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**Stronger Antinociceptive Efficacy of Opioids at the Injured Nerve
Trunk than at Its Peripheral Terminals in Neuropathic Pain**

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ABBREVIATIONS: CCI, chronic constriction injury; CNS, central nervous system; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂; DAMGO, [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin; DPDPE, D-Pen²,D-Pen⁵-enkephalin; DRG, dorsal root ganglion; ICI 174,864, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu; i.pl., intraplantar; i.t., intrathecal; norBNI, norbinaltorphimine dihydrochloride; U50,488H, *trans*-(±)3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide.

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ABSTRACT

Activation of opioid receptors on peripheral sensory neurons has the potential for safe pain control, as it lacks centrally-mediated side effects. While this approach often only partially suppressed neuropathic pain in animal models, opioids were mostly applied to animal paws although neuropathy was induced at the nerve trunk. Here we aimed to identify the most relevant peripheral site of opioid action for efficient antinociception in neuropathy. On days 2 and 14 following a chronic constriction injury (CCI) of the sciatic nerve in mice, we evaluated dose- and time-relationships of the effects of μ -, δ -, and κ -opioid receptor agonists injected either at the CCI site or intraplantarly (i.pl.) into the lesioned nerve-innervated paw, on spontaneous paw lifting and heat and mechanical hypersensitivity (using Hargreaves and von Frey tests, respectively). We found that neither agonist diminished spontaneous paw lifting, despite the application site. Heat hypersensitivity was partially attenuated by i.pl. μ -receptor agonist only, while it was improved by all three agonists applied at the CCI site. Mechanical hypersensitivity was slightly diminished by all agonists administered i.pl., whereas it was completely blocked by all opioids injected at the CCI site. These antinociceptive effects were opioid receptor type-selective and site-specific. Thus, opioids might not be effective against spontaneous pain, but improve heat and mechanical hypersensitivity in neuropathy. Importantly, efficient alleviation of hypersensitivity is governed by peripheral opioid receptors at the injured nerve trunk rather than at its peripheral terminals. Identifying the primary action site of analgesics is important for the development of adequate pain therapies.

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Introduction

Neuropathic pain can result from damage to peripheral nerves (e.g. amputation, entrapment, or compression), which triggers maladaptive alterations in the peripheral and central nervous system (CNS) (Costigan et al., 2009). Nerve injury is often associated with ongoing painful sensations (in the absence of apparent external stimulation) and by enhanced sensitivity to mechanical (e.g. touch, pressure, stroking) and thermal (e.g. heat, cold) stimuli. Neuropathic pain is particularly distressing to the patient because it usually persists beyond the initial cause cessation, and therefore, it is a disease rather than a symptom (Baron et al., 2010; Bennett, 2012).

Opioids are the most powerful analgesics, yet their application in neuropathic pain is limited by systemic and central adverse effects, including constipation, respiratory failure, nausea, cognitive impairment, dependence, and addiction (Stein et al., 2010). Importantly, activation of opioid receptors (μ , δ , and κ) on peripheral sensory neurons innervating injured tissue can decrease pain without these side effects; this has been shown following injection of systemically inactive opioid doses to inflamed tissues in animal models and in patients with postoperative or arthritic pain (Sawynok, 2003; Stein and Machelska, 2011). Notably, inhibition of the pain signal in peripheral sensory neurons may prevent the noxious input and plasticity within the CNS that gives rise to chronic pain (Walters, 2012).

However, peripheral antinociceptive effects of opioids in neuropathy were often insufficient (Machelska, 2011). Opioid effects were mostly examined in well characterized animal models utilizing nerve ligations (Kim et al., 1997; Machelska, 2011). Opioid peptides derived from immune cells accumulating at the site of nerve injury (Labuz et al., 2009, 2010) or exogenous opioids injected at this site (Truong et al., 2003; Cayla et al., 2012) reversed hypersensitivity to mechanical or heat stimuli. Nevertheless, in the majority of studies,

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opioids were applied to peripheral tissues remote from the nerve lesion site, i.e. to paws innervated by damaged nerves. Although in some studies the effects appear substantial (Obara et al., 2004, 2009; Kolesnikov et al., 2007; Guan et al., 2008; Chung et al., 2012; Hervera et al., 2012), other showed partial attenuation (Keita et al., 1995; Walker et al., 1999; Pertovaara and Wei, 2001; Martinez et al., 2002; Kabli and Cahill, 2007; Obara et al., 2007; Hervera et al., 2010, 2011; Gaveriaux-Ruff et al., 2011) or no improvement (Aley and Levine, 2002; Rashid et al., 2004; Cunha et al., 2010; Uchida et al., 2010) of mechanical or heat hypersensitivity. Behaviors indicative of spontaneous/ongoing pain have not been examined so far.

In this study, we hypothesized that activation of peripheral opioid receptors directly at the site of nerve injury rather than at the nerve peripheral terminals determines efficient antinociception in neuropathy. In a chronic constriction injury (CCI) of the sciatic nerve in mice, we comprehensively examined the dose-dependency and time-course of effects of μ -, δ -, and κ -opioid receptor agonists applied either at the nerve damage site or into hind paws, on spontaneous paw lifting and on hypersensitivity to heat and mechanical stimuli. Furthermore, we verified the opioid receptor type selectivity and the site of opioid action.

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Materials and Methods

Animals

Experiments were approved by the State animal care committee (Landesamt für Gesundheit und Soziales, Berlin) and were performed according to the *Guide for the Care and Use of Laboratory Animals* adopted by the U.S. National Institutes of Health. Male C57BL/6J mice (25-30 g, 6-8 weeks old; Harlan Laboratories, Horst, Netherlands; bred at the Charité-Campus Benjamin Franklin, Berlin) were kept in groups of 3-5 per cage, with free access to food and water, in environmentally controlled conditions (12 h light/dark schedule, $22 \pm 0.5^\circ\text{C}$, humidity 60-65%). After completion of experiments animals were killed with isoflurane (Abbott, Wiesbaden, Germany). All efforts were made to minimize the number of animals used.

Neuropathy

Chronic constriction injury was induced in deeply isoflurane-anesthetized mice by exposing the sciatic nerve at the right mid-thigh and placing three loose silk ligatures (4/0) with about 1 mm spacing around the nerve. The wound was closed with silk sutures. Sham operation was performed in a similar manner, but without nerve ligation (Labuz et al., 2009, 2010).

Assessment of Nociception

In all experiments, animals were habituated to the test cages daily (1-2 times for 15 min), starting 6 days prior to nociceptive testing. During the testing, the sequence of paws

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was alternated between animals to avoid "order" effects. Six to eight animals per group were used. The experimenter was blinded to the surgery type and drug treatments.

Mechanical Sensitivity (von Frey Test). Animals were individually placed in clear Plexiglas cubicles located on a stand with anodized mesh (Model 410, IITC Life Sciences, CA). The following calibrated von Frey filaments were used: 0.078 mN (0.0056 g), 0.196 mN (0.0076 g), 0.392 mN (0.041 g), 0.686 mN (0.059 g), 1.569 mN (0.14 g), 3.922 mN (0.28 g), 5.882 mN (0.54 g), 9.804 mN (0.66 g), 13.725 mN (1.15 g), 19.608 mN (2.35 g), and 39.216 mN (4.37 g) (Stoelting, Wood Dale, IL). The filaments were applied until they bowed, for approximately 3 s, to the plantar surface of hind paws. The up-down method (Chaplan et al., 1994) was used to estimate 50% withdrawal thresholds. Testing began using a 3.922 mN (0.28 g) filament. If the animal withdrew the paw, the just preceding weaker filament was applied. In the case of no withdrawal, the next stronger filament was applied. The maximal number of applications was 6-9, and the cut-off was 39.216 mN (4.37 g) because an uninjured paw could be elevated with the next filament (58.82 mN or 5.3 g), according to our previous studies (Labuz et al., 2009, 2010).

Spontaneous Paw Lifting. Animals were placed in cubicles described above and the frequency of spontaneous paw lifting was calculated over periods of 5 min. Paw lifting associated with locomotion, licking, rearing, or body repositioning was excluded, similar to previous studies (Bennett and Xie, 1988; Djouhri et al., 2006).

Heat Sensitivity (Hargreaves Test). Mice were individually placed in clear Plexiglas cubicles positioned on a stand with glass surface. Radiant heat generated by a high-intensity lamp bulb was applied to the plantar surface of hind paws from underneath the glass surface, and paw withdrawal latency was evaluated using an electronic timer (Model 336, IITC Life Sciences), according to Hargreaves et al. (1988). The paw withdrawal latency was determined

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by averaging two measurements separated by at least 10 s. The heat intensity was adjusted to obtain a basal withdrawal latency of about 10-12 s in uninjured paws, and the cut-off was 20 s, as previously (Cayla et al., 2012).

Substances

The following substances were used: μ -receptor agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO), δ -receptor agonist D-Pen²,D-Pen⁵-enkephalin (DPDPE; both from Bachem, Weil am Rhein, Germany), κ -receptor agonist *trans*-(\pm)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U50,488H), μ -receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), κ -receptor antagonist norbinaltorphimine dihydrochloride (norBNI; all from Sigma-Aldrich, Deisenhofen, Germany), and δ -receptor antagonist *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI 174,864; Biozol, Eching, Germany). All substances were dissolved in sterile water and diluted with 0.9% NaCl. Control groups were treated with 0.9% NaCl.

Injections

Injections were performed intraplantarly (i.pl.; in 20 μ l) in hind paws, at the site of nerve injury (CCI site; in 30 μ l), and intrathecally (in 5 μ l), under brief isoflurane anesthesia. For injections at the CCI site, a polyethylene tube was placed 2 mm from the tip around the needle to ensure the same depth of needle insertion into the middle of the scar after CCI or sham surgery, as described earlier (Labuz et al., 2009). Intrathecal injections were made between the lumbar 5 and 6 vertebrae using a 30G needle connected to a Hamilton syringe (Hamilton, Bonaduz, Switzerland), as described previously (Hylden and Wilcox, 1980).

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Experimental Protocols

The time-course of nociceptive behaviors, including spontaneous paw lifting, heat and mechanical sensitivity was evaluated in separate groups of mice, a day before, daily on days 1-7, and on day 14 after CCI or sham surgery.

The effects of opioids on nociceptive behaviors were assessed on days 2 and 14 following CCI or sham surgery. Opioid receptor agonists, DAMGO (0.125-128 μg), DPDPE (10-266 μg), and U50,488H (10-150 μg) were injected either i.pl. or at the CCI site. Their effects on heat and mechanical sensitivity were evaluated in separate groups of animals, before and 5-60 min following injections. The effects of opioids on spontaneous paw lifting were evaluated in animals examined with von Frey test, immediately after von Frey testing, to minimize the animal numbers. In additional experiments, we verified that the opioid effects on spontaneous paw lifting acquired this way were comparable to effects in animals that were not examined with von Frey test (data not shown). The frequency of paw lifting was recorded over 5 min-periods, before and 7-68 min following opioid applications.

In separate animal groups, the most effective doses of DAMGO (4-64 μg), DPDPE (266 μg), and U50,488H (75 μg) were injected at the contralateral nerve trunk (at the level corresponding to the CCI) or to the contralateral paw of CCI animals, as well as at the site of sham surgery or to the paw innervated by sham-operated nerve, and were examined 5-60 min later with the Hargreaves test. Such treatments were not evaluated with the von Frey test because von Frey thresholds in contralateral paws of CCI animals as well as in paws of sham-operated animals nearly reached the cut-off (see above and Fig. 1).

The opioid receptor selectivity and the site specificity of agonist actions were assessed 5 min after i.pl. administration or 30 min after injection at the CCI site, with the von Frey test. To verify the opioid receptor selectivity, the most effective doses of DAMGO (2-32 μg),

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DPDPE (150-266 μg), and U50,488H (50 μg) were co-injected with each opioid receptor antagonist, CTOP (1 μg), ICI 174,864 (4 μg), or norBNI (10 μg), either i.pl. or at the CCI site. To determine the site of agonist actions, (i) each agonist was applied i.pl., while the corresponding receptor antagonist was injected at the CCI site, and (ii) each agonist was injected at the CCI site, whereas the corresponding receptor antagonist was injected i.pl. or intrathecally; antagonists were applied immediately before agonists. Antagonists were used in the most effective doses we found in pilot experiments, in which each antagonist, CTOP (0.25-2 μg), ICI 174,864 (1-8 μg), and norBNI (1-10 μg) was tested with the most effective doses of the respective receptor agonist following i.pl. injections or applications at the CCI site.

Statistical Analysis

The data are expressed in raw values, as mean \pm SEM. Two-sample comparisons were made using the *t* test for normally-distributed data or Mann-Whitney test for not normally-distributed data. Changes over time (more than two time points) were evaluated using one-way repeated measurements (RM) analysis of variance (ANOVA) followed by Bonferroni test for normally-distributed data or Friedman one-way RM ANOVA followed by Dunn test for not normally-distributed data, to examine one treatment. Two-way RM ANOVA and Bonferroni test were used to compare two treatments over time. Multiple comparisons at one time point were performed using one-way ANOVA followed by Bonferroni test for normally-distributed data or Kruskal Wallis one-way ANOVA followed by Dunn test for not normally-distributed data. Dose-response relationships were analyzed using one-way ANOVA followed by linear regression. Differences were considered significant if $P < 0.05$.

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Results

Nociceptive Behaviors Following Nerve Injury

Mice with CCI significantly more frequently lifted the hind paw ipsilateral to the injury; this behavior was absent before CCI, but appeared on the first day, reaching statistical significance on day 4, which lasted up to 14 days after CCI ($P < 0.05$; Fig. 1). There was no spontaneous lifting of contralateral paws in CCI mice or of hind paws in sham-operated mice (data not shown).

Furthermore, CCI resulted in a profound heat and mechanical hypersensitivity, manifested by significantly shortened withdrawal latencies to heat and lower mechanical von Frey thresholds, respectively, in paws innervated by damaged nerves, as compared with the latencies and thresholds of contralateral paws, of both hind paws in sham-operated animals, and the latencies and thresholds before injury. Both types of hypersensitivity were observed on days 1-14 after nerve damage ($P < 0.05$; Fig. 1). There were no significant differences in heat latencies and mechanical thresholds in paws contralateral to the CCI and in paws of sham-operated mice ($P > 0.05$; Fig. 1).

Lack of Opioid Effects on Spontaneous Paw Lifting

DAMGO (2-32 μg), DPDPE (50-266 μg), and U50,488H (10-50 μg) injected i.pl. to the paw innervated by injured nerve did not reduce spontaneous paw lifting measured 7-68 min after injections, on days 2 and 14 following CCI ($P > 0.05$; Fig. 2A). Higher i.pl. doses of DAMGO (32 and 64 μg on day 2 or 64 μg on day 14) were not effective either, while the dose of 128 μg elicited paw flinching or licking, similar to U50,488H (75 μg), whereas DPDPE (270 μg) was insoluble (data not shown).

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Spontaneous paw lifting also was not affected by DAMGO (0.125-8 μg), DPDPE (10-150 μg), and U50,488H (10-50 μg) applied at the CCI site ($P > 0.05$; Fig. 2B). Higher doses of DAMGO (4 μg on day 2 or 16 μg on day 14), DPDPE (266 μg), and U50,488H (75 μg) applied at the CCI site were ineffective as well (data not shown). Vehicle applied i.pl. or at the CCI site did not alter the frequency of spontaneous paw lifting either ($P > 0.05$; Fig. 2).

Stronger Antinociceptive Efficacy of Opioids at the Injured Nerve Trunk than at Its Peripheral Terminals, in Heat and Mechanical Hypersensitivity

Heat Hypersensitivity. DAMGO (32-64 μg) injected i.pl. to the paw innervated by injured nerve dose-dependently attenuated heat hypersensitivity on days 2 and 14 after CCI ($P < 0.001$; Fig. 3). These effects peaked at 5 min and resolved at 15-30 min, as compared with control groups (Fig. 4A). Nevertheless, the hypersensitivity was only partially attenuated, as the paw withdrawal latencies after DAMGO were significantly lower than those before CCI ($P < 0.05$; Fig. 3 and Fig. 4A). In contrast, i.pl. DPDPE (50-266 μg) and U50,488H (10-50 μg) did not improve heat hypersensitivity at any neuropathy stage ($P > 0.05$; Fig. 3 and Fig. 4A). Higher i.pl. opioid doses could not be tested because they caused paw flinching or licking (DAMGO and U50,488H), or were insoluble (DPDPE), as described above.

When applied to the CCI site, all agonists, DAMGO (0.5-16 μg), DPDPE (25-266 μg), and U50,488H (25-75 μg) dose-dependently improved heat hypersensitivity ($P < 0.001$; Fig. 3). These antinociceptive effects peaked at 5 min and returned to the paw withdrawal latencies of control groups 30-45 min after injections (Fig. 4B). The most effective doses of each agonist fully reversed the sensitivity on day 2 day after CCI, although on day 14 their effects were slightly, but significantly, lower compared with latencies before CCI ($P > 0.05$; Fig. 3

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and Fig. 4B). Vehicle administered at the CCI site or i.pl. did not significantly alter paw withdrawal latencies ($P > 0.05$; Fig. 4), and there were no changes in the contralateral paws after any treatments ($P > 0.05$; data not shown).

Injection of vehicle or the most effective doses of DAMGO, DPDPE, and U50,488H at the contralateral nerve trunk or to the contralateral paw of CCI animals, as well as at the site of sham surgery or to the paw innervated by sham-operated nerve, did not significantly alter paw withdrawal latencies ($P > 0.05$; data not shown). These observations indicate that peripherally applied opioids do not attenuate heat sensitivity in the absence of nerve damage.

Mechanical Hypersensitivity. Unlike in heat hypersensitivity, the von Frey mechanical thresholds were dose-dependently elevated by all agonists, DAMGO (2-32 μg), DPDPE (50-266 μg), and U50,488H (10-50 μg) injected i.pl. to the injured nerve-innervated paws ($P < 0.001$; Fig. 5). These effects peaked at 5 min and resolved at 15-45 min (Fig. 6A). Hypersensitivity was only partially attenuated, as the thresholds after i.pl. opioids were considerably lower than thresholds before CCI ($P < 0.05$; Fig. 5 and Fig. 6A). Higher i.pl. opioid doses were not more effective, insoluble, or produced paw flinching/licking (data not shown), as described for spontaneous paw lifting.

In contrast, DAMGO (0.125-8 μg), DPDPE (10-150 μg), and U50,488H (10-50 μg) injected at the CCI site dose-dependently and fully reversed mechanical hypersensitivity at both neuropathy stages ($P < 0.001$; Fig. 5). These antinociceptive effects appeared 5-15 min, peaked at 30 min, and declined 60 min after injections (Fig. 6B).

Vehicle administered at the CCI site or i.pl. did not significantly alter von Frey thresholds ($P > 0.05$; Fig. 6), and none of the treatments affected the thresholds of contralateral paws ($P > 0.05$; data not shown). Here we did not test sham-operated animals because their von Frey thresholds nearly reached the cut-off (Fig. 1; see also methods).

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Receptor-Selectivity and Site-Specificity of Opioid Antinociception

Because opioids were the most effective in improving mechanical hypersensitivity, we examined the receptor-selectivity and site-specificity of agonist actions using the von Frey test. We used antagonists of μ - (CTOP; 1 μ g), δ - (ICI 174,864; 4 μ g), and κ -receptors (norBNI; 10 μ g) in doses that were the most effective in our pilot experiments, as described in the methods. We found that antinociception produced by the most effective i.pl. doses of DAMGO (16 μ g at 2 days or 32 μ g at 14 days), DPDPE (266 μ g), and U50,488H (50 μ g) was completely blocked by co-injected CTOP, ICI 174,864, and norBNI, respectively, at both neuropathy stages ($P < 0.05$; Fig. 7A). Likewise, antinociception produced by the most effective doses of DAMGO (2 μ g at 2 days or 8 μ g at 14 days), DPDPE (150 μ g), and U50,488H (50 μ g) administered at the CCI site was fully reversed by co-injected CTOP, ICI 174,864, and norBNI, respectively ($P < 0.05$; Fig. 7B). Importantly, following application at both sites and in both neuropathy stages, the antinociceptive effects of DAMGO were unaltered by δ - and κ -receptor antagonists, those of DPDPE were unchanged by μ - and κ -antagonists, and those of U50,488H were unaffected by μ - and δ -antagonists ($P > 0.05$; Fig. 7).

To exclude the possibility of ligand diffusion, we examined the opioid action site. We found that antinociceptive effects of i.pl. DAMGO (16 μ g at 2 days or 32 μ g at 14 days), DPDPE (266 μ g), and U50,488H (50 μ g) were not significantly altered by CTOP, ICI 174,864, and norBNI injected at the CCI site, respectively ($P > 0.05$; Fig. 8A). Similarly, the antinociceptive effects of DAMGO (2 μ g at 2 days or 8 μ g at 14 days), DPDPE (150 μ g), and U50,488H (50 μ g) applied at the CCI site were not reversed by i.pl. or intrathecally applied

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CTOP, ICI 174,864, and norBNI, respectively ($P > 0.05$; Fig. 8B). None of these treatments affected mechanical thresholds in paws contralateral to the CCI ($P > 0.05$; data not shown).

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Discussion

Our findings show that opioids acting at peripheral opioid receptors do not inhibit spontaneous paw lifting, but improve heat and mechanical hypersensitivity in neuropathy. Importantly, adequate amelioration of hypersensitivity is controlled by opioid receptors directly at the nerve lesion site. Hence, the efficacy of μ -, δ -, and κ -opioid receptor agonists was strongly enhanced after application at the nerve injury site compared with injections into paws innervated by damaged nerves. Furthermore, this antinociception was dose-dependent, opioid receptor type-selective, and site-specific since it was blocked by the respective μ -, δ -, and κ -receptor antagonists only following their co-injection with agonists, but not after application into sites remote from agonist administration.

Externally unprovoked paw lifting in animal models is thought to relate to spontaneous/ongoing pain (Djoughri et al., 2006). Although neuropathy-induced paw lifting was rare in some studies (Mogil et al., 2010; Allchorne et al., 2012), it was relatively frequent in other (Bennett and Xie, 1988; Kim et al., 1997; Djoughri et al., 2006), similar to our observations. A variable degree of nerve injury might account for these differences. We found that regardless of the application site, opioids did not attenuate paw lifting. In contrast, they ameliorated heat and mechanical hypersensitivity, having better effects against mechanical hypersensitivity. Thus, while mechanical hypersensitivity was attenuated by all three μ -, δ -, and κ -receptor agonists applied i.pl., and was completely reversed (to the pre-injury thresholds) at both neuropathy stages after opioid injections at the CCI site, the heat hypersensitivity was attenuated only by i.pl. μ -receptor agonist, and it was fully reversed at early, but not later, neuropathy stage following applications at the CCI site. While opioid actions on neuropathy-induced spontaneous pain behavior were not assessed earlier, better

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effects in mechanical than in heat hypersensitivity were previously observed for a δ -receptor agonist (Gaveriaux-Ruff et al., 2011). The reasons for these differences are unclear, as there is no comprehensive evidence on mechanisms underlying various pain modalities in neuropathy. Nevertheless, it is proposed that spontaneous/ongoing pain results from spontaneous discharges in $A\beta$ and C sensory afferents, while firing in $A\delta$ and C afferents underlies heat and mechanical sensitivity (Koltzenburg et al., 1992; Field et al., 1999; Djouhri et al., 2006). Peripheral opioid receptors are predominately expressed in dorsal root ganglion (DRG) C and $A\delta$ neurons and, to a lesser extent, in $A\beta$ neurons (Minami et al., 1995; Silbert et al., 2003; Gaveriaux-Ruff et al., 2011). Thus, if discharges in $A\beta$ fibers prevail in our model, a lower abundance of opioid receptors in these fibers, along with their degeneration after CCI (Guilbaud et al., 1993), might account for opioid ineffectiveness on spontaneous paw lifting. Alternatively, CNS opioid receptors might be more relevant to the attenuation of spontaneous pain, as discussed in a clinical study (Leung et al., 2001). Furthermore, several transducer molecules are involved in sensing heat (e.g. transient receptor potential channels) and mechanical stimuli (e.g. potassium channels) (Dubin and Patapoutian, 2010) and it would be interesting to investigate whether possible differences in interactions between these molecules and opioid receptors underlie distinct opioid efficacy in the two types of hypersensitivity.

Heat and mechanical hypersensitivity were more efficiently reduced by opioids applied at the nerve injury site than into paws in our experiments. Such comparisons have not previously been tested. In fact, the majority of earlier studies concentrated on opioid actions at the peripheral terminals of injured nerves. In line with our findings, μ -, δ -, or κ -receptor agonists applied to paws often produced moderate effects (Keita et al., 1995; Walker et al., 1999; Pertovaara and Wei, 2001; Martinez et al., 2002; Kabli and Cahill, 2007; Obara et al., 2007; Hervera et al., 2010, 2011; Gaveriaux-Ruff et al., 2011) or were ineffective (Aley and

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Levine, 2002; Rashid et al., 2004; Cunha et al., 2010; Uchida et al., 2010) against mechanical or heat hypersensitivity in various traumatic neuropathy models. In contrast, we show that both hypersensitivity types can be considerably improved by all three opioid-receptor agonists following application at the CCI site. It would be interesting to examine whether similar effects occur in other neuropathic pain models. The time-course of opioid effects in our study (with a peak at 5 min) is similar to those testing peptidergic ligands, while effects of non-peptidergic agonists usually peaked later (20-30 min), and were evaluated in rats (Keita et al., 1995; Walker et al., 1999; Martinez et al., 2002; Obara et al., 2004, 2007, 2009). Thus, the time-course of opioid actions might depend on the agonist chemical structure and the species. It remains to be elucidated which signaling pathways account for the slower onset and longer-lasting effects of opioids applied at the CCI site compared with i.pl. injections in mechanical hypersensitivity in our study. Likewise, reasons for higher DAMGO dose requirement in later versus earlier neuropathy stages (Fig. 4B, Fig. 6A and B) need to be addressed.

What could underlie the enhanced antinociceptive effects of opioids applied at the nerve injury site? A plausible factor is the availability of opioid receptors in peripheral sensory neurons. Opioid receptor protein expression has been predominately evaluated in the DRG using immunohistochemistry, while only few studies examined the receptors' presence in nerve trunk or hind paw skin (by Western blot), and μ -receptors were most often studied. Downregulation of μ -receptors in the DRG paralleled the lack of antinociceptive effects of μ -receptor agonists injected to the paw (Aley and Levine, 2002; Rashid et al., 2004), although the receptors were upregulated in the paw skin (Walczak et al., 2005), following a partial ligation of the sciatic or saphenous nerve. After spinal nerve ligation, μ -receptors were downregulated in the DRG (Kohno et al., 2005; Lee et al., 2011); i.pl. μ -receptor agonists attenuated hypersensitivity in few studies (Pertovaara and Wei, 2001; Guan et al., 2008;

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Chung et al., 2012), except for one (Cunha et al., 2010), at the time of unaltered μ -receptor levels in the paw skin (Lee et al., 2011). Following CCI, μ -receptor expression was either elevated (Truong et al., 2003) or unchanged (Kolesnikov et al., 2007) in the DRG, while μ -receptor agonists applied to paws attenuated the hypersensitivity (Martinez et al., 2002; Obara et al., 2004, 2007, 2009; Kolesnikov et al., 2007; Hervera et al., 2011, 2012; present study) or reduced A δ and C fiber excitability to mechanical stimulation after application on the skin (Schmidt et al., 2012). Interestingly, μ -receptor upregulation at the CCI site correlates with antinociceptive effects of μ -receptor agonists applied at this site (Truong et al., 2003, Cayla et al., 2012; current study). Expression of κ -receptors has so far not been examined. Only one study has addressed δ -receptors, and found their upregulation in the DRG and at the nerve injury site, as well as diminished hypersensitivity after i.pl. δ -receptor agonist injection (Kabli and Cahill, 2007). Together, the DRG levels of opioid receptors might be indicative, but not always predictive for antinociceptive effects of opioids applied peripherally in neuropathy. It is likely that opioid receptors expressed in peripheral neuronal processes are more relevant to functional effects of locally applied opioids, but this needs to be addressed with more specific methods since Western blot does not distinguish cell types in peripheral tissue; these cells include leukocytes (infiltrating injured nerve trunk) and keratinocytes and melanocytes (in the paw skin) (Austin and Moalem-Taylor, 2010; Stein and Machelska, 2011). These non-neuronal cells contain opioid receptors (Sharp, 2006; Bigliardi et al., 2009), yet their significance in pain transmission awaits investigation. Nevertheless, inflammatory processes, including blood-nerve barrier disruption at the nerve injury site (Abram et al., 2006) that can facilitate opioid access and binding to receptors and receptor signaling (Stein and Machelska, 2011), might account for effective antinociception of opioids injected at this site. On the other

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hand, immune responses in the injured nerve-innervated paw are not obvious (Perkins and Tracey, 2000; Labuz et al., 2009) and this might underlie weaker effects of i.pl. opioids.

In conclusion, both spontaneous/ongoing and evoked pain in neuropathic conditions are clinically relevant (Baron et al., 2010; Bennett, 2012). Since neuropathic pain symptoms can be mechanistically diverse (Woolf and Mannion, 1999), it might be difficult to find one treatment effective against all symptoms. Our data suggest that opioids acting at peripheral opioid receptors might not be efficacious against spontaneous pain, but improve enhanced sensitivity to heat and mechanical stimuli. Importantly, we identified functional opioid receptors directly at the site of neuronal injury as the most relevant for efficient amelioration of neuropathy-induced hypersensitivity. Their targeting might be critical for hindering the excitatory input and plasticity within the CNS that result in chronic pain. Technology-oriented research is needed to find novel ways of drug delivery to the most relevant injured tissue (Rosen and Aribat, 2005), as this should improve analgesia and decrease systemic and central adverse effects. This is additionally supported by a pilot clinical trial reporting attenuation of neuropathic pain after peripherally applied morphine in patients (Azad et al., 2000). Identification of the primary peripheral action site(s) might also apply to other analgesics since several receptors/channels undergo redistribution after nerve injury (e.g. transient receptor potential and sodium channels) (Hudson et al., 2001; Wilson-Gerwing et al., 2008).

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Authorship Contributions

Designed experiments: Machelska.

Conducted experiments: Labuz.

Analyzed or interpreted the data: Labuz, Machelska.

Wrote or contributed to the writing of the manuscript: Labuz, Machelska.

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Figure Legends

Figure 1. Development of nociceptive behaviors following nerve injury. CCI resulted in spontaneous lifting of paws ipsilateral to the CCI ($^{\dagger}P < 0.05$, compared with behavior before CCI; Friedman one-way RM ANOVA, Dunn test) and enhanced heat and mechanical hypersensitivity ($*P < 0.05$, compared with paw withdrawal latencies and thresholds before CCI, latencies and thresholds of contralateral paws of CCI animals, and of both hind paws of sham-operated animals; two-way RM ANOVA, Bonferroni test). The frequency of paw lifting was counted in periods of 5 min. Data are expressed as mean \pm SEM. N = 6-7 mice per group.

Figure 2. Lack of opioid effects on spontaneous paw lifting. DAMGO, DPDPE, and U50,488H were injected i.pl. to the paw innervated by injured nerve (**A**) or at the CCI site (**B**), on days 2 and 14 following CCI. The frequency of paw lifting was counted in periods of 5 min, before and 7-68 min after injections ($P > 0.05$, versus vehicle; two-way RM ANOVA). Data are expressed as mean \pm SEM. N = 6-8 mice per group.

Figure 3. Dose-response of opioid effects on heat hypersensitivity. DAMGO ($P < 0.001$; one-way ANOVA, linear regression), but not DPDPE and U50,488H ($P > 0.05$; one-way ANOVA), injected i.pl. to the injured nerve-innervated paw dose-dependently elevated paw withdrawal latencies on days 2 and 14 after CCI. All these opioids applied at the CCI site dose-dependently elevated paw withdrawal latencies at both neuropathy stages ($P < 0.001$; one-way ANOVA, linear regression). The effects were measured 5 min after injections and represent the same data shown at 5 min in Fig. 4. Dashed lines indicate representative latencies evaluated before CCI. Data are expressed as mean \pm SEM. N = 6-8 mice per group.

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Figure 4. Time-course of opioid effects on heat hypersensitivity. DAMGO, DPDPE, and U50,488H were injected i.pl. to the injured nerve-innervated paw (**A**) or at the CCI site (**B**), and the effects were measured before and 5-60 min after injections, on days 2 and 14 following CCI (* $P < 0.05$, versus vehicle; $^+P < 0.05$, versus latencies before CCI; two-way RM ANOVA, Bonferroni test). For the clarity of graphs, the later P values are shown only for the most effective opioid doses. Data are expressed as mean \pm SEM. $N = 6-8$ mice per group.

Figure 5. Dose-response of opioid effects on mechanical hypersensitivity. DAMGO, DPDPE, and U50,488H injected i.pl. to the injured nerve-innervated paw or at the CCI site dose-dependently elevated mechanical thresholds, on days 2 and 14 after CCI ($P < 0.001$; one-way ANOVA, linear regression). The effects were measured 5 min after i.pl. injections or 30 min after applications at the CCI site, and represent the same data shown at 5 min or 30 min, respectively, in Fig. 6. Dashed lines indicate representative thresholds evaluated before CCI. Data are expressed as mean \pm SEM. $N = 6-8$ mice per group.

Figure 6. Time-course of opioid effects on mechanical hypersensitivity. DAMGO, DPDPE, and U50,488H were injected i.pl. to the injured nerve-innervated paw (**A**) or at the CCI site (**B**), and the effects were measured before and 5-60 min after injections, on days 2 and 14 after CCI (* $P < 0.05$, versus vehicle; $^+P < 0.05$, versus thresholds before CCI; two-way RM ANOVA, Bonferroni test). For the clarity of graphs, the later P values are shown only for the most effective opioid doses. Data are expressed as mean \pm SEM. $N = 6-8$ mice per group.

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Figure 7. Receptor-selectivity of opioid-induced antinociception. **(A)** Opioid receptor-selectivity of antinociception produced by DAMGO (16 μg on day 2 or 32 μg on day 14), DPDPE (266 μg), and U50,488H (50 μg) injected i.pl. to the injured nerve-innervated paw. **(B)** Opioid receptor-selectivity of antinociception produced by DAMGO (2 μg on day 2 or 8 μg on day 14), DPDPE (150 μg), and U50,488H (50 μg) applied at the CCI site. Each agonist was co-injected with selective antagonists of μ - (CTOP; 1 μg), δ - (ICI 174,864; ICI; 4 μg), or κ -opioid receptors (norBNI; 10 μg) ($*P < 0.05$, versus vehicle; one-way ANOVA, Bonferroni test; $^\dagger P < 0.05$, versus vehicle; Kruskal Wallis one-way ANOVA, Dunn test). Experiments were performed on days 2 and 14 following CCI, and the effects were measured 5 min after i.pl. injections or 30 min after applications at the CCI site, using von Frey test. Data are expressed as mean \pm SEM. N = 6-8 mice per group.

Figure 8. Site-specificity of opioid-induced antinociception. Each agonist was tested with the corresponding opioid receptor selective antagonists, CTOP (1 μg ; μ -receptors), ICI 174,864 (ICI; 4 μg ; δ -receptors), and norBNI (10 μg ; κ -receptors). **(A)** Antinociception produced by DAMGO (16 μg on day 2 or 32 μg on day 14), DPDPE (266 μg), and U50,488H (50 μg) injected i.pl. to the injured nerve-innervated paw was unaltered by antagonists applied at the CCI site ($P > 0.05$; t test). **(B)** Antinociception produced by DAMGO (2 μg on day 2 or 8 μg on day 14), DPDPE (150 μg), and U50,488H (50 μg) injected at the CCI site was unaffected by antagonists applied i.pl. or intrathecally (i.t.; $P > 0.05$; t test). All experiments were performed on days 2 and 14 following CCI. Antagonists were applied immediately before agonists, and the effects were measured 5 min after i.pl. application of agonists or 30 min

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after injection of agonists at the CCI site, using von Frey test. Data are expressed as mean \pm SEM. N = 6 mice per group.

Figure 1

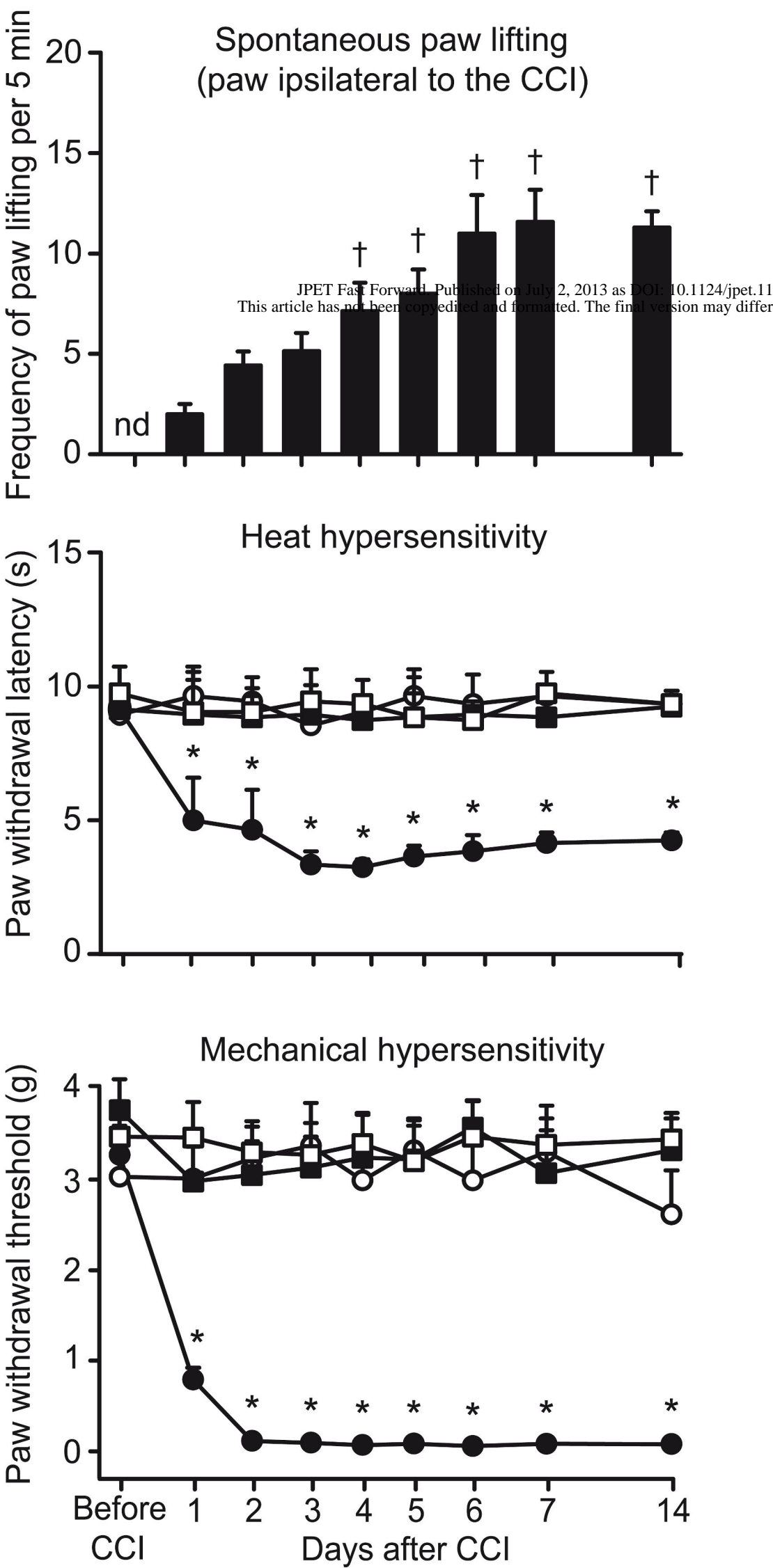


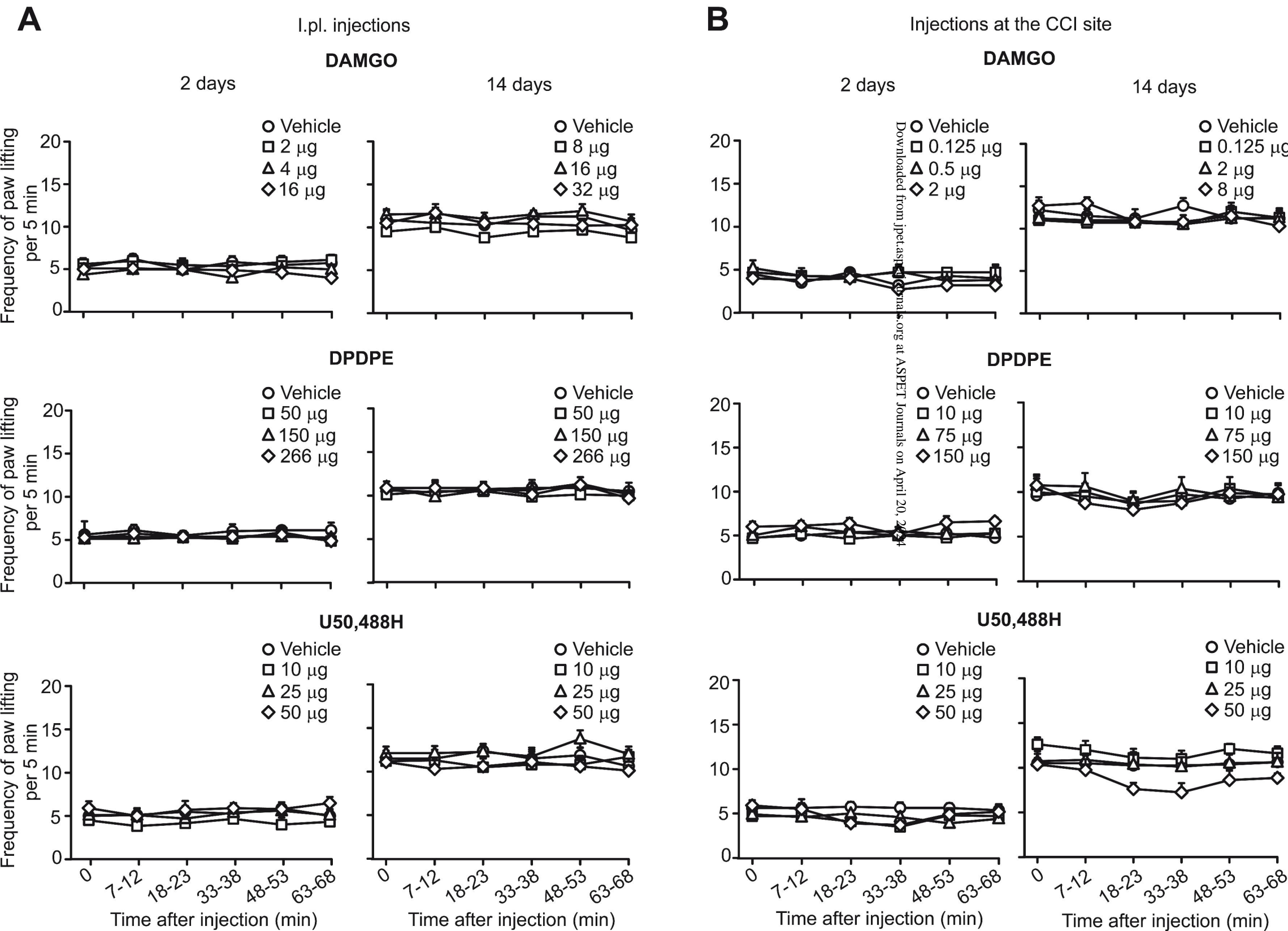
Figure 2

Figure 3

○ I.pl. injections ● Injections at the CCI site

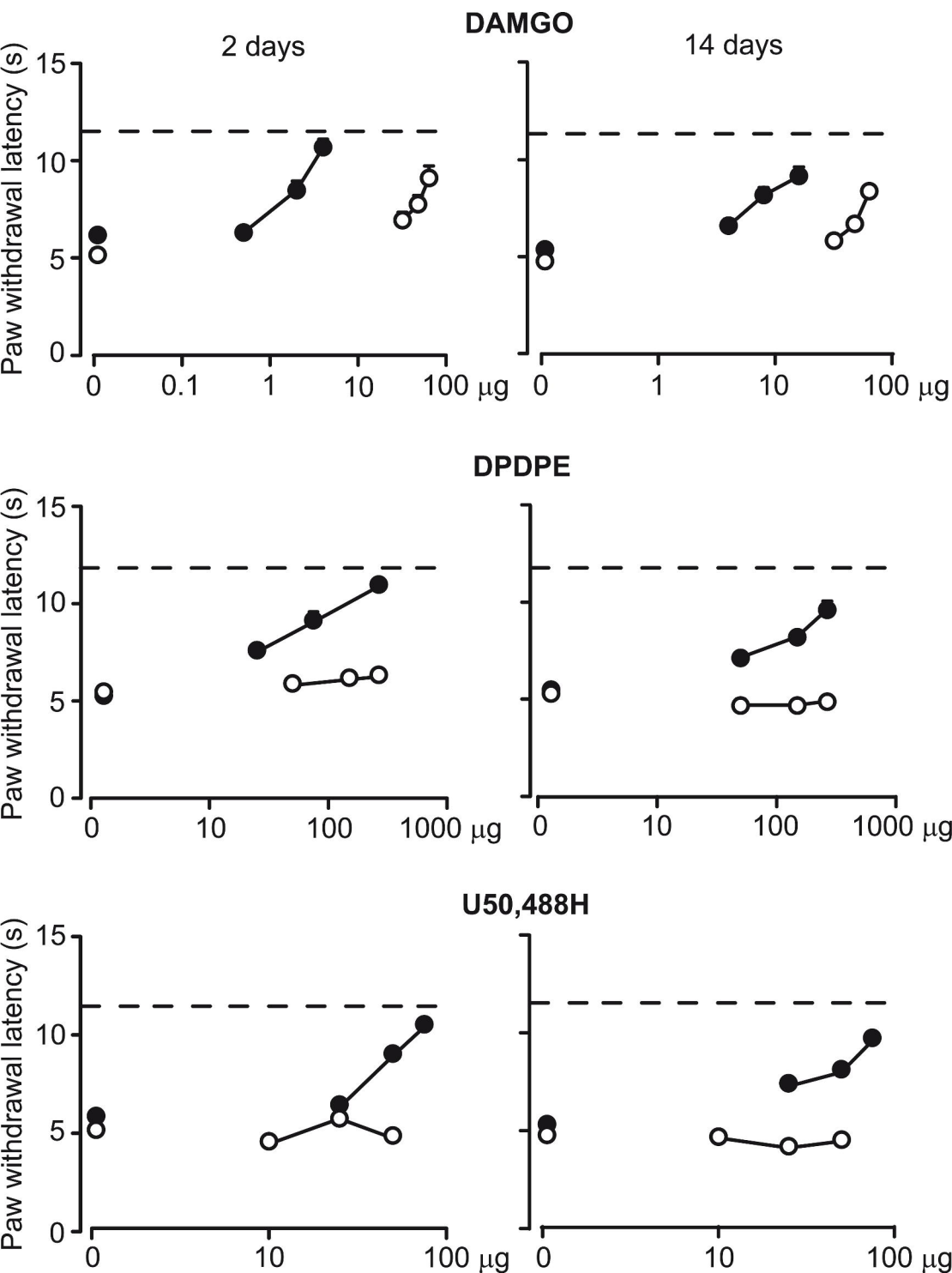


Figure 5

○ I.pl. injections ● Injections at the CCI site

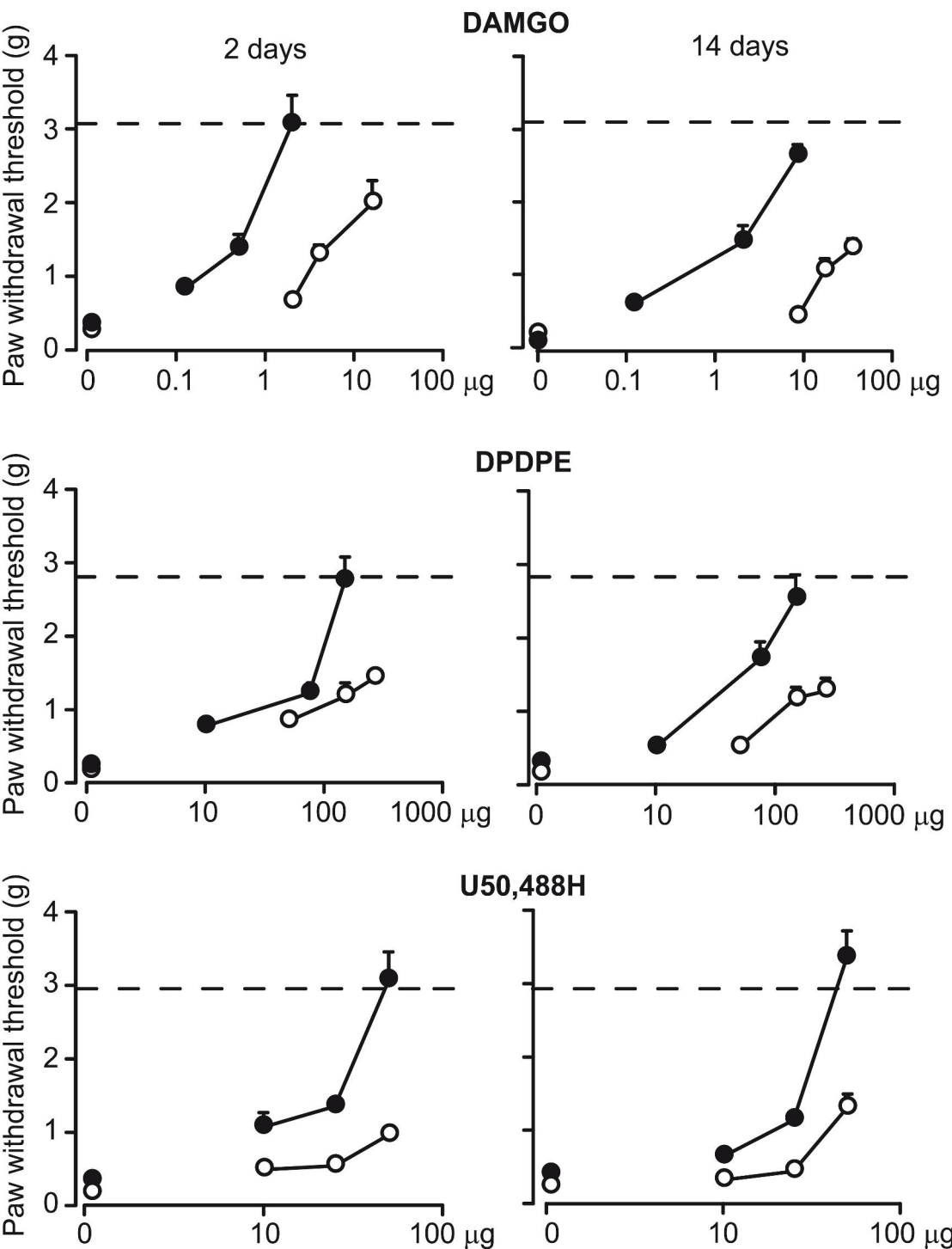


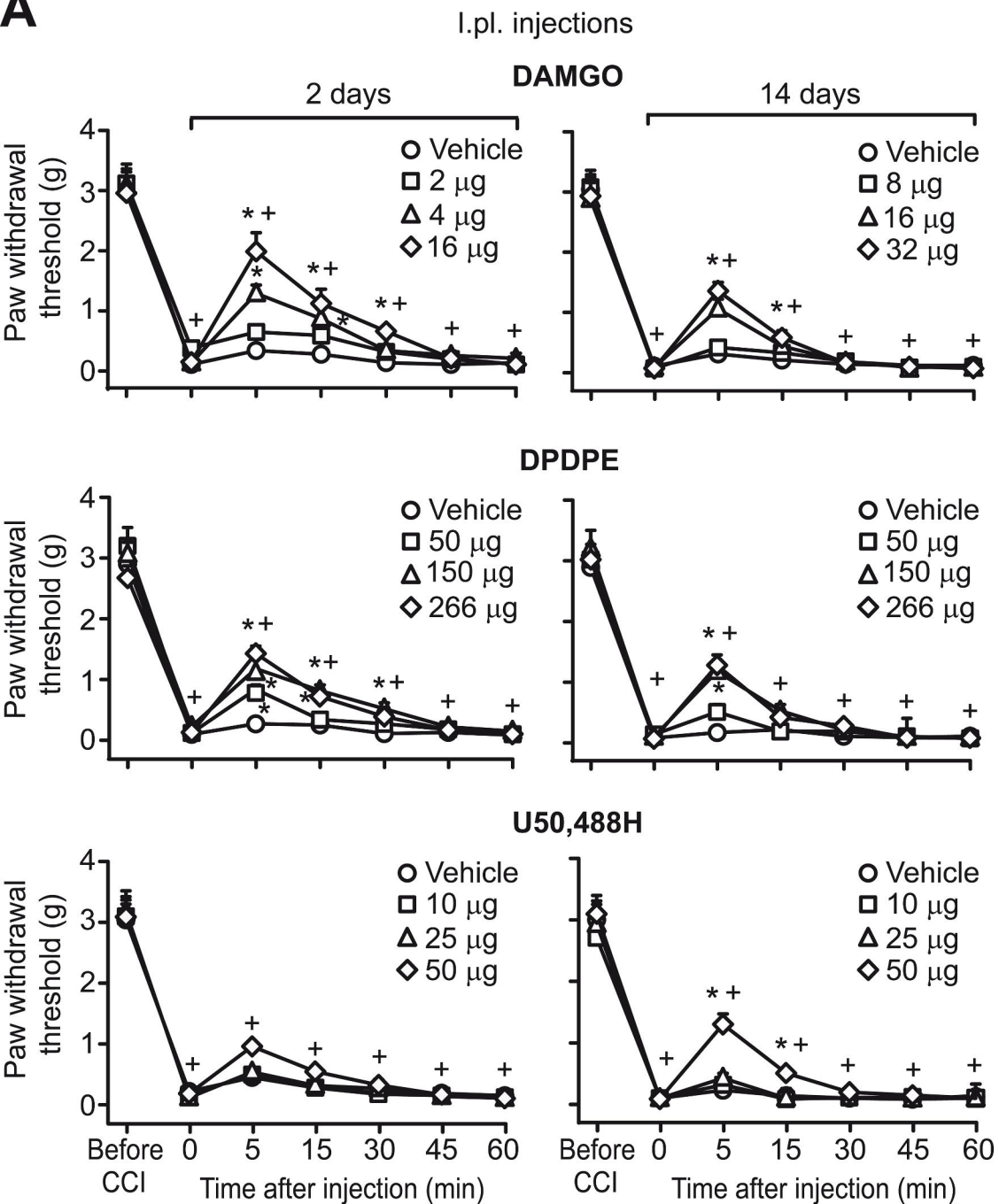
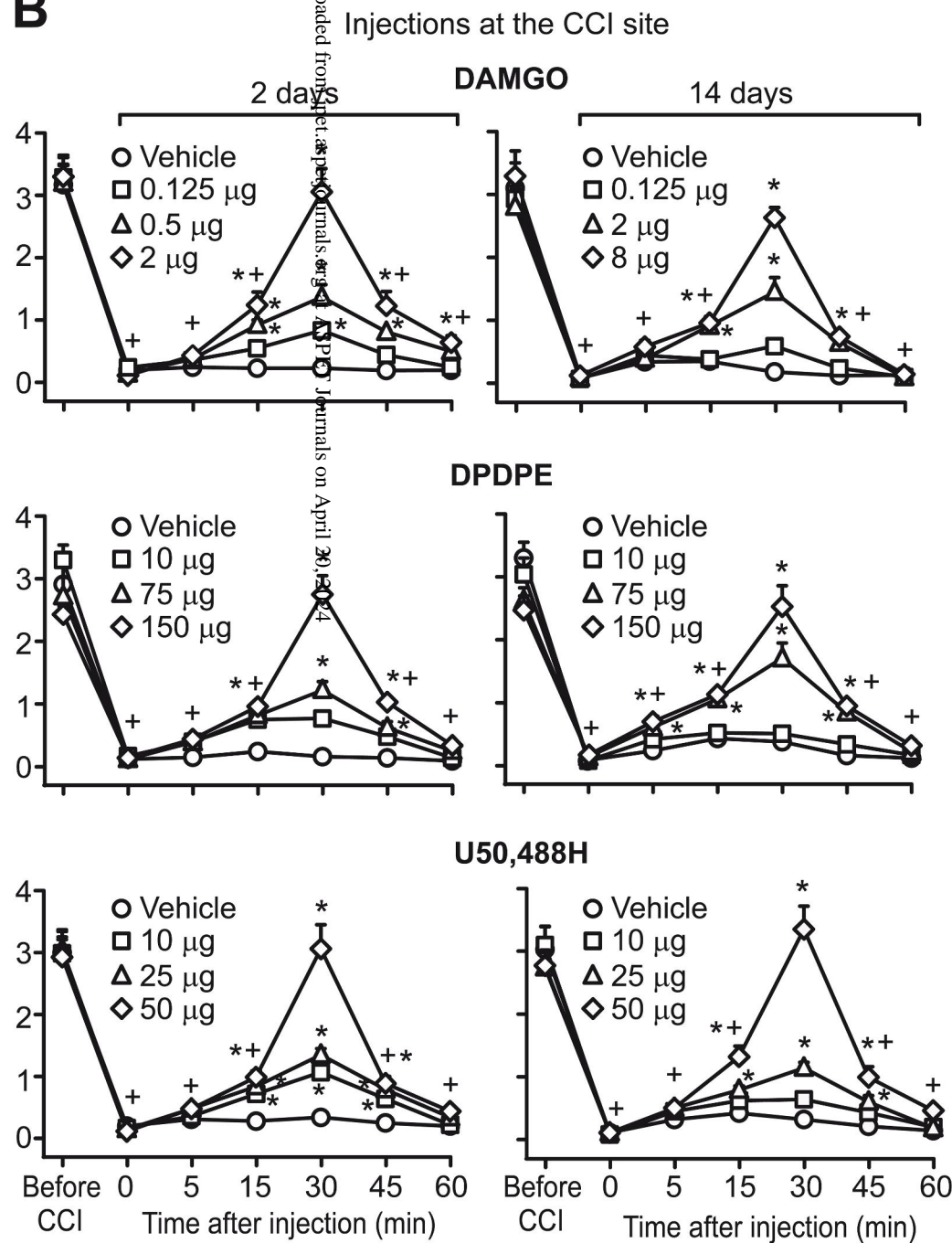
Figure 6**A****B**

Figure 7