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Persistent Peripheral Inflammation Attenuates Morphine-Induced Periaqueductal Gray Glial Cell Activation and Analgesic Tolerance in the Male Rat

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Abstract: Morphine is among the most prevalent analgesics prescribed for chronic pain. However, prolonged morphine treatment results in the development of analgesic tolerance. An abundance of evidence has accumulated indicating that central nervous system glial cell activity facilitates pain transmission and opposes morphine analgesia. While the midbrain ventrolateral periaqueductal gray (vlPAG) is an important neural substrate mediating pain modulation and the development of morphine tolerance, no studies have directly assessed the role of PAG glia. Here we test the hypothesis that morphine-induced increases in vlPAG glial cell activity contribute to the development of morphine tolerance. As morphine is primarily consumed for the alleviation of severe pain, the influence of persistent inflammatory pain was also assessed. Administration of morphine, in the absence of persistent inflammatory pain, resulted in the rapid development of morphine tolerance and was accompanied by a significant increase in vlPAG glial activation. In contrast, persistent inflammatory hyperalgesia, induced by intraplantar administration of complete Freund's adjuvant (CFA), significantly attenuated the development of morphine tolerance. No significant differences were noted in vlPAG glial cell activation for CFA-treated animals versus controls. These results indicate that vlPAG glia are modulated by a persistent pain state, and implicate vlPAG glial cells as possible regulators of morphine tolerance.

***Perspective:** The development of morphine tolerance represents a significant impediment to its use in the management of chronic pain. We report that morphine tolerance is accompanied by increased glial cell activation within the vlPAG, and that the presence of a persistent pain state prevented vlPAG glial activation and attenuated morphine tolerance.*

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***Key words:** Opioids, tolerance, pain, inflammation, astrocytes, microglia.*

Chronic pain, defined as pain lasting more than 3 to 6 months, will affect more than 1 in 3 Americans at some point in their life.[64,66] Although morphine is one of the most commonly prescribed analgesics,[76] secondary side effects (eg, tolerance) limit its efficacy for long-term chronic pain treatment.[8,28,48,49] In the absence of pain, morphine tolerance, defined as the requirement for steadily larger doses of opioids to achieve the same analgesic effect, develops quite rapidly.[19,48,54] In contrast, clinical studies have consistently reported that the latency to develop morphine tolerance is increased in chronic pain sufferers, although dose escalation is eventually required for the maintenance of analgesic efficacy.[77] Dose escalation leads to increased risk of developing additional negative side effects, including antianalgesia, addiction, withdrawal, and respiratory depression,[76] and is not always sufficient to overcome tolerance and reinstate analgesic efficacy.[77] As over 90% of chronic pain sufferers are treated with opioids,[76] including morphine, elucidation of the mechanisms by which morphine tolerance develops warrants investigation.

The midbrain ventrolateral periaqueductal gray (vlPAG) and its descending projections to the rostral ventromedial medulla (RVM) and spinal cord comprise an important neural circuit for both endogenous and exogenous opioid-mediated analgesia.[4-7,24] In rats, chronic subcutaneous injections of morphine result in tolerance to subsequent doses of morphine, an effect that is eliminated by intra-vlPAG injections of the opioid receptor antagonist naltrexone.[42] In addition, chronic

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intra-vlPAG injections of morphine induce tolerance, and this effect remains when the RVM, the primary downstream target of the PAG, is inhibited with the gamma-aminobutyric acid (GABA) agonist muscimol.[42]

An abundance of evidence has accumulated indicating that systemic morphine administration activates glial cells, including microglia and astrocytes.[31,51,52,60,70,75,84] Song and Zhou[70] reported that chronic morphine administration results in the activation of astrocytes within the cingulate cortex, hippocampus, and spinal cord, and that blockade of glial activation within the spinal cord attenuates the development of morphine tolerance. Since then, a myriad of studies have been published implicating glia activation in the development of morphine tolerance[84,85] and pain facilitation.[11,61,82] While it is clear that the activation of microglia and astrocytes contributes to the development of morphine tolerance, no studies have examined the role of activated glia within the PAG, a primary site of morphine action. Similarly, the influence of a persistent inflammatory pain state on PAG glial cell activation has not been assessed. The present study tested the hypothesis that morphine-induced increases in vlPAG glial cell activity contribute to the development of morphine tolerance, and that persistent inflammatory pain alters this activation, resulting in the attenuation of morphine tolerance.

Methods

Subjects

Weight-matched (250–350 g) male Sprague Dawley rats (Charles River, Wilmington, MA) were pair-housed on a 12:12 hour light:dark cycle. Access to food and water was available ad libitum throughout the experiments except during behavioral testing. All studies were approved by the Institutional Animal Care and Use Committee at Georgia State University and performed in strict compliance with Ethical Issues of the International Association for the Study of Pain and National Institute of Health (NIH). All efforts were made to reduce the number of animals used in these experiments and to minimize any possible suffering by the animals.

Persistent Inflammatory Hyperalgesia

In a subset of animals, persistent inflammatory hyperalgesia was induced by injection of complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO; 200 μL), suspended in an oil/saline (1:1) emulsion, into the plantar surface of the right hindpaw as previously described.[46,48,81] As intraplantar saline administration results in a short-term inflammatory response, control animals were restrained in a similar manner but did not receive an intraplantar injection.

Experiment 1: Influence of Persistent Inflammatory Pain on Morphine Tolerance

Twenty-four hours following intraplantar CFA injection or handling, animals were administered morphine (5 mg/kg, subcutaneously [s.c.]; National Institute on Drug Abuse, Bethesda, MD) or saline (1 mL/kg, s.c.) once a day for 3 consecutive days (CFA + Morphine; CFA + Saline; Handled + Morphine; Handled + Saline). The 5-mg/kg dose was chosen based on our previous studies demonstrating this to be the 50% effective dose (ED_{50}) for systemic morphine in male rats.[47,53,81] Baseline nociceptive thresholds were measured before morphine or saline injections, and 15 minutes following the first and last injection (Injection 1 and Injection 3, respectively). Tolerance was assessed on Day 5 (Day 1 being CFA administration), by injecting cumulative doses of morphine every 20 minutes, resulting in doses of 3.2, 5.6, 8.0, 10.0, and 18.0 mg/kg as previously described.[48] Nociception was assessed using the paw thermal stimulator[29] 15 minutes after each injection.[81] Briefly, for this test, the rat is placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30°C. A radiant beam of light is positioned under the hindpaw and the time for the rat to remove the paw from the thermal stimulus is electronically recorded as the paw withdrawal latency (PWL). The intensity of the beam was set to produce basal withdrawal rates of 7 to 9 seconds. A maximal PWL of 20.48 seconds was used to prevent excess tissue damage due to repeated application of the noxious thermal stimulus. Animals were acclimated to the testing apparatus (30 minutes a day for 3 consecutive days) at the start of the experiment. All behavioral testing took place between 12:00 pm and 5:00 pm (lights on at 7:00 am). All testing was conducted blind with respect to group assignment (ie, morphine or saline treatment).

Behavioral Data Analysis and Presentation

Behavioral data are expressed in raw seconds. PWL data were analyzed using repeated measures analysis of variance (ANOVA) for significant main effect of pain (CFA or Handled) and treatment (Morphine or Saline) across dose. Preplanned t-tests were used to determine specific group and dose differences when a significant main effect was observed. All values are reported as mean ± SEM; $P \leq .05$ was considered statistically significant.

Experiment 2: Anatomical Assessment of Morphine Tolerance

Twenty-four hours following intraplantar CFA or handling, animals were administered morphine (5 mg/kg, s.c.) or saline (1 mL/kg, s.c.) once a day for 3 consecutive days as described above (CFA + Morphine, CFA + Saline, Handled + Morphine, Handled + Saline). One hour following the last injection of morphine or saline, animals were given a lethal dose of pentobarbital (160 mg/kg, intraperitoneally [i.p.]) and transcardially perfused with 250 mL of .9% sodium chloride containing 2% sodium nitrite as a vasodilator to remove blood from the brain. Immediately following blood removal, 300 mL of 4% paraformaldehyde in .1 M phosphate buffer containing 2.5% acrolein (Polysciences Inc, Warrington, PA)

was perfused through the brain as a fixative. A final rinse with 250 mL of sodium chloride/sodium nitrite solution was perfused through the brain to remove any residual acrolein. Brains were removed and placed in a 30% sucrose solution and stored at 4°C until sectioning. To examine the acute effects of morphine on vlPAG glia activation, a separate group of animals received 1 s.c. injection of morphine (or saline) and were sacrificed 1 or 24 hours later. A separate group of animals (CFA + Morphine, CFA + Saline, Handled + Morphine, Handled + Saline) were decapitated immediately following treatment for Western blot analysis. Brains were removed, flash frozen in 2-methylbutane on dry ice, and stored at −80°C.

Immunohistochemistry

Hallmarks of glial cell activity include increased cytokine release that correlates with increased expression of the protein markers glial fibrillary acidic protein (GFAP; astrocytes), and CD-11b (OX-42; microglia).[60] Further, increased glial cell activity is evidenced by a profound shift in morphology that can be easily visualized using immunohistochemistry for GFAP and OX-42.[16] Perfused brains were sectioned into 25-μm coronal sections with a Leica 2000R freezing microtome (Leica, Buffalo Grove, IL) and stored free-floating in cryoprotectant-antifreeze solution[44] at −20°C. A 1:6 series through the rostrocaudal axis of each brain was processed for GFAP and OX-42 immunoreactivity using standard immunohistochemical techniques.[56] Briefly, sections were rinsed extensively in potassium phosphate-buffered saline (KPBS) immediately followed by a 20-minute incubation in 1% sodium borohydride. The tissue was then incubated in primary antibody solution (rabbit anti-GFAP 1:5,000 or rabbit anti-OX42 1:1000; Abcam, Cambridge, MA) in KPBS containing 1.0% Triton-X for 1 hour at room temperature followed by 48 hours at 4°C. After rinsing with KPBS, the tissue was incubated for 1 hour in secondary antibody (biotinylated IgG goat anti-rabbit 1:600), rinsed with KPBS, and then incubated for 1 hour in an avidin-biotin peroxidase complex (1:10; ABC Elite Kit, Vector Labs, Burlingame, CA). After rinsing in KPBS and sodium acetate (.175 M; pH 6.5), GFAP or OX-42 immunoreactivity was visualized as a black reaction product using nickel sulfate intensified 3,3′-diaminobenzidine (DAB) solution (2 mg/10 mL) containing .08% hydrogen peroxide in sodium acetate buffer. After 15 minutes, tissue was rinsed in sodium acetate buffer followed by KPBS. In a subset of sections, GFAP (rabbit anti-GFAP 1:3,000; Abcam) or OX-42 (mouse anti-CD11b 1:3000; Serotec, Raleigh, NC) was visualized using a fluorescent secondary antibody (goat anti-rabbit Dylight488 1:50 for GFAP and rabbit anti-mouse Cy3 1:50 for CD11b; Jackson Immunoresearch Laboratories, West Grove, PA). Following secondary incubation, sections were rinsed in KPBS. DAB and fluorescent sections were mounted out of KPBS onto gelatin-subbed slides, air-dried, and dehydrated in a series of graded alcohols. Tissue-mounted slides were then cleared in Xylenes and glass coverslipped using Permount (Fisher, Fair Lawn, NJ) for DAB reactions or Krystalon (EMD Chemicals Inc, Gibbstown, NJ) for fluorescence.

Western Blotting

Flash frozen brains were sectioned at 300 μm on a cryostat (Leica) and mounted onto slides. One-millimeter bilateral micropunches were taken from 6 levels for the vlPAG (Bregma −8.52, −8.28, −7.92, −7.68, −7.20, and −6.96)[58] and 6 levels of the superior colliculus (SC) (Bregma −7.68, −7.20, −6.96, −6.60, −6.24, and −5.80),[58] and homogenized in a 10-mM HEPES buffer (pH 7.2). Equal amounts of protein (2 μg) along with a standard marker (Bio-Rad, Hercules, CA) were run at 100 V for 2 hours through 10% Tris-HCl polyacrylamide gels (Bio-Rad), and electro-transferred at 4°C on ice at 250 mA for 2 hours onto polyvinylidene difluoride (PVDF) membranes (.2-μm pore size; Bio-Rad). Membranes were blocked with 5% milk in TBS-Tween 20 (1%) at 4°C overnight, and probed with rabbit anti-GFAP primary antibody (1:300,000; Abcam) in 2% milk/TBS-Tween 20 (1%) for 3 hours at room temperature followed by a 30-minute incubation in horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary (1:5000; Abcam) in 2% milk/TBS-Tween 20 (1%). Rabbit anti-β−actin primary (1:10,000; Novus Biologicals, Littleton, CO) was included as a control for protein loading. Membranes were stripped and reprobed with mouse anti-rat CD11b (OX-42, 1:700; Serotec) followed by HRP-conjugated goat anti-mouse (OX-42; 1:5000; Abcam) and HRP-conjugated goat anti-rabbit (β-actin; 1:5000; Abcam) secondaries.

Anatomical Data Analysis and Presentation

Levels of GFAP and OX-42 immunoreactivity in the vlPAG were compared across treatment groups using semiquantitative densitometry as previously described.[43,48] To determine if the observed changes in glia activation were limited to the vlPAG, sections through the SC, a region containing a high density of mu opioid receptors (MORs) but not implicated in opioid modulation of pain, were also analyzed. 12-bit grayscale images that included the region of interest (ROI) were captured using a QImaging Retiga EXi CCD camera (Surrey, BC, Canada) and iVision Image analysis software (Biovision Technologies, Exton, PA). Grayscale values for each image were inverted so that higher values were representative of increased staining levels. Images of 3 slices through each ROI for each animal were analyzed and data sampled unilaterally. Data sampling occurred by using the drawing tools in iVision to outline the ROI and using the "measure" function to determine an average grayscale pixel value for the outlined area. ROI measures were corrected for nonspecific binding by subtraction of background measure taken from gray matter adjacent to the ROI. Data were analyzed across 3 representative levels through the rostral-caudal axis of the vlPAG (Bregma −7.08, −7.68, and −8.30)[58] and SC (Bregma −7.68, −6.24, and −5.80)[58] as previously described.[48] Densitometry values are

presented as the mean ± SEM density of immunoreactivity. Data were analyzed using an ANOVA to determine significant main effects of treatment (morphine, saline) and pain (CFA, Handled). Fisher's post hoc tests were used to determine specific group differences when a significant main effect was observed; $P \leq .05$ was considered statistically significant. For Western blots, band intensities for tissue from the vlPAG and SC were visualized at 55 kD (GFAP) and 160 kD (CD11b) and quantified using ImageJ (NIH, Bethesda, MD) analysis software, as a relative intensity of GFAP or CD11b band divided by the intensity of the β−actin band. Data are expressed as the mean ratio ± SEM of protein of interest/β−actin. Data were analyzed for significant main effects of treatment (morphine, saline) and pain (CFA, Handled) using an ANOVA, and Fisher's HSD was used for post hoc analysis; $P \leq .05$ was considered statistically significant.

Results

Experiment 1: Persistent Peripheral Inflammation Attenuated Morphine Tolerance

To assess the initial analgesic potency of morphine and the degree and time course for development of morphine tolerance, PWLs were determined for both the injured (ipsilateral; right) and uninjured (contralateral; left) hindpaws at baseline, and after Day 1 and Day 3 of morphine or saline. Contralateral (uninjured) PWL did not differ between CFA + Saline and Handled + Saline groups at any time point (Fig 1A). By contrast, intraplantar CFA significantly decreased ipsilateral PWL 24, 48, and 96 hours postinjection as compared with handled controls (CFA + Saline versus Handled + Saline; Fig 1B), indicating the development of persistent hyperalgesia. Administration of morphine on Days 1 and 3 significantly increased both contralateral and ipsilateral PWLs as compared with saline controls (Figs 1A and 1B, respectively). For the contralateral paw, administration of morphine (Day 1) produced comparable levels of analgesia in the CFA + Morphine and Handled + Morphine groups (Fig 1A). However, the degree of analgesia produced by morphine on Day 3 was significantly attenuated in the Handled + Morphine versus the CFA + Morphine animals, suggesting the development of morphine tolerance (Fig 1A). In the ipsilateral hindpaw, administration of morphine on Day 1 produced antihyperalgesia in CFA-treated animals as indicated by a return to normal baseline PWL (CFA + Morphine; Handled + Saline, Fig 1B). In contrast to the decreased analgesia observed in Handled + Morphine animals on Day 3, morphine produced a significant increase in ipsilateral PWL of injured animals on Day 3 as compared to Day 1 (CFA + Morphine; Injection 1 and Injection 3, Fig 1B), indicating lack of tolerance development.

Assessment of Morphine Tolerance

Morphine tolerance, assessed on Day 5 using a cumulative dosing paradigm, was only observed in non-CFA-treated animals. As shown in Fig 2, the antinociceptive potency of morphine was significantly decreased in both the ipsilateral and contralateral hindpaws of uninjured animals that received 3 consecutive days of morphine (Handled + Morphine) as compared with uninjured animals that received saline (Handled + Saline; Figs 2A and 2C). Indeed, animals that received 3 days of saline reached 100% maximum possible analgesia (MPE) at the 8-mg/kg dose. In contrast, 100% MPE was not noted until the 18-mg/kg dose in animals that received 3 prior days of morphine. Neither the

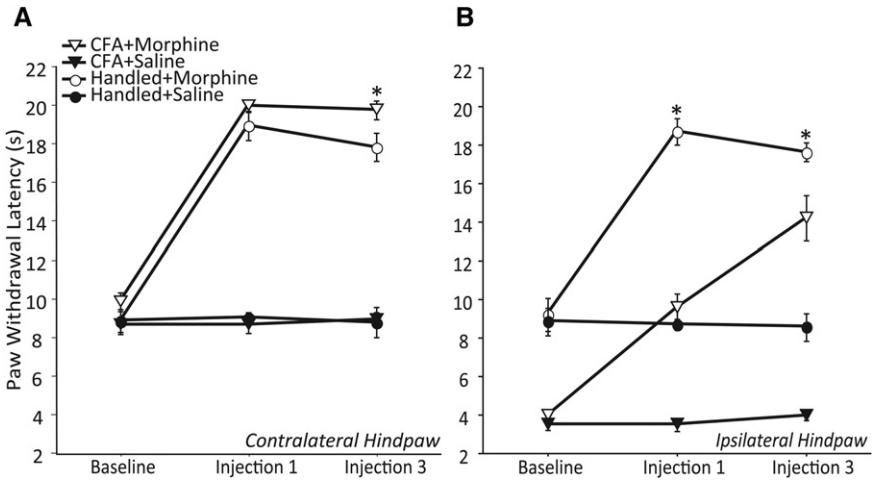

Figure 1. Contralateral **(A)** and ipsilateral **(B)** PWL (in seconds) following intraplantar CFA or handling (Baseline), and after the first and third injection of morphine or saline in CFA + Morphine (n = 6), CFA + Saline (n = 7), Handled + Morphine (n = 6), and Handled + Saline (n = 5)-treated male rats. The first and third injection of morphine caused an increase in contralateral and ipsilateral PWL as compared with saline controls ($P < .05$; **A** and **B**, respectively). Contralateral PWL did not differ between CFA + Saline and Handled + Saline groups at any time point ($P > .05$; **A**). CFA treatment caused a significant decrease in ipsilateral PWL at all time points as compared with handled controls ($P < .05$; CFA + Saline; Handled + Saline; **B**). While uninjured animals treated with morphine showed a decrease in analgesia to the third injection as compared with the first ($P < .05$; **A**), CFA-treated animals showed an increase in antihyperalgesia to the third injection ($P < .05$; **B**). Asterisks indicate significant differences between CFA + Morphine and Handled + Morphine groups.

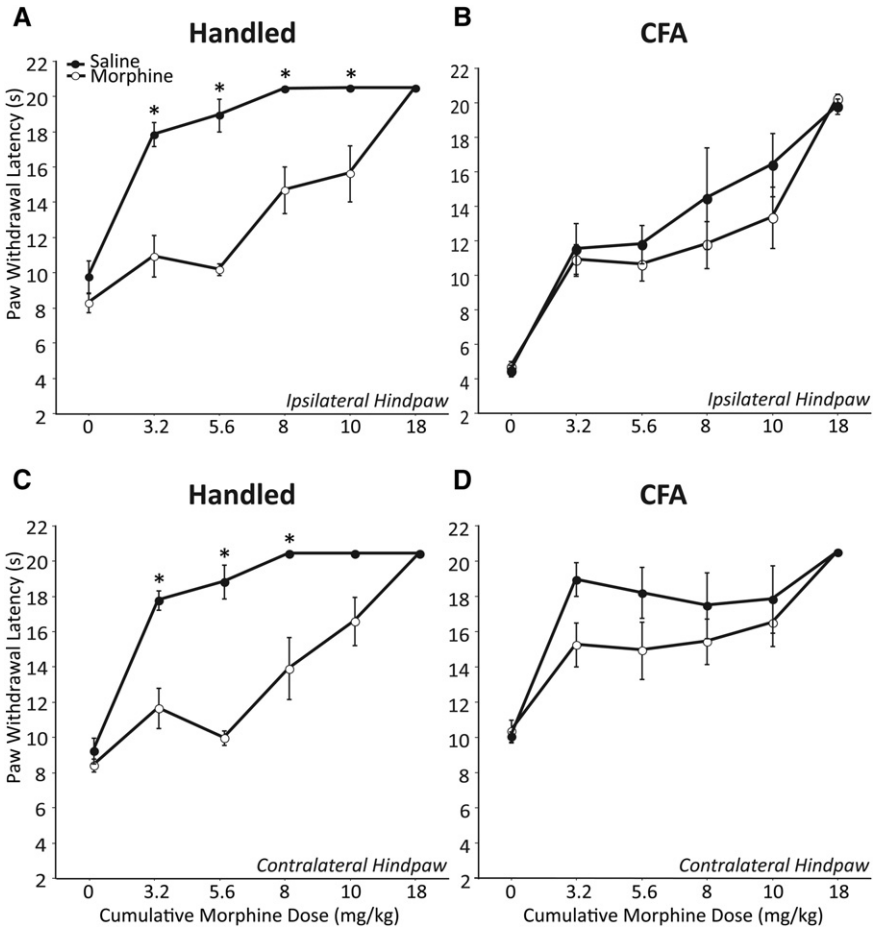

Figure 2. PWL (in seconds) as a function of cumulative doses of morphine in handled **(A** and **C)**, and CFA treated **(B** and **D)** male rats. Both ipsilateral **(A** and **B)** and contralateral **(C** and **D)** PWL data are presented. Animals received 3 consecutive days of morphine (5 mg/kg; sc, *open circles*) or saline (1 mL/kg; sc, *filled circles*). CFA + Morphine-treated animals (n = 7) did not differ from CFA + Saline treated animals (n = 4) in response to cumulative morphine on day 5 (ipsilateral; $F_{1,9} = 1.128$, $P = .3159$ and contralateral; $F_{1,9} = 1.470$, $P = .2563$). Handled + Morphine-treated animals (n = 9) showed a significant decrease in PWL in response to cumulative morphine on day 5 as compared with Handled + Saline animals (n = 5; ipsilateral; $F_{1,12} = 21.702$, $P = .0006$ and contralateral; $F_{1,12} = 20.373$, $P = .0007$). Asterisks indicate significant differences between Handled + Saline and Handled + Morphine groups.

antinociceptive nor the antihyperalgesic potency of morphine was different in CFA + Morphine-treated animals as compared with CFA + Saline-treated animals (Figs 2B and 2D), indicating lack of tolerance development. Indeed, no differences in PWLs produced by morphine were noted for all doses tested. Together these data indicate that persistent inflammatory pain attenuates the development of tolerance to morphine.

As glia activation in the spinal cord has been implicated in the development of morphine tolerance, the next series of experiments examined if glia were similarly activated within the vlPAG, and if persistent inflammatory pain altered their activation.

Experiment 2: Morphine Tolerance Is Associated With Increased Glial Cell Activation in the vlPAG

Increased activity of astrocytes, as evidenced by an increase in GFAP immunoreactivity, was only observed in non-CFA-treated animals that received morphine (Handled + Morphine, Fig 3B). A representative example of vlPAG GFAP staining in animals administered CFA + Morphine versus Handled + Morphine is shown in Fig 3A. Western blot-confirmed increased activity of astrocytes, as evidenced by an increase in relative band intensity of GFAP/β-actin, was only observed in noninjured animals that received morphine (Handled + Morphine, Fig 3C).

Similar to what was noted for astrocytes, microglia activity, as evidenced by OX-42 immunoreactivity, was significantly increased in animals that received morphine in the absence of pain (Handled + Morphine, Fig 4B). A representative example of vlPAG OX-42 staining in animals administered CFA + Morphine versus Handled + Morphine is shown in Fig 4A. A trend toward increased activity of microglia, as evidenced by an increase in relative band intensity of OX-42/β-actin, was only observed in animals that received morphine in the absence of pain (Handled + Morphine, Fig 4C); however, it did not reach statistical significance. Peripheral inflammation induced by intraplantar CFA did not elicit significant increases in vlPAG glial cell activity (Figs 3 and 4). Importantly, 1 injection of morphine (5 mg/kg) was not sufficient to alter vlPAG GFAP (Fig 5A) or OX-42 levels (Fig 5B) at 24 hours postmorphine. Similarly, no increase

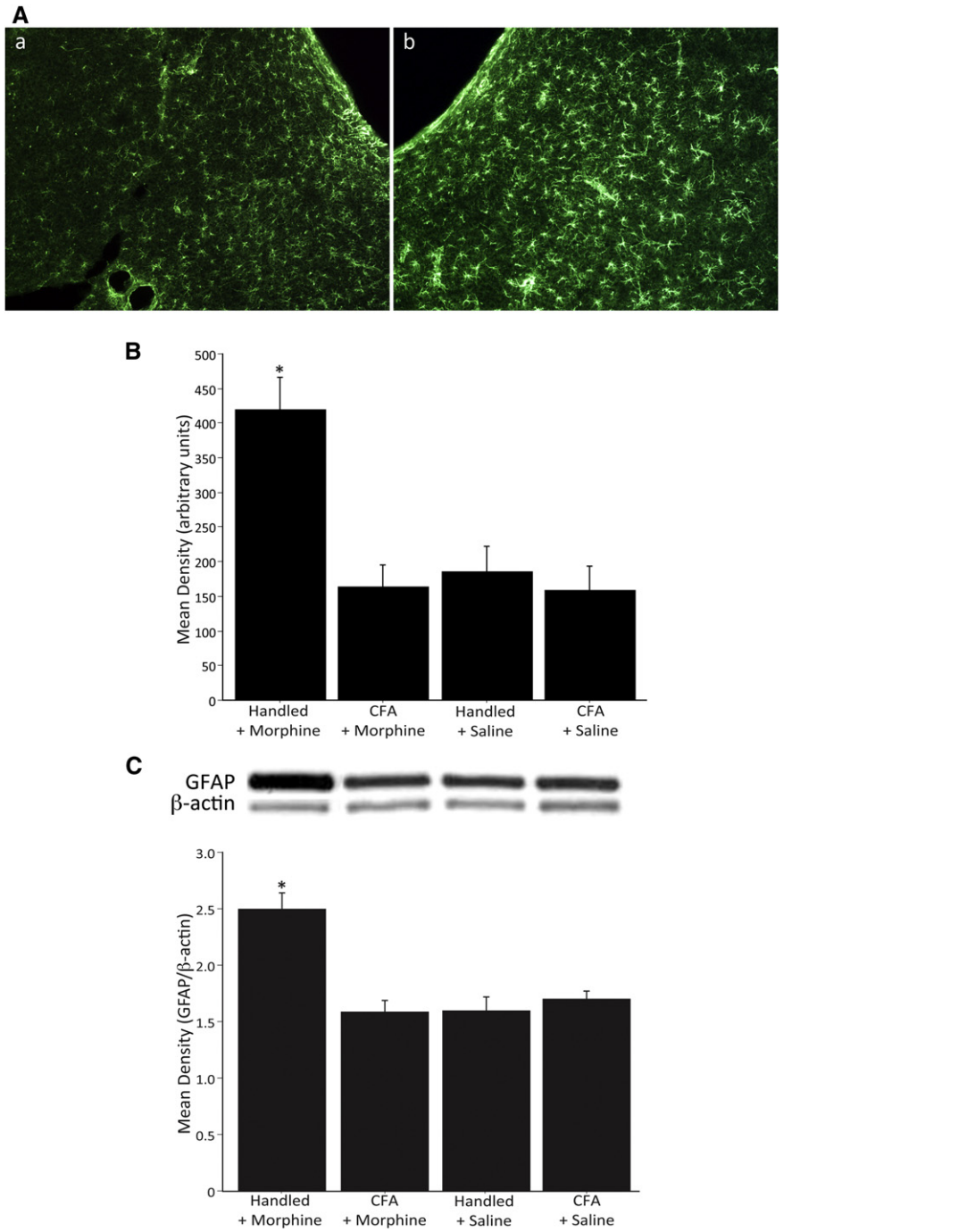

Figure 3. Representative fluorescent photomicrographs of GFAP immunoreactivity in the vlPAG of animals treated with CFA + Morphine (a) and Handled + Morphine (b) **(A)**. Densitometry of GFAP immunoreactivity in the vlPAG **(B)**. Administration of morphine, in the absence of CFA (Handled + Morphine; n = 7), resulted in a significant increase in GFAP immunoreactivity within the vlPAG ($F_{3,22}$ = 10.022, P = .0002). No differences in GFAP levels were noted for the CFA + Morphine (n = 11), CFA + Saline (n = 4) or Handled + Saline control groups (n = 4). Relative band intensity of GFAP/β-actin in the vlPAG **(C)**. Administration of morphine, in the absence of CFA (Handled + Morphine; n = 5), resulted in a significant increase in relative band intensity of GFAP/β-actin in the vlPAG ($F_{3,19}$ = 10.256, P = .0003). No differences in GFAP levels were noted for the CFA + Morphine (n = 5), CFA + Saline (n = 7) or Handled + Saline (n = 6) control groups. Asterisks indicate significant differences between Handled + Morphine group and all other groups.

in GFAP or OX-42 levels was noted 1 hour postmorphine (data not shown). No significant group differences were noted in SC GFAP or OX-42 immunoreactivity ($F_{3,23}$ = 2.089, P = .1295 and $F_{3,19}$ = 1.416, P = .2690, respectively) or protein level ($F_{3,28}$ = .232, P = .8730 and $F_{3,6}$ = 1.822, P = .2435, respectively), indicating that changes in vlPAG glial cell activity are region specific (data not shown).

Discussion

The present experiments tested the hypothesis that vlPAG glial cell activity contributes to the development of morphine tolerance. Clinical studies indicate that chronic pain attenuates the development of morphine tolerance[37,77]; however, animal studies have

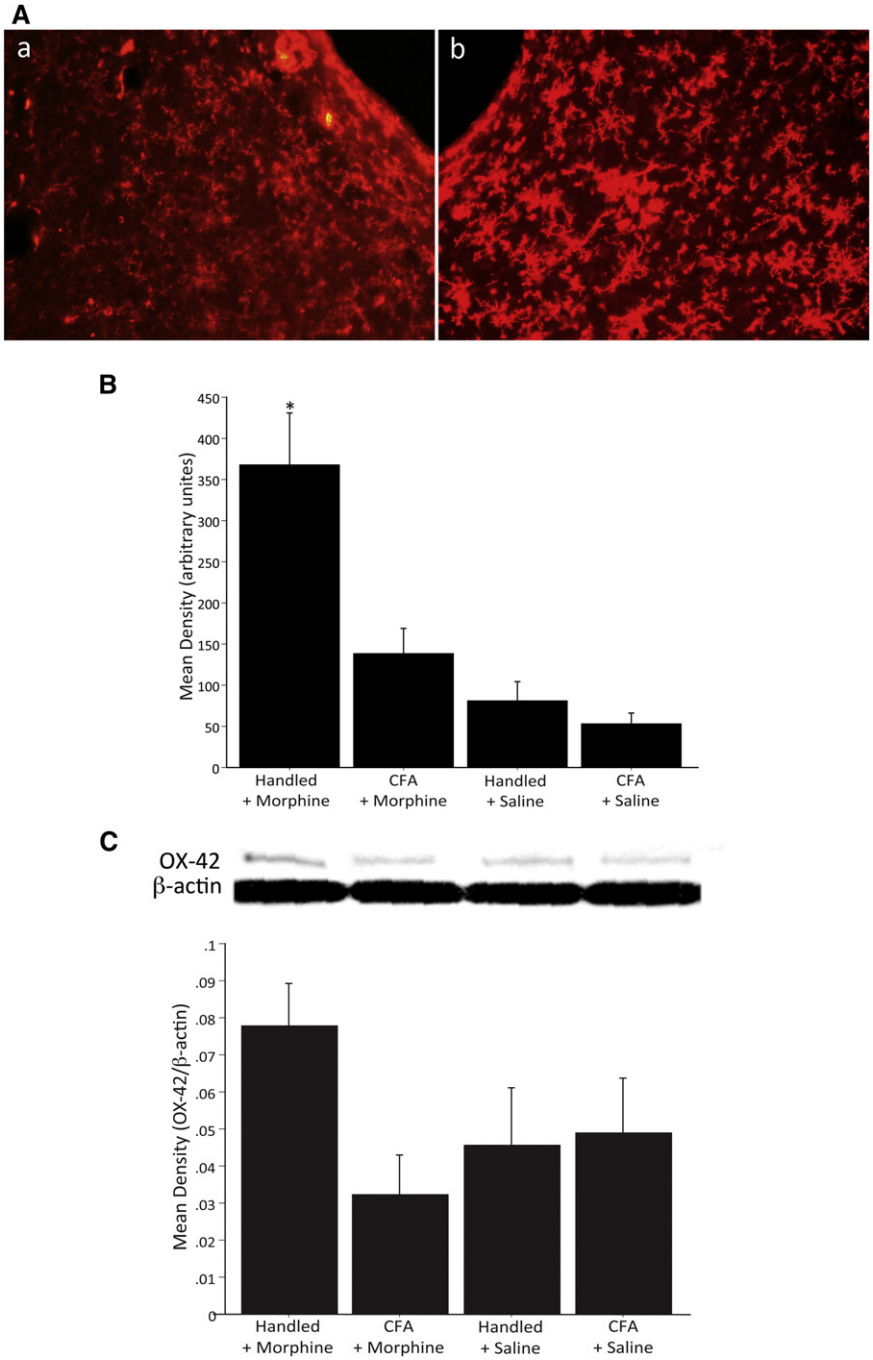

Figure 4. Representative fluorescent photomicrographs of OX-42 immunoreactivity in the vlPAG of animals treated with CFA + Morphine (a) and Handled + Morphine (b) **(A)**. Densitometry of OX-42 immunoreactivity in the vlPAG **(B)**. Administration of morphine, in the absence of CFA (Handled + Morphine; n = 8), significantly increased OX-42 immunoreactivity within the vlPAG ($F_{3,19}$ = 9.270, P = .0005). No differences in OX-42 levels were noted for the CFA + Morphine (n = 7), CFA + Saline (n = 4) or Handled + Saline (n = 4) control groups. Relative band intensity of OX-42/β-actin in the vlPAG **(C)**. Administration of morphine, in the absence of CFA (Handled + Morphine; n = 5), resulted in an increase in relative band intensity of OX-42/β-actin in the vlPAG ($F_{3,10}$ = 2.544, P = .1151); however, it did not reach significance. Asterisks indicate significant differences between the Handled + Morphine group and all other groups.

yielded variable results.[35,65,78] Therefore, the impact of persistent inflammatory hyperalgesia on morphine tolerance development and glial activation was also investigated. Here we report that 1) short-term daily administration of an ED_{50} dose of morphine was sufficient to induce morphine tolerance; 2) persistent inflammatory pain induced by intraplantar CFA significantly attenuated morphine tolerance; and 3) increased vlPAG microglia and astrocyte activity was only observed in those animals made tolerant to morphine. Together, these data suggest a potential role for vlPAG microglia and astrocytes in the development of morphine tolerance, and suggest that persistent inflammatory pain attenuates morphine tolerance by inhibiting morphine-induced vlPAG glial cell activation.

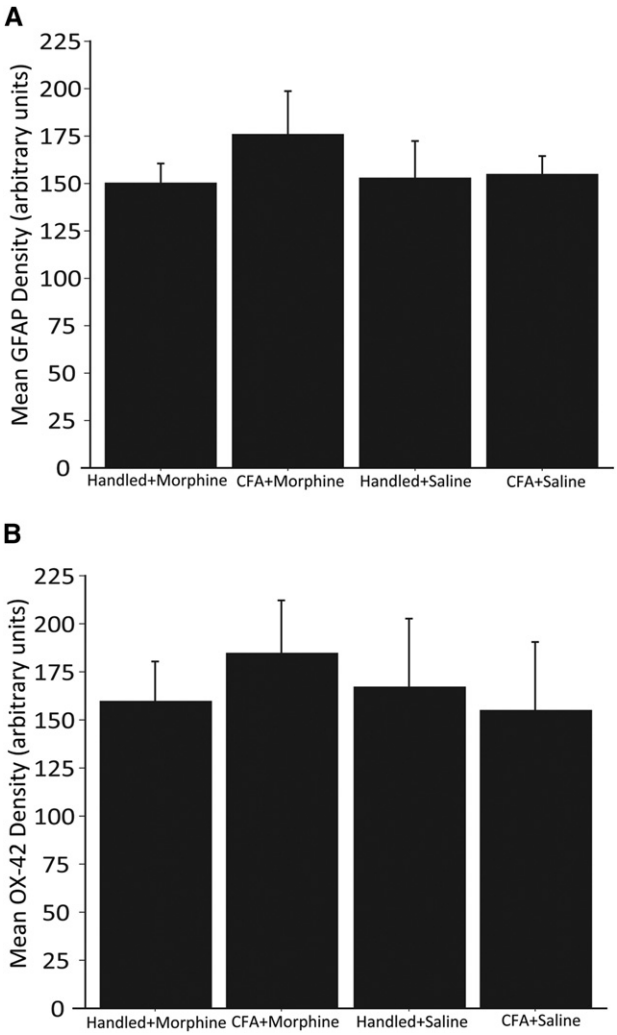

Figure 5. Densitometry of GFAP **(A)** and OX-42 **(B)** immunoreactivity in the vlPAG in Handled + Morphine (n = 3), CFA + Morphine (n = 4), Handled + Saline (n = 4), and CFA + Saline (n = 5) animals 24 hours following 1 morphine or saline injection. Neither CFA nor morphine (5 mg/kg, sc) increased vlPAG GFAP ($F_{3,12}$ = .494; P = .693) or OX-42 ($F_{3,12}$ = .162; P = .9198) levels in the vlPAG as compared to handled and saline controls.

Increases in vlPAG Microglia and Astrocyte Activity Correlate With the Development of Morphine Tolerance

Many mechanisms have been proposed to account for opioid tolerance, including decoupling, internalization, and/or downregulation of MORs,[67,68] upregulation of N-methyl-D-aspartate receptor (NMDAR) function,[1-3] downregulation of glutamate transporters,[9,59] and production of nitric oxide (a known mediator of NMDAR function).[84] These mechanisms were all thought to implicate some form of neuronal adaptation.[84] However, it is becoming increasingly clear that activated glia mediate many of these neuronal adaptations that contribute to morphine tolerance.[84] Consistent with previous reports, here we find that tolerance to morphine developed rapidly in the absence of pain.[41,42,48] Indeed, 1 ED_{50} dose of morphine (5 mg/kg) injected subcutaneously for 3 days was sufficient to induce behaviorally defined tolerance. Paralleling the development of tolerance, GFAP and OX-42 protein levels increased significantly within the vlPAG, suggesting the activation of astrocytes and microglia, respectively.

A large body of evidence has accumulated implicating opioids as activators of spinal astrocytes and microglia.[31,60,63,86] In both mice and rats, morphine increases spinal GFAP and OX-42 protein levels[20,33] as well as glially derived proinflammatory cytokines.[32,63] Inhibition of spinal glia or cytokine release increases the analgesic efficacy of morphine[32,63] and attenuates morphine tolerance.[31,51,52,60,70] Our novel findings in the vlPAG parallel the data from studies of spinal cord glia and indicate that supraspinal glial cell activity may also contribute to the development of morphine tolerance.

Under basal conditions, glia survey the environment for pathogens and debris, and they regulate ion and neurotransmitter levels in the synapse to modulate neuronal excitability.[86] The activation of glia results in the release of excitatory substances that oppose morphine analgesia (eg, proinflammatory cytokines).[86] Glial release of cytokines increases with chronicity of morphine administration,[84] making these excitatory substances key players in the development of morphine tolerance. Glially derived cytokine release, particularly tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β), results in increased density and conductance of neuronal alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)[57,71] and NMDAR,[79] decreased astrocytic glutamate transporter proteins (GLT-1, GLAST),[86] and downregulation of neuronal GABA receptors.[71] These cytokine-induced changes, among others,[39,55] effectively increase neuronal excitability. Morphine binds to neuronal MORs in the vlPAG that are primarily located on GABAergic neurons[14,18,38]; MORs binding in the vlPAG disinhibits GABAergic PAG-RVM projection neurons,[25] resulting in the net excitation of the PAG-RVM-spinal cord descending pain modulatory circuit. Glia-induced increases in the excitability of vlPAG MOR-containing neurons may act to alter the inhibitory properties of morphine, thereby decreasing analgesic efficacy and contributing to the development of morphine tolerance.

Persistent Inflammatory Pain Prevented Morphine-Induced Increases in vlPAG Glial Cell Activity and Attenuated the Development of Morphine Tolerance

The results of the present study demonstrate that the presence of persistent pain alters both the development of morphine tolerance and morphine-induced vlPAG glial cell activation. The finding that persistent peripheral inflammation attenuates morphine tolerance is consistent with the clinical literature demonstrating that opioid tolerance is attenuated in chronic pain sufferers.[17,23,26] Indeed, clinical studies have repeatedly shown that morphine tolerance develops most robustly in those individuals consuming morphine in the absence of pain.[19,54] In the present study, male rats given CFA 24 hours before the 3-day morphine administration regimen showed significant increases in analgesia to all challenge doses of morphine, as compared

with noninjured animals. Several factors may contribute to the pain-induced attenuation in morphine tolerance. First, morphine, given in conjunction with peripheral inflammation, failed to elicit the increases in vlPAG microglia and astrocyte activity observed in non-CFA-treated animals given morphine. Indeed, peripheral inflammatory pain blocked both morphine tolerance and morphine-induced glial cell activation within the vlPAG. As glia are not activated, no cytokine release would be expected, and therefore, no net change in neuronal excitability. Alternatively, cannabinoids, which are released within the PAG during peripheral pain,[80] have been shown to influence both glial activity and morphine analgesia. Second, cannabinoid receptors are robustly expressed within the vlPAG, with approximately 32% of cannabinoid receptor 1 (CB1)-expressing neurons also expressing MOR.[88] Functionally, intra-PAG administration of a CB1 agonist enhances morphine analgesia,[87] and systemic administration of cannabinoids, along with morphine, leads to the attenuation of morphine tolerance.[15,69,87] Endocannabinoids also possess potent anti-inflammatory properties,[22] which would likely block the activation of glia. Indeed, systemic administration of the cannabinoid receptor agonist WIN 55,212-2 prevents microglia and astrocyte activation and decreases the release of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in the spinal cord.[10]

In the present study, no glia activation was noted following administration of CFA alone. These results are in contrast with previous reports that peripheral pain, including that produced by CFA,[13,50,62,72] peripheral neuropathy,[16,21,27,62,73,74,83] formalin,[45] and spinal nerve ligation,[30] induces significant glia activation within the spinal cord. However, given the roles of the spinal cord and PAG in pain facilitation and pain modulation, respectively, it is not entirely surprising that there would be differential pain-induced regulation of glial activation in these 2 sites. Together, these studies suggest that inflammation elicits differential glial responses in a central nervous system region-dependent manner, and prevents morphine-induced increases in vlPAG glial cell activity.

How Opioids Activate Glia

Opioid hyperalgesia is still observed in neuronal opioid receptor (mu, delta, and kappa) knockout mice,[36] suggesting that the antianalgesic affects of morphine (eg, antianalgesia and tolerance) are mediated by non-neuronal opioid receptors. Indeed, it was recently discovered that morphine analgesia is modulated not only by classical neuronal opioid receptors but also by nonclassical glial receptor activity.[34] Opioids have been shown to bind to Toll-like receptor 4 (TLR4),[34] an innate immune receptor located on microglia and astrocytes, and an abundance of evidence has accumulated indicating that TLR4 activity opposes morphine analgesia.[34,86] Functionally, animals that receive TLR4 antagonism, as well as TLR4 knockout mice, exhibit increased responsiveness to the analgesic properties of acute morphine administration.[86] Similarly, systemic administration of TLR4 antagonists attenuates morphine tolerance.[34] To date, the specific role of TLR4 in morphine tolerance development has not been elucidated. However, given our findings that the development of morphine tolerance correlates with increased vlPAG glial cell activation, and the evidence showing that TLR4 is expressed on rat PAG glia,[12,40] future studies investigating the potential role of vlPAG TLR4 in the development of morphine tolerance are warranted.

Conclusions

There is extensive literature supporting a critical role for glial cell activation in the development of morphine tolerance. Our findings that increased vlPAG glial activity is concurrent with the development of morphine tolerance, and that pain inhibits both vlPAG glial reactivity and morphine tolerance development, suggests that vlPAG glia play a significant role in the development of morphine tolerance. Taken together, our results may provide a direct neurobiological mechanism whereby chronic inflammatory pain attenuates the development of morphine tolerance. Our results may implicate vlPAG glial cells as key regulators of this phenomenon.

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