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INTRATHECAL NEUROPEPTIDE Y REDUCES BEHAVIORAL AND MOLECULAR MARKERS OF INFLAMMATORY OR NEUROPATHIC PAIN

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Abstract

Our previous work indicates that the intrathecal administration of neuropeptide Y (NPY) acts at its cognate receptors to reduce behavioral signs of nociception in several models of inflammatory pain, including the formalin test. The present study extends these findings to a rat model of peripheral neuropathic pain, and then evaluates the hypothesis that NPY inhibits inflammation- and nerve injury-induced activation of spinal nociceptive transmission. Here we show that NPY dose-dependently reduced behavioral signs of mechanical and cold hypersensitivity in the spared nerve injury (SNI) model. Intrathecal administration of either a Y1 (BIBO3304) or a Y2 (BIIE0246) receptor antagonist dose-dependently reversed the anti-allodynic actions of NPY. To monitor the effects of NPY on the stimulus-induced activation of spinal nociresponsive neurons, we quantified protein expression of the immediate-early gene *c-fos* in lamina I–VI of the L4–L5 dorsal horn, with special attention to the mediolateral pattern of Fos immunohistochemical staining after SNI. Either tactile stimulation of the hindpaw ipsilateral to nerve injury, or intraplantar injection of noxious formalin, increased the number of Fos-like immunoreactive profiles. Tactile stimulation evoked a mediolateral pattern of Fos expression corresponding to the innervation territory of the uninjured (sural) nerve. We found that intrathecal NPY reduced both formalin- and SNI-induced Fos expression. NPY inhibition of SNI-induced Fos expression was localized to the sural (uninjured) innervation territory, and could be blocked by intrathecal BIBO3304 and BIIE0246. We conclude that NPY acts at spinal Y1 and Y2 receptors to reduce spinal neuron activity and behavioral signs of inflammatory or neuropathic pain.

Keywords

spared nerve injury; formalin; allodynia; hyperalgesia; rat; c-fos

INTRODUCTION

Neuropeptide Y (NPY) is an abundant and widespread neuroactive peptide that exerts numerous physiological actions, including pain modulation. NPY and its Y1 and Y2 receptors are located at key pain signalling centers throughout the nervous systems, particularly the dorsal

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horn of the spinal cord (Gibson et al. 1984; Ji et al. 1994). Several studies indicate that NPY, when administered at the spinal level, inhibits transient or inflammatory pain (Taylor 2005; Smith et al. 2007). For example, intrathecal administration of NPY Y2 receptor agonists reduces not only behavioral responsiveness to noxious heat (Hua et al. 1991; Taiwo and Taylor 2002), but also the hyperalgesia associated with inflammation (Taiwo and Taylor 2002; Mahinda and Taylor 2004). Much less clear are the effects of NPY receptor agonists in models of neuropathic pain. Evidence from pharmacological studies suggest that intrathecally applied Y1 and Y2 agonists attenuate the flexor reflex in axotomized animals (Xu et al. 1999), but comparable studies in behavioral models of partial peripheral nerve injury are not available. Therefore, the present studies were designed to determine whether intrathecal administration of NPY dose-dependently reduces behavioral signs of neuropathic pain, and whether such effects are mediated by Y1 or Y2 receptors. Furthermore, we asked whether intrathecal NPY inhibits formalin- and nerve injury-induced activation of spinal nociresponsive neurons. To address these questions, we mapped the population of neurons in the dorsal horn that respond to somatosensory stimulation with induction of the proto-oncogene, *c-fos* (Coggeshall 2005). We began our Fos studies with a commonly-used stimulus involving the intraplantar injection of dilute formalin. Formalin first produces a short-lived, rapid-onset period (Phase 1) of peripheral nerve activity, sympathetic activity, and pain-like behaviors (Tjolsen et al. 1992; Taylor et al. 1995b; Puig and Sorkin 1996). Phase 1 behaviors are a direct result of tissue injury and / or chemical activation of primary afferent terminals. Phase 1 is followed by a brief quiescent period essentially devoid of nociception ("interphase"), and then by a more persistent period (Phase 2) of licking, lifting and flinching behavior (Tjolsen et al. 1992). Peripheral sensitization coincides with the release of inflammatory mediators, and likely contributes to the ongoing activation of peripheral inputs that contribute to the maintenance of Phase 2 (Taylor et al. 1995b; Taylor et al. 2000). We then extended our analysis to the spared nerve injury model. This model of peripheral neuropathic pain produces robust signs of allodynia, and here we show for the first time that this is accompanied with stimulus-induced Fos expression in the dorsal horn.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles Rivers Laboratories, Inc) were housed in individual cages in a temperature controlled room on a 12-hour light/dark cycle (6am/6pm), and were given food and water *ad libitum*. All animal use protocols were approved by the IACUC of Tulane University.

Intrathecal Drug Delivery Methods

Rats (280–300 g) were fitted with intrathecal catheters to deliver drugs or saline to the lumbar spinal cord. Anesthesia was induced and maintained throughout surgery with 5% and 2–3% isoflurane, respectively.

Formalin studies—This procedure is a modification of that originally described by Storkson et al. (Storkson et al. 1996; Pogatzki et al. 2000; Taiwo and Taylor). A 32G polyurethane (PU) catheter was used to minimize morphological changes that may be associated with the use of polyethylene (PE) tubing (Sakura et al. 1996). To construct the catheter, the distal end of a 15 cm 32G PU catheter was inserted into a 15 cm piece of PE-10 tubing and the connection was sealed using super glue and 5-min epoxy, which was allowed to dry for 12 hours. A longitudinal 2–3 cm midline incision was made at the level of the iliac crest. A 23G guide needle (Lazlo Bocksai, UCSF Physiology machine shop) was advanced between the L5 and L6 vertebrae. Following confirmation of correct placement with a tail or paw flick, the 32 g PU end of the catheter, which was reinforced with a Teflon-coated stainless steel stylet, was inserted through

the needle and cranially advanced 4 cm. The 23G needle was removed and the catheter withdrawn, leaving 26 cm exteriorized. A 1-cm^2 sterilized cloth mesh (Instech Laboratories Inc., Plymouth Meeting, PA) was placed about the catheter and a drop of Dermabond (Ethicon, Inc., Somerville, NJ) secured the catheter and mesh to the underlying tissue. A 2 cm diameter loop of catheter was secured to muscle with 2 loosely tied 4-0 sutures. The stylet was removed. The catheter was tunnelled under the skin, exteriorized at a 1 cm incision at the nape, and secured to the neck muscle with 4-0 suture. The exteriorized end of the catheter was cauterized to prevent leakage. Placement was tested two days after catheterization with the intrathecal administration of 10 μl of 4% lidocaine. Animals not responding to lidocaine with temporary hind limb paralysis were euthanized and excluded from further study (~25%).

NPY antagonist studies in the SNI model—Due to the suboptimal success rate of the lumbar approach towards i.t. catheterization (~75%), subsequent studies used the classic cisternal approach (Yaksh and Rudy 1976; LoPachin et al. 1981). Under isoflurane anaesthesia, rats (290–310 g) were placed in a stereotaxic instrument (Stoelting, Wood Dale, IL) with the snout angled downwards 45° relative to the horizontal. A 1.5 cm midline incision extended from the ears to the atlas. Fascia and superficial neck muscles were bluntly dissected and retracted to expose the atlanto-occipital membrane. This membrane was punctured with the tip of a 27 g needle. A PE-10 catheter was inserted and advanced caudally 7.5 cm through the intrathecal space. When necessary, tension was applied to the tail to straighten the spine. The retractor was removed, the catheter was sutured to the neck muscle (5-0 Ethicon), and then the skin incision was closed. Finally, the external end of the catheter was cauterized to prevent any leakage or environmental contamination. Animals with signs of paralysis or other motor impairment lasting more than 24 hr were euthanized and excluded from further study (<20%).

Spared Nerve Injury (SNI) surgery

Immediately following intrathecal PU or PE catheterization, nerve injury was performed as described previously (Decosterd and Woolf 2000). An incision was made in the skin at the level of the trifurcation of the left sciatic nerve. The overlying muscles were retracted, exposing the common peroneal, tibial, and sural nerves. With care taken to avoid mechanical stimulation of the sural branch, the common peroneal and tibial nerves were loosely ligated with 6.0 silk (Ethicon, Somerville, NJ), followed by transection of 1 mm of each nerve at either end of each knot. The muscle was sutured with absorbable 5-0 sutures (Ethicon) and the wound was closed with 9mm metal clips.

Formalin studies: Drug Administration and Behavioral Testing

Drug Administration—One wk after surgery, a 4-cm piece of PE-10 tubing was connected to the intrathecal catheter assembly. The PE10 was loaded with 10 μl of drug or saline, a 1 μl air bubble, and a 10 μl saline flush. After a 30–60 min acclimation period, the rat was gently restrained, and then saline, NPY (30 μg) or morphine (20 μg) were intrathecally injected in total volume of 10 μl. Thirty min later, formalin (5% solution of 37% formaldehyde, 50μl) was subcutaneously injected into the plantar aspect of the right hindpaw, followed by behavioral measurement for 60 min.

Behavioral Testing—As previously described (Taylor et al. 1995a; Peterson et al. 1997), the number of flinches and time spent licking the injured paw during the second, third, fourth, and fifth min after injection were counted during Phase 1 (time points 1–4). Time points 1–3 and 3–5 were summed for graphical presentation. During the Interphase and Phase 2 (time points 8–60), the number of flinches and time spent licking were counted every five min, in two min bins, e.g. 8–10, 13–15, 18–20, … 58–60 (Mahinda and Taylor 2004).

Nerve Injury Studies: Drug Administration

One wk after surgery, drugs were administered as in the formalin studies. Seven d after SNI, allodynia and hyperalgesia were assessed before and after intrathecal administration of saline, NPY (3.0, 6.5, 10, or 30 μg), the Y1 antagonist BIBO3304 (1.0 μg), the Y2 antagonist BIIE0246 (1.0 or 10 μg), or a combination of NPY (30 μg) + BIBO (1.0 or 3.0 μg) or NPY (30 μg) + BIIE (0.01, 0.03, 0.3, 1.0, 3.0, or 10 μg). BIBO3304 was administered concomitantly with NPY. To take advantage of the extended duration of action of BIIE0246 (El Bahh et al. 2002), it was administered 90 min prior to NPY injection.

Some animals in this study (Fig 7–Fig 8) were not implanted with indwelling catheters. Instead, saline or drugs were administered via direct intrathecal injection (Mestre et al. 1994;Fairbanks 2003). Under light isoflurane anaesthesia (1.5%), a small midline skin incision (< 1 cm) exposed the muscle above the L5–L6 lumbar vertebrae. A 25 gauge needle (1.5 in), attached to a 50 μl Hamilton syringe, was inserted between the L5 and L6 vertebrae at an ~80 degree angle. Following contact with bone, the needle angle was lowered to ~30 degrees and advanced slightly until a reflexive tail flick was observed. Saline or drug was rapidly injected in a volume of 10 μl.

Nerve Injury Studies: Behavioral Testing

Intrathecal injections were followed by either: 1. Behavioral testing for allodynia and hyperalgesia or 2. Somatosensory stimulation to induce Fos expression. Drug administration was randomized and the observer was blind to experimental condition.

Mechanical allodynia—Animals were acclimated to their testing boxes (transparent Plexiglass boxes placed on a raised metal mesh grid) for 30–60 min. A series of 8 von Frey (VF) monofilaments (Stoelting, Inc, Wood Dale, IL) were applied to the lateral aspect of the plantar surface of the hind paw. This area is innervated by the sural branch of the sciatic nerve. A modified version of the 50% withdrawal threshold was determined using the up-down method of Dixon, modified by Chaplan et al (Chaplan et al. 1994). First, an intermediate monofilament (number 4.31, exerts ~2.0g of force) was gently applied with enough force to cause it to bend. This was repeated up to three times in distinct areas along the lateral paw. In case of a positive response (rapid withdrawal of the paw within 2 sec), a smaller filament was tested. If no response was recorded in any of the three different areas, a larger filament was tested. Occasionally, animals did not develop mechanical allodynia on the day of pharmacological testing after nerve injury (VF threshold > 5.0 g on the nerve-injured side). In such cases, von Frey testing was either terminated immediately or its data was not included in the final analysis (<5%).

Cold allodynia—Using a syringe connected to PE-90 tubing, flared at the tip to a diameter of 3½ mm, we applied a drop of acetone to the plantar paw. Surface tension maintained the volume of the drop to 10–12 μl. The length of time the animal lifted or shook its paw was recorded. The duration of paw response was recorded for 30 sec. Three observations were averaged. Occasionally, animals did not develop cold allodynia on the day of pharmacological testing after nerve injury (<3 sec). In such cases, acetone testing was either terminated or its data was not included in the final analysis (<5%).

Mechanical hyperalgesia—Next, we gently applied the sharp edge of a diaper pin to the footpad, avoiding damage to the skin. The duration of paw withdrawal was recorded for 30 sec. Three observations were averaged. If animals did not develop mechanical hyperalgesia on the day of pharmacological testing (<2.5 sec), testing was terminated or its data was excluded from the study (7%).

Fos Stimulation—We were unable to evoke Fos expression with 3 applications/min for 10 min of the number 4.34, 5.5g von Frey hair or application of 1 drop of acetone every 30 sec for 10 min (data not shown). Therefore, we utilized the method of Ma et al (Ma and Woolf 1996). Briefly, while gently restraining the rat, the experimenter manually stroked the ventrolateral aspect of the hindpaw with their thumb for 2 sec. This was repeated every 4 sec for 10 min. Animals were perfused 2 hr after formalin injection or initiation of non-noxious stimulation.

Immunohistochemistry

Animals were deeply anesthetized with an intramuscular ketamine/xylazine (Vedco, St. Joseph, MO, Henry Schein, Melville, NY, respectively) injection (1 ml/kg of 88.9 mg/ml ketamine/11.1 mg/ml xylazine) and perfused with 150–250 ml PBS followed by 250–500 ml of 10% buffered formalin. Spinal cords were removed, post-fixed for 4–16 hr, and cryoprotected in 30% sucrose overnight. Forty micron sections were cut on a freezing microtome (Leica, Bannockburn, IL). After washing 4 × 15 min in 0.1 M PB and blocking for 1 hr in normal goat serum from the Vectastain ABC-AP kit (AK-5001, Vector Labs, Burlingame, CA), sections were incubated with a rabbit anti-Fos primary antibody (1:20,000, Calbiochem, San Diego, CA) at RT for 16–24 hr or at 4° for 48–72 hr. Next, the tissue was washed again with 0.1 M PB (4 × 15 min) and incubated with a goat anti-rabbit biotinylated secondary antibody, also from the Vectastain ABC-AP kit, for 2 hr at 4°. After 3 × 5 min washes in 0.1 M PB, the tissue was incubated with an avidin/biotinylated enzyme complex from the Vectastain Elite ABC kit (PK-6100, Vector Labs) for 1 hr at 4°. The tissue was again washed 4 × 15 min in 0.1 M PB. For visualization of the Fos protein product, tissue slices were incubated in a DAB substrate kit (SK-4100, Vector Labs) for approximately 5 min, and then rinsed in 0.01 M PB for 3 × 10 min. Sections were mounted on Superfrost/Plus glass slides (Fisherbrand, Houston, TX) and then cover-slipped using Permount (Fisherbrand, Houston, TX). Using a Nikon TE2000-E microscope with Metamorph software (Version 6.1r4, Universal Imaging Corp.), digital photomicrographs of 4–5 randomly selected sections were taken from lumbar segment L4–L5. Pictures were printed and Fos-like immunoreactive (Fos-LI) profiles were manually counted by an observer blind to treatment, as described previously (Abbadie et al. 1997).

To quantify Fos-LI for mediolateral analysis, we used a technique developed by Sugimoto et al. (Sugimoto et al. 1993), based on the anatomic findings of Swett and Woolf (Swett and Woolf 1985). We measured the mediolateral extent of the dorsal horn and divided it into eight equal horizontal segments. The most medial 3 segments roughly correspond with the tibial territory, while the next 3 segments correspond to the common peroneal/sural innervation territories. After dividing the images in this manner, the neurons within the segments were manually counted by an observer blind to treatment. The most lateral two segments are not innervated by the sciatic nerve and therefore not analyzed further.

Materials

Human NPY, obtained from Anaspec (Cat #22465, San Jose, CA), was diluted in distilled water, divided into aliquots, and frozen at −80°C until use. The Y2 receptor antagonist BIIE0246 (Cat # 1700, Tocris, Ellisville, MO) was diluted in 10% DMSO and made fresh as needed. The Y1 receptor antagonist BIBO 3304 was generously provided by H. Doods (Boehringer Ingelheim, Biberach, Germany) and diluted in saline as needed. Lidocaine was obtained from Bimeda (Dublin, Ireland). Isoflurane was from Abbott Labs (Chicago, Ill).

Data Analysis

To analyze the formalin behavior studies (Fig. 1), we used GraphPad Prism 4 software (GraphPad Software, Inc. San Diego, CA), to conduct two-way ANOVAs across the entire 60

min period, with Drug as the between-subjects factor and Time as the repeated measure. When F values were significant ($p<.05$), Bonferroni's post hoc tests were used to determine differences between drugs at each time point. Separate ANOVAs were conducted for Phase 1 and Phase 2, comparing NPY and morphine to saline. Bonferroni post hoc tests were used to determine differences between drugs in each Phase.

For the dose-response SNI studies (Fig. 4), two-way ANOVAs were conducted, with Dose as the between-groups factors with five levels (saline, NPY 3 μg, NPY 6.5μg, NPY 10μg, and NPY 30 μg). Significant main effects involving Dose were further investigated with one-way ANOVA comparing the saline group with each dose of NPY from time 0–90 min. For the NPY antagonist studies (Fig. 5), we conducted one-way ANOVA with Drug as the between groups factor. When F values were significant ($P<0.05$), post hoc Bonferroni or Student's t-test was used to determine differences between two groups.

For the Fos studies, we conducted two-way ANOVA to analyze the effect of Drug and laminar region of the dorsal horn (Laminae I–II, Laminae III–IV, and Laminae V–VI) on the number of Fos-positive neurons. Further one-way ANOVAs were conducted on the effect of Drug within each laminar group. When F values were significant ($P<0.05$), post-hoc Bonferroni and Student's t-tests were used to determine the differences between two drugs groups within each laminar regions: saline vs NPY or morphine (Fig. 3); saline vs NPY or NPY/BIBO or BIBO and NPY/BIIE or BIIE (Fig. 8). All data are presented as mean ± SEM.

RESULTS

Intrathecal NPY reduces formalin-induced behavior and spinal Fos expression

Noxious stimulus-induced Fos expression is the best method to map populations of nociresponsive neurons in the dorsal horn of the awake animal (Coggeshall 2005). To test the hypothesis that intrathecal administration of NPY inhibits spinal nociceptive transmission, we evaluated formalin-induced behavior and Fos expression. Based on previous dose-response and toxicity studies, we chose an analgesic dose of NPY (30 μg) that lacks non-specific motor effects (Taiwo and Taylor 2002; Mahinda and Taylor 2004). For comparative purposes, we evaluated the effects of morphine at a dose (20 μg) that is equi-analgesic to NPY in terms of inhibition of Phase 2 formalin behavior (Yamamoto and Yaksh 1992). Morphine served as a positive control because it consistently reduces formalin-induced behavior and Fos expression (Menetrey et al. 1989).

As illustrated in Fig. 1, formalin produced overt behavioral signs of nociception with the expected biphasic profile in saline control animals, and two-way ANOVA revealed that Drug reduced flinching [$F(2,216) = 29$, $P<0.0001$] and licking [$F(2,216) = 25$, $P<0.0001$]. As observed previously (Mahinda and Taylor 2004), NPY did not significantly reduce Phase I flinching or licking ($P>0.05$), while morphine completely inhibited flinching and licking during Phase I ($P<0.05$). In contrast, both NPY and morphine blocked Phase II: one-way ANOVA revealed that Drug reduced Phase II flinching [$F(2,18) = 26$, $P<0.001$] and licking [$F(2,18) = 34$, $P<0.001$]. Qualitative assessment of gross motor behavior indicated that morphine, but not NPY, produced mild signs of sedation.

As described previously, basal Fos expression in the dorsal horn is quite low (data not shown). Fig 2 illustrates that unilateral hindpaw injection of formalin produced a robust increase in Fos expression throughout the ipsilateral L4–5 dorsal horn, particularly in laminae I–II. Fig 2 and Fig 3 illustrate that both NPY [$F(2,17) = 10.89$, $P<0.01$] and morphine [$F(2,17) = 10.89$, $P<0.01$] reduced Fos-LI by roughly 50% throughout the dorsal horn (laminae I–VI), and further post-hoc tests indicated significant effects within each laminar region. Lower numbers of Fos-

LI was observed on the contralateral side and was changed by neither NPY nor morphine (P>0.05).

Our previous study investigated the contribution of NPY Y1 and Y2 receptors to formalin nociception, and we found that BIBO partially reversed flinching behavior, while BIIE had no effect (Mahinda and Taylor, 2004). Thus, the Fos studies described below focus on the actions of NPY receptor antagonists in neuropathic pain, rather than in formalin-induced pain.

Intrathecal NPY dose-dependently attenuates behavioral signs of neuropathic pain

To test the hypothesis that NPY inhibits behavioral signs of neuropathic pain, we evaluated allodynia and hyperalgesia after intrathecal injection of saline or drug. As illustrated in Fig 4, NPY dose-dependently inhibited mechanical allodynia [$F(4,25) = 7.8$, $P<0.001$], mechanical hyperalgesia [$F(4,24) = 3.1$, $P<0.05$], and cold allodynia [$F(4,27), = 4.98$ $P<0.05$]. In general, these effects peaked at 15 min and lasted over 120 min (Fig 4A, C, E). For example, the 30 μg dose significantly reduced mechanical allodynia at the 15 min ($P<0.01$) and 60 min ($P<0.05$) time points. When responses were averaged between 15–90 min, the 30 μg dose significantly reduced mechanical allodynia ($P<0.0001$) and mechanical hyperalgesia ($P < 0.05$), and almost reached significance in reducing cold allodynia ($P = 0.063$). The 10 μg dose also reduced mechanical allodynia, but again this effect missed statistical significance ($P = 0.077$). The ED50 for NPY was 9.98 μg (mechanical allodynia) and 6.45 μg (cold allodynia) (Fig 4B, D). We could not determine ED50 for mechanical hyperalgesia due to the non-sigmoidal relationship between dose and response (Fig 4F). NPY did not change paw withdrawal behavior on the contralateral paw at any dose (data not shown).

As an additional control, we next determined whether NPY changes sensitivity in animals without nerve injury. We observed no significant difference in VF (15±0 vs 14.7±0.3), acetone (0.21±0.03 vs 0.06±0.06) or pin prick (0.36±0.07 vs 0.23±0.06) responses between the saline vs 30 μg NPY groups, respectively (P>0.05).

Both Y1 and Y2 receptors contribute to the anti-allodynic effect of intrathecal NPY

To evaluate the NPY receptor subtype that mediates the anti-allodynic effects of NPY, we tested subtype-selective Y1 and Y2 receptor antagonists (BIBO3304 and BIIE0246, respectively.) As illustrated in Fig 5A, BIBO3304 dose-dependently reversed the anti-allodynic effects of NPY [$F(4,26) = 4.04$, $P<0.05$], with complete inhibition at the 3 μg dose. Similarly, Fig 5B illustrates that BIIE0246 dose-dependently attenuated the anti-allodynic effects of NPY [$F(9,39) = 2.216$ $P<0.05$], with a maximal effect at the 0.3 and 3 μg doses (the 1 μg dose yielded an effect that missed statistical significance, $p=0.062$, $n=6$). Neither antagonist had an appreciable effect on behavioral responses elicited from the contralateral paw (data not shown).

Comparison of formalin-vs SNI-induced Fos expression

We divided the dorsal horn into 8 mediolateral subdivisions so as to more closely examine the somatotopic pattern of Fos-LI. The mediolateral distribution of Fos in the dorsal horn was quite different in formalin and SNI animals. Formalin evoked Fos-LI throughout the medial and central regions of the superficial dorsal horn (Fig 2 and Fig 6b) indicate a consistent mediolateral distribution of Fos-LI. In contrast, Fos-LI evoked by tactile stimulation + SNI occurred primarily within the central region. Thus, as illustrated in Fig. 6a and Fig 7a, the number of Fos-positive neurons was significantly lower in the medial region as compared to the lateral region (5.0 ± 1.0 vs 13.0 ± 1.6, respectively; $P<0.001$). The medial region corresponds to the innervation territory of the transected tibial nerve in our SNI model, as predicted by the seminal work of Swett and Woolf (Swett and Woolf 1985;Shields et al. 2003).

We were concerned that variability of Fos-LI in the SNI model could reduce analytical power. To address this, we roughly compared variability in total ipsilateral Fos-LI following SNI with a more established paradigm, the formalin test. We found that variability (standard deviation divided by the group mean) was 42% and 39% in the formalin and SNI groups, respectively. The similar variability indicates that SNI-induced Fos is at least as reliable a measure of spinal neuron activation as is formalin-induced Fos.

Further characterization of Fos-LI after SNI—Although nerve injury alone does not trigger Fos expression (Rodella et al. 2005), mechanical stimulation of the hindpaw after peripheral nerve injury does (Catheline et al. 1999). Fig 8 illustrates robust Fos expression in laminae I–II of nerve-injured rats at the ipsilateral side. This was greater than that evoked either on the contralateral side of SNI rats (17.9±2.1 vs 5.1±1.4; $P<0.01$, data not shown) or on the ipsilateral side of sham rats (17.9±2.1 vs 3.6± 0.4; $P<0.005$). As compared to sham, nerve injury induced a small, statistically insignificant change in contralateral Fos-LI in laminae I–II (5.1±1.5 vs 1.8±0.5; $P>0.05$).

Previous studies have reported that either intrathecal catheterization or vehicle injection bilaterally increases Fos expression in the dorsal and ventral horns (Luo et al. 1995). However, Fig 8A shows that stimulus-induced Fos expression in the setting of SNI did not differ between non-catheterized rats (17.9±2.1, second bar from the left) and intrathecally-catheterized rats given saline (18.1±2.2, third bar from the left).

NPY reduces stimulus-induced Fos expression in animals with SNI

Fig 7 and Fig 8 illustrate that drug treatment changed the number of Fos-positive neurons in laminae I–VI. Further analysis of laminar regions revealed an effect in laminae I–II [one-way ANOVA: $F(5,41) = 4.76$; $P=0.0019$], laminae III–IV [one-way ANOVA: $F(5, 41) = 3.2$; $P<0.05$], and laminae V–VI [one-way ANOVA: $F(5, 41) = 3.6$; $P<0.05$]. Further analysis in the mediolateral plane revealed an overall drug effect in the lateral region [$F(5,41) = 4.79$; $P=0.0018$] but not the medial region ($P>0.05$). Indeed, all subsequent analyses (see below) suggest that drug effects in lamina I–II were localized to the lateral aspects of the dorsal horn (Fig 8A).

Fig 7B and Fig 8A–C illustrate that, compared to saline, intrathecal NPY significantly reduced the total number of Fos-positive neurons in laminae I–II by 59% (18.1±2.2 vs 7.5±0.9; $P<0.01$), but not laminae III–IV or V–VI ($P>0.05$). As illustrated in Fig 7C and Fig 8, the Y1 receptor antagonist BIBO3304 reversed the effect of NPY, and Fos-LI in laminae I–II was similar to those of the saline group (14.2±0.9 vs 18.1±2.2, respectively; $P>0.05$). Fig 7D and Fig 8 indicates that BIBO alone had no effect in laminae I–II or III–IV, but increased Fos-LI in laminae V–VI ($P<0.05$, Fig 8C).

As shown in Fig 7E and Fig 8A, the Y2 receptor antagonist BIIE0246 also reversed the effect of NPY; Fos-LI in laminae I–II was similar to those of the saline group (13.6±1.9 vs 18.1±2.2, respectively; $P>0.05$). Fig 7F and Fig 8 indicates that BIIE alone had no significant effect as compared to saline ($P>0.05$).

DISCUSSION

NPY reduces noxious stimulus-induced behavior

Early nociception in the formalin test—The present studies demonstrate that intrathecal administration of morphine (20 μg) abolished Phase 1. This agrees with previous findings indicating that intrathecal morphine and other opioid receptor agonists, like remifentanil, decrease formalin-induced behavior (Yamamoto and Yaksh 1992; Taylor and Basbaum

2000). By comparison, the 30 μg dose of NPY only partially attenuated Phase 1 and did not disrupt motor coordination (Taiwo and Taylor 2002). The present results suggest NPY is no better than morphine at inhibiting formalin-induced nociception during Phase 1.

Persistent nociception in the formalin test—The present studies indicate that both NPY (30 μg) and morphine (20 μg) eliminated Phase 2 (Yamamoto and Yaksh 1992; Mahinda and Taylor 2004). Unlike morphine, NPY did not produce sedation. These results confirm our previous conclusion that NPY is efficacious in reducing the persistent nociception associated with formalin injection (Mahinda and Taylor 2004).

NPY reduces noxious stimulus-induced spinal Fos expression

While many studies indicate that systemic morphine reduces noxious stimulus-induced Fos expression (Menetrey et al. 1989), far fewer studies have utilized the intrathecal route of administration. One article indicated that a 10 μg dose of morphine reduced formalin-induced Fos expression by roughly 40% (Labuz et al. 2003). Here, we administered doses of morphine and NPY that produce maximal reduction in formalin-induced behavior, and asked whether NPY would be as effective as morphine in reducing Fos-LI. We found that 20 μg of morphine reduced Fos-LI in profiles of laminae I–VI by 56%, while 30 μg NPY reduced Fos by 51%. Because both phases of the formalin test substantially contribute to Fos-LI (Abbadie et al. 1997), the ability of NPY to reduce Fos to the same extent as morphine, without eliminating Phase 1, suggests that this peptide is an outstanding candidate for the interruption of spinal nociceptive transmission.

NPY reduces behavioral signs of neuropathic pain

Although Naveilhan et al. reported that intrathecal NPY decreased the heat hypersensitivity associated with partial sciatic nerve injury in mice, this study only evaluated a single dose of NPY and a single behavioral endpoint (Naveilhan et al. 2001). Our studies extend these results to the SNI model and to the rat, and show that NPY dose-dependently inhibits behavioral signs of neuropathic pain. NPY increased von Frey threshold and reduced response duration to both noxious mechanical and cold stimuli, indicating that its inhibitory effects span multiple sensory modalities. NPY inhibition does not result from non-specific behavioral effects, because these doses of NPY do not disrupt motor coordination (Taiwo and Taylor 2002).

We conclude that intrathecal NPY reduces behavioral signs of neuropathic pain. This conclusion neither supports nor contradicts the findings of Ossipov et al, who found that intracranial administration of anti-NPY antiserum or BIBO3304 into the nucleus gracilis reversed nerve injury-induced mechanical allodynia (Ossipov et al. 2002). A harmonious explanation is that NPY can either inhibit or facilitate neuropathic pain, depending on whether it is administered in the spinal cord or nucleus gracilis, respectively.

NPY Y1 receptors contribute to the anti-allodynic actions of NPY

The Y1 antagonist BIBO3304 dose-dependently reversed the anti-allodynic and antihyperalgesic actions of NPY. Furthermore, BIBO3304 reversed the inhibitory effect of NPY on Fos-LI, a molecular correlate of spinal neuron activation. We conclude that intrathecal NPY acts at spinal Y1 receptors to inhibit the activation of pain-related neurons in the dorsal horn, ultimately leading to a reduction in behavioral signs of neuropathic pain.

NPY Y2 receptors contribute to the anti-allodynic actions of NPY

The present studies indicate that the Y2 receptor antagonist BIIE0246 dose-dependently reversed the anti-allodynic effects of NPY in the SNI model of neuropathic pain. We also found that BIIE0246 reversed the inhibitory effect of intrathecal NPY on Fos-LI in the superficial

dorsal horn. Taken in combination with our Y1 antagonist data, our results suggest that NPY acts at both receptors to inhibit spinal neuron activation. Our results extend previous studies indicating that Y2-preferring agonists inhibit the flexor reflex in axotomized, anesthetized rats, an alternative model of neuropathic pain (Xu et al. 1999). In contrast to the cellular localization of Y1 receptors, spinal Y2 receptors are located on primary afferent terminals (Brumovsky et al. 2005). Because axotomy increases Y2-mediated inhibition of Ca^{2+} channel currents in dorsal root ganglion neurons (Abdulla and Smith 1999), and NPY attenuates excitatory postsynaptic currents in substantia gelatinosa cells via presynaptic Y2 receptors (Moran et al. 2004), we suggest that NPY acts at presynaptic Y2 receptors to inhibit the release of pronociceptive neurotransmitters. One such neurotransmitter could be glutamate, since Y2 agonists inhibit glutamate release from spinal synaptosomes (Martire et al. 2000).

Spinal Fos expression in the spared nerve injury model of neuropathic pain

This report is the first to validate stimulus-evoked Fos expression in the dorsal horn after SNI. Variability was no greater than that produced in a more established paradigm, the formalin test. As in other models of peripheral neuropathic pain (Catheline et al. 1999), SNI alone induced little Fos-LI. However, the application of a non-noxious mechanical stimulus to the hindpaw substantially increased the number of Fos-positive profiles in the substantia gelatinosa. Similar increases in laminae I–II Fos expression were obtained following the application of a non-noxious tactile stimulus in the sciatic nerve crush and chronic constriction injury models of peripheral neuropathic pain (Bester et al. 2000; Catheline et al. 2001).

We found that immunoreactive cells were particularly abundant in the mediolateral area innervated by central terminals of the spared sural nerve, as predicted by the anatomical studies of Swett and Woolf (Swett and Woolf 1985; Shields et al. 2003). By contrast, only a few Fos-positive profiles were found in the three medial-most subdivisions, areas of the dorsal horn likely innervated by the (cut) tibial nerve. We found that NPY significantly reduced Fos-LI in the lateral region; this was reversed by both BIBO3304 and BIIE0246. Because stimulus-induced Fos expression did not differ between non-catheterized and catheterized rats, intrathecal catheterization did not confound our results as might have occurred in previous studies (Luo et al. 1995). We conclude that NPY acts at Y1 and Y2 receptors in the region of the spinal cord that receives afferent input from the uninjured sural nerve.

Our studies revealed that intrathecal BIBO3304 not only reverses NPY-induced reduction of Fos-LI in laminae I–II, but also, when administered alone, significantly increases Fos-LI in laminae V–VI. Y1 receptors label numerous laminae within the dorsal horn, including “Type 1” small fusiform neurons of laminae I–II, and large “Type 5” multipolar lamina V neurons (Brumovsky et al. 2006). We speculate that the Y1 receptor antagonist BIBO3304 increased Fos-LI in lamina V by: 1. Indirect actions at Type I Y1 interneurons in lamina II that project to lamina V (Eckert et al. 2003); or 2. Direct actions at Type 5 Y1 projection neurons. Thus, Y1 receptor-mediated inhibition of nociceptive transmission may occur at multiple laminar levels: at laminae I–II to inhibit the actions of exogenously-administered NPY, and ultimately at deeper lamina V to inhibit the actions of endogenous neurotransmitters.

Although nerve injury produced an almost complete absence of Fos staining in the medial portion of the dorsal horn, we did observe more neurons in this region in SNI animals than in controls. We discuss two explanations for this unexpected finding. First, Sugimoto et al. found that tibial neurotomy acutely ablated intraplantar formalin-induced Fos staining in this region at one week; interestingly, staining began to normalize after just two weeks (Sugimoto et al. 1993; Sugimoto et al. 1994). We speculate that after SNI, afferent terminals from the sural innervation territory sprout into the tibial region, thus allowing peripheral stimulation of the uninjured sural nerve to activate Fos in the tibial innervation territory. Our study falls within the suggested timeframe for sprouting after peripheral nerve injury (Woolf et al. 1995; Kohama

et al. 2000). A second hypothesis for the appearance of Fos-LI in the medial dorsal horn after SNI is an increase in synaptic input, due to trans-synaptic dendritic atrophy. Sugimoto et al. (Sugimoto et al. 1990) found that chronic constriction injury was sufficient to induce transsynaptic degeneration, which, if it occurred on interneurons, would cause a loss of inhibitory tone and ultimately increase projection neuron activation. However, an important caveat to this conclusion is that not all cells that express Fos-LI are projection neurons; some are GABA- or glycinergic, and therefore are inhibitory (Blomqvist and Craig 2000).

Summary

Taken together, our results suggest that intrathecal NPY acts at Y1/Y2 receptors to inhibit spinal neuron activation, leading to antinociceptive and anti-allodynic effects. NPY inhibition of SNI-induced Fos expression was localized to the sural (uninjured) innervation territory. Such conclusions cannot be drawn from Fos-LI experiments using other common models of neuropathic pain, such as chronic constriction injury or partial sciatic nerve ligation (due to the mixed topographic distribution of spinal axon terminals of injured afferents). Our results illustrate the value of using SNI to study neuroplasticity in the dorsal horn following peripheral nerve injury. In conclusion, Y1- or Y2-selective receptor agonists may be clinically effective in the treatment of neuropathic pain.

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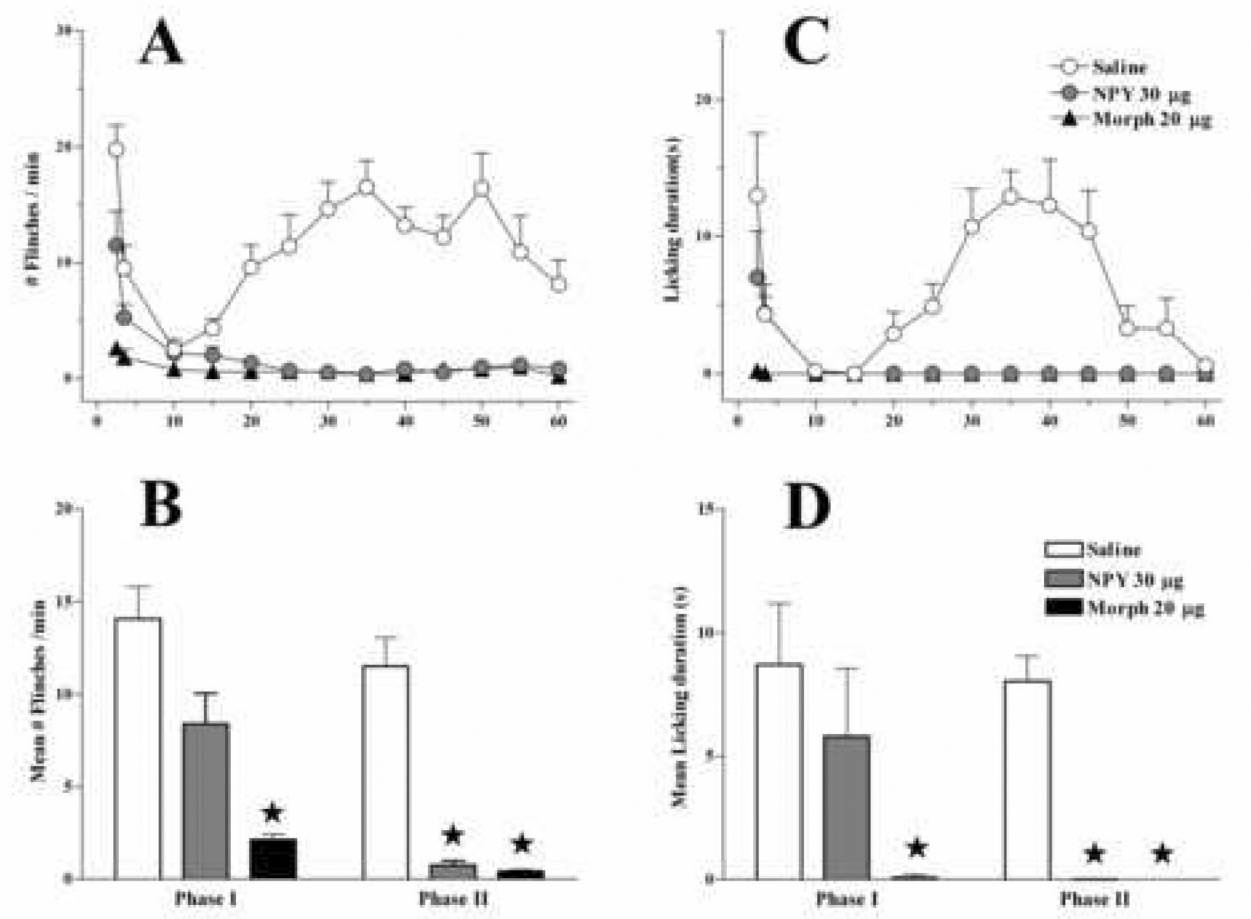

Figure 1. Intrathecal NPY reduces formalin-induced behaviors

Intraplantar administration of formalin (5% in 50 µl injected at time 0) elicits biphasic increases in both the number of flinches/min (A) and seconds spent licking the injured paw (C) following intrathecal injection of saline (n=10). Intrathecal NPY 30 min prior to formalin injection significantly reduced Phase I flinching and licking and completely blocked Phase II (n=6). Intrathecal morphine (n=5) almost completely abolished behavior during both phases. Values for Phase I represent averages from time 0–5; Phase II values are averages from time 20–55 (B, D). Values represent mean ± SEM; * = $P<0.05$ vs saline.

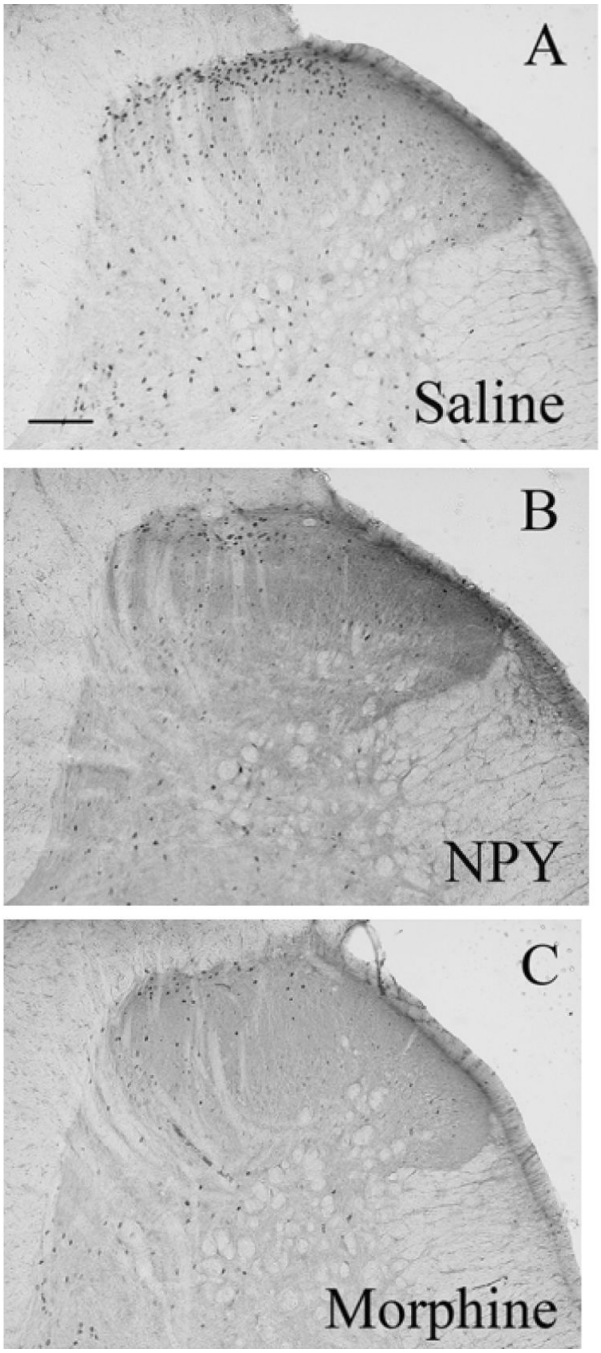

Figure 2. Formalin-induced Fos expression in the dorsal horn

These panels depict representative photomicrographs of the L4 dorsal horn for each group of animals treated with intrathecal saline (A), NPY (B) or morphine (C). Animals were perfused 2 hr after intraplantar formalin. Only the side ipsilateral to formalin injection is shown. Magnification = 100x, scale bar = 200 μm.

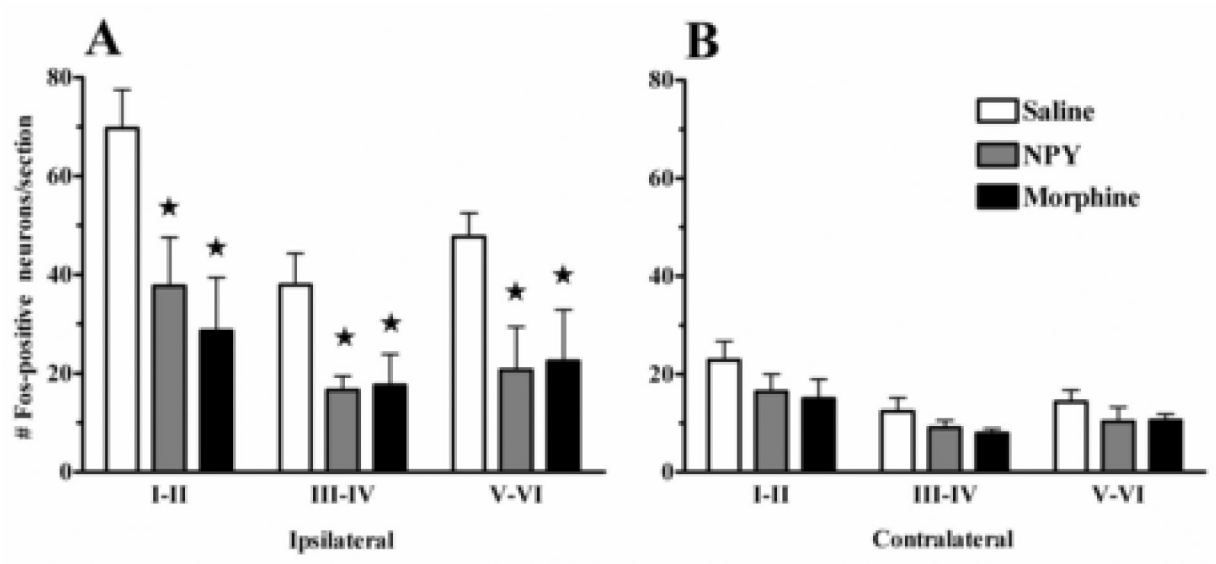

Figure 3. NPY reduces formalin-induced Fos expression in the dorsal horn

Both NPY (30 μg) and morphine (20 μg) decreased Fos expression in all laminae of the L4–L5 dorsal horn on the side ipsilateral (A) but not contralateral (B) to formalin injection. Values represent mean ± SEM. * $p<.05$ vs saline. n = 5–9.

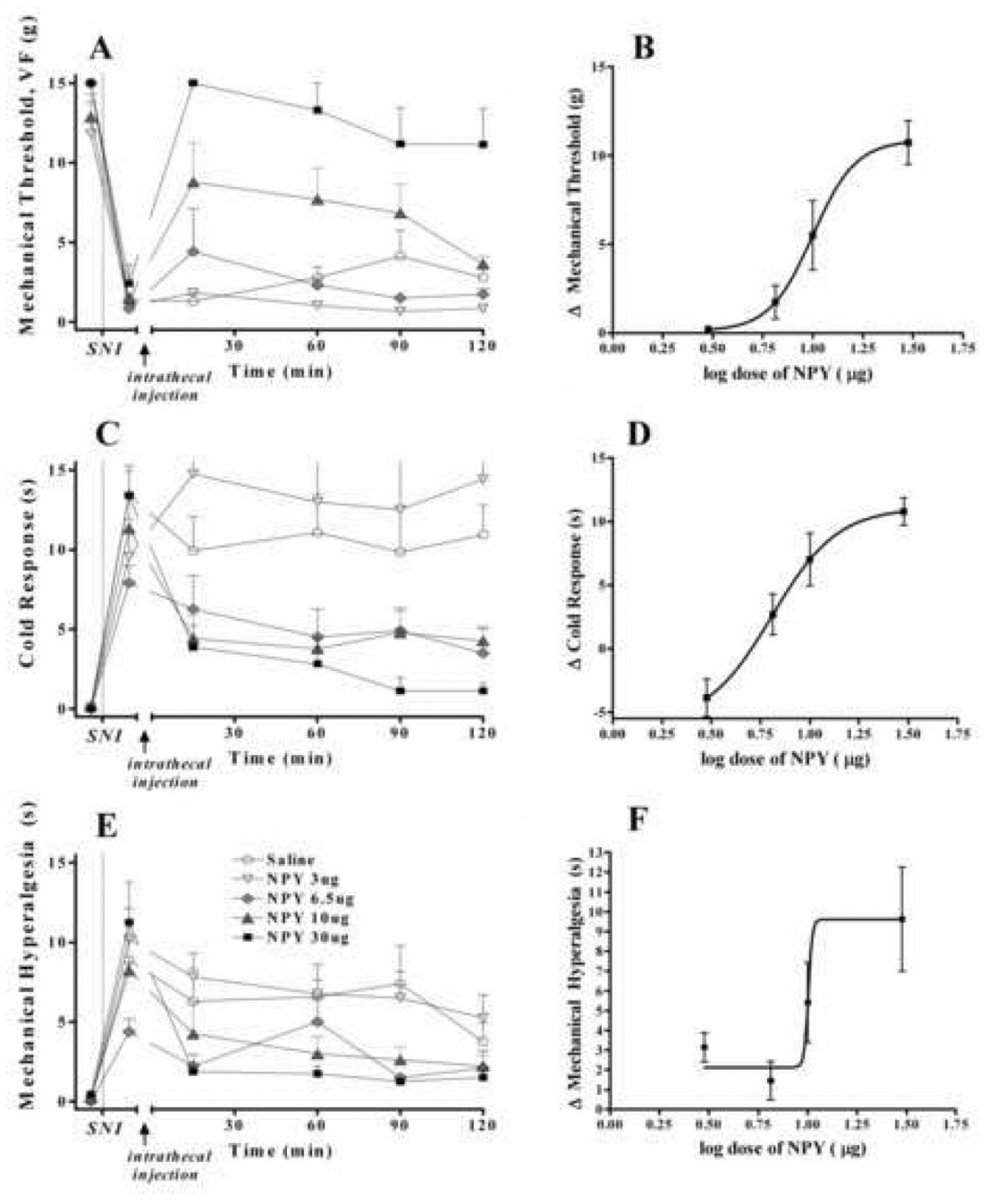

Figure 4. NPY dose dependently attenuates behavioral signs of neuropathic pain

Two wk after spared nerve injury, intrathecal administration of NPY reduced paw withdrawal responses to the plantar application of von Frey filaments (**Panels A–B**). Post-hoc comparison of baseline value with the averaged value of time points 15–90 revealed a statistically significant difference ($P<0.0001$) between saline (1.3±0.7) and 30 µg NPY (10.7±1.3). **Panels C–D** illustrate that NPY produced a strong trend towards reduction of paw withdrawal duration in response to the intraplantar application of acetone (cold response). The difference between baseline value and the averaged value of time points 15–90 for the saline group (3.0 ± 2.5) and the 30 µg NPY group (10.8±1.1) just missed statistical significance ($P=0.063$). **Panels E–F** illustrate that NPY reduced paw withdrawal responses to the plantar application of a diaper

pin (mechanical hyperalgesia). Post-hoc comparison of baseline value with the averaged value of time points 15–90 revealed a statistically significant difference ($P<0.05$) between saline (2.1 ±1.2) and 30 μg NPY (9.6±2.6). Figures B, D and F show log dose-response curves for NPY. n=4–8. Values represent mean ± SEM.

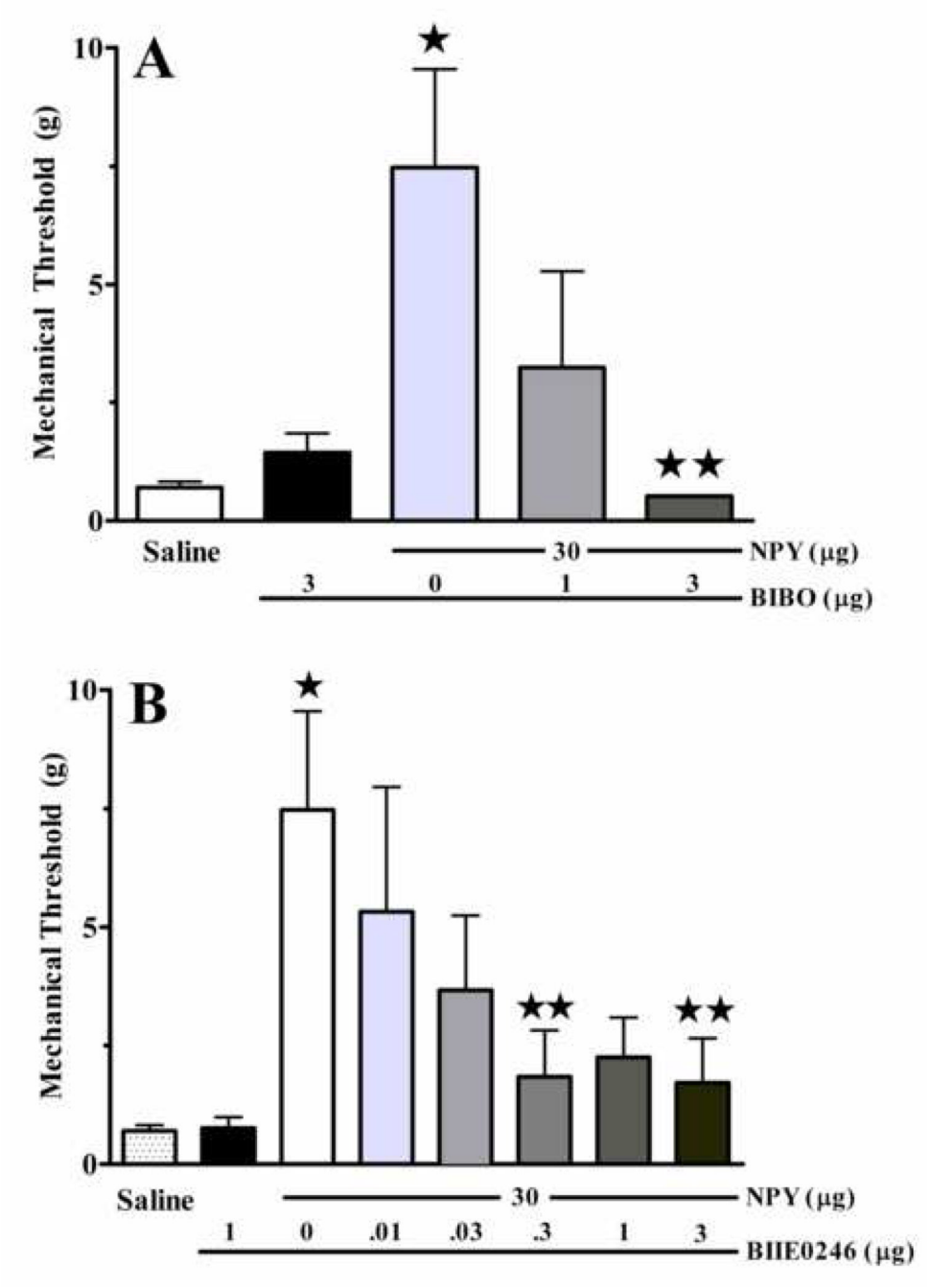

Figure 5. Both Y1 and Y2 receptors contribute to the anti-allodynic effects of NPY

Mechanical allodynia after spared nerve injury was reduced 45 min after intrathecal administration of NPY. Concurrent administration of the Y1 receptor antagonist BIBO3304 (A), or pre-administration of the Y2 receptor antagonist BIIE0246 (B), dose-dependently reversed the anti-allodynic effect of NPY. Each antagonist had little effect when administered alone. n= 4–8. Values represent mean ± SEM. * $p<0.05$ vs saline; ** $p<0.05$ vs NPY.

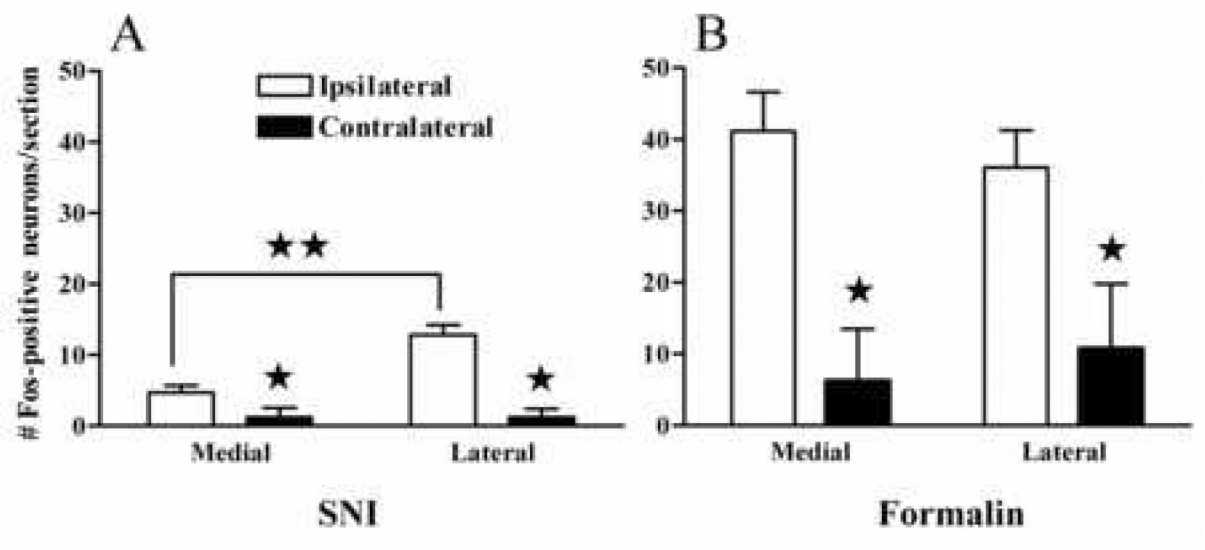

Figure 6. Distinct mediolateral patterns of Fos expression are expressed between the formalin and SNI models

Non-noxious tactile stimulation induced nearly a 3-fold increase in Fos expression in the area of the dorsal horn innervated by the common peroneal and sural nerves as compared to the area innervated by the tibial nerve (A). After formalin injection, there was no difference in Fos expression between either of the ipsilateral regions that were analyzed (B). n=7–12. Values represent mean ± SEM; *p<0.05 vs ipsilateral. **p<0.05 medial vs lateral.

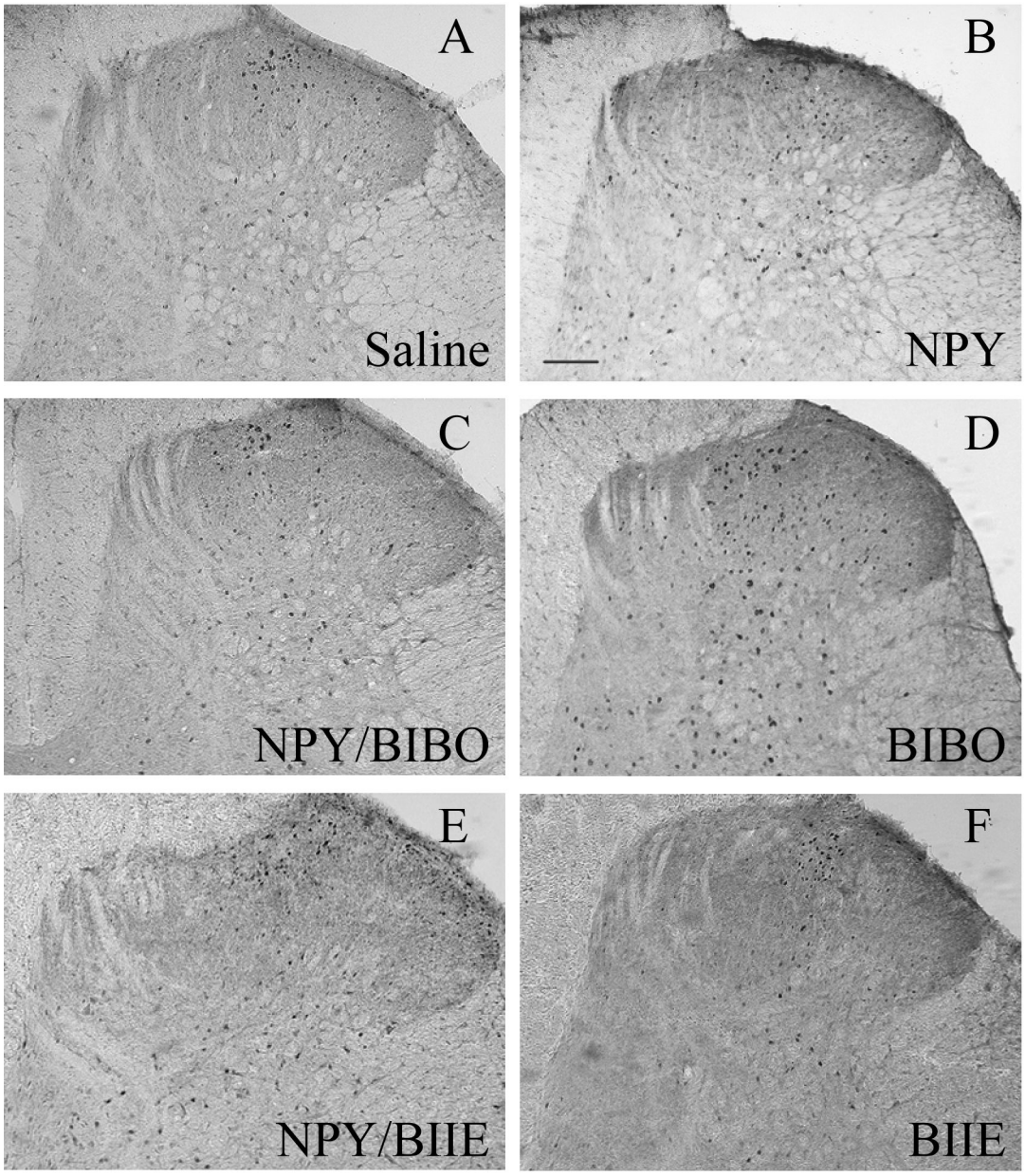

Figure 7. Tactile stimulus-induced Fos expression in rats with SNI

These panels depict representative photomicrographs of the L4 dorsal horn from rats treated with intrathecal: (A) saline; (B) NPY; (C) NPY + the Y1 receptor antagonist BIBO3304 injected together; and (D) BIBO alone; (E) NPY + the Y2 receptor antagonist BIIE0246 given 90 minutes prior and (F) BIIE0246 alone. NPY reduced Fos in the dorsal horn. Magnification = 100x, scale bar = 200 μm. Only the side ipsilateral to SNI is shown.

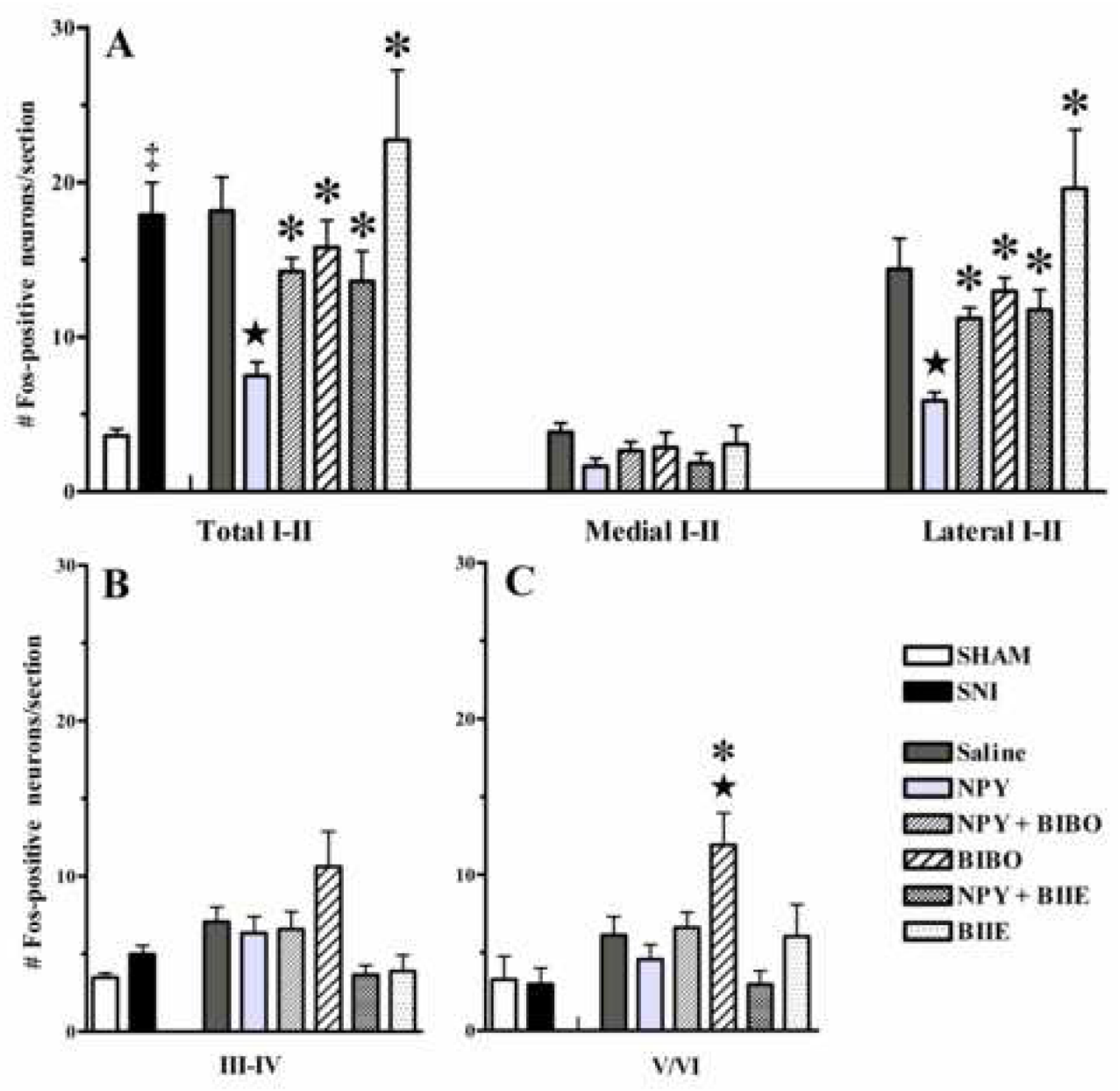

Figure 8. NPY acts at Y1 receptors to reduce non-noxious tactile stimulus-induced spinal Fos expression in animals with SNI

Panel A illustrates that sham-injured rats (open bars) express only a small number of Fos-positive neurons in laminae I–VI following mechanical stimulation. SNI (solid bars) increases the number of Fos-positive neurons in I–II. NPY decreased Fos-LI in laminae I–II of the dorsal horn. This was reversed by concomitant administration of both the Y1 receptor antagonist BIBO3304 (BIBO, 3 μg) and prior (90 min) administration of the Y2 antagonist BIIE0246 (3 μg). NPY decreased Fos-LI in the lateral region of laminae I–II, which was reversed by both BIBO3304 and BIIE0246. Similar trends were observed in the medial region but did not reach statistical significance. n=5–13. Values represent mean ± SEM; ★P<0.05 vs saline control, *P<0.05 vs NPY, ‡P<0.05 vs sham.