnormal motor function after intrathecal injection of test drugs (data not shown).

Construction of the Spinal Cord Microdialysis Probe

The technique for spinal microdialysis probe construction was modified from that in a previous study.[24] The probe was constructed using two 7-cm PE5 tubes (0.008 inch ID, 0.014 inch OD; Spectranetics, Colorado Springs, CO) and a 4.2-cm cuprophan hollow fiber (Hospal Co, Lyon, France). A Formvar-insulated Nichrome wire (0.0026 inch outer diameter; A-M System, Everett, WA) was passed through a polycarbonate tube (194 μM OD, 102 μM ID; 0.7 cm in length) and the cuprophan hollow fiber (active dialysis region), which was then connected to a PE5 catheter using epoxy glue. The middle portion of the cuprophan hollow fiber was bent to form a U-shaped loop, and both ends of the dialysis loop, which consisted of silastic tubes, were sealed with silicone. The dead space of the dialysis probe was 8 μl. During *in vitro* measurements, the recovery rates of the probes were around 40% at an infusion rate of 5 μl/min.

Behavioral Tests

The tail-flick latency was measured using the hot water immersion test (52 ± 0.5°C) with the rats placed in plastic restrainers. The average baseline tail-flick latency was 2 ± 0.5 s in naive rats, and the cut-off time was 10 s. The percentage of the maximal possible antinociceptive effect was calculated as (maximum latency − baseline latency)/(cut off latency − baseline latency) × 100. Antinociceptive dose-response curves were constructed for each study group.

Cerebrospinal Fluid Sample Collection and Measurement of Excitatory Amino Acids

One of the externalized silastic tubes was connected to a syringe pump (CMA Microdialysis AB, Solna, Sweden) and perfused with Ringer's solution (8.6 mg/ml NaCl, 0.33 mg/ml $CaCl_2$, and 0.3 mg/ml potassium chloride). The cerebrospinal fluid (CSF) dialysates were collected from the other externalized silastic tube of the microdialysis probe using a standard procedure of a 50 min washout period, followed by a 30-min sample collection period at a flow rate of 5 μl/min in a polypropylene tube on ice, and were frozen at −80°C until assayed. The concentrations of EAAs were measured by phenylisothiocyanate derivatization using a high-performance liquid chromatography (Agilent 1100; Agilent Technologies, Santa Clara, CA) with a reversed-phase ZORBAX Eclipse amino acid analysis column (4.6 × 150 mm^2, 3.5 μM) and fluorescent detector (Gilson model 121, set at 428 nm), as described previously.[25] External standards (authentic amino acids at concentrations of 156.25,

312.5, 625, 1,250, and 2,500 μM) were run at the beginning and end of each sample group. Peak heights were normalized to the standard peaks and quantified based on the linear relationship between peak height and the amount of the corresponding standard.

Preparation of Spinal Cord Total Lysate and Synaptosomal Membrane and Cytosolic Fractions and Western Blot Analysis

After drug treatment, as described in the animal preparation and intrathecal drug delivery section, rats were sacrificed by exsanguination under isoflurane (ABBOTT, Abbott Laboratories Ltd., Queenborough, Kent, United Kingdom) anesthesia and the laminectomy was performed at the lower edge of the 12th thoracic vertebra (L1–L2 spinal bony structure) and the lumbar enlargement of the spinal cord immediately removed and stored at −80°C until used for Western blotting. To prepare a total lysate, the dorsal portion of the lumbar spinal cord enlargement was homogenized in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and phosphatase inhibitors), the lysate centrifuged at 12,000*g* for 30 min at 4°C, and the supernatant used for Western blotting. To prepare cellular fractions, the dorsal portion of the lumbar spinal cord enlargement was fractionated into cytosolic, membrane, and nuclear fractions using a Cytoplasmic, Nuclear, and Membrane compartment protein extraction kit, as recommended by the manufacturer (Biochain Institute, Inc., Hayward, CA). The membrane and cytosolic fractions were checked for specificity by Western blotting with mouse anti-rat epidermal growth factor receptor (1:2,000; MBL, Naka-ku Nagoya, Japan) and anti-rat α-tubulin antibodies (1:5,000; Laboratory Frontier, Seodaemun-gu, Seoul, Korea), respectively. The protein concentrations of the samples were determined by the bicinchoninic acid assay (Pierce; Thermo Fisher Scientific Inc., Waltham, MA) using bovine serum albumin as the standard. Samples containing 20 μg of protein were adjusted to a similar volume with loading buffer (10% sodium dodecyl sulfate, 20% glycerin, 125 mM Tris, 1 mM EDTA, 0.002% bromphenol blue, 10% β-mercaptoethanol) and the proteins denatured by heating at 95°C for 5 min, separated on 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk in Tris-Tween buffer saline (50 mM Tris-HCl, 154 mM NaCl, and 0.05% Tween 20, pH 7.4), incubated overnight at 4°C with polyclonal rabbit antibodies against rat NR1, NR2A, NR2B, GluR1, or GluR2 (all 1:1,000 dilution in 5% nonfat milk in Tris-Tween buffer saline) or monoclonal mouse anti-rat postsynaptic density-95 antibodies (PSD-95; 1:5,000 dilution in 5% nonfat milk in Tris-Tween buffer saline) (all from Millipore), then incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse immunoglobulin G antibodies, as appropriate (1:2,000 in 5% nonfat milk in Tris-Tween buffer saline) (Jackson ImmunoResearch, West Grove, PA). Membrane-bound secondary antibodies were detected using Chemiluminescence^{plus} reagent (Perkin Elmer Life and Analytical Sciences, Waltham, MA) and visualized using a chemiluminescence imaging system (Syngene, Cambridge, United Kingdom). Finally, the blots were incubated for 18 min at 56°C in stripping buffer (62.6 mM Tris-HCl, pH: 6.7, 2% sodium dodecyl sulfate, 100 mM mercaptoethanol) and reprobed with monoclonal mouse anti-β-actin antibody (1:5,000; Sigma) as a loading control. The Western blot analysis was repeated three times. The density of each specific band was measured using a computer-assisted imaging analysis system (Gene Tools Match software; Syngene, Cambridge, United Kingdom).

Immunoprecipitation of PSD-95/NR1 and NR2B Subunits Complex

To determine the coassembly of PSD-95, NR1, and NR2B subunits, the coimmunoprecipitation experiments were performed by using immobilized anti-PSD–95 antibody. Anti-PSD–95 antibody (1:50; Cell Signaling, Danvers, MA) was covalently cross-linked to Dynabeads® protein A (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PSD-95/NR1 and NR2B complexes were isolated by incubating 200 μg of spinal cord dorsal horn membrane proteins solubilized in Cytoplasmic, Nuclear, and Membrane compartment protein extraction kit extraction buffer with 50 μl of Dynabeads® protein A for 1 h at room temperature. The incubation performed with normal mouse serum was used as a negative control. Dynabeads® were precipitated using a magnet, and then the beads were extensively washed with phosphate-buffered saline. Precipitated proteins were eluted with 50 μl sodium dodecyl sulfate-containing sample buffer, and 20 μl of the samples were used for Western blots as described above in Preparation of Spinal Cord Total Lysate and Synaptosomal Membrane and Cytosolic Fractions and Western Blot Analysis.

Fluorescence Immunocytochemistry and Image Analysis

For fluorescence immunocytochemistry, the lumbar spinal cord was postfixed overnight at 4°C in 4% paraformaldehyde prepared in 0.1 M phosphate buffer, pH 7.4, then cryoprotected in 30% sucrose for 2 days. It was confirmed as lumbar spinal cord by the cross anatomy, which showed nearly a circular shape with very large anterior and posterior gray horns and relatively little white matter. Sections (5 μm) were prepared, air-dried on microscope slides for 30 min at room temperature, and preincubated for 1 h with 4% normal goat serum in phosphate-buffered saline containing 0.01% Triton X-100. After three washes in ice-cold phosphate-buffered saline, the sections were incubated overnight at 4°C with unlabeled mouse monoclonal anti-rat β-III tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:100 dilution in phosphate buffered saline with Triton X-100 containing 2% normal goat serum) and rabbit polyclonal antibodies anti-rat NR1 or NR2B (both from Millipore; 1:500 dilution in phosphate buffered saline with Triton X-100 containing 2% normal goat serum). The sections were then reacted for

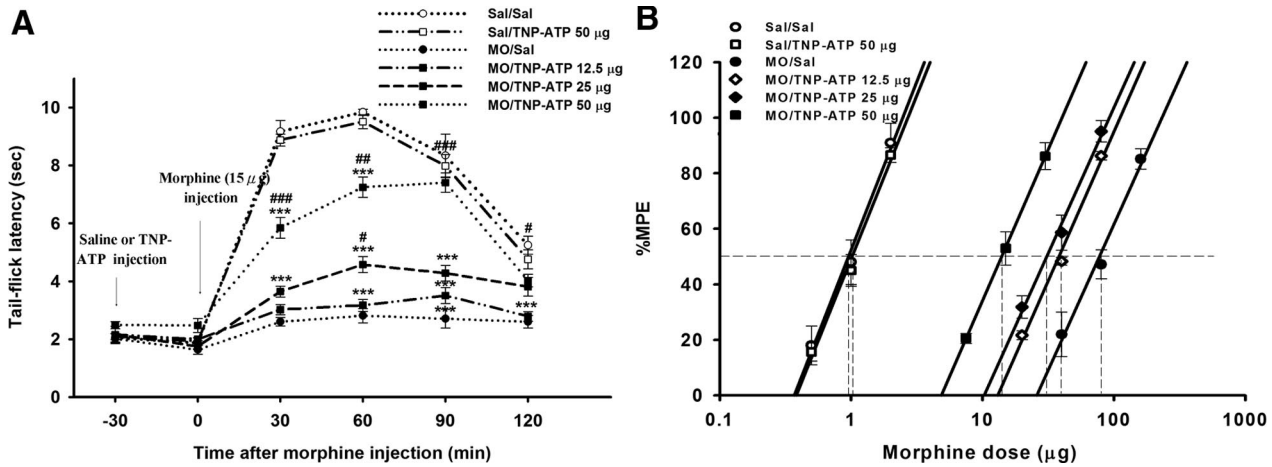

Fig. 1. 2′,3′-*O*-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) restores the antinociceptive effect of morphine in morphine-tolerant rats. (*A*) The antinociceptive effect of morphine was examined on day 5 after intrathecal infusion of either saline (1 μl/h) or morphine (15 μg/h). At 3 h after discontinuation of infusion, the rats were injected with either saline (5 μl) or TNP-ATP (12.5, 25, and 50 μg/5 μl) 30 min before morphine challenge (15 μg/5 μl), at which time the tail-flick latencies had returned to less than 3 s, then the tail-flick latency was measured every 30 min for 120 min. (*B*) Dose-response curves of the antinociceptive effect of morphine were constructed from the tail-flick test results performed on day 5. The mini-osmotic pump was disconnected for 3 h by which time the tail-flick latency had reached baseline, then different doses of morphine (0.5, 1, 2 μg for Sal/Sal and Sal/TNP-ATP 50 μg rats; 40, 80, 160 μg for MO/Sal rats; 20, 40, 80 μg for MO/TNP-ATP 12.5 and 25 μg rats; 7.5, 15, 30 μg for MO/TNP-ATP 50 μg rats) were given to rats intrathecally. The dose-response effect is expressed as the percentage of the maximal possible antinociceptive effect. The AD50 of morphine was 1.12 μg in Sal/Sal rats (95% confidence interval [CI], 1.0–1.3 μg), 1.19 μg in Sal/TNP-ATP 50 μg rats (95% CI, 1.0–1.4 μg), 90.51 μg in MO/Sal rats (95% CI, 81.3–104.5 μg), 46.54 μg in MO/TNP-ATP 12.5 μg rats (95% CI, 39.6–52.5 μg), 35.19 μg in MO/TNP-ATP 25 μg rats (95% CI, 30.1–57.9 μg), and 16.35 μg in MO/TNP-ATP 50 μg rats (95% CI, 13.2–22.8 μg) (n = 12 of each group). *** $P < 0.001$ compared with the Sal/Sal group; ### $P < 0.001$, ## $P < 0.01$, and # $P < 0.05$ compared with the MO/Sal group. All data points are presented as the mean ± SEM. MO/Sal = morphine infusion for 5 days plus saline injection on day 5 (n = 12); MO/TNP-ATP 12.5 μg = morphine infusion for 5 days plus TNP-ATP (12.5 μg/5 μl) injection on day 5 (n = 8); MO/TNP-ATP 25 μg = morphine infusion for 5 days plus TNP-ATP (25 μg/5 μl) injection on day 5 (n = 8); MO/TNP-ATP 50 μg = morphine infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection on day 5 (n = 12); Sal/Sal = saline infusion for 5 days plus saline injection on day 5 (n = 6); Sal/TNP-ATP 50 μg = saline infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection on day 5 (n = 9).

1 h at room temperature with rhodamine-labeled goat anti-rabbit immunoglobulin G antibodies (red fluorescence) and fluorescein isothiocyanate-labeled donkey anti-mouse immunoglobulin G antibodies (green fluorescence) (both from Jackson ImmunoResearch) and images were captured using an Olympus BX 50 fluorescence microscope (Olympus, Optical, Tokyo, Japan) and a δ Vision disconsolation microscopic system operated by SPOT software (Diagnostic Instruments Inc., Sterling Heights, MI). The laser wavelength was set at 488 nM for fluorescein isothiocyanate fluorescence and 568 nM for rhodamine fluorescence. Controls without primary antibody were run to confirm that the staining was specific.

Data and Statistical Analysis

All data are presented as the mean ± SEM. The statistical analysis was performed using SigmaStat 3.0 software (SYSTAT Software Inc., San Jose, CA). The appropriate paired *t* test (two-tailed) or analysis of variance (ANOVA) was used to determine the statistical significance with a criterion of *P* less than 0.05. Tail-flick latencies and EAA concentration were analyzed using two-way (time and treatment) ANOVA followed by subsequent one-way ANOVA (at each time of the experiment) with a *post hoc* Student-Newman-Keuls test. Values for the analgesic dose of 50% of the maximal possible antinociceptive effect (AD_{50}) were analyzed using a computer-assisted linear regression program SigmaPlot 10.0 (SYSTAT Software Inc.). The 95% CI was calculated using the pharmacologic calculations system PHARM/PCS version 4.2 (MicroComputer Specialists, Philadelphia, PA). For immunoreactivity data, the intensity of each test band was expressed as the optical density relative to that of the average optical density for the corresponding control band. For statistical analysis, immunoreactivity was analyzed by one-way ANOVA, followed by multiple comparisons with the Student-Newman-Keuls *post hoc* test.

Results

Treatment with the P2X Receptor Antagonist TNP-ATP Restores the Antinociceptive Effect of Morphine in Morphine-tolerant Rats

As in our previous study, morphine challenge (15 μg/5 μl, intrathecally) on day 5, at 3 h after discontinuation of drug infusion, produced a significant antinociceptive effect in saline-infused rats (Sal/Sal) ($P < 0.001$) but not in morphine-tolerant rats (MO/Sal) ($P = 0.95$) (fig. 1A). TNP-ATP alone did not produce an antinociceptive effect in either saline-infused controls ($p = 0.502$) or morphine-tolerant rats ($p = 0.962$). However, treatment with TNP-ATP (12.5, 25, 50 μg/5 μl, intrathecally) 30 min before morphine challenge (MO/TNP-ATP) dose-dependently restored the antinociceptive ef-

fect in morphine-tolerant rats ($P < 0.001$). The two-way ANOVA of these time-course curves showed significant different in tail-flick latency by treatments, by time, and for the interactions ($P < 0.001$). High-dose TNP-ATP (100 μg/5 μl) treatment produced antinociceptive effects similar to those of TNP-ATP 50 μg treatment in morphine-tolerant rats (data not shown). As shown in figure 1B, TNP-ATP treatment 30 min before morphine injection had no effect on the morphine dose–response curve in saline-infused rats (Sal/TNP-ATP), the AD_{50} being 1.12 μg in Sal/Sal rats and 1.19 μg in Sal/TNP-ATP rats. In morphine-tolerant rats, the morphine dose–response curve was shifted to the right by 81-fold (AD_{50} of 90.51 μg) compared with in saline-infused rats, and TNP-ATP (50 μg) treatment restored the antinociceptive effect of morphine in morphine-tolerant rats, shifting the AD_{50} from 90.51 (MO/Sal) to 16.35 μg (MO/TNP-ATP). Treatment with lower doses of TNP-ATP, either 12.5 or 25 μg, showed slightly restored morphine's antinociceptive effect in morphine-tolerant rats, with AD_{50} of 46.54 and 35.19 μg, respectively.

Effect of TNP-ATP on Levels of NMDA Receptor Subtypes in the Total Lysate and the Synaptosomal Membrane of Morphine-tolerant Rats

As shown in figure 2, immunoblot analysis showed that levels of NR1, NR2A, and NR2B in the spinal cord dorsal horn lysate from saline-infused rats (Sal/Sal) were unaffected by TNP-ATP treatment (Sal/TNP-ATP) (NR1, $P = 0.057$; NR2A, $P = 0.126$; and NR2B, $P = 0.957$). On day 5, long-term morphine infusion up-regulated levels of NR1 and NR2B subunits in the total lysate by approximately 50–100% (MO/Sal), and this effect was not prevented by TNP-ATP treatment (MO/TNP-ATP) ($P < 0.001$). As shown in figure 3, in morphine-tolerant rats (MO/Sal), cytosolic levels of NR1 and NR2B were no different from those in saline-infused (Sal/Sal) or saline-infused TNP-ATP-treated (Sal/TNP-ATP) rats. However, TNP-ATP treatment significantly increased cytosolic levels of NR1 and NR2B subunits in morphine-tolerant rats (MO/TNP-ATP) compared with the other groups ($P < 0.001$). In contrast, as shown in figure 3, right and bottom, increased levels of NR1 and NR2B subunits were seen in the synaptosomal membrane in morphine-tolerant rats (compare MO/Sal with Sal/Sal) ($P < 0.001$), and this effect was prevented by TNP-ATP treatment (MO/TNP-ATP) ($P < 0.001$). Expression of the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor GluR1 and GluR2 subunits in the cytosolic and synaptosomal membrane fractions was not affected by any of the treatments (data no shown) ($P = 0.672$ and 0.624, respectively). Epidermal growth factor receptor and α-tubulin markers were used to confirm the identity of the membrane and cytosolic fractions (fig. 3). Fluorescence microscopy localization of the NR1 and NR2B subunits is shown in figures 4 and 5, respectively. In morphine-tolerant rats, a robust and extensive NR1 and NR2B subunit labeling was evenly distributed throughout the entire neuron (MO/Sal), whereas labeling was cytosolic after TNP-ATP treatment (MO/TNP-ATP).

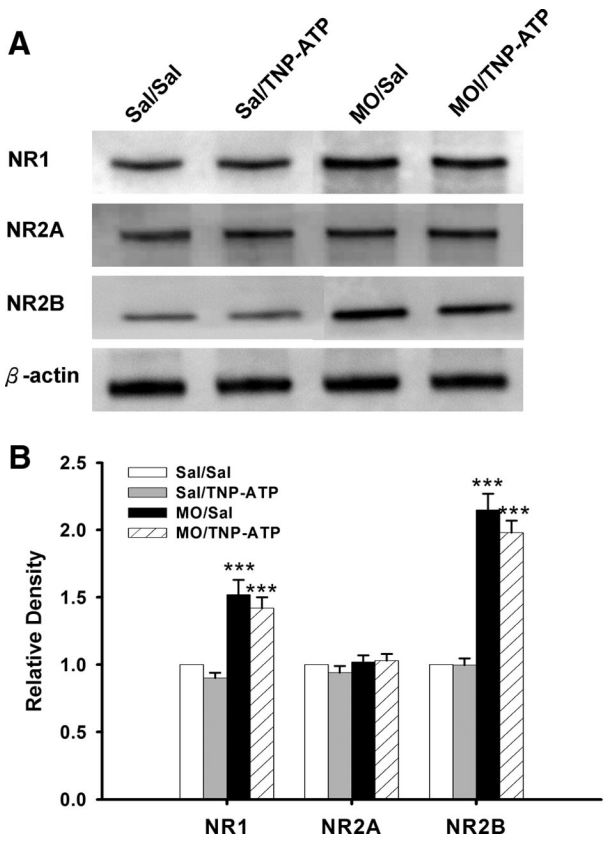

Fig. 2. Levels of *N*-methyl-D-aspartate receptors in the dorsal horn of the spinal cord after various treatments. Western blots for the NR1, NR2A, and NR2B subunits were performed on the rat spinal cord dorsal horn lysates. (*A*) Typical blots show expression of NR1, NR2A, and NR2B protein in the spinal cord dorsal horn of the four groups (n = 5 of each group). β-Actin was used as the loading control. (*B*) Pooled densitometric results for NR1, NR2A, and NR2B, with the control band intensity assigned the value of 1. *** $P < 0.001$ compared with the Sal/Sal group. MO/Sal = morphine (15 μg/h) infusion for 5 days plus saline (5 μl) injection; MO/TNP-ATP = morphine (15 μg/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection; Sal/Sal = saline (1 μl/h) infusion for 5 days plus saline (5 μl) injection; Sal/TNP-ATP = saline (1 μl/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection; TNP-ATP = 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

NR1/NR2B Antagonist Ifenprodil Treatment Attenuated the Antinociceptive Tolerance of Morphine

As shown in figure 6, on day 5, 3 h after discontinuation of morphine infusion, morphine challenge (15 μg) did not produce antinociceptive effect in morphine-tolerant rats (MO/Sal) ($P = 0.934$), whereas a significant antinociceptive effect was observed in saline-infused rats (Sal/Sal) ($P < 0.001$). However, treatment with ifenprodil (10 μg, intrathecally) 30 min before morphine challenge preserved its antinociceptive effect in morphine tolerant rats (compare MO/IFE with MO/Sal) ($P < 0.001$). Ifenprodil alone had no antinociceptive effect in either saline-infused control rats ($P = 0.543$) or morphine-tolerant rats ($P = 0.1$). As shown in figure 6B, the dose-response showed that the AD_{50} for morphine was 1.12 μg in Sal/Sal rats and 1.13 μg in Sal/IFE rats. In morphine-tolerant rats, morphine's dose–

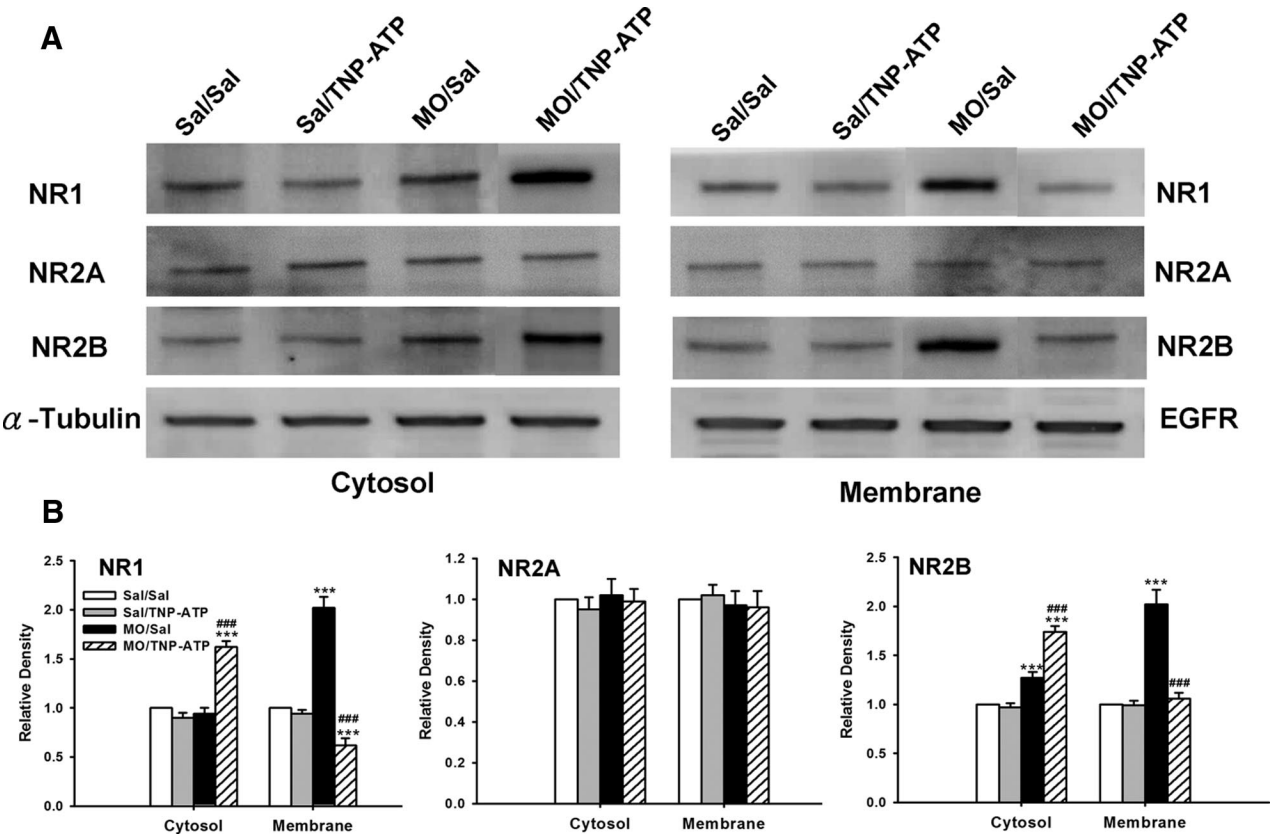

Fig. 3. 2′,3′-*O*-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) has different effects on the expression of *N*-methyl-D-aspartate receptors in the cytosolic and synaptosomal membrane fractions of morphine-tolerant rats. (*A*) Western blot analysis of NR1, NR2A, and NR2B performed on the cytosolic and synaptosomal membrane fractions of the spinal cord dorsal horn from saline-infused or morphine-infused rats injected with saline or TNP-ATP. Anti-tubulin and anti-epidermal growth factor receptor antibodies were used as the loading marker for the cytosolic and synaptosomal membrane fraction, respectively. (*B*) Densitometric measurements from five independent experiments were pooled and the band intensity for the Sal/Sal rats was assigned a value of 1. EGFR = epidermal growth factor receptor; MO/Sal = morphine (15 μg/h) infusion for 5 days plus saline (5 μl) injection; MO/TNP-ATP = morphine (15 μg/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection; Sal/Sal = saline (1 μl/h) infusion for 5 days plus saline (5 μl) injection; Sal/TNP-ATP = saline (1 μl/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection. *** $P < 0.001$ compared with the Sal/Sal group; ### $P < 0.001$ compared with the MO/Sal group (n = 5 of each group).

response curve was shifted to the right by 80-fold (MO/Sal, AD_{50} = 89.88 μg) compared with saline-infused rats (Sal/Sal, AD_{50} = 1.12 μg), and ifenprodil treatment potentiated the antinociceptive effect of morphine in morphine-tolerant rats, the AD_{50} were from 89.88 (MO/Sal) to 25.28 μg (MO/IFE).

TNP-ATP Treatment Suppresses the Morphine Challenge-evoked EAA Release in Morphine-tolerant Rats

In the CSF microdialysis experiment, TNP-ATP treatment 30 min before morphine challenge had no significant effect on CSF EAA levels in either saline-infused controls (aspartate, $P = 0.68$; glutamate, $P = 0.338$) or morphine-tolerant rats (aspartate, $P = 0.635$; glutamate, $P = 0.074$). As shown in figure 7, morphine challenge had no effect on CSF EAA levels in either saline-infused (Sal/Sal) (aspartate, $P = 0.658$; glutamate, $P = 0.868$) or saline-infused plus TNP-ATP-treated (Sal/TNP-ATP) rats (aspartate, $P = 0.949$; glutamate, $P = 0.814$). As in our previous study,[6,7] morphine challenge resulted in a significant increase in aspartate and glutamate release in morphine-tolerant rats (MO/Sal) ($P < 0.001$), and TNP-ATP treatment 30 min before morphine challenge completely blocked this morphine-evoked EAAs release in morphine-tolerant rats (MO/TNP-ATP) ($P < 0.001$). Two-way ANOVA of these time-course curves showed significant difference in EAA concentrations by treatments, by time, and for the interactions ($P < 0.001$).

TNP-ATP Treatment Down-regulates Synaptosomal Membrane PSD-95 Expression in Morphine-tolerant Rats

In figure 8, the density of the PSD-95 band on immunoblots of the synaptosomal membrane fraction from the saline-infused rat spinal cord dorsal horn (Sal/Sal) is expressed as 1. TNP-ATP treatment alone had no effect on PSD-95 expression in saline-infused rats (compare Sal/TNP-ATP and Sal/Sal). Long-term morphine infusion increased (by approximately 100%) synaptosomal membrane PSD-95 expression (MO/Sal) ($P < 0.001$) and this effect was not only prevented by TNP-ATP treatment (MO/TNP-ATP), but PSD-95 expression was lower than in the saline controls ($P < 0.001$).

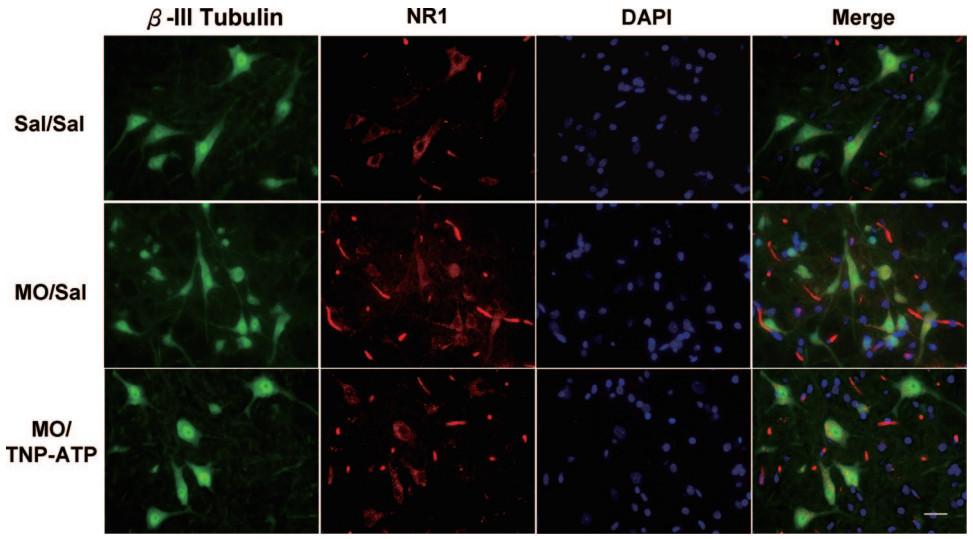

Fig. 4. 2′,3′-*O*-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) alters the distribution of *N*-methyl-D-aspartate receptor subunit NR1. The spinal cords from Sal/Sal rats and morphine-infused rats with or without TNP-ATP injection were fixed and labeled with either fluorescein isothiocyanate-labeled anti-beta-III tubulin antibody (green fluorescence for neuron) or rhodamine-labeled rabbit anti-NR1 antibody (red fluorescence) and 4′,6-diamidino-2-phenylindole (label for nucleus, blue fluorescence), and images were obtained by immunofluorescent laser scanning fluorescence microscopy. These images are representative of multiple fields examined for each treatment from four independent immunofluorescence experiments. The scale bar represents 50 μM (n = 4 of each group). DAPI = 4′,6-diamidino-2-phenylindole; Sal/Sal = saline (1 μl/h) infusion for 5 days plus saline (5 μl) injection; MO/Sal = morphine (15 μg/h) infusion for 5 days plus saline (5 μl) injection; MO/TNP-ATP = morphine (15 μg/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection.

Effect of TNP-ATP Treatment on the Coassembly of PSD-95 and NR1 and NR2B Subunits

PSD-95 provides a physical means for anchoring of NMDA receptor at the postsynaptic site, and the coassembly of PSD-95 with NR1 and NR2B in morphine-tolerant rats was examined. As shown in figure 9, an increasing of the coassembly of three proteins was noted in the morphine-tolerant rat lumbar spinal cord ($P < 0.001$). TNP-ATP treatment dose-dependently reverses the increasing of PSD-95, NR1 and NR2B expression in rats undergoing long-term intrathecal morphine infusion.

Discussion

In the present study, TNP-ATP treatment restored the antinociceptive effect of morphine and prevented the morphine-induced increase in aspartate and glutamate in the spinal CSF of morphine-tolerant rats. Moreover, we found

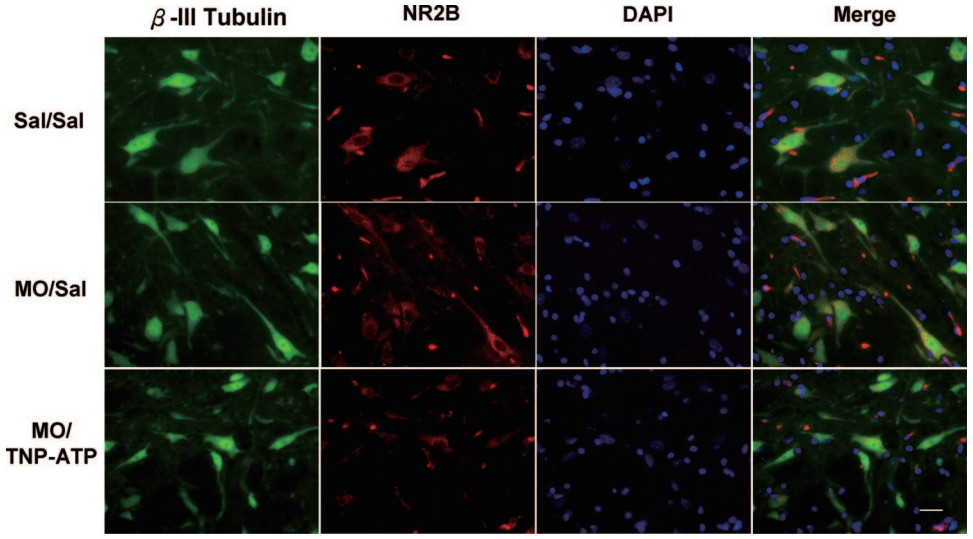

Fig. 5. 2′,3′-*O*-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate alters the distribution of *N*-methyl-D-aspartate receptor subunit NR2B. The experiment is identical to that in figure 4 but using rabbit anti-NR2B antibody. These images are representative of multiple fields examined for each treatment from four independent immunofluorescence experiments. The scale bar represents 50 μM (n = 4 of each group). DAPI = 4′,6-diamidino-2-phenylindole; TNP-ATP = 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

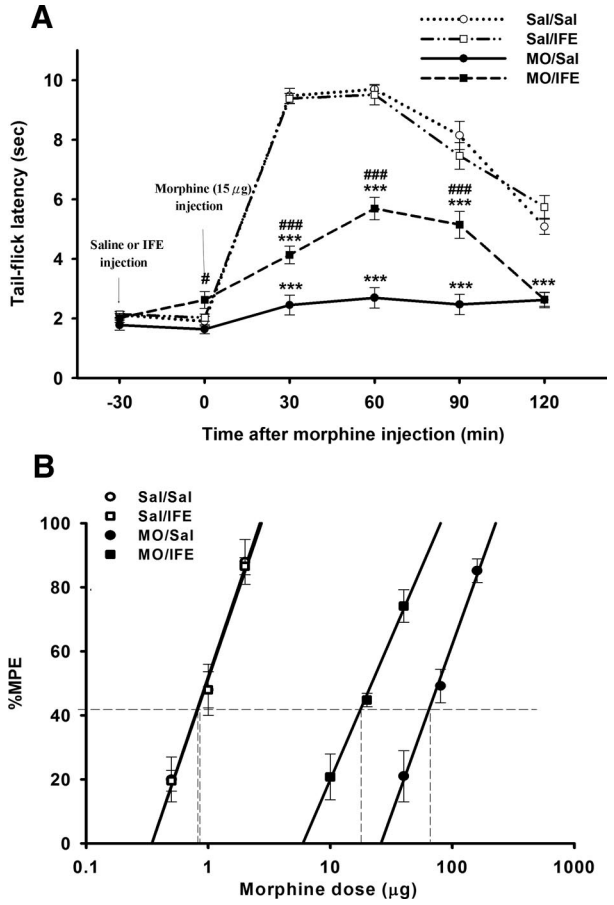

Fig. 6. Ifenprodil restores the antinociceptive effect of morphine in morphine-tolerant rats. (*A*) Morphine's antinociceptive effect was examined on day 5 after intrathecal saline (1 μl/h) or morphine (15 μg/h) infusion. At 3 h after discontinuation of infusion, rats were injected with either saline (5 μl) or ifenprodil (10 μg/5 μl) 30 min before morphine challenge (15 μg/5 μl), at which time the tail-flick latency returned to less than 3s, then the tail-flick latency was measured every 30 min for 120 min. (*B*) Dose-response curves for the antinociceptive effect of the challenge morphine were constructed as described in figure 1. The AD50 of morphine was 1.12 μg in saline-infused rats (95% confidence interval [CI], 1.0–1.3 μg), 1.13 μg in saline-infused rats pretreated with ifenprodil (95% CI, 1.0–1.3 μg), 89.88 μg in morphine-infused rats (95% CI, 77.6–112.3 μg), and 25.28 μg in morphine-infused rats pretreated with ifenprodil (10 μg) (95% CI, 20.9–34.1 μg). All data points are mean ± SEM (n = 12 of each group). *** $P <$ 0.001 compared with the Sal/Sal group; ### $P < 0.001$, # $P <$ 0.05 compared with the MO/Sal group. MO/Sal = morphine infusion for 5 days plus saline injection (n = 9); MO/IFE = morphine infusion for 5 days plus ifenprodil injection (n = 12); MPE = maximal possible antinociceptive effect; Sal/IFE = saline infusion for 5 days plus ifenprodil injection (n = 9); Sal/Sal = saline infusion for 5 days plus saline injection (n = 6).

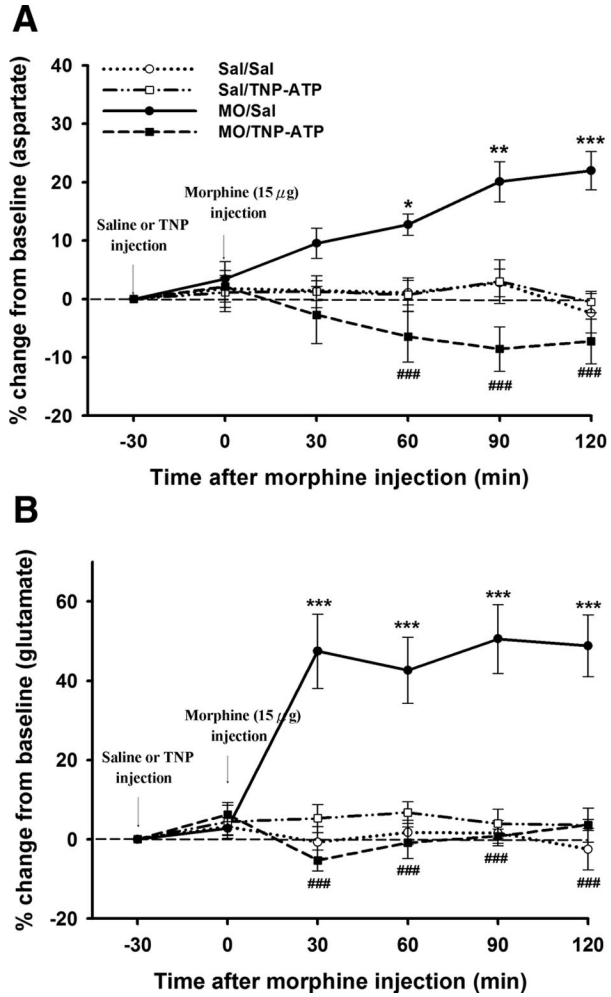

Fig. 7. 2′,3′-*O*-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate suppresses the morphine-evoked excitatory amino acid release in morphine-tolerant rats. Rats were treated as in figure 1; then, after morphine challenge, cerebrospinal fluid dialysates were collected for measurement of aspartate (*A*) and glutamate (*B*). The average of the two consecutive CSF dialysates (30 min each) just before drug injection was used as the basal concentration (100%). Results are presented as the mean ± SEM of the % change from the control data. *** $P <$ 0.001, ** $P <$ 0.01, * $P <$ 0.05 compared with the Sal/Sal group; ### $P <$ 0.001 compared with the MO/Sal group. MO/Sal, morphine (15 μg/h) infusion for 5 days plus saline (5 μl) injection (n = 10); MO/TNP-ATP, morphine (15 μg/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection (n = 10); Sal/Sal, saline (1 μl/h) infusion for 5 days plus saline (5 μl) injection (n = 6); Sal/TNP-ATP, saline (1 μl/h) infusion for 5 days plus TNP-ATP injection (50 μg/5 μl) (n = 6); TNP-ATP = 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

that long-term morphine infusion up-regulated expression of the NMDA receptor NR1 and NR2B subunits in the total lysate of the lumbar enlargement of the spinal cord, and this was unaffected by TNP-ATP treatment. However, TNP-ATP treatment significantly increased the amount of cytosolic NR1 and NR2B; in contrast, it reversed the increase of NR1 and NR2B expression in the synaptosomal fraction of morphine-tolerant rat spinal cords. Moreover, treatment with NMDA receptor NR1/NR2B antagonist ifenprodil produced an effect similar to that of TNP-ATP; it also potentiated the antinociceptive effect of morphine. Therefore, the 5.5-fold leftward shift in the AD_{50} of morphine in toler-

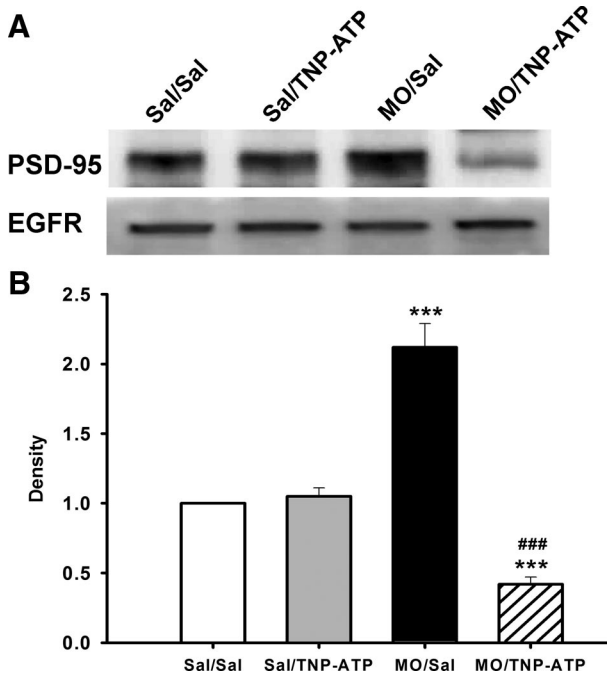

Fig. 8. 2′,3′-*O*-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate treatment reverses the increase in postsynaptic density-95 (PSD-95) expression after long-term morphine infusion. (*A*) Western blot for PSD-95 in the synaptosomal membrane fraction of the different treatment groups; epidermal growth factor receptor was used as the internal standard. (*B*) Relative band densities of PSD-95 after the different treatments; the results are the mean ± SEM (n = 4 for each group). The band intensity of the Sal/Sal rats was assigned a value of 1. *** $P < 0.001$ compared with the Sal/Sal group; ### $P < 0.001$ compared with the MO/Sal group. EGFR = epidermal growth factor receptor; MO/Sal = morphine (15 μg/h) infusion for 5 days plus saline (5 μl) injection; MO/TNP-ATP = morphine (15 μg/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection; PSD-95 = postsynaptic density-95; Sal/Sal = saline (1 μl/h) infusion for 5 days plus saline (5 μl) injection; Sal/TNP-ATP = saline (1 μl/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection; TNP-ATP = 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

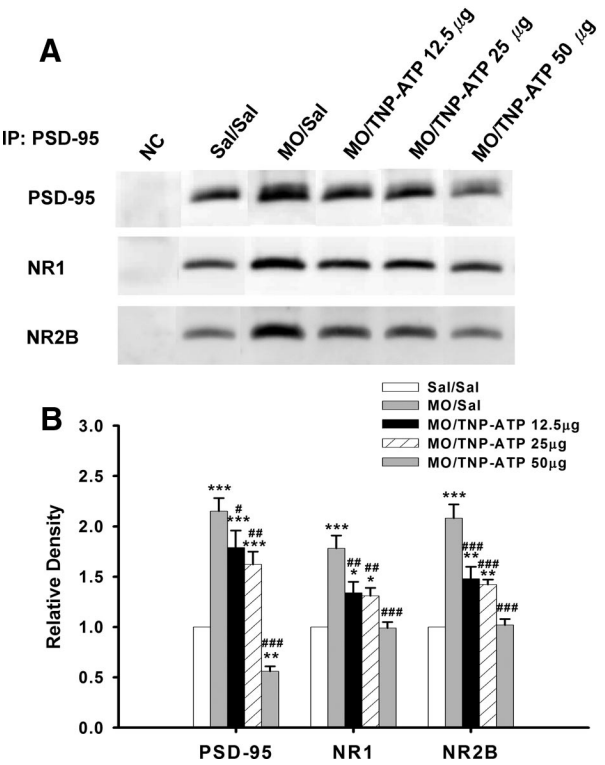

Fig. 9. 2′,3′-*O*-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate treatment dose-dependently down-regulates postsynaptic density-95 (PSD-95), NR1, and NR2B coprecipitated complex expression in morphine-tolerant rats. (*A*) Immunoprecipitation of PSD-95-NR1-NR2B complex in the synaptosome of different treatment groups. (*B*) Quantification of the coprecipitated complex density of different treatments. The results are expressed as mean ± SEM (n = 4 for each group). The band intensity of Sal/Sal rats was assigned a value of 1. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with the Sal/Sal group; ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ compared with the MO/Sal group. IP = immunoprecipitation; NC = negative control; MO/Sal = morphine (15 μg/h) infusion for 5 days plus saline (5 μl) injection; MO/TNP-ATP 12.5 μg = morphine (15 μg/h) infusion for 5 days plus TNP-ATP (12.5 μg/5 μl) injection; MO/TNP-ATP 25 μg = morphine (15 μg/h) infusion for 5 days plus TNP-ATP (25 μg/5 μl) injection; MO/TNP-ATP 50 μg = morphine (15 μg/h) infusion for 5 days plus TNP-ATP (50 μg/5 μl) injection; Sal/Sal = saline (1 μl/h) infusion for 5 days plus saline (5 μl) injection; TNP-ATP = 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

ant rats by TNP-ATP treatment might be *via* regulation of NMDA expression and synaptic excitatory amino acid concentration in morphine-tolerant rats. In addition, the up-regulation of PSD-95 in the synaptosomal fraction was also observed in the morphine-tolerant rat spinal cords, and this effect was reversed by TNP-ATP treatment. Quantification of the coprecipitated complex revealed that treatment of TNP-ATP dose-dependently down-regulates PSD-95, NR1, and NR2B expression in morphine-tolerant rats. Taken together, the treatment of TNP-ATP in inhibition of NMDA receptor NR1 and NR2B subunits expression on the postsynaptic membrane may be involved, at least in part, in the loss of PSD-95 expression.

Glutamate and aspartate have been shown to be involved in nociception transmission in the spinal cord.[26] In previous studies and our recent studies, the results failed to demonstrate an increase in CSF EAA levels during induction of morphine tolerance.[7,27,28] However, posttreatment with naloxone evoked a significant and time-dependent increase in the CSF dialysate glutamate and taurine concentration but not other amino acids in rats receiving long-term morphine infusion.[27] Likewise, we demonstrated that morphine challenge induced an increase of glutamate and aspartate in the CSF dialysates of morphine-tolerant rats; it was also accompanied by a loss of morphine's analgesic effect,[7,28] and coadministration of morphine with the NMDA antagonist not only attenuated morphine tolerance development but also blocked morphine-induced spinal EAAs release.[28] The sustained potentiation of NMDA receptor-mediated responses may be through μ-opioid receptor-mediated protein kinase

C activation.[29] This evidence suggests a positive feedback control between opioid and glutamatergic receptors, particularly the NMDA receptors. It is known that long-term morphine infusion induces tolerance and G_i-protein uncoupling, and the morphine challenge in our present study may act *via* G_s-protein signal transduction and result in an excitatory effect of morphine on NMDA receptors.[30,31] Thus, the increase of EAA concentration by morphine challenge in the present study might be reflecting a direct action of morphine on NMDA receptor sensitization after long-term morphine exposure. Coadministration of morphine with various drugs, such as the NMDA antagonist MK-801, gabapentin, or amitriptyline, preserves the antinociceptive effect of morphine by lowering CSF EAA levels.[7,28,32] In the present study, we also found that short-term intrathecal morphine challenge induced an increase in glutamate and aspartate levels in tolerant rat spinal CSF dialysates and loss of the antinociceptive effect of morphine, and that TNP-ATP treatment prevented the morphine-evoked EAA increase in the CSF. These findings suggest that the restoration of the antinociceptive effect of morphine by TNP-ATP might partly result from a reduction in spinal EAA release.

Activation of NMDA receptors has been shown to play a crucial role in the development of tolerance to the analgesic effect of morphine.[4] Pharmacological analysis has demonstrated that blockade of NMDA receptor hyperfunction effectively prevents the development of morphine tolerance.[33,34] The competitive NMDA receptor antagonist LY274614 prevents antinociceptive tolerance to the highly selective μ-opioid agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin.[35] In the present study, we also demonstrated that posttreatment with NMDA receptor specific antagonist ifenprodil (10 μg) restored the antinociceptive effect of morphine in morphine-tolerant rats. Studies involving alterations in synaptic NMDA receptor expression, including antisense and transgenic knockdown of NMDA receptors, support the idea that NMDA receptor activation is important for morphine-induced plasticity and provide strong evidence that a unique pharmacological state is required for inhibition of behavioral adaptations.[12,36] Yang *et al.*[37] demonstrated that the amount of NMDA receptors at the synapse regulates synaptic responses and pain sensitivity. The present study showed that long-term morphine infusion increased NR1 and NR2B expression in the synapse and that this correlated with development of morphine tolerance, in agreement with a previous report that morphine tolerance is associated with time-dependent up-regulation of the NR1 subunit in the spinal cord dorsal horn compared with the saline control group.[38] It would seem that enhancement of NR1 expression at the synapse strengthens NMDA receptor-mediated synaptic transmission and thus increases NMDA receptor-evoked intracellular signals, leading to central sensitization and behavioral manifestations.[12,39] In morphine-tolerant rats, treatment with the P2X receptor antagonist TNP-ATP significantly decreased synaptic NR1 and NR2B subunit expression and decreased the morphine-evoked EAA release and restored the antinociceptive effect. The rapid dynamic change in synaptic NR1/NR2B in neurons was associated with decreased PSD-95 expression.

The PSD protein family, including PSD-95, is critical for anchoring NMDA receptor NR2 subunits in the postsynaptic membrane and mediates the triggering of many physiologic and pathophysiological functions *via* NMDA receptor activation.[40,41] Previous studies have demonstrated a critical role for the interaction of PSD-95 with NMDA receptors in receptor trafficking to the neuron surface, synaptic localization, and intracellular signaling.[42–44] Cotransfection with PSD-95 and NR1/NR2A or NR1/NR2B subunit clones results in increased NR2A and NR2B subunit expression *via* interaction of the C-terminal threonine/serine/valine/valine motif of the NR2 subunit with PSD-95 and results in increased cell-surface expression of the assembled NR1/NR2A and NR1/NR2B subtypes.[45–47] In addition, binding of PSD-95 to the NR2B C-terminal serine/threonine-X-valine motif reduces receptor endocytosis from the neuron surface and stabilizes NR2B-containing NMDA receptors in the synapse,[42,43] thereby increasing the residence time of receptors at the cell surface. These studies suggest that PSD-95 plays a crucial role in the trafficking, membrane targeting, and internalization of NMDA receptor complexes. In our present study, PSD-95 expression was increased after long-term morphine infusion, and this effect was inhibited by short-term TNP-ATP treatment before morphine challenge. Quantification of the immunoprecipitated complex densities of PSD-95/NR1/NR2B revealed a significant increase in morphine-tolerant rats; this phenomenon was dose-dependently down-regulated by the TNP-ATP treatment. This suggests that a lower level of PSD-95 results in loss of stability of NR1 and NR2B subunits in the synapse, which reduces the communication/coupling of NMDA receptors with intracellular signaling cascades. The underlying mechanisms between P2X receptor and PSD-95 interaction need further investigation.

P2X receptors play a crucial role in facilitating pain transmission at peripheral and spinal sites, as both peripheral sensory neurons and spinal cord dorsal horn neurons can be depolarized by ATP.[48,49] Studies have indicated that P2X and μ-opioid receptors are functionally coupled in sensory neuron.[50] Extracellular ATP-evoked P2X receptor inward current inhibited opioid sensitivity in neurons cocultured with fibrosarcoma cells.[51] Translocation and activation of protein kinase C enhance postsynaptic neuron excitability in morphine-tolerant rats.[10,52,53] Moreover, activation of protein kinase C showed significant potentiation of Ca^{2+} signal and inward cation current (predominantly Na^+), as well through $P2X_3$ receptor in DT-40 3KO and human embryonic kidney 293 cells.[54] Up-regulation of $P2X_3$ receptor expression is seen after chronic constriction injury of the sciatic nerve and provokes ectopic sensitivity to ATP.[55,56] Recent reports using gene knockout, antisense oligonucleotides, or the selective $P2X_3$ antagonist A-317491 all point to a crucial role of $P2X_3$ receptors in chronic inflammatory and neuro-

pathic pain.[20,57,58] It is noteworthy that P2X receptor agonist-induced nociception can be inhibited by intrathecal administration of NMDA receptor antagonists.[59] A direct interaction between the purinergic and glutamatergic receptor systems in mediating nociceptive processing in the spinal cord is further supported by evidence that P2X receptor activation can stimulate glutamate release in spinal dorsal horn neurons.[60] In the present study, we found that treatment with the P2X receptor antagonist TNP-ATP preserves morphine's antinociceptive effect in morphine tolerant rats; the mechanisms might be involved in a significant reduction of synaptosomal NR1 and NR2B expression and morphine-evoked EAA release from presynaptic nerve terminals in morphine-tolerant rats. The above results provide direct evidence for an interaction between the purinergic and NMDA receptor systems.

TNP-ATP is one of the potent P2X receptor antagonists and is selective for $P2X_1$, $P2X_3$, and $P2X_{2/3}$ receptors.[61] Intrathecal administration of TNP-ATP attenuates α,β-meATP-induced hyperalgesia in mice and the pronociceptive effect of formalin and capsaicin.[59,62] In our present study, intrathecal treatment with TNP-ATP (63 nM) alone produced no antinociceptive effect. Although previous studies indicated that intrathecal administration of low doses of TNP-ATP (1–10 nM) produces a partial, but significant, antinociceptive effect in mice,[62] and intradermal administration of larger doses (100–300 nM) produces significant attenuation (approximately 50%) of short-term formalin-induced paw flinching.[63] Intraperitoneal administration of sufficient doses of TNP-ATP (100 μM/kg) can completely block visceral nociception in the abdominal constriction assay.[64] These diverse results might be due to differences in the doses of TNP-ATP, animal models, and relevant site of action. The differences need further investigation.

In conclusion, our present study demonstrates that TNP-ATP treatment restores the antinociceptive effect of morphine in morphine-tolerant rats possibly by inducing internalization of NR1 and NR2B from the synaptosomal membrane into the neuron cytosol, thus reducing NMDA receptor-mediated intracellular signaling and EAA release in the CSF after morphine challenge. The synaptic trafficking of glutamate receptor subunit NR1 and NR2B may be modulated by the synaptic scaffolding proteins PSD-95.

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