Supporting Information

Chakrabarti et al. 10.1073/pnas.1009923107

SI Materials and Methods

Experimental Animals. Experiments used male and female adult (250–300 g) Sprague–Dawley rats (Charles River). All experimental procedures were in strict accordance with guidelines established by the Animal Care and Use Committee of State University of New York Downstate Medical Center.

Determination of Stage of Estrous Cycle. Histology of vaginal smears was used to evaluate stage of cycle. Predominance of small leukocytes was indicative of diestrous; a predominance of large round nucleated cells was indicative of proestrous.

Adult Ovariectomy. Established procedures were used to ovariectomize female rats (1). Briefly, the ovarian bundles were tied off with 4-O silk sutures, excised, and removed from the body cavity. The cutaneous incisions were closed with 5-O silk sutures.

Membrane Preparation. Spinal tissue was homogenized in 20 mM Hepes at pH 7.4, containing 10% sucrose, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, and protease inhibitors, 1 mM Benzamidine, 0.2 g/L Bacitracin, 2 mg/L Aprotinin, 3.2 mg/L each of trypsin inhibitor from soybean and Leupeptin, 20 mg/L each of *N*-tosyl-L-phenylalanine chloromethyl ketone, N^a-*p*-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride and complete mixture inhibitor tablet per 50 mL (Roche Molecular Biochemicals). Supernatants from a low-speed spin (1,000 × *g* for 10 min) were centrifuged at a higher speed (30,000 × *g*) for 40 min. Membrane pellets were resuspended in the same buffer without sucrose and stored in aliquots at −80 °C for future use.

Immunoprecipitation. Membranes were solubilized in the above Hepes buffer containing 150 mM NaCl, 1% Nonidet P-40 (Nonidet P-40), 0.5% Na-deoxycholate, 0.1% Na-dodecyl sulfate and 10% glycerol, agitated 60 min at 4 °C and centrifuged (14,000 × *g* for 20 min at 4 °C). Clear supernatants were used for protein assay and immunoprecipitation (IP). Spinal cord membranes were subjected to two sequential IP procedures. The immunoprecipitations used either an N-terminally directed anti-KOR antibody that was generated against aa 1–70 (Santa Cruz Biotechnology) or an anti-MOR antibody that was generated against the carboxyl-terminal 50 aa (2) (generously provided by Thomas Cote). The first IP was performed in solution as described (3) by using 400–600 μg of solubilized membranes. Immunoprecipitates so obtained were incubated overnight at 4 °C with antibody-immobilized columns that were prepared and eluted according to the manufacturer (Pierce). Column eluate were heated in sample buffer, electrophoresed by using 4–12% Bis-Tris gels (Invitrogen) under nonreducing conditions, electrotransferred onto nitrocellulose membranes and used for MOR and KOR Western blot analysis.

Western Blot Analysis. Standard procedures for Western blot analyses were used as described by this laboratory (3). MOR Western blot analyses used the same anti-MOR antibody that was used for MOR IP. In contrast to the antibody used for KOR IP, KOR Western blots used a rabbit polyclonal antibody generated against aa 262–275 of KOR (Pierce). The anti-dynorphin A (1–17) antibody that was used cross-reacts minimally (<0.5%) with dynorphin 1–13 and does not recognize dynorphin 1–8. Secondary antibody used was a peroxidase-labeled donkey anti-rabbit antibody. Antibody–substrate complex was visualized by using a Supersignal West Dura kit (Pierce). The chemiluminescence generated was quantified by using a GBox (CCD camera; Syngene) and quantified using the syngene software (Syngene). Specificity of Western blot signals was demonstrated via their diminution/elimination when using antisera that had been preabsorbed by using affinity columns constructed with their respective blocking peptides. Sample pairs, obtained from spinal cord membranes of male and female (proestrous), ovariectomized and male, or proestrous and diestrous female rats, were processed, electrophoresed, blotted, and quantified in parallel.

Cross-Linking Heterodimeric MOR/KOR to EM2 or Dynorphin. Membranes from female (proestrous) spinal cord were incubated in vitro with either EM2 or dynorphin and cross-linked with 5 mM DSS (20 min at room temperature; Pierce). This reaction was terminated by using a quench buffer (15 min at room temperature), followed by solubilization and sequential IP using anti-KOR antibodies. To investigate the binding of endogenous dynorphin to MOR/KOR, 5 μg of morphine was injected intrathecally to release endogenous dynorphin (4), 30 min after which the spinal cord was quickly removed and incubated with 5 mM DSS for 20 min. Thereafter, KOR IP and dynorphin and KOR Western blot analyses ensued as described above.

Implantation of i.t. Cannulae. A permanent indwelling cannula was inserted into the lumbar spinal cord subarachnoid space as described originally (5) and routinely performed in this laboratory (4). In brief, a saline-filled catheter (PE-10; Clay Adams) was inserted through an incision in the atlanto-occipital membrane, slowly introduced into the spinal cord subarachnoid space (8.0 cm), and secured in place. The cephalic portion of the catheter was externalized through the skin above the skull area where it was relatively inaccessible to the paws. Only animals that appeared to be free of infection upon gross inspection were used. Motoric integrity was assessed in all experimental groups by using the righting reflex and the inclined plane test. Those exhibiting motor impairment following surgery were eliminated from the study.

Intrathecal Administration of Drugs. Affinity-purified anti-dynorphin antibodies or nor-BNI were administered in 5–10 μL over a 60-s period to the subarachnoid space of the lumbar spinal cord via a permanent indwelling i.t. cannula. Complete delivery was ensured by flushing the cannula with an additional 10 μL of saline. Thereafter, tail flick latencies were determined at various intervals and compared with predrug thresholds. Neither the saline vehicle used to administer nor-BNI nor preadsorbed anti-dynorphin antibodies altered spinal morphine antinociception.

Assessment of Tail Flick Latency. Tail flick latency to radiant heat was quantified by using a Tail Flick Analgesia Meter (IITC) (6–8). Intensity of the radiant heat was adjusted such that baseline values average ≈3.5 s. A cutoff of 10 s latency prevented any untoward consequences to the tail.

Statistical Analysis. Significance of differences in the magnitude of Western blot signals was assessed by using the two-tailed Student's *t* test. A mixed linear model was used to assess effects on tail flick latency of drug treatment, stage of estrus cycle, and their interaction. There was a significant treatment by stage of cycle interaction effect ($F_{(2,36)} = 7.53$, $P = 0.002$), significant main effects for stage of cycle ($F_{(1,36)} = 14.3$, $P < 0.001$), and for treatment ($F_{(2,36)} = 20.2$, $P < 0.001$). Simple effects analyses between treatment groups showed significant difference between proestrous and diestrous groups for effects of morphine plus nor-BNI treatment ($P = 0.034$) and for effects of morphine plus anti-

dynorphin antibody treatment ($P < 0.001$) but not for treatment with morphine alone ($P = 0.568$). Simple effects analyses within the diestrous group showed that neither i.t. nor-BNI nor anti-dynorphin antibodies significantly altered spinal morphine antinociception (omnibus test $P > 0.2$ for both). In contrast, during proestrous, i.t. treatment with either not-BNI or anti-dynorphin antibodies significantly reduced spinal morphine antinociception ($P < 0.001$ for both treatments).

1. Wayneforth H, Flecknell P (1992) *Experimental and Surgical Techniques in the Rat* (Academic, London).
2. Chalecka-Franaszek E, Weems HB, Crowder AT, Cox BM, Côté TE (2000) Immunoprecipitation of high-affinity, guanine nucleotide-sensitive, solubilized mu-opioid receptors from rat brain: Coimmunoprecipitation of the G proteins G(alpha o), G(alpha i1), and G(alpha i3). *J Neurochem* 74:1068–1078.
3. Chakrabarti S, Chang A, Gintzler AR (2010) Subcellular localization of mu-opioid receptor G(s) signaling. *J Pharmacol Exp Ther* 333:193–200.
4. Liu NJ, von Gizycki H, Gintzler AR (2007) Sexually dimorphic recruitment of spinal opioid analgesic pathways by the spinal application of morphine. *J Pharmacol Exp Ther* 322:654–660.
5. Yaksh TL, Rudy TA (1976) Chronic catheterization of the spinal subarachnoid space. *Physiol Behav* 17:1031–1036.
6. Schmauss C (1987) Spinal k-opioid receptor-mediated antinociception is stimulus specific. *Eur J Pharm* 137:197–205.
7. Stewart PE, Hammond DL (1993) Evidence for delta opioid receptor subtypes in rat spinal cord: Studies with intrathecal naltriben, cyclic[D-Pen2, D-Pen5] enkephalin and [D-Ala2, Glu4]deltorphin. *J Pharmacol Exp Ther* 266:820–828.
8. D'Amour FE, Smith DL (1941) A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 72:74–79.