Cellular/Molecular

Opioid-Induced Hyperalgesic Priming in Single Nociceptors

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Clinical μ-opioid receptor (MOR) agonists produce hyperalgesic priming, a form of maladaptive nociceptor neuroplasticity, resulting in pain chronification. We have established an in vitro model of opioid-induced hyperalgesic priming (OIHP), in male rats, to identify nociceptor populations involved and its maintenance mechanisms. OIHP was induced in vivo by systemic administration of fentanyl and confirmed by prolongation of prostaglandin E2 (PGE2) hyperalgesia. Intrathecal cordycepin, which reverses Type I priming, or the combination of Src and mitogen-activated protein kinase (MAPK) inhibitors, which reverses Type II priming, both partially attenuated OIHP. Parallel in vitro experiments were performed on small-diameter (<30 μm) dorsal root ganglion (DRG) neurons, cultured from fentanyl-primed rats, and rats with OIHP treated with agents that reverse Type I or Type II priming. Enhancement of the sensitizing effect of a low concentration of PGE2 (10 nM), another characteristic feature of priming, measured as reduction in action potential (AP) rheobase, was found in weakly isolectin B4 (IB4)-positive and IB4-negative (IB4-) neurons. In strongly IB4-positive (IB4+) neurons, only the response to a higher concentration of PGE2 (100 nM) was enhanced. The sensitizing effect of 10 nM PGE2 was attenuated in weakly IB4+ and IB4− neurons cultured from rats whose OIHP was reversed in vivo. Thus, in vivo administration of fentanyl induces neuroplasticity in weakly IB4+ and IB4− nociceptors that persists in vitro and has properties of Type I and Type II priming. The mechanism underlying the enhanced sensitizing effect of 100 nM PGE2 in strongly IB4+ nociceptors, not attenuated by inhibitors of Type I and Type II priming, remains to be elucidated.

Key words: excitability; fentanyl; isolectin B4; neuroplasticity; nociceptor; sensitization

Introduction

Opioid analogues remain among the most effective treatments for moderate-to-severe pain (Manchikanti et al., 2012; Cheung et al., 2014; Ballantyne, 2015; Bicket et al., 2019; Pisanu et al., 2019; Preuss et al., 2020). They can, however, produce serious adverse effects, including analgesic tolerance, addiction, opioid-induced hyperalgesia (OIH), and pain chronification (Ossipov et al., 2005; Chu et al., 2006; De Felice and Porreca, 2009; Roeckel et al., 2016; Mercadante et al., 2019), which limits their use and contributes to their abuse. Even when opioid analogues are used briefly, in the perioperative period, they may lead to increased, postoperative pain and opioid requirements, up to a year later (Chia et al., 1999; van Gulik et al., 2012; Rashiq and Dick, 2014; Yildirim et al., 2014; Lyons et al., 2015; Chang et al., 2018; Rong et al., 2019), indicating that opioids can produce long-lasting alterations in pain processing, well beyond the duration of opioid exposure. We have established a preclinical model of the transition from acute to chronic pain, hyperalgesic priming, here referred to as priming. Priming is a form of long-lasting nociceptor neuroplasticity manifested by a left-shift in dose dependence and marked prolongation of mechanical hyperalgesia in response to pronociceptive mediators, prototypically prostaglandin E2...
(PGE$_2$), but unchanged baseline nociceptive threshold (Aley et al., 2000; Parada et al., 2003, 2005; Reichling and Levine, 2009; Araldi et al., 2015, 2017a, 2018bc, 2019; Ferrari et al., 2015, 2019; Khomula et al., 2017). Thus, while in control rats PGE$_2$-induced hyperalgesia is of short duration (<4 h), in the primed state hyperalgesia is produced by lower concentrations of PGE$_2$ (Parada et al., 2003, 2005) and is still present, unattenuated, 4 h later (Aley et al., 2000).

We have recently shown that μ-opioid receptor (MOR) agonists, including two commonly used clinical opioids, fentanyl, and oxymorphone (Araldi et al., 2019; Ferrari et al., 2019), as well as two biased MOR agonists, PZM21 and TRV130 (Araldi et al., 2018a), can produce both OIH and opioid-induced hyperalgesic priming (OIHP). We have recently established an *in vitro* model of OIH, neuroplasticity induced by *in vivo* administration of fentanyl that persists in cultured DRG neurons, differentially affecting peptidergic and non-peptidergic nociceptors (Khomula et al., 2019). However, despite a growing literature obtained from *in vivo* experiments, lack of an *in vitro* model of OIH has made it difficult to study underlying mechanisms at the cellular level, in identified nociceptor populations.

Primary afferent nociceptors can be divided into three categories based on isolectin B4 (IB4)-binding status: strongly IB4-positive (IB4+), weakly IB4+, and IB4-negative (IB4−; Kashiba et al., 2001; Fang et al., 2006). IB4+ neurons depend on glial-derived neurotrophic factor (GDNF), and express Ret, whereas IB4− neurons depend on NGF for survival and express TrkA (Molliver et al., 1997; Bennett et al., 1998; Kashiba et al., 1998). Weakly IB4+ neurons also express TrkA (Kashiba et al., 2001; Fang et al., 2006). Nociceptors can also be classified into non-peptidergic and peptidergic (Bennett et al., 1996; Molliver et al., 1997; Wooll and Ma, 2007). Strongly IB4+ nociceptors belong to the non-peptidergic class, whereas weakly IB4+ and IB4− nociceptors are peptidergic (Kashiba et al., 2001; Fang et al., 2006). These classes differ in electrophysiological properties (Stucky and Lewin, 1999), physiological function (Snider and McMahon, 1998), and their role in OIH (Araldi et al., 2015, 2018c; Ferrari et al., 2019).

To establish an *in vitro* model of OIH we (1) produced OIH *in vivo*, assessed at the nociceptor peripheral and central terminal as prolongation of PGE$_2$-induced mechanical hyperalgesia, in rats treated with systemic fentanyl; (2) established, *in vivo*, a protocol to reverse OIH and also evaluate whether systemic fentanyl-induced priming occurs in peptidergic and/or nonpeptidergic neurons; (3) employed, *in vitro*, concentration dependence of PGE$_2$-induced sensitization, in populations of cultured DRG neurons (i.e., strongly and weakly IB4+, and IB4−) from rats primed *in vivo* with systemic fentanyl, to reveal *in vitro* correlates of OIH at the single cell level; and (4) tested putative maintenance mechanisms of OIH in nociceptor populations classified by their IB4-binding status (based on *in vitro* correlates, in cultured DRG neurons from animals in which OIH was reversed *in vivo*).

**Materials and Methods**

**Animals**

Experiments were performed on 220–420 g male Sprague Dawley rats (Charles River Laboratories). Experimental animals were housed three per cage, under a 12/12 h light/dark cycle, in a temperature-controlled and humidity-controlled animal care facility at the University of California, San Francisco. Food and water were available *ad libitum*. Nociceptive testing was performed between 9 A.M. and 5 P.M. All experimental protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Effort was made to minimize number of animals used and their suffering.

**Nociceptive threshold testing**

Mechanical nociceptive threshold was measured using an Ugo Basile Analgesymeter (Randall-Selitto paw-withdrawal device, Stoeling), which applies a linearly increasing mechanical force to the dorsum of a rat’s hind paw, with a dome-shaped plinth, as previously described (Taiwo et al., 1989; Taiwo and Levine, 1989; Araldi et al., 2015, 2017a; Ferrari and Levine, 2015). Rats were placed in cylindrical acrylic restrainers that provide ventilation, and lateral ports that allow extension of their hind legs for the assessment of nociceptive threshold, while minimizing restraint stress. To acclimatize rats to the testing procedure, they were placed in restrainers for 40 min before starting training sessions (three consecutive days of training) and for 30 min before experimental manipulations. Nociceptive threshold was defined as the force, in grams, at which the rat withdrew its paw. Baseline nociceptive threshold for each individual rat was defined as the mean of three readings taken before the first experimental treatment. In each experiment, only one paw per rat was used. Each experiment was performed on a different group of rats (one paw/rat, six rats/group). The individuals performing behavioral experiments (D.A. and I.J.M.B.) were blinded to treatments.

**Drugs and routes of administration**

The following compounds were used in these experiments: cordycepin 5′-triphosphate sodium salt (a protein translation inhibitor), fentanyl citrate salt (a MOR agonist), SU6656 (a Src family kinase inhibitor), U0126 (a MAPK/extracellular signal-regulated kinase [ERK] inhibitor), and PGE$_2$ (a direct-acting nociceptor-sensitizing agent), all of which were purchased from Sigma-Aldrich.

A stock solution of PGE$_2$ (1 μg/ml) was prepared in 100% ethanol and additional dilutions made with physiological saline (0.9% NaCl), to prepare the final solution used in experiments (100 ng/μl), which yielded a final ethanol concentration of 2%. In previous *in vivo* control experiments, we have shown that ethanol in this concentration had no effect on mechanical nociceptive threshold (Ferrari et al., 2016). Fentanyl and cordycepin were dissolved in saline. SU6656 and U0126 were dissolved in 100% dimethylsulfoxide (DMSO; Sigma-Aldrich) and further diluted in saline containing 2% Tween 80 (Sigma-Aldrich). The final concentration of DMSO and Tween 80 was ~2%.

Intradermal administration of PGE$_2$ (100 ng/μl) was performed on the dorsum of the hindpaw using a 30-gauge hypodermic needle adapted to a 50 μl Hamilton syringe by a segment of PE-10 polyethylene tubing (Becton Dickinson).

Intrathecal administration of PGE$_2$, cordycepin, the combination of SU6656 and U0126, and the combination of cordycepin, SU6656, and U0126 was performed in rats briefly anesthetized with 2.5% isoflurane (Phoenix Pharmaceuticals) in 97.5% O$_2$, using a 29-gauge hypodermic needle (300 unit/ml syringe; Walgreens Pharmacy) inserted into the subarachnoid space, between the L4 and L5 vertebrae. The maximum intrathecal volume administered was 20 μl; cordycepin (4 μg) was dissolved in saline and injected intrathecally in a volume of 20 μl, when followed by intradural PGE$_2$ (100 ng/μl), and in a volume of 10 μl when followed by intrathecal PGE$_2$ (400 ng/10 μl; Araldi et al., 2018c). The combination of SU6656 (10 μg) and U0126 (10 μg) was injected intrathecally in a final volume of 20 or 10 μl (these drugs were mixed in the syringe at the moment of injection), followed by PGE$_2$ injected intradurally (100 ng/5 μl) or intrathecally (400 ng/10 μl), respectively. The combination of all three inhibitors (cordycepin (4 μg/6 l), SU6656 (10 μg/6 l), and U0126 (10 μg/6 l)) was injected intrathecally followed, 10 min later, by intradural PGE$_2$ (100 ng/5 μl). To verify whether the inhibitors reversed systemic fentanyl-induced priming permanently, PGE$_2$ was again injected intradurally (100 ng/5 μl) or intrathecally (400 ng/20 μl), 4, 14, and 28 days after the inhibitors. The intrathecal site of injection was confirmed by a sudden flick of the rat’s tail, a reflex that is evoked by accessing the subarachnoid space followed by bolus intrathecal injection (Mestre et al., 1994).
Systemic (subcutaneous) administration of fentanyl was performed at the pate of the neck (Araldi et al., 2018c, 2019). Rats received an injection of fentanyl (30 μg/kg, s.c.) diluted in saline and administered subcutaneously (100 μl/100 g body weight).

**Administration of saporins**

**IB4-saporin**

IB4-saporin, an IB4+ nociceptor neurotoxin (Advanced Targeting Systems), was diluted in saline and a dose of 3.2 μg, in a volume of 20 μl, administered intrathecally, 14 d before systemic administration of fentanyl (30 μg/kg). The dose and timing of IB4-saporin administration were chosen based on previous reports from our group and others (Vulcanova et al., 2001; Nishiguchi et al., 2004; Joseph et al., 2008, 2010; Araldi et al., 2015, 2016a, 2017b, 2018c).

**SSt-saporin**

SSt-saporin, a neurotoxin specifically targeting neurons that express substance P (SP) receptors (Advanced Targeting Systems), was diluted in saline and a dose of 100 ng in a volume of 20 μl administered intrathecally 14 d before rats received a systemic fentanyl (30 μg/kg). The addition of [Sar9, Met(O2)11]-substance P-saporin (SSP-saporin) to the SP-conjugated to saporin makes the agent more stable and potent than when SP alone is bound to saporin. The dose and time point of the treatment (14 d before fentanyl) were based on previous studies (Wiley et al., 2007; Choi et al., 2012), where there was observed loss of intrinsic lumbar dorsal horn neurons expressing neurokinin 1 receptor (NK1R), a receptor for SP, in Laminae I-III but not in deeper laminae (Khasabov et al., 2002; Vierck et al., 2003; Wiley et al., 2007; Choi et al., 2012; Weisshaar and Winkelstein, 2014; Kras et al., 2015; Araldi et al., 2016b, 2017b, 2018b, c), thus disrupting signaling dependent on peptidergic nociceptors (Seki et al., 2005).

We used rats treated with unconjugated saporin as controls, since previous studies demonstrated that intrathecal administration of saporin alone has no effects on NK1R staining in the spinal cord (Hwang et al., 2003) and does not decrease IB4 labeling in Lamina II of the dorsal horn (Joseph et al., 2008). The control group received 3.2 μg of unconjugated saporin, diluted in 20 μl of saline, intrathecally. This dose (3.2 μg) is equal to the IB4-saporin dose and higher than SSP-saporin (100 ng).

To inject unconjugated saporin, IB4-saporin, or SSP-saporin, rats were briefly anesthetized with 2.5% isoflurane (Phoenix Pharmaceuticals) and then a 29-gauge hypodermic needle (300 units/ml syringe, Walgreens Pharmacy) was inserted, on the midline, into the cisterna magna (FM, in both IB4 – SSP-saporin treated groups, t0 = 1.3; p = 0.24, for the SSP-saporin-treated group, and t0 = 0.5; p = 0.66, for the IB4-saporin-treated group, when the mechanical nociceptive threshold is compared before and 14 d after intrathecal saporins; paired Student’s t test).

**Culture of DRG neurons**

Primary cultures of dorsal root ganglion (DRG) neurons were made from adult male Sprague Dawley rats (220–235 g), as described previously (Ferrari et al., 2016, 2018; Khomula et al., 2017; Araldi et al., 2018c). In brief, under isoflurane anesthesia, rats were decapitated, and the dorsal of the vertebral column surgically removed; L4 and L5 vertebrae. Rats regained consciousness ~2 min after stopping anesthesia. There was no effect of unconjugated saporin, IB4-saporin, or SSP-saporin on mechanical nociceptive threshold per se (t0 = 0.6; p = 0.59, for the unconjugated saporin-treated group, t0 = 1.3; p = 0.24, for the SSP-saporin-treated group, and t0 = 0.5; p = 0.66, for the IB4-saporin-treated group), when the mechanical nociceptive threshold is compared before and 14 d after intrathecal saporins; paired Student’s t test).

**In vitro patch-clamp electrophysiology**

Cultured DRG neurons were used in *in vitro* electrophysiology experiments 24–96 h after DRG dissociation and plating. DRG from at least three rats (separate culture preparations) were used for each experimental series. Within the text, *n* refers to the number of neurons. Cells were identified as neurons by their double biurefringent plasma membranes (Cohen et al., 1968; Landowne, 1993). While small, medium, and large sized DRG neurons were routinely observed in the same preparation, this study focused on cells with a soma diameter <30 μm (small DRG neurons), predominantly representing C-type nociceptors (Harper and Lawson, 1985; Gold et al., 1996b; Petruska et al., 2000, 2002; Woolf and Ma, 2007). After mounting a coverslip with cells in the recording chamber, the culture medium was replaced with Tyrode’s solution containing 140 mM NaCl, 4 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM glucose, and 10 mM HEPES and adjusted to pH 7.4 with NaOH; osmolality is 310 mOsm/kg (Ferrari et al., 2016, 2018; Khomula et al., 2017; Araldi et al., 2018c). Tyrode’s solution was used as external perfusion solution in the *in vitro* experiments. Drugs used in *in vitro* were diluted to their final concentration in this solution just before application. The volume of the recording chamber is 150 μl. The perfusion system is gravity-driven, flow rate of 0.5–1 ml/min. All experiments were performed at room temperature (20–23°C).

Whole-cell patch-clamp recordings, in current clamp mode, were made to assess changes in the excitability of cultured DRG neurons. Holding current was adjusted to maintain membrane potential at ~70 mV. Rheobase, the minimum magnitude of a current step needed to elicit an action potential (AP), was determined from a protocol using increasing square wave current pulses. To estimate the rheobase, a series of current steps with 200-pA increments was applied until APs were elicited. The protocol was then adjusted to five to six pulses with step size of 5–10% of the rheobase estimate (two to three subthreshold stimuli and two to three with AP generation, to avoid overstimulation/desensitization; Ferrari et al., 2018; Khomula et al., 2019).

Recording electrodes were fabricated from borosilicate glass capillaries (0.84/1.5 mm i.d./o.d., Warner Instruments, LLC) using a Flaming/Brown P-87 puller (Sutter Instrument Co). Recording electrode resistance was ~3 MΩ after being filled with a solution containing the following: 130 mM KCl, 10 mM HEPES, 10 mM EGTA, 1 mM CaCl2, 5 mM MgATP, and 1 mM Na-GTP; pH 7.2 (adjusted with Tris-base), 300 mOsm (measured by Wescor Vapro 5520 osmometer, ELLTech Group; Ferrari et al., 2018). Junction potential was not adjusted. Series resistance was below 20 MΩ at the end of recordings and was not compensated. Recordings were made with an Axon MultiClamp 700 B amplifier, filtered at 10 kHz, and sampled at 20 kHz using Axon Digidata 1550B controlled by pCLAMP 11 software (all from Molecular Devices LLC).

Drugs were applied at least 10 min after the establishment of whole cell configuration, at which time baseline current was stable.

**Identification of priming in vitro**

We have previously shown that PGE2 sensitizes small-diameter DRG neurons, putative nociceptors, *in vitro* (Gold et al., 1996b,c, 1998). Within 1–2 min, sensitization can be detected in “whole-cell” patched neurons, in current clamp mode, as a reduction of rheobase, the minimal sustained current required to generate an AP, for a saturating concentration of PGE2 (1 μM), in both IB4– and IB4+ small-diameter DRG neurons (Gold et al., 1996b; Ferrari et al., 2018). Since it is not practical to hold cells “clamped” for 4 h, to reveal priming, we relied on another *in vivo* feature of hyperalgesic priming, a more than one order magnitude leftward shift (to lower values) for the dose dependence of PGE2-induced hyperalgesia (Parada et al., 2005). Since only ~60% of cultured DRG neurons are responsive to PGE2 (Gold et al., 1996b), it would not be possible to distinguish between responsive and nonresponsive neurons by their response to a low concentration of PGE2 response to a higher concentration is required. Previously, it has been shown that the dose dependence for the *in vitro* sensitizing effect of PGE2 ranges from concentrations of 1 nM to 1 μM (Gold et al., 1996b). Therefore, we chose two PGE2 concentrations, 10 and 100 nM, to examine our hypothesis that priming *in vivo* induces a leftward shift of the dose response curve in primed DRG neurons.
Fluorescence imaging of in vitro IB4 binding

The bright-field imaging system consisted of an inverted microscope (Eclipse TE-2000, Nikon Instruments Inc) with epi-fluorescence using a xenon lamp (Lambda LS, Sutter Instruments Co) for excitation (Ferrari et al., 2018). Illumination was controlled by a Lambda 10-2 filter wheel and Lambda SC Smart Shutter controllers (Sutter Instruments Co); an Andor Clara Interline CCD camera (Andor Technology Ltd.) was used for high-resolution digital image acquisition. MetaFluor software (Molecular Devices LLC) provided computer interface and controlled the whole system as well as being used for image processing. A Plan Fluor objective (20 × UV, NA 0.50; Nikon Instruments Inc) was used for both fluorescent and transmitted light imaging with phase contrast illumination.

Cells were incubated in Tyrode’s solution supplemented with 10 μg/ml IB4 conjugated to Alexa Fluor 488 dye (Invitrogen Life Technologies) for 10–12 min, in the dark. After washout, fluorescent images were captured during the first 15 min of each experiment using a standard GFP filter set (Chroma Technology). Cells demonstrating bright fluorescence and a halo around the neuronal plasma membrane were recognized as IB4+ (strongly positive, if intensity above 40% of maximum for the selected field of view, and weakly positive if intensity in the range 20–40% of maximum), whereas those having intensity below 20% of maximum were considered as IB4− (Fang et al., 2006; Khomula et al., 2013, 2017, 2019; Ferrari et al., 2016).

Data analysis

All data are presented as mean ± SEM of n independent observations. In all figures bars show mean and error bars show SEM. Statistical comparisons were made using GraphPad Prism 8.0 statistical software (GraphPad Software Inc); p < 0.05 was considered statistically significant.

In behavioral experiments, the dependent variable was mechanical paw-withdrawal threshold. Each data point was the mean of three readings. All magnitudes were expressed as percentage change from baseline. Average mechanical nociceptive threshold before systemic fentanyl was 143.4 ± 1.3 g and 4 d after was 142.8 ± 1.14 (n = 60 rats; t(59) = 0.48, p = 0.64, paired Student’s t test).

As described in detail in the figure legends, the following statistical tests were used: paired (Fig. 1) and unpaired (Fig. 5C) Student’s t test, two-way repeated-measures ANOVA followed by Bonferroni’s post hoc test (Figs. 1–3), one-way ANOVA followed by Tukey’s post hoc test (Figs. 6D, 7D, 8), two-way ANOVA followed by Holm–Sidák’s post hoc test (Figs. 5A, 6D, 6A–C, 7A–C).

Results

Systemic fentanyl induces Type I and Type II priming at the nociceptor peripheral terminal

We recently demonstrated the presence of OIH and OIHP, in vivo, at the nociceptor peripheral terminal after a single systemic (subcutaneous) injection of an analgesic dose of fentanyl (30 μg/kg; Khomula et al., 2019). We also demonstrated that OIH, induced by systemic fentanyl, is attenuated by administration of cordycepin at the peripheral terminal (Khomula et al., 2019), indicative of Type I priming (Ferrari et al., 2013). More recently, we identified a second type of hyperalgesic priming, referred to as Type II (Araldi et al., 2015, 2018c). In distinction to the maintenance mechanisms for Type I priming (Ferrari et al., 2013), Type II is dependent on the combined activation of Src and MAP kinases (Araldi et al., 2017a). While we recently demonstrated that OIH induced by systemic fentanyl is reversed by cordycepin (Khomula et al., 2019), the maintenance mechanism of OIH has not been tested. Therefore, we evaluated, using the same in vivo model, whether hyperalgesic priming in the peripheral terminal is Type I (reversed by cordycepin) or Type II (reversed by the combination of Src and MAPK inhibitors).

Rats received a systemic injection of fentanyl (30 μg/kg, s.c.) followed, 4 d later by intrathecal injection of vehicle (20 μl), cordycepin (4 μg/20 μl), the combination of Src (SU6656, 10 μg/10 μl) and MAPK (U0126, 10 μg/10 μl) inhibitors, or a combination of all three drugs [cordycepin (4 μg/6 μl), SU6656 (10 μg/6 μl), and U0126 (10 μg/6 μl)]. Ten minutes later, PGE2 (100 ng) was injected intradermally and mechanical nociceptive threshold evaluated a further 30 min and 4 h later. When measured 30 min after injection, PGE2-induced hyperalgesia in all groups. However, the prolongation of PGE2 hyperalgesia, measured at the fourth hour, was attenuated in the groups treated with cordycepin, SU6656 + U0126 or the combination of all three inhibitors (F(3,20) = 10.8, p = 0.0002, when the vehicle-treated and inhibitors-treated groups are compared at the fourth hour after intradermal PGE2 two-way ANOVA multiple comparisons test; Fig. 1A). To determine whether intrathecal administration of inhibitors was able to permanently attenuate priming induced by systemic fentanyl, the same groups of rats again received intradermal PGE2 (100 ng), 4 d (Fig. 1B), 14 d (Fig. 1C), and 28 d (Fig. 1D) after vehicle or inhibitors. In the groups treated with cordycepin, the combination of SU6656 + U0126, and the combination of all three inhibitors, the prolongation of PGE2-induced hyperalgesia was attenuated, at all time points evaluated [4 d, (Fig. 1B) F(3,20) = 23.6, p < 0.0001; 14 d (Fig. 1C) F(3,20) = 4.1, p = 0.02; and 28 d (Fig. 1D) F(3,20) = 7.9, p = 0.0011, when the vehicle-treated group is compared with all the inhibitors-treated groups at the fourth hour after intradermal PGE2 two-way ANOVA multiple comparisons test]. These results provide confirmation that the reversal effects of cordycepin, and the combination of Src and MAPK inhibitors persisted for at least 28 d. In contrast, in the vehicle-treated group, priming did not reveal any attenuation out to day 32 after induction, as demonstrated by unattenuated magnitude of PGE2-induced hyperalgesia at 4 h. Altogether, these findings indicate that in the peripheral terminal of the nociceptor, maintenance of OIHP produced by a single systemic administration of fentanyl (30 μg/kg), shares mechanisms with both Type I and Type II priming. Of note, the combination of all three inhibitors (cordycepin + SU6656 + U0126) did not produce additional attenuation of PGE2-induced hyperalgesia, at 4 h, in systemic fentanyl-primed rats, when it was compared with the cordycepin-treated and SU6656 + U0126-treated groups.

Priming induced by systemic fentanyl in the central terminal of the nociceptor is Type II

Systemic fentanyl at an analgesic dose (30 μg/kg) induces both Type I and Type II priming in the peripheral terminal of the nociceptor (Fig. 1). We next investigated the type of priming that systemic fentanyl induces in the central terminal. Rats received systemic fentanyl (30 μg/kg, s.c.) and 4 d later, vehicle (10 μl), cordycepin (4 μg/10 μl), or the combination of Src (SU6656, 10 μg/5 μl) and MAPK (U0126, 10 μg/5 μl) inhibitors was administered intrathecally, followed, 10 min later by PGE2 (400 ng/10 μl), injected at the same site. Mechanical nociceptive threshold was evaluated 30 min and 4 h after intrathecal PGE2. Measured 30 min after its administration, intrathecal PGE2 induced hyperalgesia in all groups. The prolongation of hyperalgesia induced by intrathecal PGE2 was markedly attenuated in the group treated with the combination of SU6656 + U0126, but not in the vehicle-treated and cordycepin-treated groups (F(2,15) = 49.9,
p < 0.0001, when the combination of SU6656 + U0126-treated group is compared with the vehicle-treated and the cordycepin-treated groups at the fourth hour after intrathecal PGE2; two-way ANOVA multiple comparisons test (Fig. 2A). To verify whether intrathecal treatment with inhibitors permanently reversed priming induced by systemic fentanyl in the central terminal of the nociceptor, PGE2 (400 ng) was again injected 4 d after intrathecal injection of vehicle, cordycepin, SU6656 + U0126, or the combination of all three inhibitors (Fig. 2B). 14 d (Fig. 2C), and 28 d (Fig. 2D) after vehicle and inhibitors. The prolongation of PGE2-induced hyperalgesia was still inhibited in the group treated with the combination of SU6656 + U0126 but not in the vehicle-treated and cordycepin-treated groups (F(3,25) = 41.6, p < 0.0001, when the combination of SU6656 + U0126-treated group was compared with the vehicle-treated and the cordycepin-treated groups at the fourth hour after intradermal PGE2; two-way repeated-measures ANOVA followed by Bonferroni post hoc test). These findings support the suggestion that systemic fentanyl induces both Type I and Type II priming, in the peripheral terminal of the nociceptor (n = 6 paws/6 rats per group).
after intrathecal PGE₂; two-way ANOVA multiple comparisons test; Fig. 2B). Since treatment with cordycepin did not affect the prolongation of PGE₂-induced hyperalgesia in systemic fentanyl-primed rats, this group was not tested at the follow-up time points. In the group of rats treated with the combination of SU6656 + U0126, the prolongation of PGE₂-induced hyperalgesia was still inhibited at days 14 and 28 after its administration [14d (Fig. 2C) $F_{(1,10)} = 56.4$, $p < 0.0001$; and 28 d (Fig. 2D) $F_{(1,10)} = 14.3$, $p = 0.0036$, when the vehicle-treated group is compared with the combination of SU6656 + U0126-treated group.
Mechanical nociceptive threshold was not different from pre-fentanyl baseline in all saporin-treated groups, measured 30 min after its administration. However, the prolongation of PGE2-induced hyperalgesia at the fourth hour was markedly attenuated in the group treated with SSP-saporin, but not in the saporin-control-treated and IB4-saporin-treated groups.

Support the suggestion that at the central terminal, systemic fentanyl requires IB4 peptidergic nociceptors to develop priming. Four days later, at which time mechanical nociceptive threshold was not different from pre-fentanyl baseline, PGE2 was injected intradermally and the mechanical nociceptive threshold evaluated 30 min and 4 h after its injection. Prolongation of PGE2-induced hyperalgesia was present in all saporin-treated groups measured 30 min after its injection. Also, prolonged hyperalgesia induced by intradermal PGE2 was present in all groups treated with saporin (F(2,15) = 0.69, p = 0.52, when all saporin-treated groups are compared at the fourth hour after intradermal PGE2; two-way repeated-measures ANOVA followed by Bonferroni post hoc test), indicating that in the peripheral terminal of the nociceptor systemic fentanyl does not require IB4+ and SP peptidergic nociceptors to develop priming.

Seven days after systemic fentanyl and 3 d after intradural PGE2, rats were tested for priming in the central terminal of the nociceptor. PGE2 (400 ng/20 μl) was injected intrathecally and mechanical nociceptive threshold evaluated 30 min and 4 h later. Intrathecal PGE2-induced hyperalgesia was present in all saporin-treated groups, measured 30 min after its administration. However, the prolongation of PGE2-induced hyperalgesia at the fourth hour was markedly attenuated in the group treated with SSP-saporin, but not in the saporin-control-treated and IB4-saporin-treated groups (F(2,15) = 16.0, ***p = 0.0002, when the SSP-saporin-treated group is compared with the saporin (control)-treated and the IB4-saporin-treated groups at the fourth hour after intrathecal PGE2; two-way repeated-measures ANOVA followed by Bonferroni post hoc test). These findings support the suggestion that at the central terminal, systemic fentanyl requires IB4+ peptidergic nociceptors to induce priming (n = 6 paws/6 rats per group).

Table 1. Effect of PGE2 on small DRG neurons of different IB4-binding classes from control animals

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<th></th>
<th>Strongly IB4+</th>
<th>Weakly IB4+</th>
<th>IB4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells tested with PGE2</td>
<td>10</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>% of neurons responsive to PGE2 [number of responsive cells]</td>
<td>60% (6 cells)</td>
<td>71% (10 cells)</td>
<td>67% (6 cells)</td>
</tr>
<tr>
<td>% reduction in rheobase induced by 10 nm PGE2</td>
<td>13 ± 5</td>
<td>12 ± 4</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>% reduction in rheobase induced by 100 nm PGE2</td>
<td>23 ± 3</td>
<td>24 ± 4</td>
<td>27 ± 7</td>
</tr>
<tr>
<td>Baseline rheobase, pA</td>
<td>384 ± 56</td>
<td>313 ± 45</td>
<td>294 ± 47</td>
</tr>
<tr>
<td>(including neurons not tested with PGE2)</td>
<td>(n = 20)</td>
<td>(n = 23)</td>
<td>(n = 20)</td>
</tr>
</tbody>
</table>

at the fourth hour after intrathecal PGE2; two-way ANOVA multiple comparisons test). These data support the suggestion that systemic fentanyl induces Type II but not Type I priming in nociceptor central terminals, which is in accordance with our previous findings (Araldi et al., 2018b), while Type I priming only occurs at the peripheral terminal.

Involvement of peptidergic nociceptors in priming at the central terminal

Maintenance of Type I and Type II priming can occur in different classes of nociceptors. For example, Type I, induced by inflammatory mediators, occurs in IB4+ nonpeptidergic nociceptors (Joseph and Levine, 2010), while Type II priming, induced by intradermal DAMGO, was prevented by intrathecal administration of SSP-saporin (Araldi et al., 2018b), known to disrupt signaling pathway of peptidergic (mostly IB4+) nociceptors (Seki et al., 2005). In the present experiments, we evaluated the role of these two nociceptor populations in priming induced by systemic fentanyl, in the peripheral and central nociceptor terminals. Saporin-control, IB4-saporin [destroys IB4+ (mostly nonpeptidergic) nociceptors], or SSP-saporin was injected intrathecally and, 14 d later, systemic fentanyl (30 μg/kg) was administered subcutaneously. Four days later, PGE2 was injected intradermally and the mechanical nociceptive threshold evaluated 30 min and 4 h after its injection. Prolongation of PGE2-induced hyperalgesia was present in all saporin-treated groups (F(2,15) = 0.69, p = 0.52, when the control (saporin), the SSP-saporin-treated and the IB4-saporin-treated groups are compared at the fourth hour after intradermal PGE2; two-way repeated-measures ANOVA multiple comparisons test]. These data support the suggestion that at the central terminal, systemic fentanyl requires IB4+ peptidergic nociceptors to induce priming (n = 6 paws/6 rats per group).
ANOVA; Fig. 3A], indicating that fentanyl-induced priming in the nociceptor peripheral terminal is not dependent on SP peptidergic or IB4+ nociceptors, compatible with the role of a different class of nociceptors (Cavanaugh et al., 2011; Araldi et al., 2018c). Seven days after systemic fentanyl and 3 d after intradermal PGE2, priming was tested in the central terminal. Rats received PGE2 (400 ng) intrathecally and mechanical nociceptive threshold was evaluated 30 min and 4 h later. In the group treated with SSP-saporin, but not...
the fourth hour after intrathecal PGE2; two-way repeated-measures ANOVA; Fig. 3B), indicating that priming induced by systemic fentanyl, in the central terminal of the nociceptor, is dependent on peptidergic nociceptors. These data support the suggestion that more than one population of nociceptors is involved in OIH induced by systemic fentanyl.

Enhanced PGE2-induced sensitization of sensory neurons cultured from fentanyl-primed rats
To further understand the populations of nociceptors in which priming occurs, we translated our in vivo to an in vitro priming model by examining the effect of systemic fentanyl on PGE2-induced sensitization of cultured DRG neurons. Since it is not practical to hold cells clamped for 4 h, to reveal priming, we relied on another in vivo feature of hyperalgesia, a more than one order magnitude leftward shift (to lower values) for the dose dependence of PGE2-induced hyperalgesia (Parada et al., 2005). Since only ~60% of cultured DRG neurons are responsive to PGE2 (Gold et al., 1996b), it would not be possible to distinguish between responsive and nonresponsive neurons by their response to a low concentration of PGE2; response to a higher concentration is required. Therefore, we chose two PGE2 concentrations, 10 and 100 nM, to examine our hypothesis that priming induces a leftward shift of the dose-response curve in primed DRG neurons.

In cultures of DRG neurons derived from opioid naive animals, corresponding magnitudes of sensitization were 11 ± 2% (n = 22) for 10 nM PGE2 and 24 ± 2% (n = 17) for 100 nM PGE2; 67% of small DRG neurons (soma diameter <30 μm; see Materials and Methods) examined (22 out of 33) were responsive to PGE2. Average baseline rheobase of examined neurons was 310 ± 41 pA (n = 33). In DRG neurons from control animals, no significant difference was found between mean rheobase values of small DRG neurons responsive (314 ± 55 pA) and nonresponsive (282 ± 81 pA) to PGE2 (unpaired t test, t23 = 0.3). Considering that DRG neurons in different IB4-binding classes have different electrophysiological properties (Stucky and Lewin, 1999) and are differentially

Figure 5. Fentanyl-induced priming in vivo is associated with enhanced PGE2-induced sensitization of small DRG neurons, in vitro. Rats were primed by the systemic administration of fentanyl (30 μg/kg, s.c.) 8 d before preparing neuronal cultures, to parallel our in vivo protocol. 4 d before injection of fentanyl and 4 d after administering of vehicle or reversal agents. Patch-clamp electrophysiology recordings were made in small-diameter DRG neurons from fentanyl-primed and control (opioid naive) groups (depicted in all panels by the white and dark blue bars, correspondingly), after 24 h in culture. A–C: bars show pooled magnitudes of decrease in rheobase, relative to preadministration baseline (measured before the first application of PGE2), and 5 min after application (10 and 100 nM); 10 nM PGE2 was applied first, and then, 10 min later, its concentration was increased to 100 nM (cumulative concentration dependence). Symbols show individual values. Only those neurons with a change in rheobase of net <10%, for the higher or lower concentration, were considered for analysis. Some neurons were not tested with the higher concentration because of loss of the patch (but only used if they showed at least 10% reduction in rheobase for the lower concentration), therefore, regular ANOVA was used for analysis. A. Reduction of rheobase in response to 10 and 100 nM PGE2, analyzed regardless of IB4-binding status. In DRG neurons from primed rats the effect of both concentrations of PGE2 was significantly greater than in controls [two-way ANOVA; effect of PGE2, concentration, p = 0.0015; F(2,35) = 10.8; not significant (ns) interaction; p = 0.32, F(1,35) = 1.0; Holm–Sidak’s post hoc test: t23 = 3.3, **adjusted p < 0.001 for 10 nM; t23 = 3.6, ***adjusted p = 0.0007 for 100 nM]. Number of cells in control group: n = 22 for 10 nM and n = 17 for 100 nM; in primed group: n = 19 for both 10 and 100 nM. B. Reduction of rheobase in response to PGE2 (10 nM), in neurons separated by IB4-binding intensity into: strongly IB4+ (‘strong’), weakly IB4+ (‘weak’), and IB4– classes (‘neg’). Effect of priming was statistically significant [two-way ANOVA; effect of condition F(1,31) = 23.0, p < 0.0001; effect of IB4-binding status F(2,31) = 1.1, p = 0.34; interaction F(2,31) = 1.2, p = 0.33]. Holm–Sidak’s post hoc test revealed a statistically significant increase in the effect of PGE2, in weakly IB4+ (F(1,31) = 4.1, ***adjusted p = 0.0008) and IB4– (F(1,31) = 2.9, *adjusted p = 0.011) classes, indicating a leftward shift in the concentration-response curve for PGE2 in these neurons. In strongly IB4+ neurons, the effect of priming was not statistically significant (F(1,31) = 1.5, adjusted p = 0.14). Number of cells (strong/weak/neg) in control group: 6/10/6, in primed group: 6/7/6. C. Reduction of rheobase in response to 100 nM PGE2, in strongly IB4+ neurons from the primed group of rats was significantly enhanced compared with the control group (n = 6 per group; two-tailed unequal Student’s t test: t6 = 3.6, *p = 0.005), indicating a different change in this neuronal population associated with in vivo priming. D. Baseline rheobase (before administration of PGE2) in three neuronal classes with different IB4-binding status. PGE2-sensitive and PGE2-insensitive neurons were included in the analysis of baseline rheobase. Differences between primed and control groups as well as between classes were not statistically significant [two-way ANOVA: effect of IB4-binding status, not significant (ns), p = 0.92, F(2,31) = 0.40; effect of condition: ns, p = 0.68, F(2,31) = 0.17; interaction: ns, p = 0.53, F(2,31) = 0.64]. Number of cells (strong/weak/neg) in control group: 20/21/20, in primed group: 18/24/18.

the saporin (control)-treated and IB4-saporin-treated groups, prolongation of PGE2-induced hyperalgesia was markedly attenuated [F(2,31) = 16.0, p = 0.0002, when the saporin (control), the SSP-saporin-treated and the IB4-saporin-treated groups are compared at
affected by exposure to fentanyl (Khomula et al., 2019), we analyzed their response to PGE2 separately (Table 1). Fractions of neurons responsive to PGE2 and magnitude of sensitization by 10 nM PGE2 were similar under control conditions; ANOVA did not reveal a significant difference between baseline rheobase ($p = 0.42, F_{(2,60)} = 0.89$).

In cultured small-diameter DRG neurons derived from animals primed with fentanyl in vivo (primed group) reduction of rheobase induced by both concentrations of PGE2 (10 and 100 nM) was significantly greater than in neurons from control animals (two-way ANOVA: effect of condition: $p < 0.0001, F_{(1,73)} = 38.2$; Holm–Sidak's post hoc: $t_{(73)} = 5.3$, adjusted $p < 0.0001$ for 10 nM; $t_{(73)} = 3.6$, adjusted $p = 0.0007$ for 100 nM; Figs. 4A,B, 5A). And, when neurons were separated according to their IB4-binding status, and analyzed, the effect of 10 nM PGE2 (Fig. 5B), our primary endpoint, a significant effect of priming was confirmed (two-way ANOVA: effect of condition $F_{(1,35)} = 23.0, p < 0.0001$). Holm–Sidak's post hoc test revealed a significant increase in the effect of PGE2 in weakly IB4+ ($t_{(35)} = 4.1$, adjusted $p = 0.0008$) and IB4− ($t_{(35)} = 2.9$, adjusted $p = 0.011$) classes. In strongly IB4+ neurons, the difference between primed and control groups was not statistically significant for 10 nM PGE2 ($t_{(35)} = 1.5$, adjusted $p = 0.14$). However, for these neurons we found a significant increase in sensitization by the higher concentration (100 nM) of PGE2 (two-tailed unpaired Student's $t$ test: $t_{(10)} = 3.6, p = 0.005$; Fig. 5C).

Of note, we did not see a significant effect of priming on rheobase of neurons of any IB4-binding status (two-way ANOVA: effect of IB4-binding status, not significant (ns), $p = 0.92, F_{(2,117)} = 0.40$; effect of condition: ns, $p = 0.68, F_{(1,117)} = 0.17$; interaction: ns, $p = 0.53, F_{(2,117)} = 0.64$; Fig. 5D), or when the three classes were combined (control vs priming, two-tailed unpaired Student's $t$ test: $t_{(122)} = 0.34, p = 0.73$). This is in agreement with the results of our current (Fig. 1) and previous (Ferrari et al., 2013; Hendrich et al., 2013; Araldi et al., 2015, 2018c; Khomula et al., 2019) behavioral studies, which demonstrated no effect of priming on baseline mechanical nociceptive threshold.

These findings confirm our initial hypothesis that priming-like neuroplasticity could be observed in vitro, in nociceptors cultured from primed rats, and identify nociceptor populations in which this occurs. While all three nociceptor populations were affected by fentanyl, leftward shift in dose dependence of PGE2-induced sensitization was only observed in weakly IB4+ and IB4− neurons.

![Figure 6. Effect of in vivo treatment of fentanyl-primed rats with the reversal agent for Type I priming on PGE2-induced sensitization of small DRG neurons. Rats were primed by the systemic administration of fentanyl (30 µg/kg, s.c.) 8 d before preparing neuronal cultures (4 d before intrathecal cordycine, followed by an additional 4 d before culture preparation). Recordings were made in small-diameter DRG neurons from fentanyl-primed animals either treated (reversal group) or not treated (primed group) with cordycine (depicted in A–C by the blue and dark blue bars, correspondingly) after 24 h in culture. In A–D, bars show pooled magnitudes of decrease in rheobase, relative to baseline, after PGE2 application (10 and 100 nM), measured and analyzed in the same way as described in Figure 5 and Materials and Methods. Symbols show individual values. In A–C, values for primed group were repeated from Figure 5A–C for the purpose of comparison. A, Reduction of rheobase in response to 10 and 100 nM PGE2, analyzed regardless of IB4-binding status. In neurons from primed rats the effect of 10 nM but not 100 nM PGE2 was significantly greater than in the reversal group (two-way ANOVA: effect of condition: $F_{(1,168)} = 12.5, p = 0.0007$; Holm–Sidak's post hoc: $t_{(30)} = 3.2$, **adjusted $p = 0.008$ for 10 nM; $t_{(35)} = 1.8$, adjusted $p = 0.07$, not significant (ns), for 100 nM). Number of cells in primed group: $n = 19$ for both 10 and 100 nM in reversal group; $n = 19$ for 10 nM and $n = 15$ for 100 nM. B, Reduction of rheobase in response to 100 nM PGE2 in weakly IB4+ ("weak") and IB4− ("neg") neurons. Attenuation of PGE2-induced sensitization after in vivo cordycine was statistically significant in both neuronal populations (two-way ANOVA: effect of condition $F_{(1,122)} = 16.3, p = 0.0006$; Holm–Sidak's post hoc: $t_{(20)} = 3.2$, **adjusted $p = 0.008$ for weakly IB4+; $t_{(25)} = 2.5$, #adjusted $p = 0.02$ for IB4−). Number of cells (weak/neg) in reversal group: 6/6, in primed group: 7/6. C, Reduction of rheobase in response to 100 nM PGE2 in strongly IB4+ ("strong") and merged weakly IB4+ and IB4− ("weak and neg") neurons from primed and reversal groups. Two-way ANOVA revealed statistically significant interaction ($F_{(1,38)} = 8.5, p = 0.007$), indicating differential effects on the three different neuronal classes. Indeed, statistically significant attenuation in reversal compared with primed group occurred in weakly IB4+ and IB4− but not in strongly IB4+ neurons (Holm–Sidak's post hoc: $t_{(20)} = 0.9$, adjusted $p = 0.38$ for strong, not significant (ns); $t_{(25)} = 3.7$, **adjusted $p = 0.002$ for weak and neg). Number of cells (strong/weak and neg): 6/13 in primed group, 6/9 in reversal group. D, Reduction of rheobase in response to 100 nM PGE2 in strongly IB4+ ("strong"), weakly IB4+ ("weak"), and IB4− ("neg") neurons from reversal group. Values in strongly IB4+ class were significantly greater than in weakly IB4+ and IB4− neurons (one-way ANOVA: $F_{(2,120)} = 11.8$, ***$p = 0.0002$; Tukey's post hoc: $q_{(12)} = 6.3$, adjusted $p = 0.002$ for strongly IB4+ vs weakly IB4+; $q_{(12)} = 5.2$, adjusted $p = 0.008$ for strongly IB4+ vs IB4−; $q_{(12)} = 1.3$, adjusted $p = 0.63$, not significant (ns), for weakly IB4+ vs IB4−). Number of cells: 6 strong, 4 weak, 5 neg.)
Intrathoracic cordycepin attenuates priming-induced enhancement of PGE2 sensitization of nociceptors, in vitro

Using our in vitro model of fentanyl-induced priming, we determined whether administration of cordycepin, which partially reversed priming in vivo, has a similar effect on priming-like neuroplasticity in cultured DRG neurons. We prepared cultures of DRG neurons derived from animals that were primed in vivo with fentanyl and 4 d later received intrathoracic cordycepin (cordycepin group), used in our behavior experiments to attenuate Type I priming (Fig. 1). DRG neuron cultures were prepared 4 d after administering cordycepin, a time at which attenuation of priming was observed in behavioral experiments (Fig. 1). Small-diameter DRG neurons cultured from the cordycepin-treated group received PGE2 (10 nm) and then, 10 min later, PGE2 100 nm, and magnitude of the reduction in rheobase induced was compared with that in DRG neurons from primed rats (Figs. 4B, C, D).

First, we compared the effects of PGE2, 10 and 100 nm, on small DRG neurons, regardless of IB4-binding status (Fig. 6A). We found a significant attenuation of the sensitizing effect of PGE2 in the cordycepin-treated group compared with the primed group, for 10 nm but not for 100 nm PGE2 (two-way ANOVA; effect of condition: F(1,68) = 12.5, p = 0.0007; Holm–Sidak’s post hoc: t(68) = 3.2, adjusted p = 0.04 for 10 nm; t(68) = 1.8, adjusted p = 0.07, for 100 nm). This finding supports the suggestion that in vivo administration of cordycepin attenuates priming in nociceptors, in vitro, similar to our in vivo observation.

To assess possible IB4-binding status-specific changes induced by in vivo administration of cordycepin, we took into account our finding of an enhanced effect of 10 nm PGE2 on weakly IB4+ and IB4− populations, and the enhanced effect of only 100 nm PGE2 on strongly IB4+ neurons. We found significant attenuation of the sensitizing effect of 10 nm PGE2 in weakly IB4+ and IB4− neurons after cordycepin was administered in vivo to primed animals (two-way ANOVA; effect of condition: F(1,21) = 16.3, p = 0.0006; Holm–Sidak’s post hoc: t(21) = 3.2, adjusted p = 0.008 for weakly IB4+; t(21) = 2.5, adjusted p = 0.02 for IB4−; Fig. 6B). In contrast, the enhanced sensitizing effect of 100 nm PGE2 in populations of putative nociceptors. However, enhanced sensitization by the high dose of PGE2 was found in strongly IB4+ nociceptors. Together, our behavioral and in vitro findings support the suggestion that fentanyl-induced priming develops in the nociceptor.
strongly IB4+ neurons was not significantly different between neurons from primed and cordycepin-treated primed rats (two-way ANOVA: statistically significant effect of interaction: $F_{(1,30)} = 8.5, p = 0.007$; Holm–Sidak’s post hoc: $t_{(30)} = 0.9$, adjusted $p = 0.38$ for strongly IB4+; $t_{(30)} = 3.7$, adjusted $p = 0.002$ for weakly IB4+ and IB4− combined; Fig. 6C). In strongly IB4+ neurons, from cordycepin treated rats, the sensitizing effect of 100 nM PGE2 was significantly greater than in weakly IB4+ and IB4− neurons (one-way ANOVA: $F_{(1,12)} = 11.8, p = 0.002$; Tukey’s post hoc: $q_{(12)} = 6.3$, adjusted $p = 0.002$ for strongly IB4+ vs weakly IB4+; $q_{(12)} = 5.2$, adjusted $p = 0.008$ for strongly IB4+ vs IB4−; $q_{(12)} = 1.3$, adjusted $p = 0.63$ for weakly IB4+ vs IB4−; Fig. 6D). These results support the suggestion that maintenance mechanisms of enhanced sensitization by PGE2 are dependent on Type I priming in weakly IB4+ and IB4− nociceptor populations.

Intrathecal Src plus MAPK inhibitors attenuates priming-induced enhancement of PGE2 sensitization of nociceptors, in vitro

Using our in vitro model of fentanyl-induced priming, we next determined whether in vivo administration of agents that reverse Type II priming (Fig. 1) also attenuates enhanced PGE2-induced sensitization in DRG neurons cultured from fentanyl-treated animals, and which nociceptor populations were affected. Animals were primed with fentanyl, in vivo, and 4 d later received the combination of Src and MAPK inhibitors (intrathecally; Fig. 1A). DRGs were harvested for culturing 4 d later. Reduction of rheobase induced by 10 and 100 nM PGE2 in small-diameter DRG neurons from these cultures were compared with corresponding values in cultures from the primed group of animals (Figs. 4B,D, 7). We found a significant attenuation of the sensitizing effect of PGE2 in this reversal group compared with the primed group, when neurons were analyzed regardless of IB4-binding status (two-way ANOVA; effect of condition: $F_{(1,196)} = 16.6, p = 0.0001$; Holm–Sidak’s post hoc: $t_{(70)} = 3.3$, adjusted $p = 0.003$ for 10 nM; $t_{(70)} = 2.5$, adjusted $p = 0.02$ for 100 nM; Fig. 7A). When IB4-binding status was considered, we found significant attenuation of the sensitizing effect of PGE2 (10 nM), in weakly IB4+ and IB4− neurons (two-way ANOVA; effect of condition: $F_{(2,21)} = 15.5, p = 0.0008$; Holm–Sidak’s post hoc: $t_{(12)} = 3.4$, adjusted $p = 0.005$ for weakly IB4+; $t_{(21)} = 2.2$, adjusted $p = 0.04$ for IB4−; Fig. 7B). The sensitizing effect of PGE2 (100 nM) in strongly IB4+ neurons was not significantly different between reversal and primed groups (two-way ANOVA; statistically significant effect of interaction: $F_{(1,33)} = 8.8, p = 0.006$; Holm–Sidak’s post hoc: $t_{(21)} = 0.74$, adjusted $p = 0.46$ for strongly IB4+; $t_{(33)} = 4.1$, adjusted $p = 0.0005$ for weakly IB4+ and IB4− combined; Fig. 7C). However, the sensitizing effect of 100 nM PGE2 in strongly IB4+ neurons was significantly greater than in weakly IB4+ and IB4− neurons (one-way ANOVA: $F_{(1,15)} = 11.2, p = 0.0010$; Tukey’s post hoc: $q_{(15)} = 4.8$, adjusted $p = 0.011$ for strongly IB4+ vs weakly IB4+; $q_{(15)} = 6.5$, adjusted $p = 0.010$ for strongly IB4+ vs IB4−; $q_{(15)} = 1.7$, adjusted $p = 0.48$ for weakly IB4+ vs IB4−; Fig. 7D). These results, along with the results of the previous section (Fig. 6), confirm a significant functional difference between strongly IB4+ nociceptors versus weakly IB4+ and IB4− nociceptors in how they were affected by systemic fentanyl, in vivo. While in both reversal groups, PGE2-induced sensitization in strongly IB4+ neurons remained unattenuated, compared with the primed group, in weakly IB4+ and IB4− neurons, it was significantly attenuated. Overall, the in vitro effect of combined in vivo inhibition of Src and MAPK on all three classes was qualitatively similar to that found for in vivo cordycepin (Fig. 6). Of note, enhanced sensitization by PGE2 in both weakly IB4+ and IB4− nociceptor populations shared maintenance mechanisms with both Type I and Type II priming.

In summary, we have demonstrated, in vivo, the existence of long-lasting nociceptor neuroplasticity, induced by systemic administration of an analgesic dose of fentanyl. This neuroplasticity, which involves changes in nociceptor function, is responsible for both hyperalgesic priming and, as previously shown, for OIH (Khomula et al., 2017). Here, for the first time, we have created an in vitro model for studying OIH. Using this model, we provide evidence for specific changes in electrophysiological properties of nociceptors, in their response to PGE2, which correlates with the presence of hyperalgesic priming in vivo and reversal (partial permanent attenuation) by cordycepin or the combination of Src and MAPK inhibitors. The combination of behavioral and in vitro cellular approaches allowed us to provide evidence of different roles for classes of nociceptors in hyperalgesic priming induced in vivo by systemic fentanyl. Additionally, our in vitro results identified a novel form of neuroplasticity in strongly IB4+ nociceptors, characterized by increased change in rheobase induced by the high but not low concentration of PGE2, which is not reversed by either cordycepin or the combination of Src and MAPK inhibitors. Studies are ongoing to elucidate the in vivo correlate of this novel form of nociceptor neuroplasticity. We suggest that the residual PGE2-induced hyperalgesia (~50%), which was still present at the peripheral nociceptor terminal at the fourth hour, in rats treated with inhibitors of both Type I and Type II priming (Fig. 1), is mediated by neuroplasticity in strongly IB4+ nociceptors.

Discussion

Opioid analgesics, especially of the fentanyl class, can produce a hypersensitivity to noxious stimuli (OIH) and long-lasting pain.
exacerbation (chronification; Zylicz and Twycross, 2008; Silverman, 2009; Yi and Przybylkowski, 2015). Since the action of MOR agonists on nociceptors plays an important role in their pronociceptive effects (Scherrer et al., 2009; Corder et al., 2017; Streicher and Bilsky, 2017), we focused on cellular mechanisms of OIH, using in vitro patch-clamp electrophysiology.

Three forms of OIH have been reported. Type I is reversed by cordycepin (Ferrari et al., 2013, 2019; Araldi et al., 2018c), Type II by the combination of a Src and MAPK inhibitor (Araldi et al., 2017a, 2018c), and a third type, produced by biased MOR agonists, is neither Type I nor Type II (Araldi et al., 2018a). In the case of fentanyl, different nociceptor populations and distinct intracellular mechanisms are involved in OIH induced in the setting of different routes of administration and doses (i.e., subanalgesic and analgesic; Araldi et al., 2018c). Our present in vivo findings show that an analgesic dose of fentanyl induces priming in the peripheral terminal of the nociceptor, sharing maintenance mechanisms with both Type I and Type II priming.

Consistent with our in vivo findings, we observed enhanced PGE$_2$-induced sensitization (reduction of rheobase) in small-diameter DRG neurons cultured from fentanyl-primed rats, confirming our hypothesis that priming-like neuroplasticity can be observed in vitro. Enhanced sensitization by the lower concentration of PGE$_2$, which was our primary endpoint, was only observed in weakly IB4$^+$ and IB4$^-$ neurons. In contrast, in strongly IB4$^+$ neurons we found a significant increase in sensitization by the higher but not the lower concentration of PGE$_2$. Of note, we did not observe an effect of priming on pre-PGE$_2$ rheobase, paralleling the lack of change in baseline nociceptive threshold in primed animals, in behavioral studies (Parada et al., 2005; Araldi et al., 2018c; Ferrari et al., 2019). Our finding of priming-like neuroplasticity in cultured DRG neurons, which have lost their terminals in peripheral tissues and spinal dorsal horn, during culture preparation, support the suggestion that the priming state in nociceptors also exists in the soma.

A significant attenuation of the enhanced sensitizing effect of the low concentration (10 nM) of PGE$_2$ was observed in weakly IB4$^+$ and IB4$^-$ DRG neurons (both peptidergic) cultured from rats whose fentanyl-induced priming was reversed in vivo by agents that reverse Type I (cordycepin) or Type II (the combination of a Src and a MAPK inhibitors) priming. These findings support the suggestion that both Type I and Type II priming develop, in the same or different nociceptor populations, identified in vitro as weakly IB4$^+$ and IB4$^-$ (peptidergic) nociceptors. However, underlying intracellular mechanisms, allowing coexistence of both Type I and Type II priming in the same neuron, still need to be investigated. One might suggest a convergent signaling event, downstream, affected by Type I and Type II reversal agents. For instance, protein translation in DRG neurons requires ERK signaling, and Src activation and PKA/PKC activation may also lead to ERK activation (Hu and Gereau, 2003; Kawasaki et al., 2004; Uttam et al., 2018), supporting the suggestion that Type II reversal agents might also inhibit protein translation (required for Type I priming) downstream of ERK signaling. However, Type II priming at the central terminal was not sensitive to cordycepin (Fig. 2; Araldi et al., 2018c). Similarly, Type I priming is insensitive to agents that reverse Type II priming (Araldi et al., 2018c; Ferrari et al., 2019). Together, these findings support the suggestion that there is no overlap between mechanisms of Type I and Type II priming.

Alternatively, it is possible that different neuronal subpopulations, within weakly IB4$^+$ and IB4$^-$ peptidergic nociceptors mediate Type I and Type II priming. In this case, however, one would expect an additive attenuating effect of reversal agents of Type I and Type II priming in vivo (as they would act on different neurons), which we did not observe (Fig. 1). The prominent attenuating effect of SSP-saporin on fentanyl-induced priming, only at the nociceptor central terminal (Fig. 3), indicates that different populations of nociceptors mediate priming, and in particular Type II priming, at the central and peripheral terminal. The fact that OIH, in the nociceptor peripheral terminal, was not affected by either IB4-saporin or SSP-saporin treatment could be attributed to the involvement of a different class of nociceptors, negative for both nonpeptidergic and peptidergic markers, as suggested in our previous study in which intrathecal fentanyl-induced priming in the nociceptor peripheral terminal was not prevented by treatment with IB4-saporin or SSP-saporin (Araldi et al., 2018c). Of note, nonpeptidergic IB4$^-$ neurons account for ~20% of TRPV1$^+$ neurons, as shown in mice (Cavanaugh et al., 2011).

The enhanced sensitizing effect of the higher concentration of PGE$_2$, in strongly IB4$^+$ (non-peptidergic) neurons was not attenuated by drugs that reverse Type I or Type II priming, identifying a novel form of neuroplasticity in these nociceptors. It is possible that neuroplasticity in this class of nociceptors contributes to the remaining PGE$_2$-induced hyperalgesia, at the fourth hour, in rats treated with a combination of drugs that reverse both Type I and Type II priming that did not attenuate priming any more than inhibition of either Type I or Type II priming alone.

Although we use only mechanical stimulation to reveal enhanced and prolonged hyperalgesia, we do not expect that priming has selectivity to stimulus modality. Indeed, our in vitro findings suggest that priming is associated with enhanced reduction of threshold for electrical excitability in response to PGE$_2$. Thus, any depolarizing stimulus capable of activating a primed nociceptor would require lower stimulus magnitude to trigger AP generation after sensitization.

Observed changes in rheobase implicate changes in ion channel function. It is very likely that the function of channels mediating the effects of PGE$_2$ are affected, including voltage-gated sodium, potassium and calcium channels (Gold et al., 1996a, 1998; Kopp and Cichá, 1999; Gu et al., 2008; Fan et al., 2011; for review, see Kawabata, 2011). We do not, however, think that priming directly affects properties of ion channels involved in AP generation, rather it alters the PGE$_2$ signaling pathway (Aley et al., 2000; Khasar et al., 2008; for review, see Reichling and Levine, 2009; Reichling et al., 2013).

Once sensitized, any part of a primed nociceptor may contribute to a prolongation of hyperalgesia and in this way contribute to pain chronification. For instance, tissue damage, like a surgical incision or wound, is associated with production of sensitizing agents, resulting in hyperalgesia. In the case of priming, when nociceptors remain sensitized for a much longer time after administration of sensitizing agents, pain would be expected to be prolonged as well. Increased and prolonged nociceptor activity in the presence of a stimulus that leads to activation of the nociceptor likely will facilitate central sensitization. Of note, since priming by itself does not alter pain sensitivity, it is unlikely to lead to central sensitization.

Along with central sensitization, hyperalgesic priming is recognized as an integral part of the “pain memory” paradigm (Ji et al., 2003; Price and Inyang, 2015) and serves to illuminate a critical role of the nociceptor in pain chronification. Studies have
demonstrated the progressive nature of chronic pain (Price and Inyang, 2015), as well as OIH, which has been tightly linked to OIH in our studies (Khomula et al., 2019). Use of opioids for analgesia in a clinical setting, from the perspective of our OIH model may be viewed as the triggering event for induction of a pain memory, thus providing important clinical parallels that can help explain mechanisms and suggest potential pathways to resolution of neuroplasticity that drives chronicity.

In conclusion, our in vitro results indicate that fentanyl induces neuroplasticity in nociceptors of all three IB4-binding categories; neuroplasticity in weakly IB4+ and IB4− (peptidergic) nociceptors likely contributes to Type I and Type II OIH in vivo, which differs from our previous finding for OIH induced by fentanyl, where only a role of weakly IB4+ nociceptors was observed (Khomula et al., 2019). Of note, strongly IB4+ nociceptors, which develop a novel type of neuroplasticity in fentanyl-primed animals, also revealed robust fentanyl-induced sensitization in cultures from opioid naïve animals, which does not account for fentanyl induced OIH, since it did not become greater in cultures from primed animals. In contrast, weakly IB4+ and IB4− nociceptors were sensitized by fentanyl in cultures from in vivo primed animals, sensitization that was reversed by cordycepin, but only in weakly IB4+ neurons (Khomula et al., 2019). On the other hand, their enhanced PGE2-induced sensitization, attributed to priming, was attenuated by cordycepin and the combination of Src and MAPK inhibitors, in both peptidergic classes; they also reveal differences between neuroplasticity in weakly IB4+ and IB4− nociceptors, dissected by the effect of intra-thecal cordycepin on their sensitization by fentanyl, in vitro.

References


