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Systemic Administration of Propentofylline, Ibudilast, and (+)-Naltrexone Each Reverses Mechanical Allodynia in a Novel Rat Model of Central Neuropathic Pain

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Abstract: **Central neuropathic pain (CNP) is a debilitating consequence of central nervous system damage for which current treatments are ineffective. To explore mechanisms underlying CNP, we developed a rat model involving T13/L1 dorsal root avulsion. The resultant dorsal horn damage creates bilateral below-level (L4-L6) mechanical allodynia. This allodynia, termed spinal neuropathic avulsion pain, occurs in the absence of confounding paralysis. To characterize this model, we undertook a series of studies aimed at defining whether spinal neuropathic avulsion pain could be reversed by any of 3 putative glial activation inhibitors, each with distinct mechanisms of action. Indeed, the phosphodiesterase inhibitor propentofylline, the macrophage migration inhibitory factor inhibitor ibudilast, and the toll-like receptor 4 antagonist (+)-naltrexone each reversed below-level allodynia bilaterally. Strikingly, none of these impacted spinal neuropathic avulsion pain upon first administration but required 1 to 2 weeks of daily administration before pain reversal was obtained. Given reversal of CNP by each of these glial modulatory agents, these results suggest that glia contribute to the maintenance of such pain and enduring release of macrophage migration inhibitory factor and endogenous agonists of toll-like receptor 4 is important for sustaining CNP. The markedly delayed efficacy of all 3 glial modulatory drugs may prove instructive for interpretation of apparent drug failures after shorter dosing regimens.**
Perspective: ***CNP that develops after trauma is often described by patients as severe and intolerable. Unfortunately, current treatments are not effective. This work suggests that using pharmacologic treatments that target glial cells could be an effective clinical treatment for CNP.***

Key words: *Avulsion, toll-like receptor 4, D-naltrexone, glia, pain.*

Central neuropathic pain (CNP) is a common and debilitating consequence of a variety of different central nervous system traumas, including spinal cord injury (SCI), stroke, traumatic brain injury (TBI), and multiple sclerosis (MS). The percentage of patients that develop central pain following trauma range from 8% in stroke, 25% in MS, 50% in TBI, and up to 66% in SCI.[9,56,68] Mechanisms underlying CNP are poorly understood, and current pharmacotherapies for treating this type of pain are not effective.

CNP is extremely difficult to treat, and current therapies have limited response rates, provide only minor pain relief, and often have intolerable side effects. Classes of drugs commonly used to treat CNP include tricyclic antidepressants, calcium channel trafficking inhibitors, anticonvulsants, and opioids.[24] Using SCI as an example, a clinical SCI study found that gabapentin, which inhibits calcium channel trafficking,[35,45] was no more effective than the active placebo and that the tricyclic

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antidepressant amitriptyline was slightly more effective than active placebo but resulted in undesirable side effects (nausea, bladder problems, constipation).[63] Another clinical study found that the anticonvulsant drug lamotrigine did not relieve central pain in patients with MS.[10] Although opioids are one of the most widely used and effective central pain treatments,[85] the side effects and addiction problems that can arise from chronic use cause many pain patients to discontinue treatment.[6] In addition, recent literature has shown that morphine given shortly after SCI can have deleterious effects on recovery.[36] One commonality among all of these pathologies is that their mechanisms of action are thought to be largely neuronal, but it is well known that glial cells that become activated after a traumatic inflammatory event play a large role in the induction and maintenance of a variety of chronic pain states of peripheral origin.[13,27] Examining the potential role of glia in mechanisms underlying CNP may potentially lead to the development of more efficacious CNP treatments as well as improve our understanding of the mechanisms that underlie this type of pain.

Because the complexities of central nervous system traumas make them extremely difficult to treat, it is important to consider not only neuronal targeting compounds but also those treatments that target other cell types such as glia and macrophages. The current series of studies examined the effects of administering 3 different putative glial activation inhibitors, propentofylline (PPF; a phosphodiesterase [PDE] inhibitor),[55,71] ibudilast (some PDE actions but considered predominantly as a macrophage migration inhibitory factor [MIF] inhibitor),[18] and (+)-naltrexone (a non-opioid toll-like receptor 4 [TLR4] antagonist),[39] in a novel model of unilateral T13/L1 dorsal root avulsion SCI. Dorsal root avulsions are common after automobile and motorcycle accidents[7,14] and can cause below-level pain.[20,23] Our avulsion model creates a discrete dorsal horn spinal cord lesion resulting in bilateral below-level neuropathic pain (termed spinal neuropathic avulsion pain [SNAP]) but does not cause the paralysis, gross white matter damage, or urinary tract infections that are inherent, at least in part, in most models of stroke, TBI, MS, and contusion and hemisection SCI.[84] We developed this avulsion model in order to study pain behavior in isolation from such complicating factors so that we may better understand the specific mechanisms underlying CNP.

Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado, Boulder. The care and use of the animals also conformed to guidelines of the International Association for the Study of Pain. Pathogen-free male Sprague Dawley rats (325–350 g; Harlan Laboratories, Madison, WI) were used for all experiments. Rats were pair-housed prior to surgery and then single-housed after surgery with standard rat chow and water available ad libitum. Housing was in a temperature-controlled room that was maintained at 23 ± 2°C with a 12-hour light/dark cycle (lights on at 7:00 AM). The rats were allowed a minimum of 1 week to habituate to the colony room before initiating the experiment. All procedures were done during the light cycle.

Drugs

PPF (a gift from MediciNova, San Diego, CA, and Solace/Patheon UK Ltd, Wiltshire, England) was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories, Abbott Park, IL) and administered at a dose of 10 mg/kg per intraperitoneal (i.p.) injection. Controls received i.p. equivolume (1 mL/kg) saline. Ibudilast (MN-166; a gift from MediciNova) was dissolved in 100% corn oil (Mazola) and administered at 10 mg/kg per subcutaneous (s.c.) injection. Controls received s.c. equivolume (.5 mL/kg) corn oil. (+)-Naltrexone (a gift from NIDA and NIAAA, Rockville, MD) was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories) and administered at 6 mg/kg per s.c. injection. Controls received equivolume (1 mL/kg) saline. All drugs were given systemically as they are all known to cross the blood-brain barrier.[25,39,46]

SNAP Surgery

Unilateral (left) T13/L1 dorsal root avulsion was performed under isoflurane anesthesia, as previously described in detail.[83] Briefly, laminectomy was performed at the T12 vertebral level and the dura mater was incised over the dorsal root entry zone. The T13 and L1 dorsal rootlets were carefully isolated and then clamped at the dorsal root entry zone and briskly pulled out (avulsed). Sterile saline-moistened surgical sponge was placed over the exposed spinal cord to protect it, the muscle was sutured in layers with sterile 3-0 silk, and the skin was closed with stainless steel wound clips. Immediately following surgery, rats were single-housed in a cage with foam padding for a few hours to protect their spinal cord from further trauma due to the brief ataxic period that follows recovery from anesthesia. Sham operated rats were treated identically, except for avulsing of the rootlets. Combi-Pen-48 antibiotic (.2 mL; Bimeda, Inc, Le Sueur, MN) was administered at the time of surgery and daily for 4 days after surgery.

Low-Threshold Mechanical Allodynia Testing

Prior to surgery, rats were habituated to the testing environment for 4 consecutive days prior to recording of behavioral responses. All von Frey assessments were performed blind with respect to drug and surgery assignments. Assessment of von Frey thresholds occurred before surgery (baseline) and across a time course beginning 2 weeks after surgery. The von Frey test was performed on the plantar surface of each hind paw as previously described in detail.[53] A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL) were sequentially applied to the left and right hind paws in random order, each for 8 seconds

at constant pressure to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by log10 (milligrams ×10). The range of monofilaments used in these experiments (.407–15.136 g) produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity (expressed as log10 [milligrams ×10]).[15] The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function.[34] This method normalizes the withdrawal threshold for parametric analyses.[34] The general von Frey testing rubric is as follows. Rats were baselined before surgery and were allowed a 14-day recovery period. Rats were tested on a weekly basis on days 14, 21, and 28 postsurgery. Drug administration began after the day 28 behavioral test, with one exception [(+)-naltrexone], in which drug administration began on day 32 postsurgery because of extenuating circumstances. Behavioral testing then occurred once per week until the conclusion of the study, except for when a more detailed time course was necessary to define when reversal of enhanced mechanical reactivity (allodynia) began to occur. In these studies, behavioral testing occurred every 2 to 3 days after drug administration.

Pharmacologic Manipulations

PPF Time Courses

Daily PPF dosing began after development of SNAP was confirmed by von Frey testing 14, 21, and 28 days after surgery. PPF (10 mg/kg) or equivolume vehicle (saline) was administered once daily for either 14 or 35 days. PPF was administered between 4:00 PM and 6:00 PM, and behavioral testing occurred approximately 15 hours later (7:00 AM–9:00 AM), so as to parallel previous publications.[73] This delayed testing time point is standard when looking at glial effects of PPF in order to allow time for second messenger signaling cascades to exert their effects.[31,54,60,72,74] Rats were behaviorally tested to define their mechanical response thresholds every 2 to 7 days until the final behavioral test 15 hours after the last dose of PPF or vehicle.

Ibudilast Time Course Later in the Development of SNAP

Daily ibudilast dosing began after development of SNAP was confirmed by von Frey testing 14, 21, and 28 days after surgery. Ibudilast (10 mg/kg) or equivolume vehicle (corn oil) was administered once daily for 35 days. Ibudilast was administered between 8:00 AM and 10:00 AM each morning, and testing occurred 1 to 2 hours later, so as to parallel previous publications.[47] Rats were behaviorally tested to define their mechanical response thresholds every 7 days until the final behavioral test 1 to 2 hours after the last dose of ibudilast or vehicle.

Ibudilast Time Course Early in the Development of SNAP

Daily ibudilast dosing began after development of SNAP was confirmed by von Frey testing 14 days postsurgery. Ibudilast (10 mg/kg) or equivolume vehicle (corn oil) was administered once daily for 21 days. Ibudilast was administered between 8:00 AM and 10:00 AM each morning and testing occurred 1 to 2 hours later, as described above. Rats were behaviorally tested to define their mechanical response thresholds every 7 days until the final behavioral test after the last dose of ibudilast or vehicle.

(+)-Naltrexone Time Course

Daily (+)-naltrexone dosing began after development of SNAP was confirmed by von Frey testing 14, 21, 28, and 32 days after surgery. Given its relatively short half-life compared to ibudilast and PPF, (+)-naltrexone (6 mg/kg) or equivolume vehicle (saline) was administered 3 times a day for 14 days. (+)-Naltrexone was administered at approximately 9:00 AM, 12 NOON, and 3:00 PM and testing occurred 1 hour after the second injection, based on our previous studies.[39] Rats were behaviorally tested to define their mechanical response thresholds every 7 days until the final behavioral test after the last dose of (+)-naltrexone or vehicle.

(+)-Naltrexone Drug Cessation Time Course

Daily (+)-naltrexone dosing began after development of SNAP was confirmed by von Frey testing 14, 21, and 28 days after surgery. Given its relatively short half-life compared to ibudilast and PPF, (+)-naltrexone (6 mg/kg) or equivolume vehicle (saline) was administered 3 times a day for 6 days. (+)-Naltrexone was administered at approximately 9:00 AM, 12 NOON, and 3 PM and testing occurred 1 hour after the second injection, based on our previous studies.[39] Rats were behaviorally tested to define their mechanical response thresholds daily until the final behavioral test after the last dose of (+)-naltrexone or vehicle on day 33. Rats were then behaviorally tested every 2 to 6 days, at approximately 1:00 PM, until they returned to predrug allodynia levels. Because we already observed that (+)-naltrexone had no effect on sham operated rats in the previous (+)-naltrexone study, and to conserve drug, sham groups were not included in this study.

Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Ipsilateral and contralateral behavioral data were analyzed individually. Behavioral measures were normalized as described above, and group differences were analyzed by comparing area under the curve (AUC), as previously described by Jones and Sorkin.[42] AUC values (GraphPad Prism 5.01; GraphPad software Inc, San Diego, CA) were calculated from absolute threshold values, from 3.56 (the lowest threshold response value in the data set) up to the threshold response of each rat across time. Decreased AUC reflects

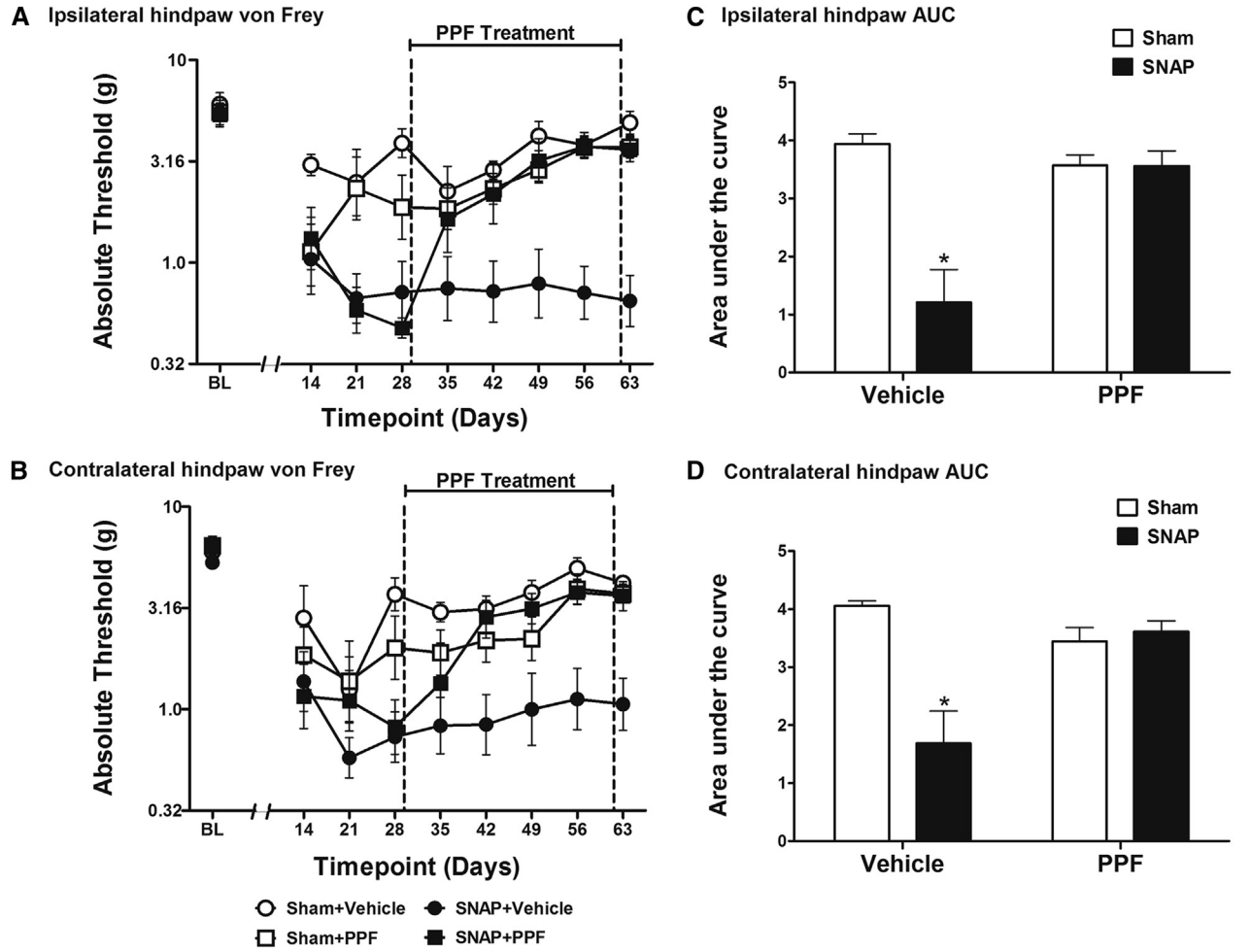

Figure 1. Assessment of the effects of PPF on SNAP. Rats were tested for mechanical allodynia across a time course on both the ipsilateral (**A**) and contralateral (**B**) hind paws. Rats that received PPF (10 mg/kg, i.p.) for 35 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hind paws. Data are presented as mean ± SEM and analyzed using a 2-way ANOVA on the AUCs; n = 6 to 8 per group. *$P < .05$ compared to all other groups.

an increase in mechanical allodynia. For all studies, the AUC measures across time were collapsed into a single time point for each animal; thus, there are no repeated measurements. For baseline measurements, a 2-way analysis of variance (ANOVA) at that single time point was the statistic used. For predrug statistics, a t-test was performed on the AUC values as there were only 2 groups, sham and SNAP. For statistics during the drug administration period, a 2-way ANOVA was then performed on the AUC values, with the exception of the studies examining the effects of (+)-naltrexone on SNAP. For the (+)-naltrexone drug time course in Fig 5, a 1-way ANOVA was used because it is not a 2 × 2 design. For the study looking at behavior after stopping (+)-naltrexone administration in Fig 6, a t-test was used because there are only 2 groups, SNAP + Vehicle and SNAP + (+)-Naltrexone. The appropriate AUC statistic was performed on days 35 to 63 for Fig 1, days 30 to 42 for Fig 2, days 35 to 63 for Fig 3, days 21 to 35 for Fig 4, days 35 to 46 for Fig 5, days 29 to 33 for Figs 6C and 6D, and days 35 to 42 for Figs 6E and 6F. A Bonferroni post hoc test for multiple comparisons was used where appropriate. For all tests, $P < .05$ was considered statistically significant.

Results

Effect of Administering Propentofylline on SNAP

In this first study, once-daily PPF was administered i.p. at 10 mg/kg for 35 days beginning 28 days postsurgery. No differences were observed between groups in the response thresholds recorded for the hind paw ipsilateral (Fig 1A) or contralateral (Fig 1B) to the avulsion injury presurgery (baseline [BL]). No differences were observed between the SNAP groups on either the ipsilateral or contralateral hind paw predrug, recorded 14, 21, and 28 days after surgery, that is, prior to initiation of PPF treatment. PPF had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to avulsion. The SNAP group was significantly more allodynic than the sham group predrug (days 14–28) on both the ipsilateral ($t_{30} = 4.396$, $P < .001$) and contralateral ($t_{29} = 2.9$, $P < .01$) hind paws. The 2-way ANOVA comparing the AUC of Sham + Vehicle, Sham + PPF, SNAP + Vehicle, and SNAP + PPF over the drug treatment time course (days 35–63) showed a

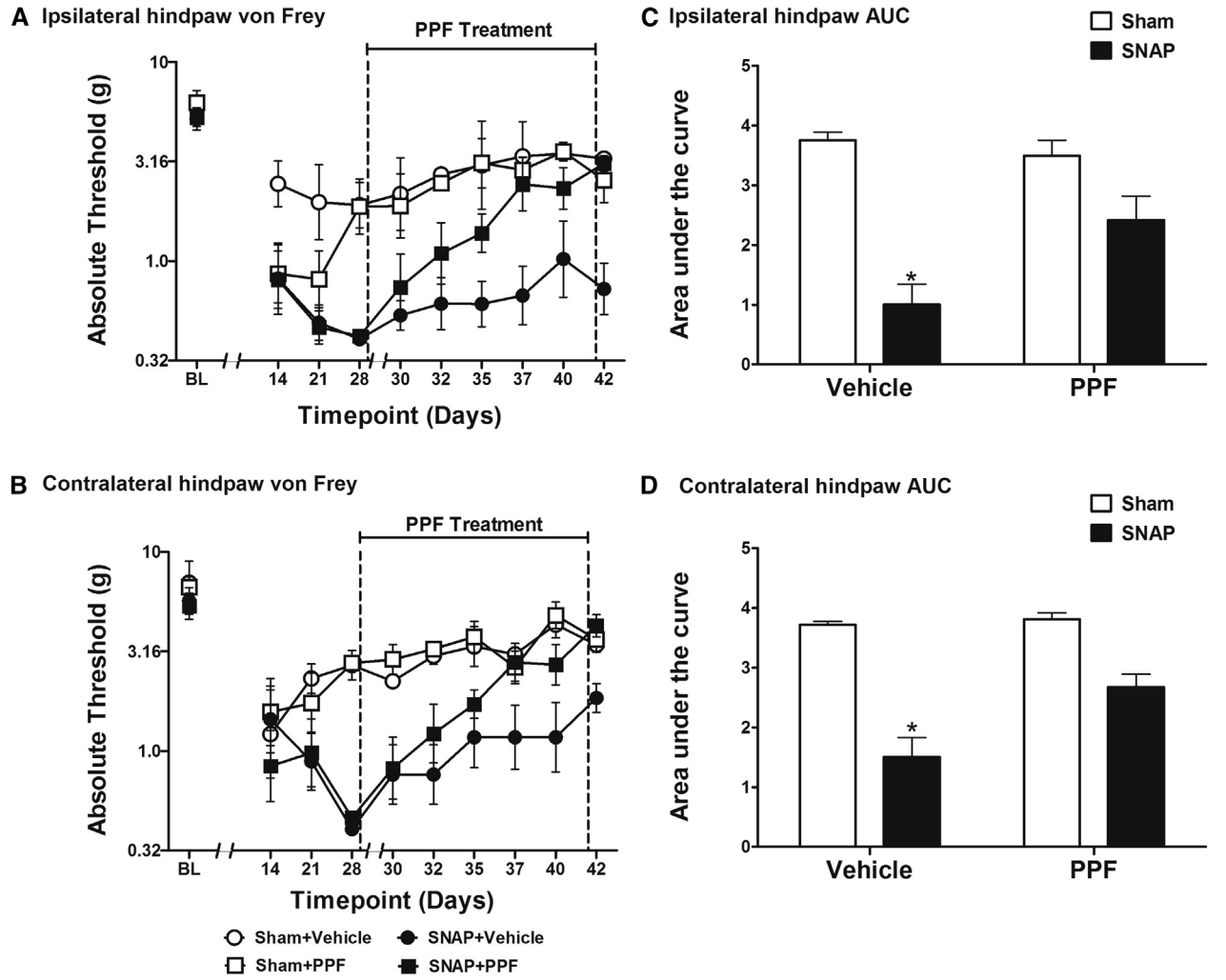

Figure 2. Detailed time course of the effects of PPF on SNAP. Rats were tested for mechanical allodynia across a time course on both the ipsilateral (**A**) and contralateral (**B**) hind paws. Rats that received PPF (10 mg/kg, i.p.) for 14 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hind paws. Data are presented as mean ± SEM and analyzed using a 2-way ANOVA on the AUCs; n = 6 per group. *$P < .05$ compared to all other groups.

significant interaction in both the ipsilateral ($F_{1,32} = 16.29$, $P < .001$; Fig 1C) and contralateral ($F_{1,32} = 15.78$, $P < .001$; Fig 1D) hind paws. There was also a significant main effect of surgery in both the ipsilateral ($F_{1,32} = 16.57$, $P < .001$) and contralateral ($F_{1,32} = 11.94$, $P < .01$) hind paws as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,32} = 8.603$, $P < .01$) and contralateral ($F_{1,32} = 4.232$, $P < .05$) hind paws. Bonferroni post hoc analysis of the AUCs revealed that the SNAP + Vehicle group was significantly more allodynic than all other groups ($P < .05$) in both the ipsilateral and contralateral hind paws. Furthermore, there were no significant differences between the other 3 groups in both the ipsilateral and contralateral hind paws ($P > .05$).

Detailed Time Course of Administering Propentofylline on SNAP

Given that PPF was able to reverse SNAP, the next step was to determine how quickly PPF reversed the pain. To answer this question and to provide a replication of the full reversal of allodynia reported above, PPF was administered for 14 days and tested every 2 to 4 days over the drug time course. No differences were observed between groups in the response thresholds recorded for the hind paw ipsilateral (Fig 2A) or contralateral (Fig 2B) to the avulsion injury presurgery (BL). No differences were observed between the SNAP groups on either the ipsilateral or contralateral hind paw predrug, recorded 14, 21, and 28 days after surgery, that is, prior to initiation of PPF treatment. PPF had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. The SNAP group was significantly more allodynic than the sham group predrug (days 14–28) on both the ipsilateral ($t_{22} = 4.155$, $P < .001$) and contralateral ($t_{22} = 4.154$, $P < .001$) hind paws. The 2-way ANOVA comparing the AUC of Sham + Vehicle, Sham + PPF, SNAP + Vehicle, and SNAP + PPF over the drug treatment time course (days 30–42) showed a significant interaction in both the ipsilateral ($F_{1,20} = 7.854$, $P < .05$; Fig 2C) and contralateral ($F_{1,20} = 6.996$, $P < .05$; Fig 2D) hind paws. There was

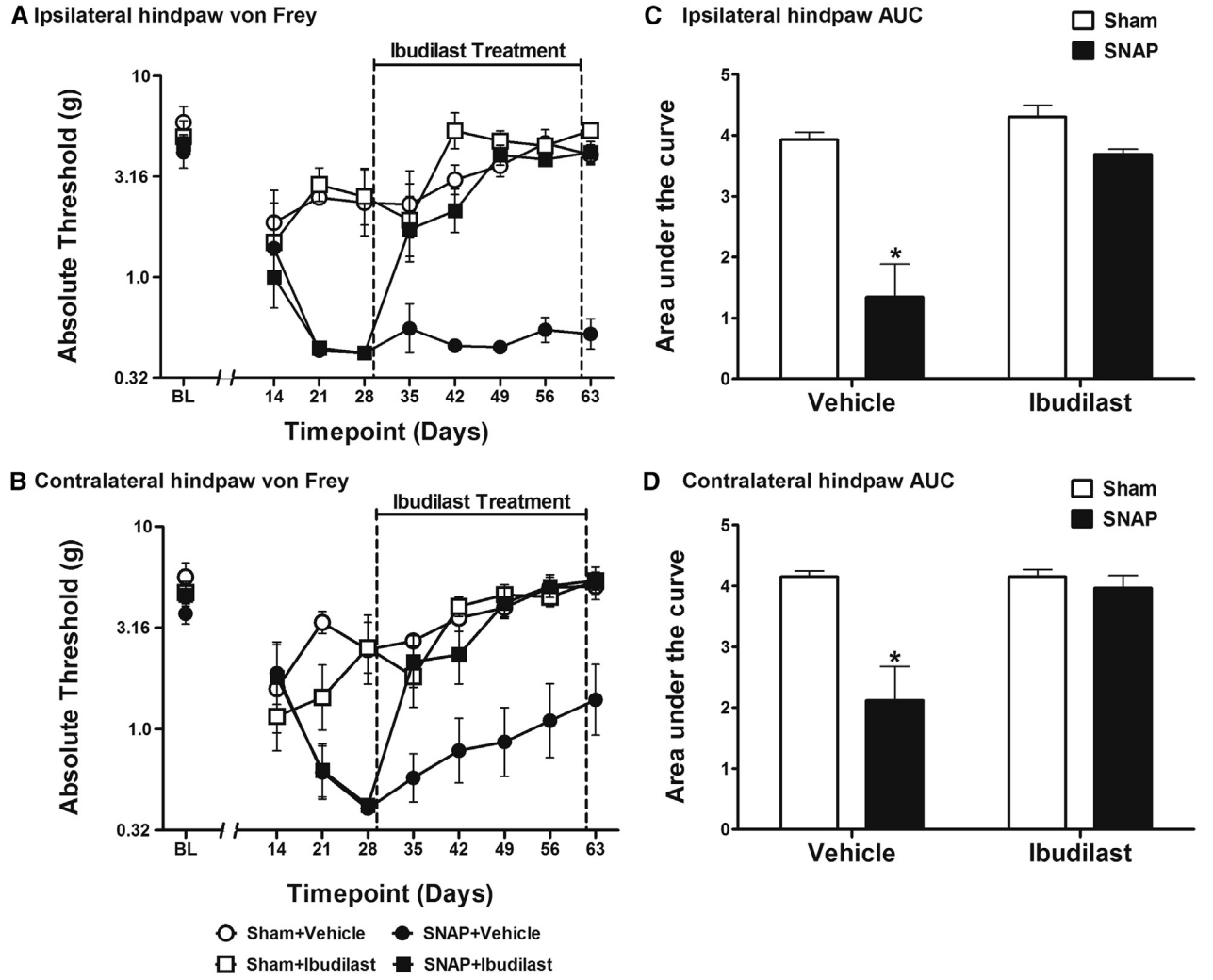

Figure 3. Assessment of the effects of ibudilast administered late in the development of SNAP. Rats were tested for mechanical allodynia across a time course on both the ipsilateral (**A**) and contralateral (**B**) hind paws. Rats that received ibudilast (10 mg/kg, s.c.) for 35 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hind paws. Data are presented as mean ± SEM and analyzed using a 2-way ANOVA on the AUCs; n = 6 to 8 per group. *$P < .05$ compared to all other groups.

also a significant main effect of surgery in both the ipsilateral ($F_{1,20}$ = 40.65, $P < .0001$) and contralateral ($F_{1,20}$ = 67.61, $P < .0001$) hind paws as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,20}$ = 7.442, $P < .05$) and contralateral ($F_{1,20}$ = 9.668, $P < .01$) hind paws. Bonferroni post hoc analysis of the AUCs revealed that the SNAP + Vehicle group was significantly more allodynic than all other groups ($P < .05$) in both the ipsilateral and contralateral hind paws. Furthermore, there were no significant differences between the other 3 groups in both the ipsilateral and contralateral hind paws ($P > .05$). Replicating the effects reported above, PPF again completely reversed allodynia, such that response thresholds for the SNAP + PPF group were comparable to those of sham controls.

Effect of Administering Ibudilast Late in the Development of SNAP

To define whether similar results could be achieved using a different putative glial activation inhibitor with a distinct mechanism of action, ibudilast was chosen because it is known to have MIF inhibitor and TLR4 inhibitor mechanisms of action for neuropathic pain reversal[64] beyond its action as a PDE inhibitor.[47] Ibudilast was administered s.c. once daily at 10 mg/kg for 35 days beginning 28 days postsurgery. No differences were observed between groups in the response thresholds recorded for the hind paw ipsilateral (Fig 3A) or contralateral (Fig 3B) to the avulsion injury presurgery (BL). No differences were observed between the SNAP groups on either the ipsilateral or contralateral hind paw predrug, recorded 14, 21, and 28 days after surgery, prior to initiation of ibudilast dosing. Ibudilast had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. The SNAP group was significantly more allodynic than the sham group predrug (days 14–28) on both the ipsilateral (t_{26} = 6.366, $P < .0001$) and contralateral (t_{27} = 3.767, $P < .001$) hind paws. The 2-way ANOVA comparing the AUC of Sham + Vehicle, Sham + Ibudilast, SNAP + Vehicle, and SNAP + Ibudilast over the drug

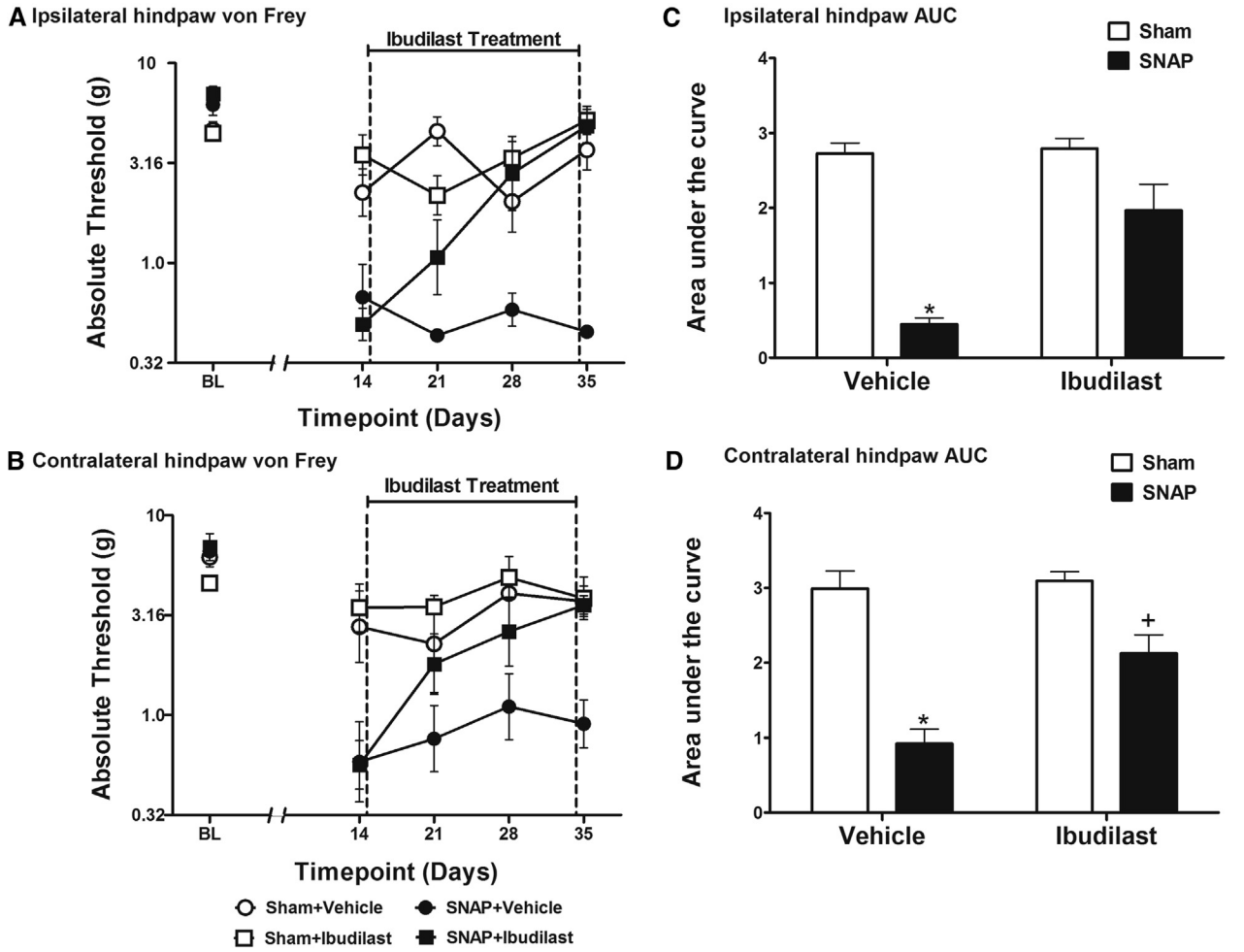

Figure 4. Assessment of the effects of ibudilast administered early in the development of SNAP. Rats were tested for mechanical allodynia across a time course on both the ipsilateral (**A**) and contralateral (**B**) hind paws. Rats that received ibudilast (10 mg/kg, s.c.) for 21 days beginning 14 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hind paws. Data are presented as mean ± SEM and analyzed using a 2-way ANOVA on the AUCs; n = 5 to 6 per group. *$P < .05$ compared to all other groups, †$P < .05$ compared to Sham + Vehicle and Sham + Ibudilast.

treatment time course (days 35–63) showed a significant interaction in both the ipsilateral ($F_{1,28} = 11.01$, $P < .01$; Fig 3C) and contralateral ($F_{1,28} = 9.033$, $P < .01$; Fig 3D) hind paws. There was also a significant main effect of surgery in both the ipsilateral ($F_{1,28} = 28.92$, $P < .0001$) and contralateral ($F_{1,28} = 13.03$, $P < .01$) hind paws as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,28} = 20.97$, $P < .0001$) and contralateral ($F_{1,28} = 8.975$, $P < .01$) hind paws. Bonferroni post hoc analysis of the AUCs revealed that SNAP + Vehicle group was significantly more allodynic than all other groups ($P < .05$) in both the ipsilateral and contralateral hind paws. Furthermore, there were no significant differences between the other 3 groups in both the ipsilateral and contralateral hind paws ($P > .05$).

Effect of Administering Ibudilast Early in the Development of SNAP

Because 2 different putative glial activation inhibitors could reverse established chronic SNAP, here it was tested whether ibudilast would prove effective when administration began at an earlier time point after surgery. To examine this issue, ibudilast was administered once daily s.c. at 10 mg/kg for 21 days beginning 14 days postsurgery. No differences were observed between groups in the response thresholds recorded for the hind paw ipsilateral (Fig 4A) or contralateral (Fig 4B) to the avulsion injury presurgery (BL). Ibudilast had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. The 2-way ANOVA comparing the AUC of Sham + Vehicle, Sham + Ibudilast, SNAP + Vehicle, and SNAP + Ibudilast over the drug treatment time course (days 21–35) showed a significant interaction in both the ipsilateral ($F_{1,20} = 12.79$, $P < .01$; Fig 4C) and contralateral ($F_{1,20} = 7.268$, $P < .05$; Fig 4D) hind paws. There was also a significant main effect of surgery in both the ipsilateral ($F_{1,20} = 58.24$, $P < .0001$) and contralateral ($F_{1,20} = 55.06$, $P < .001$) hind paws as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,20} = 15.18$, $P < .001$) and contralateral ($F_{1,20} = 10.20$, $P < .01$) hind paws. Bonferroni post hoc analysis of the AUCs revealed that SNAP + Vehicle group was

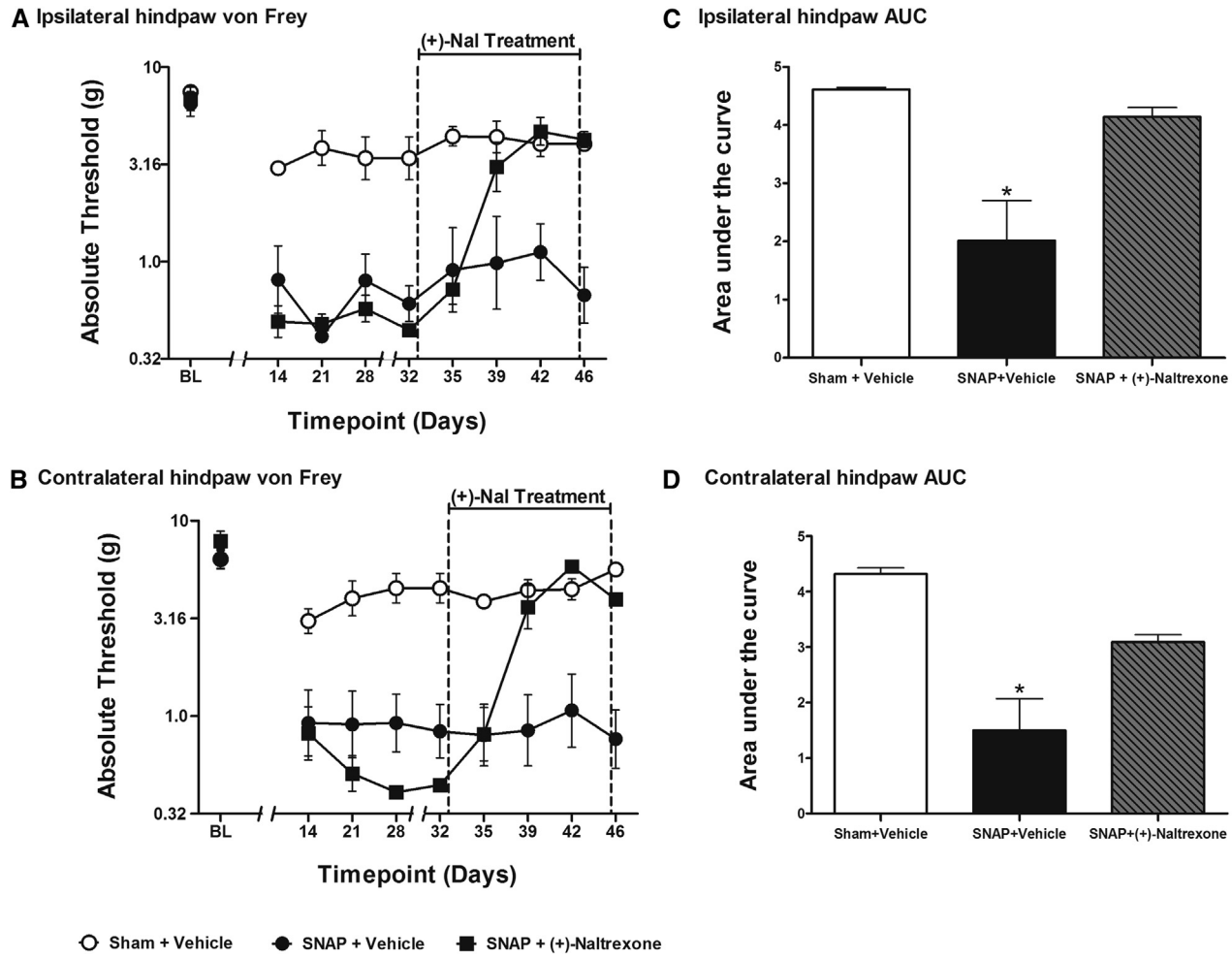

Figure 5. Assessment of the effects of (+)-naltrexone on SNAP. Rats were tested for mechanical allodynia across a time course on both the ipsilateral (**A**) and contralateral (**B**) hind paws. Rats that received (+)-naltrexone (6 mg/kg/inj, s.c.) for 14 days beginning 32 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hind paws. Data are presented as mean ± SEM and analyzed using a 1-way ANOVA on the AUCs; n = 6 per group. *$P < .05$ compared to all other groups.

significantly more allodynic than all other groups ($P < .05$) in both the ipsilateral and contralateral hind paws. Furthermore, there were no significant differences between the other 3 groups in the ipsilateral hind paw ($P > .05$). Although the ipsilateral and contralateral hind paws of the SNAP + Ibudilast group did not statistically differ, the contralateral hind paw of the SNAP + Ibudilast group, given its tighter SEMs, was found to be statistically different from both the Sham + Vehicle group and the Sham + Ibudilast group ($P < .05$), supportive of a modestly reliable but incomplete reversal of allodynia contralaterally.

Effect of Administering (+)-Naltrexone on SNAP

The success of ibudilast and PPF, above, raises the question of whether a glial modulator with no known PDE activity can also resolve SNAP. Because one of the mechanisms of action of ibudilast is as a TLR4 inhibitor,[64] this study sought to define whether TLR4 inhibition would be sufficient to resolve SNAP. Given that TLR4 is activated by endogenous substances released by cellular stress, damage, and death,[11,38] inhibition of TLR4 was chosen for testing given the neuropathology associated with SNAP. (+)-Naltrexone was chosen for testing as it is a nonopioid, blood-brain barrier permeable, highly selective TLR4 antagonist[75] that has been shown to reverse peripheral neuropathic pain.[39] Here we administered 3 times daily (+)-naltrexone s.c. at 6 mg/kg for 14 days beginning 32 days postsurgery. To extend the detailed time course reported for PPF above, behavior was again recorded every 3 to 4 days of (+)-naltrexone dosing so as to define the rapidity with which allodynia reversal would occur in the absence of PDE inhibition. No differences were observed between groups in the response thresholds recorded for the hind paw ipsilateral (Fig 5A) or contralateral (Fig 5B) to the avulsion injury presurgery (BL). No differences were observed between the SNAP groups on either the ipsilateral or contralateral hind paw predrug recorded 14, 21, 28, and 32 days after surgery, that is, prior to initiation of (+)-naltrexone dosing. (+)-Naltrexone had no effect on response thresholds of sham operated rats, which showed mild and

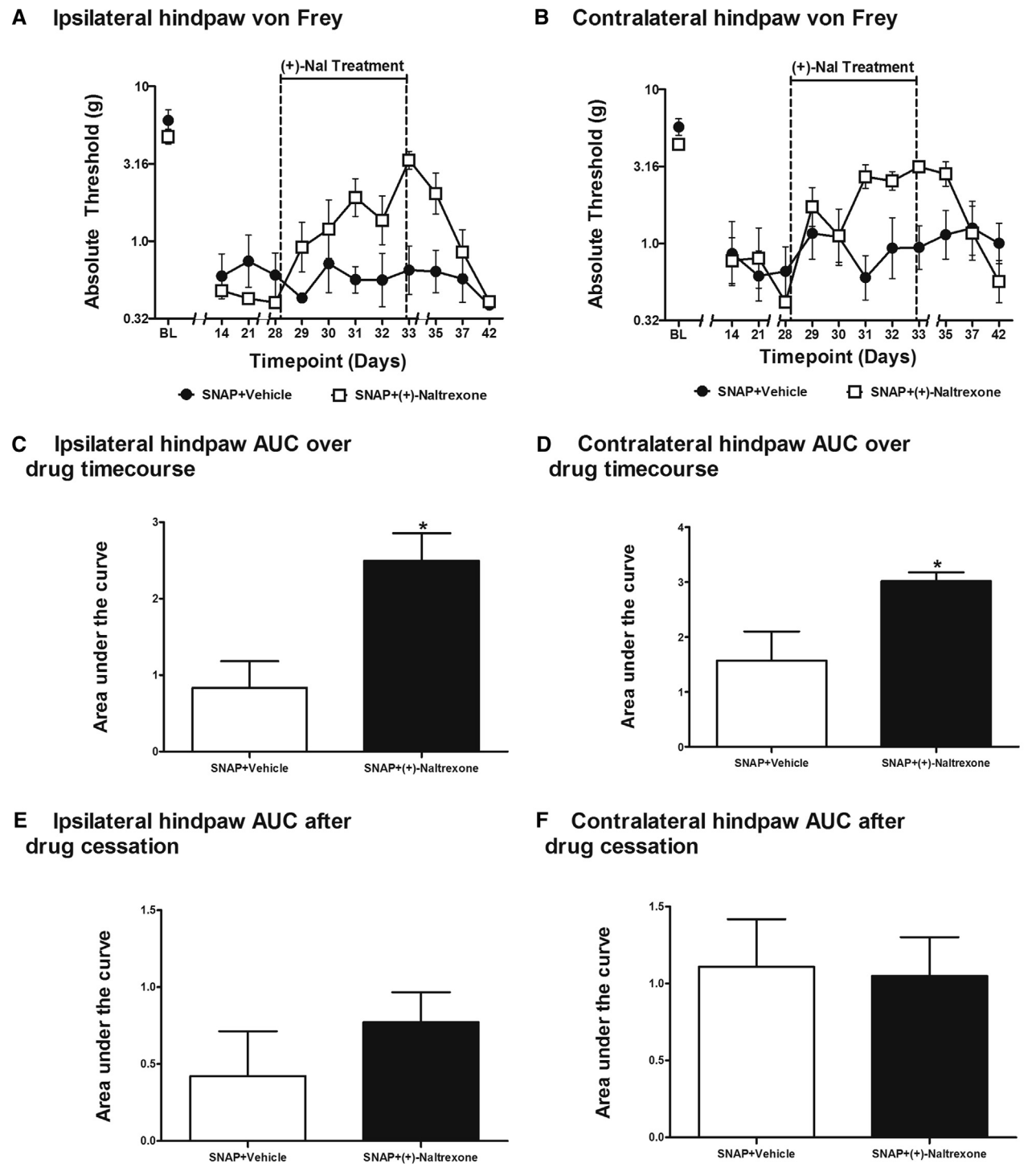

Figure 6. Assessment of the effects of ceasing (+)-naltrexone on SNAP. Rats were tested for mechanical allodynia across a time course on both the ipsilateral (**A**) and contralateral (**B**) hind paws. Rats that received (+)-naltrexone (6 mg/kg/inj, s.c.) for 6 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hind paws. After stopping (+)-naltrexone administration on day 33, rats that were receiving (+)-naltrexone returned to predrug allodynia thresholds in both the ipsilateral (**E**) and contralateral (**F**) hind paws. Data are presented as mean ± SEM and analyzed using a t-test on the AUCs; n = 6 per group. *$P < .05$ compared to all other groups.

transient allodynia compared to SNAP. The SNAP group was significantly more allodynic than the sham group predrug (days 14–32) on both the ipsilateral ($t_{16} = 6.928$, $P < .0001$) and contralateral ($t_{16} = 7.444$, $P < .0001$) hind paws. The 1-way ANOVA comparing the AUC of Sham + Vehicle, SNAP + Vehicle, and SNAP + (+)-Naltrexone over the drug treatment time course (days 35–46) was significant in both the ipsilateral ($F_{2,15} = 11.76$, $P < .001$; Fig 5C) and contralateral ($F_{2,15} = 16.82$, $P < .001$; Fig 5D) hind paws. Bonferroni post hoc analysis of the AUCs revealed that SNAP + Vehicle group was significantly more allodynic

than all other groups ($P < .05$) in both the ipsilateral and contralateral hind paws. Furthermore, there were no significant differences between the other 2 groups in both the ipsilateral and contralateral hind paws ($P > .05$).

Effect of Ceasing (+)-Naltrexone Administration on SNAP

To determine whether the reversal of allodynia observed with such glial modulators may be sustained after elimination of the drug, a final study was undertaken using (+)-naltrexone as a test compound for this purpose. Here we administered 3 times daily (+)-naltrexone s.c. at 6 mg/kg for 6 days beginning 28 days postsurgery and then stopped administering the drug after day 33 and continued to record behavior until the rats were back at predrug allodynia pain thresholds. No differences were observed between groups in the response thresholds recorded for the hind paw ipsilateral (Fig 6A) or contralateral (Fig 6B) to the avulsion injury presurgery (BL). No differences were observed between the SNAP groups on either the ipsilateral or contralateral hind paw predrug recorded 14, 21, and 28 days after surgery, that is, prior to initiation of (+)-naltrexone dosing. The t-test comparing the AUC of SNAP + Vehicle and SNAP + (+)-Naltrexone over the drug treatment time course (days 29–33) was significant in both the ipsilateral ($t_9 = 3.267$, $P < .01$; Fig 6C) and contralateral ($t_9 = 2.833$, $P < .05$; Fig 6D) hind paws. The t-test comparing the AUC of SNAP + Vehicle and SNAP + (+)-Naltrexone over the drug cessation period (days 35–42) was not significant in either the ipsilateral ($P > .05$; Fig 6E) or contralateral ($P > .05$; Fig 6F) hind paw.

Discussion

Here we show that 3 different putative glial inhibitors with distinct mechanisms of action, PPF, ibudilast, and (+)-naltrexone, are all able to reverse SNAP. Thus, converging lines of evidence from testing multiple inhibitors commonly assumed to have some glial mechanisms of action suggest a role for glia in this phenomenon. Importantly, none of these inhibitors reversed allodynia on first administration but required multiple days of treatment to achieve full reversal. Taken together, these data suggest that treating CNP with inhibitors that have some action on glia may prove to be a fruitful strategy for improving clinical pain control in such cases.

One strategy for controlling CNP is increasing cyclic adenosine monophosphate by inhibiting PDEs, which hydrolyze cyclic adenosine monophosphate and/or cyclic guanosine monophosphate.[8] PDE inhibitors reverse chronic constriction injury (CCI)-induced allodynia and hyperalgesia,[79] and rolipram has been shown to decrease proinflammatory cytokine levels in TBI[3] and SCI.[59] Further, selective PDE4 inhibitors decrease hind paw allodynia from compression SCI by reducing immune cell infiltration/activation and free radical formation.[5] PPF is a methyl xanthine–selective PDE4 inhibitor,[72] an isoform expressed in both microglia and astrocytes.[51] Although neurons express PDE4, PDE4 is the major isoform expressed in immune and inflammatory cells.[41] Further, microglial activation is regulated by PDE4,[66] and PPF decreases release of proinflammatory cytokines in microglia.[65] In addition, systemic PPF decreases both microglia and astrocyte activation in a rat peripheral neuropathy model.[72] The present study is the first to show that *systemic* PPF is effective in reversing allodynia in a CNP model. The only other neuropathic pain studies using PPF administered it intrathecally in a rat model of hemisection SCI, which also reversed mechanical allodynia.[28] Notably, the half-life of PPF and its active metabolite is very short, ~1 hour[72]; therefore, the antiallodynic effects seen in the current studies at ~15 hours post administration suggest that persistent intracellular alterations have occurred, in keeping with the conclusions of Sweitzer et al.[72] Studies have shown that increases in cyclic adenosine monophosphate results in increases of the anti-inflammatory cytokine interleukin (IL)-10,[2,21,69] including from microglia.[86] Notably, IL-10 has been recognized for its therapeutic potential in SCI.[76,89] The resulting ratio of decreased proinflammatory signaling to increased anti-inflammatory signaling could explain the sustained pain reversal seen even when propentofylline is not present at the time of testing.

Although ibudilast, like PPF, is a PDE inhibitor (inhibits PDE 1, 2, 3, and 4 in rat),[46] PDE inhibition alone cannot account for the anti-inflammatory effects of ibudilast.[18] Therefore, there must be an alternative mechanism.[64] Ibudilast is now known to also inhibit macrophage MIF[64] and is effective in treating spinal nerve ligation,[47] SCI (present data),[33] and CCI pain.[33] MIF stimulates the release of IL-1 and tumor necrosis factor (TNF) and is required for IL-1 and TNF-induced mitogen-activated protein kinase activation.[77] MIF itself is now recognized as a proinflammatory cytokine[1] and has been implicated in multiple central neuropathies including stroke,[80] MS,[32] TBI,[78] and SCI.[57] In studies examining the role of MIF in SCI, MIF knockout mice with compression SCI recovered motor function faster than wild-type controls,[57] and a clinical pilot study found that SCI patients with chronic pain had higher levels of serum MIF compared to both noninjured controls and SCI patients who did not have a history of chronic pain.[70] It is important to note that these increased circulating levels of MIF were seen long after the initial injury, providing evidence that there is enduring release of MIF that could then be sustaining the CNP state we observe in SNAP, which is reversed by ibudilast administration. Taken together, these studies suggest that because MIF is up-regulated in multiple CNP models, MIF inhibitors are potential candidates for successful CNP therapeutics.

Another potential CNP treatment that has not been as well studied is (+)-naltrexone, a selective TLR4 antagonist, as evidenced by in vivo and in vitro studies as well as in silico modeling.[37,39,40,75] In contrast to PPF and ibudilast, (+)-naltrexone has no known PDE or MIF inhibitory actions. Although we have shown that (+)-naltrexone reverses peripheral CCI pain,[39] no one has shown reversal of CNP using (+)-naltrexone until now. (+)-Naltrexone is unique compared to the (–)-naltrexone isomer in that it does not bind classic μ-opioid

receptors.[75] As a result, (+)-naltrexone does not cause many of the undesirable side effects seen with many other opiate-based analgesics,[39,75] making it a more desirable treatment option. Because TLR4 in the central nervous system is found predominantly on glial cells[37] and blocking TLR4 reverses allodynia, it is very likely that the pain reversal seen here after (+)-naltrexone administration is glially mediated. Importantly, as observed with PPF and ibudilast, it took multiple days of (+)-naltrexone administration to see full reversal, indicating that acute dosing with glial modulators may not be ideal for treating established CNP. In addition, the antiallodynic effects of (+)-naltrexone gradually dissipated once the drug was no longer being administered, which has also been observed previously for PPF[29] and ibudilast.[47] This suggests that although these drugs are perhaps able to attenuate glial activation during administration, they are not inducing permanent changes that can overcome the proinflammatory environment and persistent glial activation caused by the original injury and subsequent central sensitization.

One reason that many drugs fail clinically could be that they are not given for a sufficiently long duration, and there are examples in the literature of this with all 3 of the drugs tested here. Reversal of spinal nerve ligation–induced allodynia failed to occur when PPF was administered for only 7 days,[58] whereas it took 10 days of daily PPF administration to both prevent and reverse spinal nerve ligation–induced allodynia.[72] In the CCI and spinal nerve ligation models, only transient pain reversal occurred with 1 day of systemic ibudilast, and ~5 days of dosing was required for full reversal of CCI allodynia.[47] Similarly, only transient reversal (~1 hour) of allodynia occurs with a single intrathecal injection of (+)-naltrexone, whereas chronic intrathecal infusion of (+)-naltrexone completely reversed allodynia only when administered for 4 days.[39] In the current studies, it took multiple days of systemic administration to see significant allodynic reversal with all 3 of the drugs tested, which suggests that had these drugs been administered as a single injection or for only a couple of days, they would have failed.[81]

Furthermore, treatments are often more effective if they are administered early on in pain development. For instance, disease-modifying antirheumatic drugs for rheumatoid arthritis are effective for treating pain and other symptoms only if administered within the first 3 months symptoms appear.[50] Another example of successful early pharmacologic intervention is in migraine patients, where administering triptans within the first 20 to 120 minutes after symptom onset can actually prevent allodynia from developing.[12] Unfortunately, there are many cases in which pain patients do not or are not able to seek treatment until they have been in chronic pain for months. Thus, it is important to find therapeutics that are effective not only when administered on initial pain development but also after the symptoms have become chronic. Here we gave ibudilast in the early stages of pain development as well as in a later stage of chronic pain development, and saw complete reversal of allodynia in both cases. These data suggest that glia are likely involved both in the early stages of neuropathic pain development and in the long-term maintenance of chronic neuropathic pain, and that at least ibudilast has the potential to treat patients at various stages of their pain progression. Ibudilast has recently been identified as a TLR4 antagonist[40] (K.W.J., unpublished observations, 2012), and ibudilast as well as PPF and (+)-naltrexone was able to treat below-level contralateral pain as well, increasing their attractiveness as therapeutics.

Although the studies here focus on the *glial* mechanisms underlying CNP, we cannot rule out the fact that neurons and other cell types are also likely involved. Chew et al report increased ipsilateral bilateral glial activation in the dorsal horn following L3-L6 dorsal root avulsion[16] and increased ipsilateral mechanical hypersensitivity following L5 dorsal and ventral root avulsion,[17] which lends support to what we see in the current studies. However, they also see significant increases in bilateral infiltrating macrophages in the dorsal horn, which could also be helping to maintain the neuropathic pain.[16,17] Inhibiting α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors attenuates mechanical allodynia and neuronal hyperexcitability following SCI.[30] In addition, injecting *N*-methyl-D-aspartate (NMDA) near a contusion SCI site delays functional recovery,[22] and administering NMDA antagonists shortly after SCI can improve functional recovery and increase pain thresholds.[44] A recent study found that coadministering PPF and a neuronal NMDA receptor inhibitor had additive effects on attenuating peripherally induced chronic pain.[54] It is thought that MIF triggers extracellular signal–regulated kinase/NMDA-dependent plasticity in sensory neurons,[1] and it has been shown that ibudilast suppresses apoptosis in nerve cells during ischemic brain injury.[48] Lastly, there are studies that report that TLR4 is expressed in primary sensory neurons and can contribute to neuropathic pain.[19]

Because the drugs used here were administered systemically and are putative glial inhibitors, we recognize that their actions may not be exclusively on glia and could be interacting with other cell types, including resident peripheral immune cells or cells recruited to the injured spinal tissue. PPF suppresses the production of TNF-α[43] and reactive oxygen species in macrophages[4] and can also counteract neutrophil activation by blocking the removal of adenosine.[88] Both PPF and ibudilast can prevent kainite-induced cell death in oligodendroglia,[87] and ibudilast can also inhibit platelet aggregation in the presence of endothelial cells.[62] Furthermore, ibudilast reduces inflammatory cell infiltration into the dorsal spinal cord in experimental autoimmune encephalomyelitis,[26] which could also be happening in SNAP, along with attenuating resident microglial activation. Because TLR4 can also be expressed on neurons[19] and peripheral[49,52] or recruited immune cells,[67] (+)-naltrexone could be exerting some effects on these cells as well. Since we used a pain model of central origin, and multiple pain studies using these compounds suggest that the antiallodynic effects are glially mediated,[33,39,47,61,72,73,82] it is likely that these drugs exert at

least some of their antiallodynic effects by inhibiting glial activation. However, these observations highlight the importance of considering the contributions of neurons, peripheral/infiltrating immune cells, and glia to CNP.

In conclusion, it is clear that glia play an important role in the pathogenesis of CNP. We were able to show, using our dorsal root avulsion model of SCI (SNAP), that administering putative glial inhibitors reverses SCI-induced allodynia both at the onset of pain and after chronic neuropathic pain had developed, although it takes at least a week of daily dosing to achieve full reversal. Treating CNP with inhibitors that have some mechanistic action on glial cells has the potential to optimize treatment and dramatically increase the quality of life for thousands of chronic pain patients.

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