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Chronic Morphine-Induced Changes in Signaling at the A_3 Adenosine Receptor Contribute to Morphine-Induced Hyperalgesia, Tolerance, and Withdrawal[S]

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ABSTRACT

Treating chronic pain by using opioids, such as morphine, is hampered by the development of opioid-induced hyperalgesia (OIH; increased pain sensitivity), antinociceptive tolerance, and withdrawal, which can contribute to dependence and abuse. In the central nervous system, the purine nucleoside adenosine has been implicated in beneficial and detrimental actions of morphine, but the extent of their interaction remains poorly understood. Here, we demonstrate that morphine-induced OIH and antinociceptive tolerance in rats is associated with a twofold increase in adenosine kinase (ADK) expression in the dorsal horn of the spinal cord. Blocking ADK activity in the spinal cord provided greater than 90% attenuation of OIH and antinociceptive tolerance through A_3 adenosine receptor (A_3AR) signaling. Supplementing adenosine signaling with selective A_3AR agonists blocked OIH and antinociceptive tolerance in rodents of both sexes. Engagement of A_3AR in the spinal cord with an ADK inhibitor or A_3AR agonist was associated with reduced dorsal horn of the spinal cord expression of the NOD-like receptor pyrin domain-containing 3 (60%–75%), cleaved caspase 1 (40%–60%), interleukin (IL)-1β (76%–80%), and tumor necrosis factor (50%–60%). In contrast, the neuroinhibitory and anti-inflammatory cytokine IL-10 increased twofold. In mice, A_3AR agonists prevented the development of tolerance in a model of neuropathic pain and reduced naloxone-dependent withdrawal behaviors by greater than 50%. These findings suggest A_3AR-dependent adenosine signaling is compromised during sustained morphine to allow the development of morphine-induced adverse effects. These findings raise the intriguing possibility that A_3AR agonists may be useful adjunct to opioids to manage their unwanted effects.

SIGNIFICANCE STATEMENT

The development of hyperalgesia and antinociceptive tolerance during prolonged opioid use are noteworthy opioid-induced adverse effects that reduce opioid efficacy for treating chronic pain and increase the risk of dependence and abuse. We report that in rodents, these adverse effects are due to reduced adenosine signaling at the A_3AR, resulting in NOD-like receptor pyrin domain-containing 3–interleukin-1β neuroinflammation in spinal cord. These effects are attenuated by A_3AR agonists, suggesting that A_3AR may be a target for therapeutic intervention with selective A_3AR agonist as opioid adjuncts.

Introduction

Acute morphine exposure stimulates μ-opioid receptor–mediated release of the purine nucleoside adenosine from spinal synaptosomes (Cahill et al., 1993). Released adenosine binds four known receptors: G_i protein alpha subunit-coupled A_1AR and A_3AR and $G_s$protein alpha subunit-coupled A_{2A}AR and A_{2B}AR (Fredholm et al., 2001). In the central nervous system (CNS), adenosine signaling exerts potent neuroprotective and anti-inflammatory effects (Borea et al., 2009) and is controlled by the balance of extracellular release and

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intracellular uptake and metabolism (Borea et al., 2009). This balance is governed by the intracellular enzyme adenosine kinase (ADK), in which high levels of ADK drive adenosine uptake, reduce extracellular adenosine, and compromise adenosine signaling (Boison, 2013). Inhibiting ADK promotes greater extracellular adenosine levels and signaling (Boison, 2013). Hence, ADK acts as the essential "upstream" regulator of adenosine neurotransmission (Boison, 2013).

Our understanding of the interactions between adenosine signaling and opioids, such as morphine, in the CNS is largely limited to A_1AR and A_{2A}AR. Adenosine signaling through A_1AR is important for acute morphine antinociceptive effects evidenced by the loss of morphine antinociception after spinal administration of A_1AR antagonists (Sweeney et al., 1987a,b; Suh et al., 1997) or in $A_1AR^{-/-}$ mice (Wu et al., 2005). However, reduced A_1AR and A_{2A}AR signaling plays a role in the detrimental effects of opioids. Prolonged use of opioids such as morphine can lead to the development of paradoxical painful hypersensitivity (opioid-induced hyperalgesia; OIH), reduced antinociceptive response to opioids over time (antinociceptive tolerance) (Collett, 1998; Angst and Clark, 2006), and the development of psychologic and physical symptoms upon opioid cessation (withdrawal) that undermine the effective use of opioids to treat chronic pain conditions (Collett, 1998; Angst and Clark, 2006). Previous studies reported that prolonged morphine treatment decreases adenosine signaling at A_1AR in the brainstem that corresponded with dependence and addiction behaviors (Zarrindast et al., 1999; Wu et al., 2013). Accordingly, A_1AR agonists or ADK inhibitors attenuate naloxone-precipitated opioid withdrawal behaviors, whereas A_1AR antagonists exacerbate these effects (Zarrindast et al., 1999; Wu et al., 2013). The expression of morphine-induced withdrawal behaviors was attenuated by A_{2A}AR agonists and exacerbated by A_{2A}AR antagonists (Kaplan and Sears, 1996). However, therapeutic use of A_1AR or A_{2A}AR agonists or ADK inhibitors as adjuncts to opioids is not a viable therapeutic approach because of documented A_1AR and A_{2A}AR-mediated cardiovascular side effects (Kiesman et al., 2009; Zylka, 2011).

In contrast, the role of A_3AR in acute or chronic morphine action has not been studied. A_3ARs are expressed by CNS neurons, astrocytes, and microglia, including the spinal cord dorsal horn, and they exert anti-inflammatory effects (Janes et al., 2016). Orally bioavailable, small molecule, receptor subtype-selective A_3AR agonists, such as IB-MECA, have been developed (Kim et al., 1994; Jacobson, 1998; Tosh et al., 2012) and advanced to phase II/III clinical trials for several inflammatory conditions and cancer with a good safety profile (Silverman et al., 2008; Fishman et al., 2012; Stemmer et al., 2013). We have recently reported evidence suggesting that A_3AR agonists reduce the activation of the NOD-like receptor pyrin domain-containing 3 (NLRP3) inflammasome in the spinal cord linking beneficial effects of A_3AR agonists to the inhibition of NLRP3 inflammasome signaling (Wahlman et al., 2018). NLRP3 inflammasome is a critical complex for the processing of interleukin-1β (IL-1β) (Tsuchiya and Hara, 2014). Over the last several years, compelling evidence supports that underlying the development of opioid-induced hyperalgesia, tolerance, withdrawal, dependence, and reward is the imbalance of inflammatory cytokine IL-1β and anti-inflammatory cytokine IL-10 signaling that leads to increased neuronal excitability in the CNS (Hutchinson et al., 2011; Grace et al., 2015; Roeckel et al., 2016). Accordingly, genetic and pharmacological approaches that block IL-1β production and signaling or increase IL-10 signaling have been beneficial in preclinical animal studies (Johnston et al., 2004; Shavit et al., 2005; Hutchinson et al., 2011). More recently, Grace et al. (2016) demonstrated that repeated administration of morphine in rodents can activate NLRP3 inflammasome in the spinal cord.

Collectively, these data led us to investigate whether adenosine signaling at the A_3AR is compromised during prolonged morphine treatment and contributes to the dysregulation of NLRP3 inflammasome activity in the spinal cord, leading to the development of morphine-induced adverse effects. Our findings demonstrate that chronic morphine increases ADK activity and decreases endogenous adenosine at the A_3AR in the spinal cord. Selective A_3AR agonists were found to attenuate morphine-induced adverse effects by downregulating NLRP3 activation in the spinal cord. Our findings identify A_3AR as a target for therapeutic intervention and provide novel mechanistic insights involved in the pathogenesis of morphine-induced hyperalgesia, tolerance, and withdrawal.

Materials and Methods

Experimental Animals. Male and female Sprague Dawley rats (200–225 g), male CD-1 mice (20–30 g), and male C57BL\6 wild-type mice (20–30 g) were purchased from Harlan Laboratories (Indianapolis, IN), and male $A_3AR^{-/-}$ mice (20–30 g) were obtained from Merck (Whitehouse Station, NJ). Animals were housed in a climate-controlled room on a 12-hour light/dark cycle with food and water provided ad libitum. All experiments were performed in accordance with the guidelines of the International Association for the Study of Pain as well as the regulations of the U.S. National Institutes of Health and those of Italy (DM 116192) and the European Union (OJ of EC L 358/112/18/1986). Experimental protocols were approved by the Saint Louis University Institutional Animal Care and Use Committee, the University of Adelaide Animal Ethics Committee (Ethics approval number M-60-2009), the University of Messina Review Board for the Care of Animals and Institutional Animal Care, the University of Arizona Institutional Animal Care and Use Committee, and the University of Calgary Animal Care Committee in accordance with the Canadian Council on Animal Care. All animals were sex-, age-, and weight-matched and then randomly assigned to treatment groups. To assure reproducibility, animals were split into two to three batches with an equal number of animals in each group, and behavioral experiments and corresponding biochemical assays were started and performed on different days with experimenters blinded to treatment conditions.

Test Compounds. Using a structure-based approach in the design of second-generation A_3AR agonists as illustrated in the modeled A_3AR binding complementarity of MRS5698 and MRS5980

ABBREVIATIONS: A_1AR, adenosine receptor subtype 1; A_{2A}AR, adenosine receptor subtype 2A; A_3AR, adenosine receptor subtype 3; ADK, adenosine kinase; CCI, chronic constriction injury of the sciatic nerve; Cl-IB-MECA, 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide; CNS, central nervous system; IB-MECA, 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide; IFN-γ, interferon-γ; IL, interleukin; NLRP3, NOD-like receptor pyrin domain-containing 3; OIH, opioid-induced hyperalgesia; P2XR, purinergic cation channel; TNF, tumor necrosis factor.

(Supplemental Fig. 1), we have achieved >1000-fold receptor subtype selectivity for A_3AR over other adenosine receptor. The selectivity for A_3AR of these second-generation A_3AR agonists greatly exceeds the selectivity of IB-MECA and Cl-IB-MECA, which display 50–250-fold selectivity over the other three receptor subtypes in rodents (Tosh et al., 2014, 2015). MRS5698 (1*S*,2*R*,3*S*,4*R*,5*S*)-4-(6-((3-chlorobenzyl) amino)-2-((3,4-difluorophenyl) ethynyl)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide and MRS5980 (1*S*,2*R*,3*S*,4*R*,5*S*)-4-(2-((5-chlorothiophen-2-yl)ethynyl)-6-(methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1 carboxamide were synthesized as previously described (Tosh et al., 2012). MRS5980 is a more water-soluble congener of MRS5698 (Tosh et al., 2012). The remaining following compounds are purchased commercially: MRS1523 (3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate; Millipore-Sigma, St. Louis, MO), IB-MECA (1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide; Tocris Bioscience, Bristol, UK), morphine sulfate (a kind gift from Mallinckrodt Pharmaceuticals, St. Louis, MO), ABT-702 dihydrochloride (5-(3-bromophenyl)-7-(6-morpholinopyridin-3-yl)pyrido[2,3-*d*]pyrimidin-4-amine dihydrochloride; Tocris), and naloxone (Millipore-Sigma).

Delivery of Test Agents. For morphine administration, rats were lightly anesthetized with 3% isoflurane, maintained on 2% isoflurane in 100% O_2, and an osmotic minipump (Alzet 2001; Alzet Osmotic Pumps, Cupertino, CA) primed to deliver 1.0 μl/h saline vehicle or morphine at 75 μg/μl/h (~8.2–9 mg/kg per day) over 6 days was subcutaneously implanted in the interscapular region as previously described (Muscoli et al., 2010). After implantation, the animals were singly housed for the remainder of the experiment.

Intrathecal administration of test agents (10 μl) or their vehicle (3% DMSO; 10 μl) was done through chronic intrathecal cannulas implanted as previously described (Muscoli et al., 2010) followed by sterile physiologic saline (12 μl) flush. Animals were singly housed following intrathecal catheter implantation surgery and allowed to recover for 7 days before receiving injections.

Test substances or their vehicle (0.5% methylcellulose/10% DMSO) given by oral gavage were delivered in a 0.2-ml dosing volume.

Defining Estrous Cycle Stage. Given the multiday nature of the design in freely cycling females, a vaginal smear was taken after the last behavioral time point and stage of estrous defined by cytology as described (Byers et al., 2012). All animals displayed a normal 4- to 5-day estrous cycle.

Development of Morphine-Induced Thermal Hyperalgesia and Antinociceptive Tolerance in Rats

Thermal Hyperalgesia. The development of thermal hyperalgesia was measured in rats by the Hargreaves method (Hargreaves et al., 1988) by using a Basile Plantar Test (Model 37370; Ugo Basile, Monvalle VA, Italy). The cutoff latencies (20 seconds) were set to prevent tissue injury and the heat intensity (infrared, 60) was set so as to elicit a 16–18-second baseline withdrawal latency as previously described (Muscoli et al., 2010). A significant ($P < 0.05$) reduction in paw-withdrawal latency (seconds) over the infusion period time compared with baseline is characterized as thermal hyperalgesia.

Antinociceptive Tolerance. Rats received acute intraperitoneal injections of morphine (6 mg/kg) during the period of infusion with morphine or saline on days 1, 3, and 6 (Muscoli et al., 2010). Nociceptive responses were measured by using the tail-flick assay (D'Amour and Smith, 1941) 30 minutes after the acute injection of morphine, a time point known to provide a maximal antinociceptive response to acute morphine (Muscoli et al., 2010). The latencies for tail withdrawal from a noxious radiant heat source (Model 37360; Ugo Basile) were measured to determine the changes in the antinociceptive effect of the acute morphine challenge as previously described (Muscoli et al., 2010). The cutoff latencies (rats, 10 seconds, and mice, 15 seconds) were set to prevent tissue injury, and the heat intensity (infrared, 65) was set so as to elicit a 2–4-second baseline withdrawal latency. Tolerance to the morphine antinociceptive effect was indicated by a significant ($P < 0.05$) reduction in tail-flick latencies 30 minutes after the acute morphine challenge. Data are reported as the percentage of maximal possible antinociceptive effect (%MPE) with 100% being complete morphine analgesia as calculated by the following equation:

$$\%\text{MPE} = (\text{response latency} - \text{baseline latency})/ \times (\text{cutoff latency (10 or 15 sec)} - \text{baseline latency}) \times 100.$$

Naloxone-Precipitated Withdrawal. Opioid withdrawal behaviors were measured as previously described (Liu et al., 2011). Chronic morphine withdrawal was induced in male Balb/c mice (19–26 g) by repeated intraperitoneal injections of morphine given twice daily (morning and afternoon) for 3 consecutive days with an escalating dose schedule as follows: day 1, 7.5 and 15 mg/kg; day 2, 30 and 30 mg/kg; and day 3, a single dose of 30 mg/kg. A_3AR agonists or their vehicle (10% DMSO in saline) were coinjected with morphine during the treatment paradigm. A group of control mice received an equal number of saline injections over 3 days; these mice also received the vehicle used for the A_3AR agonists. Naloxone (10 mg/kg, i.p.) or its vehicle (saline) was injected 1 hour after the last morphine or saline injection on day 3. The animals were placed into individual Plexiglas observation cylinders (25 × 11 cm), and the incidence of jumping, front paw shakes, and hunching indicating withdrawal were recorded for 30 minutes.

Antiallodynic Tolerance in the Chronic Constriction Injury Model. Mechano-allodynia was assessed on day 0 prior surgery by probing the plantar aspect of the hind paw with calibrated von Frey filaments (mice: 0.04–2.00 g; rats: 1.4–26 g; Stoelting, Wood Dale, IL) according to the "up-and-down" method (Dixon, 1980), and a paw withdrawal threshold (grams) was calculated as previously described (Janes et al., 2014). Chronic constriction injury (CCI) to the sciatic nerve of the left hind leg in mice was then performed under general anesthesia (Bennett and Xie, 1988). Briefly, CD-1 mice were anesthetized with 3% isoflurane/O_2 and maintained on 2% isoflurane/O_2 during surgery. A small incision (1–1.5 cm in length) was made in the middle of the lateral aspect of the left thigh to expose the sciatic nerve, which was loosely ligated around the entire diameter of the nerve at two distinct sites (spaced 1 mm apart) using silk sutures (6.0).

For daily injection studies, after daily baseline behavior measurements were completed, animals received a subcutaneous injection of morphine or saline and coinjections of test compounds or their vehicle and were returned to their cage. Mechano-allodynia was then reassessed 1 hour after morphine (time of peak antiallodynic effect). Data are reported as the percentage of reversal of mechano-allodynia, with 100% being pain behavior on day 0 (d0) prior to CCI and 0% being pain behavior on day 7 (d7) when mechano-allodynia was at a peak, using the following equation:

$$\%\text{Reversal of mechano} - \text{allodynia} = \left(\text{PWT(g)}_t - \text{PWT(g)}_{d7}\right)/\left(\text{PWT(g)}_{d0} - \text{PWT(g)}_{d7}\right) \times 100$$

For minipump studies, after measuring the degree of mechano-allodynia on day 7, minipumps were implanted to deliver morphine or saline with test compound or their vehicle. Mechano-allodynia was reassessed over 14 days corresponding to the life of the minipump. Data are reported as paw withdrawal threshold (grams).

Liquid chromatography–mass spectroscopy/mass spectroscopy–multiple reaction monitoring of plasma morphine metabolites. Morphine concentrations were determined at Mallinckrodt Pharmaceuticals from 25 μl plasma by using methods previously described (Zou et al., 2009; Kole et al., 2011). Liquid chromatography–mass spectroscopy/mass spectroscopy analysis was performed on a Waters Acquity UPLC system connected to a Sciex API 4000 Q-Trap Mass Spectrometer, utilizing the Turbo Ion Spray source in positive-ion multiple-reaction-monitoring mode. Analyst version 1.6 was used to

calculate concentrations of analytes, which were reported in nanograms per milliliter.

Western Blot Analyses. The dorsal horn of the rat lumbar enlargement of the spinal cord (L4-L6) was harvested, flash frozen in liquid nitrogen, and stored at −80°C. Samples were homogenized in extraction buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EGTA; 0.1 mM EDTA; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluorid; 15 μg/ml trypsin inhibitor; 3 μg/ml pepstatin A, 2 μg/ml leupeptin, 40 μg/ml benzamidine; 1 mM sodium orthovanadate; Millipore-Sigma) at the highest setting for 2 minutes and then centrifuged for 15 minutes at 4°C, as previously described (Paterniti et al., 2012). Protein concentrations were measure by bicinchoninic acid assay (Thermo Fisher Scientific, Carlsbad, CA). Proteins were denatured in Laemmli buffer and boiled for 5 minutes. The proteins (20–40 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose or polyvinylidene fluoride membranes. Membranes were blocked for 2 hours at room temperature in 5% nonfat dried milk or 1% bovine serum albumin in 1X PBS, pH 7.4, depending on manufacturer's protocol for antibody, and subsequently probed with the following specific antibodies: anti-ADK (1:500, PA5-27399; Thermo Fisher Scientific), anti-NLRP3 (1:400, sc-66846; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-caspase 1 (1:500, sc-1597; Santa Cruz Biotechnology) in 1X PBS, 2.5% nonfat dried milk, or 0.1% bovine serum albumin and 0.1% Tween-20 at 4°C overnight. The bound antibodies were then dectected with peroxidase-conjugated bovine anti-mouse IgG secondary antibody (1:3000; Jackson ImmunoResearch, West Grove, PA) and peroxidase-conjugated goat anti-rabbit IgG (1:3000; Jackson ImmunoResearch) for 1 hour at room temperature. Peroxidase-conjugated antibodies were visualized by enhanced chemiluminescence (Bio-Rad, Hercules, CA) and documented and quantified for band densitometry by using Chemidoc XRS+ documentation system and ImageLab software (BioRad). Each membrane was then probed for β-actin (1:5000; Millipore-Sigma) for use as endogenous loading controls.

Cytokine ELISA. The levels of cytokines in spinal cord lysates were assessed by using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer's protocol.

Statistical Analysis. All data collected were analyzed and expressed as mean ± S.D. for n animals, as noted. No animal behavior or biochemical data have been excluded. Data collected from each animal was considered a single biologic unit and analyzed by two-tailed, Welch's-corrected unpaired t test, two-way ANOVA with Bonferroni comparisons, or one-way ANOVA with Dunnett's comparisons as noted. All data were analyzed by using GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego, CA, www.graphpad.com). Significant differences were defined at $P < 0.05$.

Results

Antinociceptive Effects of Acute Morphine Are Not Mediated by Adenosine Signaling at the A_3AR. Intrathecal administration of MRS1523, a selective A_3AR antagonist (Kreckler et al., 2006), did not prevent the acute antinociceptive actions of morphine used at a dose previously shown to exert near to maximal antinociception when tested in the thermal tail flick assay (D'Amour and Smith, 1941) (Fig. 1A). Likewise, the acute antinociceptive effects of morphine were similar in A_3AR knockout mice when compared with age and sex-matched wild-type mice (Fig. 1B). Doses of MRS1523 were chosen from our previously published work (Wahlman et al., 2018).

ADK-Dependent Alterations in A_3AR Signaling Contribute to OIH and Antinociceptive Tolerance. In agreement with our previous study (Muscoli et al., 2010), subcutaneous infusion of morphine over 7 days led to a time-dependent reduction in thermal paw withdrawal latencies,

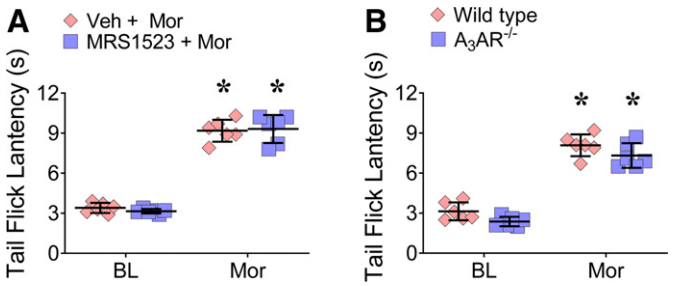

Fig. 1. The acute antinociceptive effects are not dependent on A_3AR signaling. (A) The antinociceptive effects of an acute dose of morphine (3 mg/kg) in naïve mice were not altered by an intrathecal injection of the A_3AR antagonist MRS1523 (1 nmol) given 15 minutes before morphine [$F(1,20) = 0.45$, $P = 0.51$, $\eta_p^2 = 0.31$, $n = 6$/group]. (B) The antinociceptive effects of acute morphine were similar in $A_3AR^{-/-}$ mice and their control wild-type mice [$F(1,20) = 1.3 \times 10^{-29}$, $P = 1.0$, $\eta_p^2 = 1.3 \times 10^{-29}$, $n = 6$/group]. Results are mean ± S.D. and analyzed by two-tailed repeated measures two-way ANOVA with Bonferroni comparisons. *$P < 0.05$ vs. baseline (BL). Mor, morphine; Veh, vehicle.

indicating the development of OIH (Fig. 2A). Moreover, sustained administration of morphine over the same period decreased the antinociceptive effects to an acute intraperitoneal dose of morphine in a time-dependent manner, indicating the development of antinociceptive tolerance (Fig. 2B). A twofold increase in ADK expression in the dorsal horn of the spinal cord was observed at time of peak OIH and tolerance indicative of morphine-induced alterations of adenosine signaling (Fig. 2C). Attenuating ADK activity with daily intrathecal injections of the potent (IC_{50} = 1.7 nM) and highly selective nonnucleoside ADK inhibitor, ABT-702 (Lee et al., 2001), prevented the development of OIH and antinociceptive tolerance (Fig. 2, A and B). Daily intrathecal coinjections of MRS1523 reduced the beneficial effects of ABT-702 by greater than 50% (Fig. 2, A and B).

To test whether supplementing A_3AR signaling in the spinal cord would attenuate OIH and antinociceptive tolerance, we used the well characterized A_3AR agonist, IB-MECA (Kim et al., 1994), at a dose that we have previously reported (Ford et al., 2015). Daily intrathecal injections of IB-MECA during morphine infusion blocked the development of OIH and antinociceptive tolerance in male rats (Fig. 3, A and B). Oral administration (a preferred clinical route) of IB-MECA (Fig. 3, C and D) or the more selective A_3AR agonist, MRS5698 (Little et al., 2015; Tosh et al., 2015) (Fig. 3, E and F), during morphine infusion also blocked the development of OIH and antinociceptive tolerance in male rats. The effects of orally administered A_3AR agonists were attenuated by daily intrathecal injection of MRS1523 (Fig. 3, C–F). Moreover, we found similar attenuation of OIH and antinociceptive tolerance in female rats given oral MRS5698 (Fig. 3, G and H). In contrast to their effects on OIH and antinociceptive tolerance, the acute antinociceptive effects of morphine were not potentiated by either IB-MECA or MRS5698 (Fig. 3I).

A_3AR Agonists Attenuate Morphine-Induced NLRP3 Activation and IL-1β Production and Increase IL-10. We next examined whether the beneficial effects of A_3AR agonists on OIH and antinociceptive tolerance are associated with alterations in morphine-induced NLRP3/IL-1β processing pathways in the spinal cord. At peak OIH and antinociceptive tolerance, the expression of NLRP3 (Fig. 4, A and D) and cleaved caspase 1 (Fig. 4, B and E) in the dorsal spinal

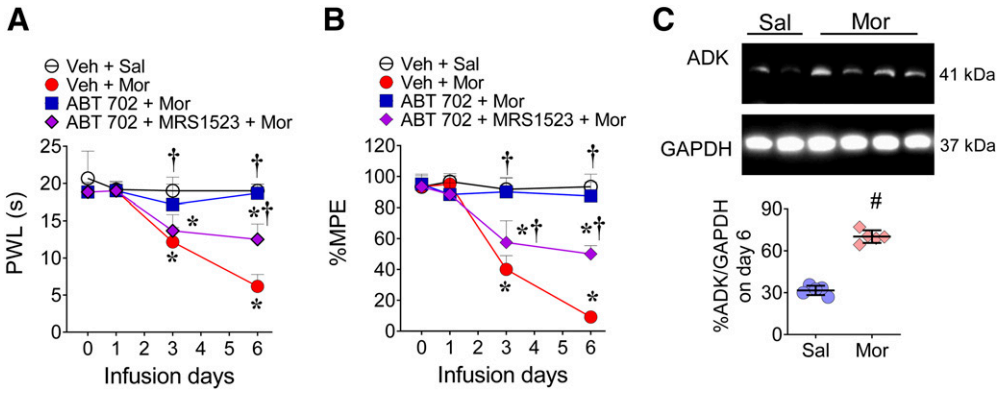

Fig. 2. Adenosine signaling at A_3AR in spinal cord is altered during sustained morphine treatment. Morphine infusion, but not saline, caused the time-dependent development of (A) thermal hyperalgesia and (B) antinociceptive tolerance that was attenuated by daily intrathecal administration of ABT-702 (30 nmol/day) but not its vehicle. Daily intrathecal injections of the A_3AR antagonist, MRS1523 (1 nmol/day), given 15 minutes before intrathecal ABT-702 reduced the effects of ABT-702. (C) When compared on day 6 with male rats infused with saline, ADK expression increased in the dorsal horn of the spinal cord harvested from male rats given morphine infusion. Results are mean ± S.D. and analyzed by two-tailed (A and B) repeated-measures two-way ANOVA with Bonferroni comparisons or (C) unpaired t test with Welch's correction. (A) $F(9,80) = 14$, $P = 3.0 \times 10^{-13}$, $\eta_p^2 = 0.67$; $n = 6$/group; (B) $F(9,60) = 39$, $P = 2.0 \times 10^{-11}$, $\eta_p^2 = 0.83$; $n = 6$/group; and (C) $t(7.4) = 15.4$, $P = 7.2 \times 10^{-7}$, $d = 9.7$; $n = 5$/group. $^*P < 0.05$ vs. day 0; $^\dagger P < 0.05$ vs. Veh + Mor; $^\#P < 0.05$ vs. Sal. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mor, morphine; %MPE, % maximal possible effect; PWL, paw withdrawal latency; Sal, saline; Veh, vehicle.

cord of rats infused with morphine were increased by greater than ninefold and threefold, respectively. This was accompanied by three- to fourfold increase in IL-1β and threefold increase in tumor necrosis factor (TNF) in the spinal cord (Fig. 4, C and F). Inhibiting ADK with intrathecal administration of ABT-702 (Fig. 4, A–C) reduced the expression of NLRP3 (threefold; Fig. 4A), cleaved caspase 1 (twofold; Fig. 4B), IL-1β (5.8-fold; Fig. 4C), and TNF (2.3-fold; Fig. 4C) in the dorsal spinal cord of rats infused with morphine. In contrast to the inflammatory cytokines, IL-10 expression was increased with ABT-702 (threefold; Fig. 4C). Intrathecal injection of MRS1523 attenuated the beneficial effects of ABT-702 on the expression of NLRP3 (twofold), cleaved caspase 1 (twofold), IL-1β (fourfold), and TNF (1.7-fold) (Fig. 4, A–C), and increased IL-10 expression in response to ABT-702 was reduced by 1.6-fold. Oral delivery of MRS5698 had similar effects as ABT-702 did on the expression of NLRP3 (Fig. 4D), caspase 1 (Fig. 4E), and cytokines (Fig. 4F).

A_3AR Agonists Attenuate Morphine-Induced Antiallodynic Tolerance in Neuropathic Pain Models. Opioids are regarded as second- or third-line treatments for neuropathic pain partly because of the development of opioid adverse effects, yet many patients receive these drugs because first-line treatments are often ineffective (Volkow and McLellan, 2016). We tested whether A_3AR agonists would attenuate morphine-induced adverse effects during treatment in a mouse model of traumatic nerve injury–induced neuropathic pain caused by chronic constriction of the sciatic nerve (CCI) (Chen et al., 2019). Subcutaneous injection of morphine at the time of peak CCI-induced mechano-allodynia (day 7 post-CCI) (Chen et al., 2019) reversed allodynia by approximately 90% (95% CI: 84%–94%; Fig. 5A). This antiallodynic effect gradually disappeared after repeated morphine injections (antiallodynic tolerance; Fig. 5A). Coadministration of morphine with a low dose of IB-MECA or MRS5698, which by itself had no antinociceptive effect, blocked the development of antiallodynic tolerance (Fig. 5A). In the same animals, tolerance to morphine antinociceptive effects on the normal thermal pain threshold (tail flick assay) was also blocked (Fig. 5B). These effects were not associated with alterations in plasma levels of morphine metabolites, morphine-3-glucuronide, and morphine-6-glucuronide (Fig. 5C), suggesting that morphine pharmacokinetics were unaltered.

To strengthen our findings, we used an additional experimental paradigm coupled with another higher selective A_3AR agonist, MRS5980. On day 7 post-CCI in mice, subcutaneous infusion of morphine infusion via a 2-week osmotic minipump almost completely reversed mechano-allodynia within 1 day of the start of the infusion; however these antiallodynic effects were lost in a time-dependent manner, indicating the development of tolerance (Fig. 5D). Coinfusion of the highly selective A_3AR agonist, MRS5980, maintained the antiallodynic effects of morphine over 14 days until end of study (Fig. 5D). No drug effects were observed in the contralateral paw (Fig. 5E). At the low dose used here, MRS5980 had no effect when given alone (Fig. 5, D and E).

A_3AR Agonists Attenuate Morphine Withdrawal Behavior. As previously reported (Liu et al., 2011), mice treated for 3 days with escalating doses of morphine followed by an acute dose of naloxone displayed robust withdrawal behaviors: jumping, front paw shaking, and hunched or prayer postures (Fig. 6A). Daily coadministration of IB-MECA, MRS5698, or MRS5980 with morphine reduced the incidences of these withdrawal behaviors by two- to fourfold (Fig. 6B).

Discussion

Chronic pain is a significant problem afflicting 1.5 billion people worldwide (Goldberg and McGee, 2011), with an

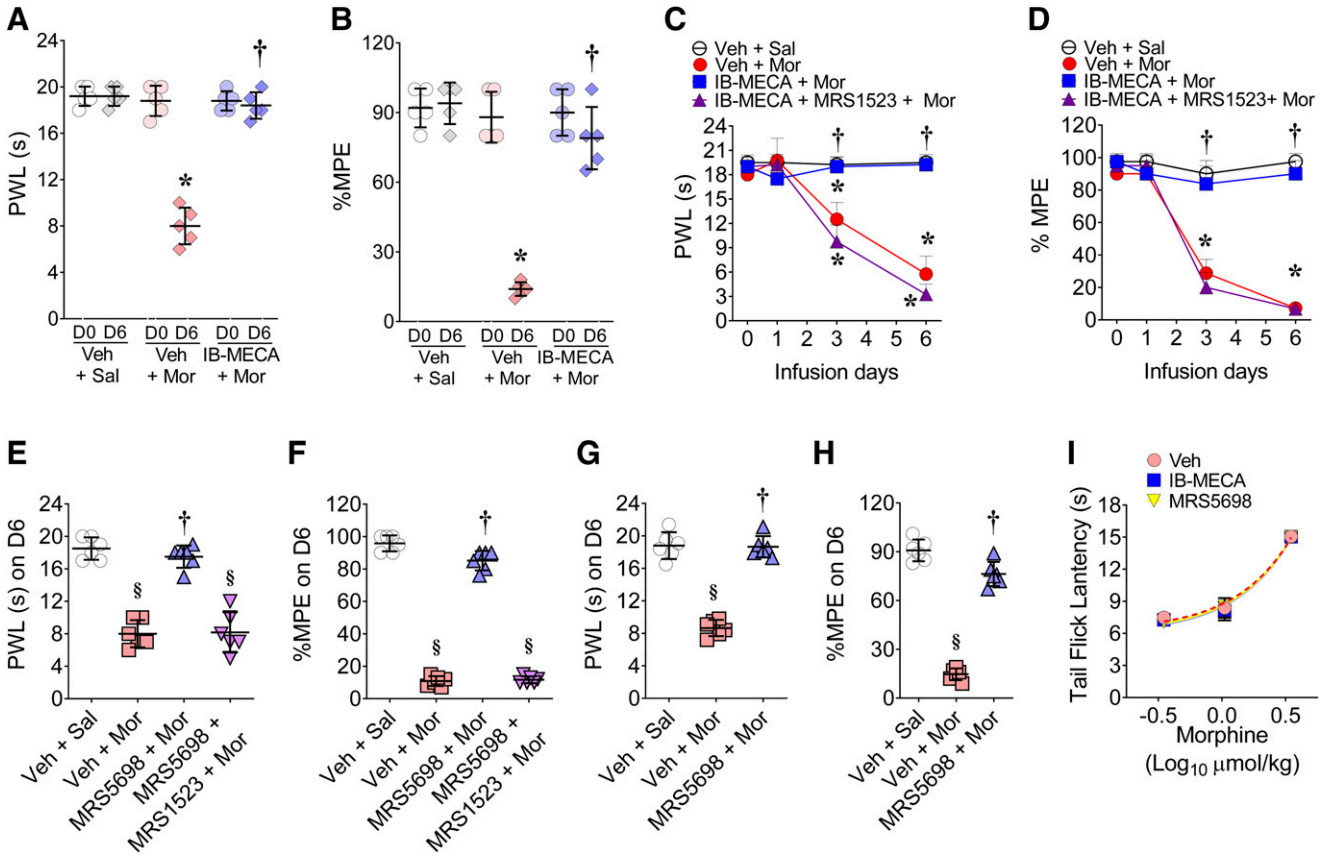

Fig. 3. A_3AR agonists attenuate morphine-induced hyperalgesia and antinociceptive tolerance. The thermal hyperalgesia (A) and antinociceptive tolerance (B) that developed in male rats receiving morphine, but not saline, infusion was prevented in rats given daily intrathecal administration of IB-MECA (30 nmol/day). Thermal hyperalgesia (C and E) and antinociceptive tolerance (D and F) were also attenuated by daily oral administration of IB-MECA or MRS5698 (0.3 mg/kg per day). These effects of IB-MECA and MRS5698 (C and F) were ablated by daily injection of the A_3AR agonists with MRS1523 (intrathecal; 1 nmol/day). In female rats, MRS5698 (0.3 mg/kg per day) also attenuated thermal hyperalgesia (G) and antinociceptive tolerance (H) (n = 6/group). (I) The acute antinociceptive dose response of morphine in naïve male rats to a noxious thermal stimulus (tail-flick) was not altered in rats given an oral dose of IB-MECA or MRS5698 (0.3 mg/kg) with morphine. Results are mean ± S.D. and analyzed by two-tailed repeated-measures two-way ANOVA with Bonferroni comparisons (A–D), one-way ANOVA with Dunnett's comparisons (E–H), or the least sum of square method by a three-parameter, nonlinear analysis (I) (Hill-slope = 1). (A) $F(2,8) = 109$, $P = 1.6 \times 10^{-6}$, $\eta_p^2 = 0.99$, n = 5/group; (B) $F(2,8) = 34$, $P = 1.2 \times 10^{-4}$, $\eta_p^2 = 0.97$, n = 5/group; (C) $F(9,36) = 19$, $P = 2.5 \times 10^{-11}$, $\eta_p^2 = 0.95$, n = 4/group; (D) $F(9,36) = 44$, $P = 8.1 \times 10^{-17}$, $\eta_p^2 = 0.98$, n = 4/group; (E) $F(3,20) = 62$, $P = 2.6 \times 10^{-10}$, $\eta^2 = 0.90$, n = 6/group; (F) $F(3,20) = 686$, $P = 2.5 \times 10^{-20}$, $\eta^2 = 0.99$, n = 6/group; (G) $F(2,15) = 112$, $P = 9.7 \times 10^{-10}$, $\eta^2 = 0.94$, n = 6/group; and (H) $F(2,15) = 262$, $P = 2.1 \times 10^{-12}$, $\eta^2 = 0.97$, n = 6/group. *$P < 0.05$ vs. day 0 and †$P < 0.05$ vs. Veh + Mor. Mor, morphine; %MPE, % maximal possible effect; PWL, paw withdrawal latency; Sal, saline; Veh, vehicle.

annual financial impact exceeding 1 trillion dollars. Its treatment is notoriously difficult and often involves prescribing opioids. However, the long-term use of opioids, such as morphine, is limited by emergence of OIH, antinociceptive tolerance, and withdrawal that can contribute to dependence and abuse liability (Collett, 1998; Angst and Clark, 2006). Despite these serious side effects, there remains a strong reliance on opioids for pain management (Volkow and McLellan, 2016), making continued investigation of the molecular underpinnings of their adverse effects essential. Our findings are the first to suggest that adenosine signaling through A_3AR is disrupted during prolonged morphine treatment as a consequence of increased ADK expression in the spinal cord. This disruption was evident from our findings that inhibiting ADK attenuated OIH and antinociceptive tolerance with effects partially mitigated by intrathecal injections of the A_3AR antagonist, MRS1523. This partial attenuation suggests that other adenosine receptors contribute to beneficial effects of ABT-702, most likely A_1AR, which mediates many protective properties of adenosine (Fredholm et al., 2011; Zylka, 2011; Chen et al., 2013). Accordingly, and perhaps the most noteworthy, our data show that supplementing A_3AR signaling with A_3AR agonists blocked OIH and tolerance in uninjured and nerve-injured rodents. Moreover, our data support previous findings implicating that morphine withdrawal may be dependent on disruption of adenosine signaling (Kaplan and Sears, 1996; Zarrindast et al., 1999; Wu et al., 2013). In addition to the reported beneficial effects of A_1AR and $A_{2A}AR$ activation on preventing the development of opioid-dependence behaviors (Kaplan and Sears, 1996; Zarrindast et al., 1999; Wu et al., 2013), our data demonstrate that A_3AR is capable of attenuating naloxone-precipitated withdrawal, warranting further exploration of this pathway in the context of dependence and reward mechanisms. Because IB-MECA (CF101; piclidenoson) and Cl-IB-MECA (CF102; namodenoson) are already in clinical trials for cancer and inflammatory diseases and have good safety profiles (Müller and Jacobson, 2011), using A_3AR agonists as opioid adjuncts may represent a viable approach to combat the adverse effects in the clinic without the adverse cardiovascular effects exerted

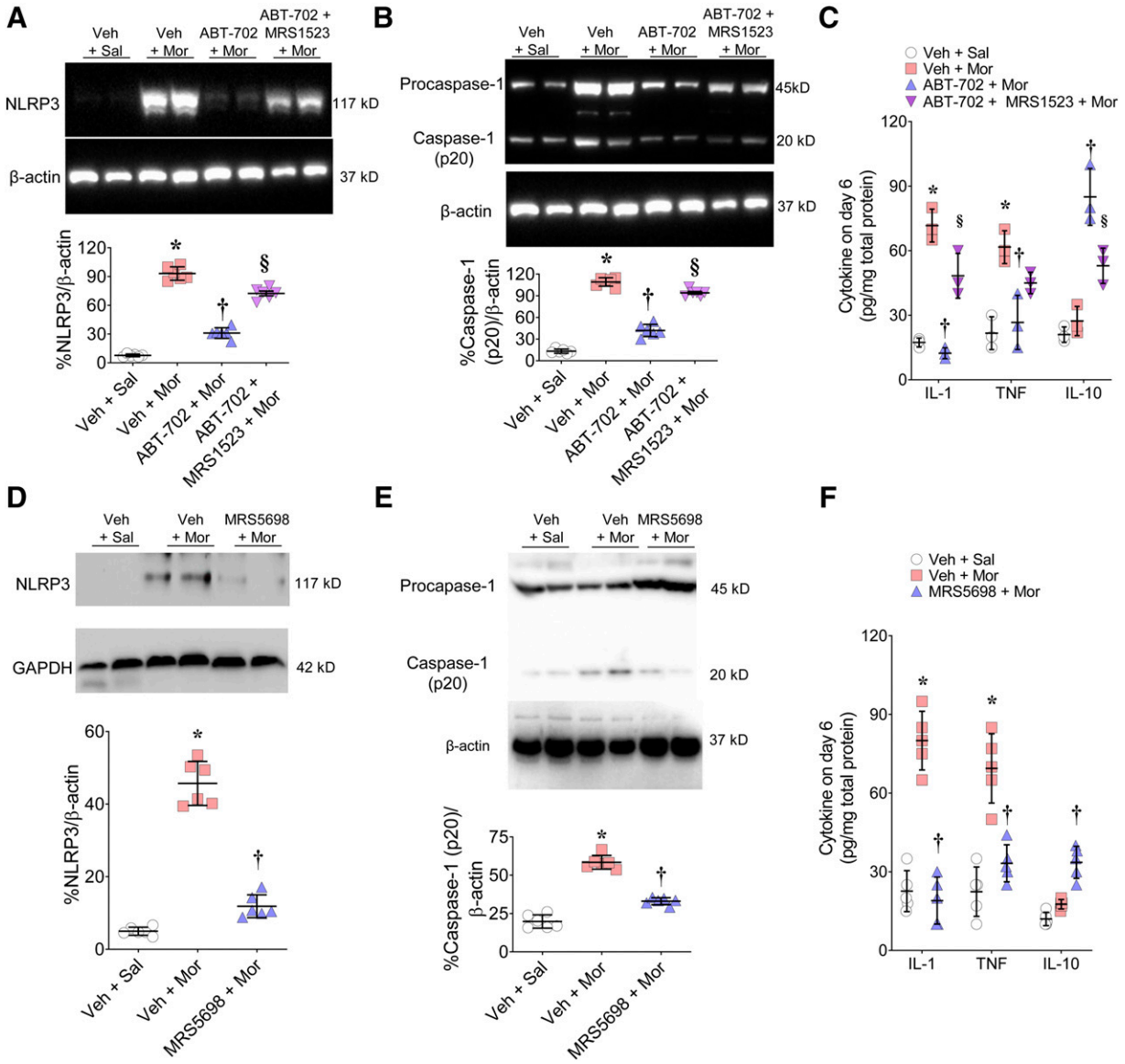

Fig. 4. ADK inhibitor and A_3AR agonist attenuate morphine-induced NLRP3 inflammasome activation and neuroinflammation. When compared with saline-infused rats, the expression of NLRP3 (A), cleaved caspase 1 (B), and IL-1β and TNF (C) increased by day 6 in the spinal cords harvested from rats morphine infusion. Daily intrathecal ABT-702 (30 nmol/day), but not its vehicle, attenuated these events (A–C) and increased IL-10 (C). Inhibition of A_3AR signaling with MRS1523 (1 nmol/day) abrogated the effects of ABT-702 (A–C). Oral administration of MRS5698 (0.3 mg/kg per day) attenuated morphine-induced NLRP3 (D), cleaved caspase 1 (E), and IL-1β and TNF (F) and increased IL-10 expression (F). The vehicle used for the A_3AR agonist was 10% DMSO in 0.5% methylcellulose. Results are mean ± S.D. and analyzed by two-tailed, one-way ANOVA with Dunnett's comparisons. (A) $F(3,20) = 308$, $P = 6.8 \times 10^{-17}$, $\eta^2 = 0.98$, $n = 6$/group; (B) $F(3,20) = 355$, $P = 1.7 \times 10^{-17}$, $\eta^2 = 0.98$, $n = 6$/group; (C) IL-1β - $F(3,8) = 52$, $P = 1.3 \times 10^{-5}$, $\eta^2 = 0.95$, $n = 3$/group; TNF: $F(3,8) = 13$, $P = 0.0017$, $\eta^2 = 0.83$, $n = 3$/group; IL-10: $F(3,8) = 34$, $P = 6.9 \times 10^{-5}$, $\eta^2 = 0.95$, $n = 3$/group; (D) $F(2,15) = 179$, $P = 3.5 \times 10^{-11}$, $\eta^2 = 0.96$, $n = 6$/group; (E) $F(2,15) = 158$, $P = 8.3 \times 10^{-11}$, $\eta^2 = 0.95$, $n = 6$/group; (F) IL-1β: $F(2,12) = 66$, $P = 3.3 \times 10^{-7}$, $\eta^2 = 0.92$, $n = 5$/group; TNF: $F(2,12) = 29$, $P = 2.5 \times 10^{-5}$, $\eta^2 = 0.83$, $n = 5$/group; IL-10: $F(2,12) = 40$, $P = 64.9 \times 10^{-6}$, $\eta^2 = 0.95$, $n = 5$/group. $^*P < 0.05$ vs. Veh + Sal; $^\dagger P < 0.05$ vs. Veh + Mor; $^\S P < 0.05$ vs. ABT-702 + Mor. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mor, morphine; Sal, saline; Veh, vehicle.

by A_1AR and A_{2A}AR agonists (Kiesman et al., 2009; Zylka, 2011).

As we have stated, the development of OIH and antinociceptive tolerance with prolonged morphine treatment are partly dependent on the development of neuroinflammation within the spinal cord (Grace et al., 2015; Roeckel et al., 2016). During neuroinflammation, the reduction of extracellular adenosine because of enhanced intracellular ADK expression and activity (Aronica et al., 2013) coupled with the release of ATP (Fiebich et al., 2014) creates an imbalance between antiinflammatory adenosine signaling at A_1AR/A_3AR and proinflammatory purinergic signaling at purinergic G protein–coupled receptor/P2XR (Rodrigues et al., 2015). For example, ATP activation of P2X7R elicits NLRP3 oligomerization and activation of caspase 1 (Tsuchiya and Hara, 2014), activation of P2X4R modulates microglial activity, and activation of purinergic G protein–coupled receptor 1 increases glutamatergic signaling in neurons and increases reactivity and cell-to-cell calcium flux in astrocytes (Rodrigues et al., 2015). In turn, neuroinflammatory cytokines, such as IL-1β, drive increased ADK expression to maintain this imbalance (Aronica et al., 2011). Morphine-induced IL-1β signaling in the spinal cord

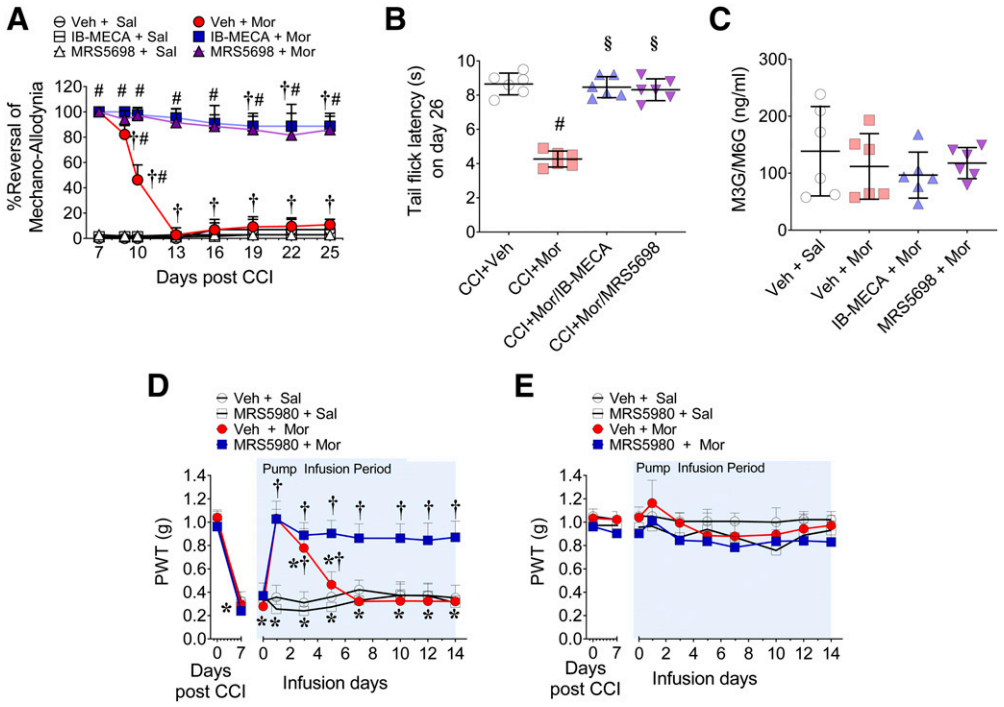

Fig. 5. A_3AR agonists prevent antiallodynic tolerance in mice with nerve injury–induced neuropathic pain. (A) Subcutaneous morphine (3 mg/kg) but not saline injections at the time of peak mechano-allodynia (day 7 post-CCI) reversed CCI-induced allodynia in mice, but this reversal gradually disappeared with daily morphine injections (antiallodynic tolerance). Coinjection of IB-MECA or MRS5698 (0.3 mg/kg per day), but not vehicle (10% DMSO in saline), prevented antiallodynic tolerance. By themselves, these low doses of A_3AR agonists had no effect on allodynia. (B) The antinociceptive response to an acute dose of morphine (3 mg/kg, i.p) on day 26 at 30 minutes postinjection in mice that received daily injections of morphine were significantly reduced when compared with mice that received saline injections over the same timeframe indicative of antinociceptive tolerance. However, antinociceptive tolerance did not develop in mice treated with IB-MECA or MRS5698 combined with morphine. (C) Plasma morphine metabolite (M3G/M6G) concentrations measured by HPLC 30 minutes after acute morphine. Mechano-allodynia in the paws ipsilateral (D) and contralateral (E) to CCI in mice that received saline or morphine infusions for 1 week with MRS5980 (0.3 mg/kg per day) or vehicle (10% DMSO in 0.5% methylcellulose) beginning at peak pain on day 7. Results are mean ± S.D. and analyzed by two-tailed, repeated-measures two-way ANOVA with Bonferroni comparisons (A, D, and E) or two-tailed, one-way ANOVA with Dunnett's comparisons (B and C). (A) $F(3,161) = 34$, $P = 1.9 \times 10^{-57}$, $\eta_p^2 = 0.97$, $n = 3$ (Veh + Sal), 4 (Veh + Mor and MRS5698 + Mor), and 6 (IB-MECA + Mor, IB-MECA + Sal and MRS5698 + Sal); (B) $F(3,20) = 76$, $P = 4.0 \times 10^{-11}$, $\eta^2 = 0.92$, $n = 6$/group; (C) $F(3,20) = 0.61$, $P = 0.62$, $\eta^2 = 0.08$, $n = 6$/group; (D) $F(27,162) = 25$, $P = 2.1 \times 10^{-44}$, $\eta_p^2 = 0.96$, $n = 6$ (Veh + Sal, Veh + Mor) and 5 (MRS5980 + Mor, MRS5980 + Mor); (E) $F(27,162) = 1.4$, $P = 0.098$, $\eta_p^2 = 0.59$, $n = 6$ (Veh + Sal, Veh + Mor) and 5 (MRS5980 + Mor, MRS5980 + Mor). $^*P < 0.05$ vs. day 0; $^\dagger P < 0.05$ vs. day 7; $^\#P < 0.05$ vs. Veh + Sal or CCI + Sal; $^\S P < 0.05$ vs. CCI + Mor. M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; Mor, morphine; PWT, paw withdrawal threshold; Sal, saline; Veh, vehicle.

leads to the development of OIH, tolerance, and withdrawal (Johnston et al., 2004; Shavit et al., 2005; Hutchinson et al., 2011). Exogenous IL-1β has been shown to counterregulate morphine analgesia, whereas its inhibition prolongs the beneficial effects of morphine (Shavit et al., 2005). The development of OIH and tolerance is associated with increased excitatory glutamate neurotransmission (Lee et al., 2011; (Garzón et al., 2012)), and IL-1β increases glutamatergic signaling at the synapse by enhancing presynaptic glutamate release (Yan and Weng, 2013) and reducing glial glutamate uptake (Sama et al., 2008). Morphine-induced IL-1β in the spinal cord has also been found to contribute to opioid withdrawal behaviors (Hutchinson et al., 2011). Although we have not explored the mechanisms whereby A_3AR agonists block withdrawal, inhibitory effects on IL-1β are likely based on our current understanding (Liu et al., 2011).

IL-1β can dampen the effects of IL-10 signaling during inflammation. For example, in monocyte/macrophage culture models, IL-1β and another caspase 1–dependent inflammatory cytokine, IL-18, synergize with TNF to enhance

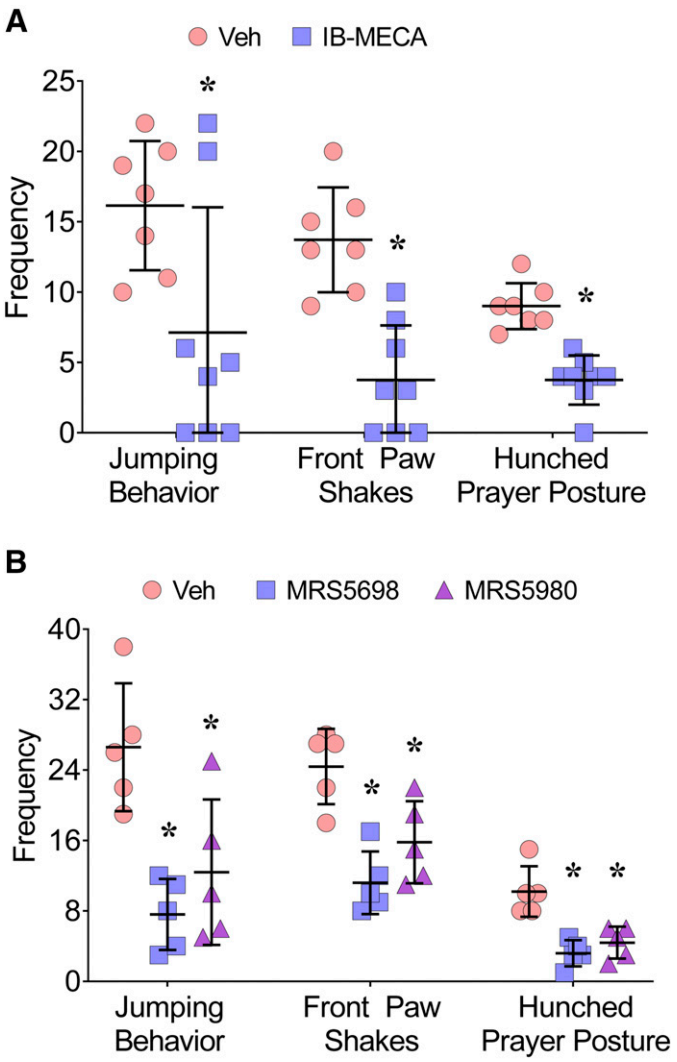

Fig. 6. A_3AR agonists attenuate morphine withdrawal. (A) Incidence of jumping, front paw shakes, and hunching withdrawal behaviors in male mice that received escalating morphine over 3 days with daily subcutaneous IB-MECA (0.1 mg/kg; $n = 8$) or vehicle ($n = 7$/group) [jumping: $t(10.75) = 2.5$, $P = 0.029$, $d = 1.3$; paw shaking: $t(12.86) = 5.1$, $P = 2.2 \times 10^{-4}$, $d = 2.6$; hunching: $t(12.93) = 6.0$, $P = 4.5 \times 10^{-5}$, $d = 3.1$]. (B) Incidence of jumping, front paw shakes, and hunching withdrawal behaviors in male mice that received escalating morphine over 3 days with daily subcutaneous MRS5698 (0.1 mg/kg; $n = 5$), MRS5980 (0.1 mg/kg; $n = 5$), or vehicle ($n = 5$) [jumping: $F(2,12) = 11$, $P = 0.0022$, $\eta^2 = 0.64$; paw shaking: $F(2,12) = 13$, $P = 0.0010$, $\eta^2 = 0.68$; hunching: $F(2,12) = 15$, $P = 4.9 \times 10^{-4}$, $\eta^2 = 0.72$]. The IB-MECA, MRS5698, and MRS5980 doses are below those that have any antinociceptive effect when given alone. Results are mean ± S.D. and analyzed by (A) Welch's corrected two-tailed t test or (B) two-tailed, one-way ANOVA with Dunnett's comparisons. *$P < 0.05$ vs. Veh. Veh, vehicle.

interferon-γ (IFN-γ) production (Raices et al., 2008). IFN-γ, in turn, has been shown to downregulate IL-10 expression (Donnelly et al., 1995) and alter its anti-inflammatory signaling by driving the coupling of IL-10 receptor signaling from signal transducer and activator of transcription 3 (STAT3)-dependent signaling, which is predominantly anti-inflammatory, to STAT1-dependent signaling, which led to increased expression of TNF and IL-1β (Herrero et al., 2003). Interestingly, activation of A_3AR in mouse macrophate (RAW264.7) and human monocyte (THP-1) cells by Cl-IB-MECA has been reported to increase phosphorylation of STAT1 and reduce the inflammatory responses of IFN-γ signaling (Barnholt et al., 2009). To counterregulate the effects of IL-1β on IL-10, activation of anti-inflammatory IL-10 signaling has been shown to reduce the release of IL-1β by blocking NLRP3 activation (Sun et al., 2019). In a recent study, procyanidins attenuated NLRP3 expression in morphine-treated BV-2 microglia, and when used in mice, they attenuated NLRP3 expression and tolerance (Cai et al., 2016). Our findings suggest that it is likely that the ability of an ADK inhibitor or A_3AR agonist to attenuate OIH and antinociceptive tolerance is associated with the relief of suppressive regulation on IL-10 signaling exerted by NLRP3 activation and ensuing IL-1β signaling. Once relieved, IL-10 can provide reported antinociceptive (Johnston et al., 2004; Wu et al., 2018) and anti-inflammatory effects (Lin et al., 2010) to mitigate OIH and tolerance. Whether these effects are directly exerted by A_3AR signaling or mediated by other inflammatory pathways, such as IFN-γ, affected by A_3AR signaling is the subject of ongoing studies.

It is well recognized that the antinociceptive effects of morphine are due to adenosine signaling at the A_1AR in the spinal cord (Sweeney et al., 1987a,b; Suh et al., 1997; Wu et al., 2005). In contrast to the documented role of the A_1 adenosine receptor subtype in morphine's antinociceptive effects, antinociceptive effects of acute morphine were not due to A_3AR signaling, in that responses were not altered in the presence of an A_3AR antagonist or lost in the A_3AR knockout mouse. Accordingly, the antinociceptive effects of acute morphine appear to remain dependent on A_1AR. In summary, our work has defined a novel pathway involved in the development of morphine-induced adverse events and has provided a starting point to consider evaluation of A_3AR agonists that are already in clinical trials as adjunct to opioids.

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Authorship Contributions

Participated in research design: Hutchinson, Bennett, Vanderah, Salvemini.

Conducted experiments: Doyle, Largent-Milnes, Chen, Staikopoulos, Esposito, Dalgarno, Fan, Cuzzocrea, Trang, Hutchinson, Vanderah.

Contributed new reagents or analytic tools: Tosh, Jacobson.

Performed data analysis: Doyle, Largent-Milnes, Hutchinson.

Wrote or contributed to the writing of the manuscript: Doyle, Largent-Milnes, Cuzzocrea, Jacobson, Trang, Hutchinson, Bennett, Vanderah, Salvemini.

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