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Spinal A₃ adenosine receptor activation acutely restores morphine antinociception in opioid tolerant male rats

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Abstract

Opioids are potent analgesics, but their pain-relieving effects diminish with repeated use. The reduction in analgesic potency is a hallmark of opioid analgesic tolerance, which hampers opioid pain therapy. In the central nervous system, opioid analgesia is critically modulated by adenosine, a purine nucleoside implicated in the beneficial and detrimental actions of opioid medications. Here, we focus on the A₃ adenosine receptor (A₃AR) in opioid analgesic

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AUTHOR CONTRIBUTIONS

Conceptualization, Validation, Investigation, Analysis, Writing, H.L.P.; Conceptualization, Validation, Investigation, Analysis, C.X.; Investigation, Analysis, C.F.; Investigation, Analysis, R.D.; Investigation, Y.K.; Investigation, S.S.; Investigation, Analysis, N.B.; Supervision, K.J.; Supervision, C.A.; Conceptualization, Supervision, D.S.; Conceptualization, Validation, Writing, Supervision, Funding acquisition, T.T. All authors read and approved the manuscript.

COMPETING INTERESTS

Dr. Salvemini is founder of BioIntervene, Inc., a company developing A₃AR agonists for clinical use. Dr. Trang is co-founder of AphioTx Inc., which is developing pannexin-1 channel-targeted therapies. All other authors have declared that no conflict of interest exists.

DECLARATION OF TRANSPARENCY

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

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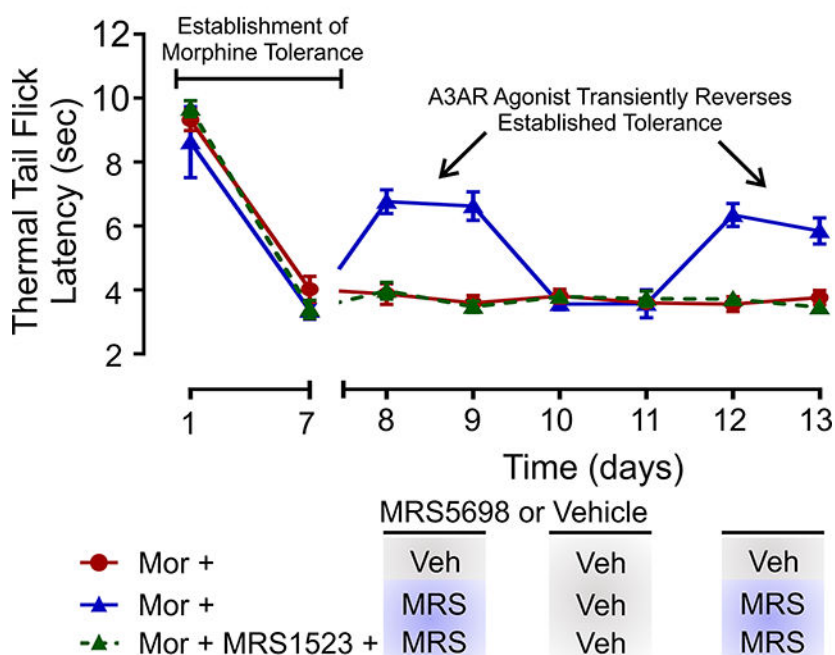
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tolerance. Intrathecal administration of the A₃AR agonist MRS5698 with daily systemic morphine in male rats attenuated the reduction in morphine antinociception over 7 days. In rats with established morphine tolerance, intrathecal MRS5698 partially restored the antinociceptive effects of morphine. However, when MRS5698 was discontinued, these animals displayed a reduced antinociceptive response to morphine. Our results suggest that MRS5698 acutely and transiently potentiates morphine antinociception in tolerant rats. By contrast, in morphine-naïve rats MRS5698 treatment did not impact thermal nociceptive threshold or affect antinociceptive response to a single injection of morphine. Furthermore, we found that morphine-induced adenosine release in cerebrospinal fluid was blunted in tolerant animals, but total spinal A₃AR expression was not affected. Collectively, our findings indicate that spinal A₃AR activation acutely potentiates morphine antinociception in the opioid tolerant state.

Graphical Abstract

“On/Off/On” MRS5698 Treatment Paradigm



Keywords

adenosine; analgesia; opioids; opioid tolerance; RRID:AB_10711040; RRID:AB_10751536; RRID:AB_141844; RRID:AB_2039711; RRID:AB_2535792; RRID:AB_2636996; RRID:AB_449329; RRID:AB_476743; RRID:AB_521594; spinal cord

1 | INTRODUCTION

Opioid medications are used to manage a variety of pain conditions, but their pain-relieving effects diminish with repeated use. The reduction in analgesic potency is a feature of opioid analgesic tolerance and is a major barrier to effective pain management (Mercadante et

al., 2019). Few strategies exist to address the problem of opioid tolerance, and as patients struggle to manage pain, they are often prescribed higher doses or switched to more potent opioid medications. Dose escalation increases opioid use liability and may contribute to opioid use disorder (Babu et al., 2019; Hayes et al., 2020). Novel strategies for overcoming opioid tolerance are urgently needed to improve the clinical utility and safety of opioid medications for treating pain.

Interactions between the opioid and adenosine systems critically modulate opioid analgesia within the central nervous system. Adenosine is a purine nucleoside that exerts its effects via four receptor subtypes: G_i-protein coupled A₁AR and A₃AR, and G_s-protein coupled A_{2A}AR and A_{2B}AR (Boison et al., 2010; Fredholm et al., 2005, 2011). Early evidence indicated that acute morphine administration in rats drives adenosine release in the spinal cord (Cahill et al., 1993; M. Sweeney et al., 1987) and adenosine signaling through A₁AR is critical for morphine antinociception (Suh et al., 1997; M. Sweeney et al., 1987; M. I. Sweeney et al., 1987; Wu et al., 2005). By contrast, prolonged morphine treatment decreases extracellular adenosine and impairs adenosine A₁AR signaling (Nelson et al., 2009; Zarrindast et al., 1999). Reduced signaling at A₁AR and A_{2A}AR contributes to adverse opioid effects, including opioid tolerance, dependence, and withdrawal (Kaplan & Sears, 1996; Wu et al., 2013; Zarrindast et al., 1999). However, targeting A₁AR and A_{2A}AR as opioid adjuncts or standalone analgesics is not viable because of negative side effects, most notable of which are adverse cardiovascular events (Boison, 2013; Kiesman et al., 2009; Zylka, 2011).

In search for adenosine-targeted therapies, attention is shifting to A₃AR because of its low expression in cardiac tissue and its cardioprotective and cerebroprotective effects (Abbracchio et al., 1997; Fishman et al., 2012; Lopes et al., 2003; Ru et al., 2011; Von Lubitz et al., 1999). Phase II/III clinical trials for cancer and several autoimmune conditions have reported good safety outcomes with small molecule A₃AR selective agonists, such as IB-MECA and MRS5698 (Fishman et al., 2002, 2012; Silverman et al., 2008; Stemmer et al., 2013; Tosh et al., 2012). Preclinical studies reveal that A₃AR is expressed along key pain signaling pathways in the spinal cord and brainstem, and its activity has anti-inflammatory effects within the nervous system (Chen et al., 2012; Little et al., 2015; Salvemini & Jacobson, 2017; Trang et al., 2015). In nerve-injured rats, selective A₃AR agonists alleviate neuropathic pain without disrupting normal nociceptive processing (Chen et al., 2012; Janes et al., 2014, 2015; Little et al., 2015). Recent evidence also indicates that chronic morphine disrupts A₃AR-dependent signaling in the spinal cord (Doyle et al., 2020). Intervention with A₃AR selective agonists to normalize adenosine signaling effectively attenuates morphine-induced hyperalgesia, tolerance, and withdrawal (Doyle et al., 2020). In the present study, we further examine the interaction between chronic morphine treatment and A₃AR, uncovering that spinal A₃AR activation transiently restores morphine antinociception in opioid tolerant animals.

2 | METHODS

2.1 | Animals

Adult male Sprague Dawley rats (aged 6–8 weeks, Charles River Laboratories, Sherbrooke, QC, Canada) were housed in standard cages under a 12:12 light/dark cycle (lights on at 08:00 hr) with ad libitum access to standard rodent chow pellets and water. A total of 321 rats were used in experiments contributing to this paper. Sample size calculation using G*Power revealed target group size of 5 (effect size 0.5, $\alpha = 0.05$). An average sample size of six per group was used dependent on animal cohort sizes and variability in results. Rats were lightly anesthetized with a 1.5% isoflurane O₂ mixture during intrathecal injections. Rats were handled and exposed to equipment prior to behavioral experiments for acclimatization and were randomly allocated to different test groups. All experiments were approved by the University of Calgary Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2 | Drugs

Morphine sulfate (Professional Compounding Centers of America, London, ON, Canada) was prepared in saline (Sodium Chloride solution 0.9% w/v, Sigma-Aldrich, St. Louis, MO, USA). Drugs were commercially purchased: IB-MECA (1-deoxy-1-[6-[[[(3-iodophenyl)methyl] amino]-9H-purin-9-yl]-N-methyl- β -D-ribofuranuronamide, N6-(3-Iodobenzyl)adenosine-5'-N-methyluronamide; Sigma-Aldrich, St. Louis, MO, USA), MRS5698 ((1S,2R,3S,4R,5S)-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; R&D Systems, Minneapolis, MN, USA), and MRS1523 (3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate; Sigma-Aldrich, St. Louis, MO, USA). Drugs were dissolved in DMSO with saline.

2.3 | Intrathecal drug administration

Intrathecal injections of IB-MECA, MRS5698, or vehicle control were performed as described by De la Calle and Paino (De la Calle & Paño, 2002). Briefly, animals were placed on a cylindrical Falcon tube such that the spinal column was curved at the level of the L3-L5 vertebrae. Drugs or vehicle control were delivered to the intrathecal space between L3-L4 and L4-L5 vertebrae via lumbar puncture with a 30-gauge needle connected to a Hamilton syringe. Intrathecal delivery of drugs or vehicle (3% DMSO) was administered in a 10 μ l volume. To control for intrathecal delivery, morphine-treated rats were intrathecally injected with vehicle, while saline-treated (morphine-naïve) rats were intrathecally injected with IB-MECA or MRS5698 as drug controls.

2.4 | Morphine treatment and nociceptive testing

Morphine sulfate was administered intraperitoneally (i.p.) once daily for 7 consecutive days at 15 mg/kg body weight to establish systemic antinociceptive tolerance (Burma et al., 2017; Leduc-Pessah et al., 2017). Control animals were given an equivalent volume of saline daily for 7 consecutive days. On each day, baseline nociceptive threshold was measured

15 min before, and then assessed 30 min after, intraperitoneal injection of morphine or saline using the thermal tail-flick test (Ugo Basile, Varese, Italy). In this test, an infrared thermal stimulus was applied to the ventral surface of the tail. The latency, in seconds (s), for removal of the tail from the stimulus was measured and expressed as tail-flick latency. A maximum cut-off time of 10 s was used to prevent tissue damage. The intensity of the heat stimulus was set to 80 infrared intensity. Parameters for assessment remained consistent for each experimental measurement. The tail-flick latency for each animal was calculated as the average of three consecutive measurements.

2.5 | Intrathecal IB-MECA or MRS5698 injection and acute morphine antinociception

Acute response to a single injection of morphine was performed on day 1 to characterize the timecourse of morphine-induced antinociception at 30, 60, 90, and 120 min after morphine injection. Intrathecal IB-MECA (3 or 50 nmol, i.t.) or MRS5698 (3 or 50 nmol, i.t.) was administered at the time of systemic morphine injection. A submaximal dose of morphine (7.5 mg/kg; i.p.) was given to discern whether IB-MECA or MRS5698 potentiates acute morphine antinociception. As control, rats received intrathecal vehicle injection with a submaximal dose of systemic morphine.

2.6 | Daily intrathecal IB-MECA or MRS5698 injections and chronic morphine antinociception

In another animal cohort, intrathecal IB-MECA (50 nmol, i.t.) or MRS5698 (3 or 50 nmol i.t.) was co-administered with daily systemic morphine (15 mg/kg, i.p.) over 7 days. Control animals received systemic saline (i.p.) and/or intrathecal vehicle injection. Thermal antinociception was assessed prior to (baseline) and at 30 min post-injections each day using the tail-flick test.

2.7 | Intrathecal MRS5698 in animals with established morphine antinociceptive tolerance

To establish morphine tolerance, rats were injected daily with morphine (15 mg/kg, i.p.) for 7 consecutive days. On days 8–12, morphine-tolerant rats continued to receive systemic morphine (15 mg/kg, i.p.), but also daily intrathecal injection of either MRS5698 (3 or 50 nmol) or vehicle. Control animals received systemic saline (i.p.) and/or intrathecal vehicle injections.

In separate experiments, we tested the transient effects of MRS5698 (50 nmol) using an “on/off/on” drug administration paradigm in morphine-tolerant rats. On days 8 and 9, rats received intrathecal MRS5698 (“on”) and systemic morphine; on days 10 and 11, these rats were injected with intrathecal vehicle (“off”) and systemic morphine; and on days 12 and 13, intrathecal MRS5698 (“on”) was again administered with systemic morphine. A₃AR selective antagonist MRS1523 (25 nmol) was intrathecally administered 15 min prior to MRS5698. As an injection control, intrathecal vehicle was administered with systemic morphine on days 8–13. In all experiments, thermal antinociception was assessed using the tail-flick test prior to (baseline) and at 30 min post-injections each day.

2.8 | Dose-response experiments

To determine morphine potency, escalating doses of morphine (2.5, 5, 15, 30, 50, 75 mg/kg) were administered intraperitoneally every 30 min and the response to morphine was assessed by the thermal tail-flick test until a maximal level of antinociception (10 s, cut-off time) was achieved. The tail-flick latencies obtained at baseline and at each dose were used to calculate the percent of maximum possible effective dose (% MPE) in order to produce morphine dose-response curves and calculate the median effective dose (ED₅₀) for each animal. Dose-response curves and ED₅₀ values were compared between treatment groups. Morphine dose-response experiments were conducted on day 8 or day 13 of experiments. Rats were injected with either intrathecal vehicle or MRS5698 concomitantly with the first injection of systemic morphine; subsequent injections for the dose-response were performed only with escalating doses of systemic morphine.

2.9 | Adenosine measurements in cerebral spinal fluid (CSF)

CSF was collected from adult male Sprague Dawley rats (175–225 g) treated with either a single subcutaneous (s.c.) injection of morphine (6 mg/kg) or after 7 days of daily morphine treatment (6mg/kg; s.c.). As a control, rats received either a single or 7 day s.c. injection of saline. CSF samples were collected from the cisterna magna 30 min after morphine or saline injection (Mousseau et al., 2018; Pegg et al., 2010). In brief, rats were anesthetized with 2% isoflurane and positioned in a stereotaxic frame. Ear bars were placed in the external auditory meatus, the scalp was shaved, and a midline scalp incision was performed to expose the cervico-spinal muscle and the atlanto-occipital membrane. Using a 1 ml syringe connected to a 27G needle, the atlanto-occipital membrane was punctured, and CSF was gently aspirated into an Eppendorf tube containing ABT-702 (50 nM, Tocris Biosciences), ARL-67156 (1 μM, Sigma), and protease inhibitors (Sigma) and immediately flash frozen on dry ice. Adenosine levels were analyzed using a fluorometric Adenosine Assay kit (Abcam) according to the manufacturer's instructions and fluorescence levels were measured using a FilterMax F5 Multimode Microplate Reader (Molecular Devices). For calculations, CSF adenosine levels were corrected to the mean fluorescent value of a blank and the amount of adenosine/well (pmol) was derived from a trendline equation based on standard curve data. All samples were diluted to fit within standard value range and are presented as adenosine concentration (μM) in CSF.

2.10 | Western blotting

Spinal cords were isolated from rats on the last day of treatment (D7 or D12) using hydraulic extrusion. The lumbar region (L3–L5) was isolated and hand homogenized with microfuge tube homogenization sticks in 250 μl of cold RIPA buffer containing 50 mM TrisHCl, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na₃VO₄, 1 U/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, protease inhibitors, and phosphatase inhibitors. Samples were incubated on ice for 30 min with periodic vortexing, and then centrifuged at 4°C for 30 min at 12,000 rpm. Supernatants were collected from samples and total protein was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were heated at 95°C for 5 min in loading buffer (350 mM Tris, 30% glycerol, 1.6% SDS, 1.2% bromophenol 25 blue, 6% β-mercaptoethanol) then

electrophoresed on a 10% polyacrylamide gel and transferred onto nitrocellulose membrane. After blocking, membranes were probed with primary antibodies. Rabbit α -A₃AR (1:1,000, RRID:AB_2039711), and mouse α - β -Actin (1:10,000, RRID:AB_476743).

Membranes were washed in TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween 20) and incubated for 1 hr at room temperature in fluorophore-conjugated secondary antibodies (α -rabbit and α -mouse conjugated IR Dyes 1:5,000, Mandel Scientific, Guelph, ON). Membranes were imaged using the LICOR Odyssey Clx Infrared Imaging System (Mandel Scientific). Band intensities were quantified using Image J from original full-length images. Band intensities were first normalized to β -actin as a loading control and expressed relative to the average of the control samples (Sal or Mor + Veh).

2.11 | Immunohistochemistry

Rats were anesthetized with pentobarbital (Bimeda-MTC Animal Health Inc.) and perfused transcardially with phosphate-buffered saline (PBS) followed by ice-cold 4% paraformaldehyde (PFA, Sigma, P6148). The lumbar spinal cord (L3–L5) was removed, postfixed overnight at 4°C, and placed in 30% sucrose solution at 4°C. Spinal cord was sectioned using a microtome (Leica, SM2010R) at a thickness of 40 μ m. Free-floating spinal cord sections were incubated in blocking solution (3% normal donkey serum) for 2 hr at room temperature and then incubated 48 hr at 4°C with primary antibodies: rabbit anti-A₃AR (1:100, RRID:AB_10751536) mouse anti-GFAP (1:200, RRID:AB_449329), mouse anti-NeuN (1:500, RRID:AB_10711040), and goat anti-Iba1 (1:500, RRID:AB_521594). Sections were then incubated with fluorochrome-conjugated secondary antibodies: donkey anti-rabbit Alexa 488 (1:1,000, Invitrogen, A-21206, RRID:AB_2535792), donkey anti-mouse Alexa 568 (1:1,000, abcam, ab175472, RRID:AB_2636996), and donkey anti-goat Alexa 647 (1:1,000, Invitrogen, A-21447, RRID:AB_141844) for 3 hr at room temperature. Images were obtained using a Leica SP8 confocal microscope (Table 1).

2.12 | Data and statistical analysis

All data are presented as the mean \pm SEM. Statistical analyses of results were performed with GraphPad Prism 6 software using unpaired *t* test (two-sided), or ordinary one-way ANOVA with post hoc Sidak's test. Timecourse and daily antinociception experiments were analyzed using a two-way repeated measure ANOVA with post hoc Dunnett, Tukey or Sidak's test. *p* < 0.05 was deemed significant. *p* values were automatically adjusted for multiple comparisons in Graphpad. No outliers were identified and no data were excluded from analysis. Further statistical details are reported in figure legends.

3 | RESULTS

3.1 | Spinal A₃AR activation does not alter acute morphine antinociception in naïve animals

To investigate the importance of spinal A₃AR in morphine antinociception, rats were treated with an intrathecal injection of selective A₃AR agonist, MRS5698 (Figure 1a). First, we examined whether intrathecal MRS5698 affects acute morphine antinociception. In these experiments, a submaximal dose of morphine (7.5 mg/kg, i.p.) produced an antinociceptive

response in the thermal tail-flick test that peaked at 30 min post-injection and declined over the course of 120 min (Figure 1b). Neither the magnitude nor timecourse of acute morphine-induced antinociception was affected by an intrathecal injection of MRS5698 (3 nmol and 50 nmol) (Figure 1b). In morphine-naïve (saline-treated) rats, intrathecal MRS5698 had no impact on baseline thermal nociceptive responses, indicating that MRS5698 alone is not antinociceptive (Figure 1b).

3.2 | Spinal A₃AR activation potentiates morphine antinociception in tolerant animals without preventing the loss of morphine potency

To establish morphine antinociceptive tolerance, rats were treated with morphine (15 mg/kg, i.p.) once daily for 7 consecutive days (Figure 1a). Thermal tail-flick latency was assessed before and 30 min after morphine injection. On day 1, morphine treatment induced a significant increase in tail-flick latency (Figure 1c) and elevated cerebrospinal fluid (CSF) levels of adenosine as compared with saline-treated control rats (Figure 1d). The antinociceptive effect decreased with repeated morphine exposure, such that on days 6 and 7 an injection of morphine had no significant impact on thermal nociceptive threshold (Figure 1c) and CSF adenosine levels 30 min after morphine injection were no different from saline-treated rats (Figure 1d). Furthermore, thermal tail-flick latency following morphine injection in these tolerant rats was indistinguishable from saline-treated control rats (Figure 1c). By contrast, when intrathecal MRS5698 (3 or 50 nmol) was co-administered daily with morphine, antinociception persisted to day 7 (Figure 1c).

Repeated morphine exposure results in a significant reduction in antinociceptive potency, which is a hallmark of morphine tolerance. To assess morphine potency, we performed a cumulative dose-response in which escalating doses of systemic morphine were administered every 30 min until a maximal antinociceptive effect was reached in the thermal tail-flick test (Figure 1e,f). Determination of potency occurred on day 8, 24 hr after the last injection of morphine and intrathecal MRS5698 or vehicle. We found that rats treated with morphine for 7 days required higher doses of morphine to achieve a maximal antinociceptive response than morphine-naïve (saline-treated) rats. This reduction in antinociceptive potency was reflected by a rightward shift in the morphine dose-response curve (Figure 1e) and a significant increase in the morphine ED₅₀ (Figure 1f). Likewise, rats treated with morphine and MRS5698 over 7 days required higher morphine doses, as demonstrated by a similar shift in morphine dose-response and increased ED₅₀. We confirmed these results using another A₃AR agonist IB-MECA (3 or 50 nmol), which attenuated the progressive decline in antinociception resulting from daily morphine treatment (Figure 1h), but also did not prevent the increase in morphine ED₅₀ (Figure 1e,f) or affect acute morphine response (Figure 1g). Thus, daily co-administration of MRS5698 or IB-MECA with morphine did not alter the potency or efficacy of morphine in rats that were opioid tolerant.

In determining morphine potency, rats in the above experiments were not given an intrathecal injection of MRS5698 or IB-MECA immediately prior to escalating doses of morphine on day 8. It is possible that the actions of these A₃AR selective agonists may be acute, and therefore, transiently augment morphine-induced antinociception in tolerant rats. We tested this possibility in dose-response experiments wherein MRS5698 (50 nmol)

was administered with the first escalating dose of systemic morphine (Figure 2a). MRS5698 was used in subsequent experiments because of its greater A₃AR selectivity: MRS5698 is >10,000-fold more selective for A₃AR than A₁AR or A_{2A}AR subtypes (Tosh et al., 2012), while IB-MECA has 50-fold greater selectivity for A₃AR compared to A₁AR or A_{2A}AR subtypes (Gallo-Rodriguez et al., 1994; Jacobson et al., 1993). In morphine-tolerant rats, we found that an intrathecal injection of MRS5698 on day 8 reduced the rightward shift in morphine dose-response (Figure 2b) and attenuated the increase in morphine ED₅₀ (Figure 2c). By contrast, morphine-tolerant rats that received intrathecal vehicle injection prior to dose-response experiments displayed a blunted antinociceptive response to escalating doses of morphine. The response to morphine was independent of whether rats had received prior 7 days of MRS5698 or vehicle. In morphine-naïve (saline-treated) rats, intrathecal MRS5698 had no effect on morphine antinociceptive potency. Taken together, our findings indicate that activating spinal A₃AR acutely potentiates morphine antinociception in tolerant animals.

3.3 | Spinal A₃AR activation partially restores morphine antinociception in tolerant animals

We next examined whether spinal A₃AR activation restores morphine antinociception in rats with established tolerance (Figure 3). Rats were rendered morphine tolerant with 7 days of morphine treatment. On days 8–12, rats received a systemic injection of morphine (15 mg/kg) with intrathecal MRS5698 (3 or 50 nmol) or vehicle (Figure 3a). In morphine tolerant rats, the combination of morphine and MRS5698 produced a dose-dependent increase in tail-flick latency; this antinociceptive effect was comparable across days 8–12 (Figure 3b). By contrast, rats treated with morphine and intrathecal vehicle continued to display a blunted morphine response (Figure 3b). Intrathecal MRS5698 or vehicle injection had no effect on nociceptive threshold in morphine-naïve (saline-treated) rats (Figure 3c).

Morphine dose-response experiments were performed on day 13 (Figure 3d,e). Consistent with the above results (Figure 2), morphine tolerant rats given intrathecal MRS5698 with the first escalating dose of morphine displayed a greater antinociceptive response than rats receiving intrathecal vehicle (Figure 3d,e). Prior MRS5698 treatment on days 8–12 had no impact on the morphine dose-response, and MRS5698 administered in the absence of morphine did not affect thermal nociceptive threshold (Figure 3b) or alter morphine dose-response (Figure 3d,e).

Next, we asked whether repeated morphine exposure or MRS5698 treatment affects spinal A₃AR protein levels. Western blot analysis of L3-L5 spinal cord homogenates revealed that total A₃AR expression is comparable in rats treated with saline or morphine for 7 days (Figures 4a and S1). Spinal A₃AR expression was also not affected by co-administration of MRS5698 with morphine on days 1–7 or 8–12 (Figure 4b,c).

3.4 | MRS5698 transiently potentiates morphine antinociception in morphine-tolerant animals

Our results indicate that MRS5698 acutely restores morphine antinociceptive response in opioid tolerant rats. To further test this, we used an “on/off/on” MRS5698 treatment

paradigm (Figure 5a). In these experiments, rats were exposed to systemic morphine over 7 days to induce antinociceptive tolerance. On days 8 and 9, morphine-tolerant rats receiving intrathecal MRS5698 (“on”) together with systemic morphine displayed a partial antinociceptive response (Figure 5b). This response to morphine was absent when intrathecal vehicle (“off”) was administered on days 10 and 11, whereas MRS5698 injection (“on”) on days 12 and 13 reinstated the morphine antinociceptive response. The actions of MRS5698 were blocked by intrathecal injection of A₃AR antagonist MRS1523, indicating acute MRS5698 potentiation of morphine antinociception is A₃AR dependent.

4 | DISCUSSION

Opioids produce potent analgesia, but their pain-relieving effects diminish with repeated use. Here, we show that treatment with A₃AR agonists, IB-MECA, or MRS5698, transiently restores antinociception in morphine-tolerant rats. These compounds possess high A₃AR selectivity (Jacobson et al., 1993; Tosh et al., 2012) and demonstrate good safety and efficacy profiles in phase II/III clinical trials for cancer and inflammatory conditions (Fishman et al., 2012; Silverman et al., 2008; Stemmer et al., 2013). Our findings suggest that A₃AR-targeted approaches may circumvent the need for dose escalation in opioid tolerance, possibly decreasing the risk of adverse effects associated with higher opioid doses.

A key observation in this study is that intrathecal MRS5698 and IB-MECA treatment attenuated the progressive decline in antinociception when administered daily with morphine and transiently restored antinociceptive responses when given to rats with established morphine tolerance. Rats treated with morphine and an A₃AR selective agonist for 7 days required greater amounts of morphine than naïve rats to achieve maximal antinociception. The requirement for higher doses was comparable to rats exposed to morphine only, indicating that neither daily MRS5698 nor IB-MECA treatment prevented the reduction in morphine potency. It is important to note that these dose-response experiments were performed on day 8 by exposing rats to progressively higher doses of morphine in the absence of MRS5698 and IB-MECA. We therefore suspected that selective A₃AR agonist treatment may be exerting an acute effect on morphine antinociception in the tolerant state. Along these lines, when MRS5698 was administered with morphine in dose-response experiments, it attenuated the increase in ED₅₀. By using an intrathecal MRS5698 “on/off/on” dosing paradigm, we confirmed in tolerant rats that morphine response was partially and transiently restored in the presence of MRS5698, an effect blocked by the A₃AR antagonist MRS1523. We ruled out a direct A₃AR-mediated antinociceptive effect because neither compound administered in the absence of morphine affected thermal nociceptive threshold, consistent with previous reports that MRS5698 and IB-MECA are not antinociceptive per se (Chen et al., 2012; Little et al., 2015).

Another important observation is that intrathecal MRS5698 and IB-MECA differentially potentiated morphine antinociception in tolerant rats: there was no impact on morphine response when A₃AR agonists were administered to naïve rats. Similarly, spinal delivery of MRS5698 or IB-MECA suppresses opioid-induced hyperalgesia and alleviates withdrawal without altering acute morphine antinociception (Doyle et al., 2020). This state-dependent

effect has also been reported in neuropathic rats wherein A₃AR agonist treatment augmented morphine antinociceptive efficacy and potency (Doyle et al., 2020), as well as prevented or reversed mechanical allodynia in the absence of opioids (Chen et al., 2012; Little et al., 2015). These findings are consistent with the notion that opioid tolerance and neuropathic pain share a common adenosine-mediated mechanism (Joseph et al., 2010; Mayer et al., 1999). Indeed, Sandner-Kiesling et al. (2001) reported that impaired opioid-induced adenosine release in the spinal cord may account for diminished opioid efficacy and potency in the treatment of neuropathic pain (Sandner-Kiesling et al., 2001). By contrast, interventions that increase spinal adenosine levels by inhibiting adenosine kinase, or that supplement A₃AR adenosine signaling, augment morphine antinociceptive responses in neuropathic animals (Doyle et al., 2020). These interventions also prevent or reverse neuropathic pain in the absence of opioid treatment (Chen et al., 2012; Little et al., 2015).

In rodents, μ -receptor activation critically contributes to morphine-induced spinal adenosine release and this release is modulated by interactions between μ - and δ -receptors (Cahill et al., 1995; Cahill et al., 1996; Sweeney et al., 1989). Neither δ - nor κ -receptor activation alone is sufficient to cause adenosine release from spinal cord synaptosomes (Cahill et al., 1995, 1996). In humans, adenosine release has also been reported following intrathecal administration of morphine and fentanyl, potent μ -receptor agonists (Eisenach et al., 2004). While acute morphine exposure induces adenosine release (Cahill et al., 1993), repeated exposure pathologically alters spinal pain processing and opioid signaling (Ferrini et al., 2013; Gintzler & Chakrabarti, 2006; Rivat & Ballantyne, 2016). In opioid tolerant rats, we found that morphine-induced CSF adenosine release and antinociceptive responses were blunted. However, total CSF adenosine levels were comparable with saline control rats, suggesting that it is morphine-induced adenosine release rather than a change in basal adenosine levels that is affected in morphine-tolerant rats. However, previous studies have reported that chronic opioid administration decreases extracellular levels of adenosine in the brain (Nelson et al., 2009; Wu et al., 2013).

Although we found that total spinal A₃AR protein levels were not affected by repeated morphine or MRS5698 treatment, it is possible that cell-specific changes in A₃AR expression, localization, and/or Gi-coupled signaling may contribute to the A₃AR agonist response in morphine-tolerant rats. A₃AR is expressed on endothelial cells, inflammatory cells, neurons, and glia (Abbracchio et al., 1997; Jacobson, 1998; Lopes et al., 2003). Within the rat spinal dorsal horn, A₃AR may be found on neurons and astrocytes with lower levels detected in microglia (Figure S2), whereas higher A₃AR expression has been reported in brain microglia (Butovsky et al., 2014). However, single-cell RNA sequencing failed to detect A₃AR in spinal sensory neurons (Häring et al., 2018). A₃AR agonists are known to suppress microglia reactivity (Hammarberg et al., 2003; Lee et al., 2006), reduce neuronal excitability and dampen astrocyte activity (Brand et al., 2001; Janes et al., 2015; Wahlman et al., 2018; Wittendorp et al., 2004). Interactions among these various cell types within the spinal circuitry underlies the development of opioid tolerance. Thus, future studies directed at teasing apart the cell-specific effects of A₃AR will provide a more detail mechanistic understanding.

Another limitation of this study is that experiments were performed only in male rats. However, our group recently determined that chronic morphine impairs spinal A₃AR signaling in male and female rats. In both sexes, inhibiting spinal adenosine kinase or activating A₃AR preserved morphine antinociception, attenuated opioid-induced hyperalgesia, and reduced withdrawal (Doyle et al., 2020). Thus, A₃AR activity critically modulates response to repeated opioid treatment and it is a convergent mechanism in both males and females.

In conclusion, our findings show that A₃AR activation transiently restores morphine antinociception in opioid tolerant rats. These results provide a mechanistic twist—the presence of MRS5698 is required to preserve morphine antinociception. This information could optimize A₃AR-targeted therapies and address the need for dose escalation in opioid tolerant individuals, decreasing the risk of adverse effects associated with higher opioid doses. Another notable advantage of targeting A₃AR is that agonists of this receptor do not engage reward centers, which means their addiction liability may be low, and A₃AR-mediated antinociceptive effects do not diminish over time (Little et al., 2015). These are important therapeutic features when considering A₃AR agonists as adjuncts for restoring or enhancing opioid pain relief in opioid tolerant individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

Data sets and materials generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Significance

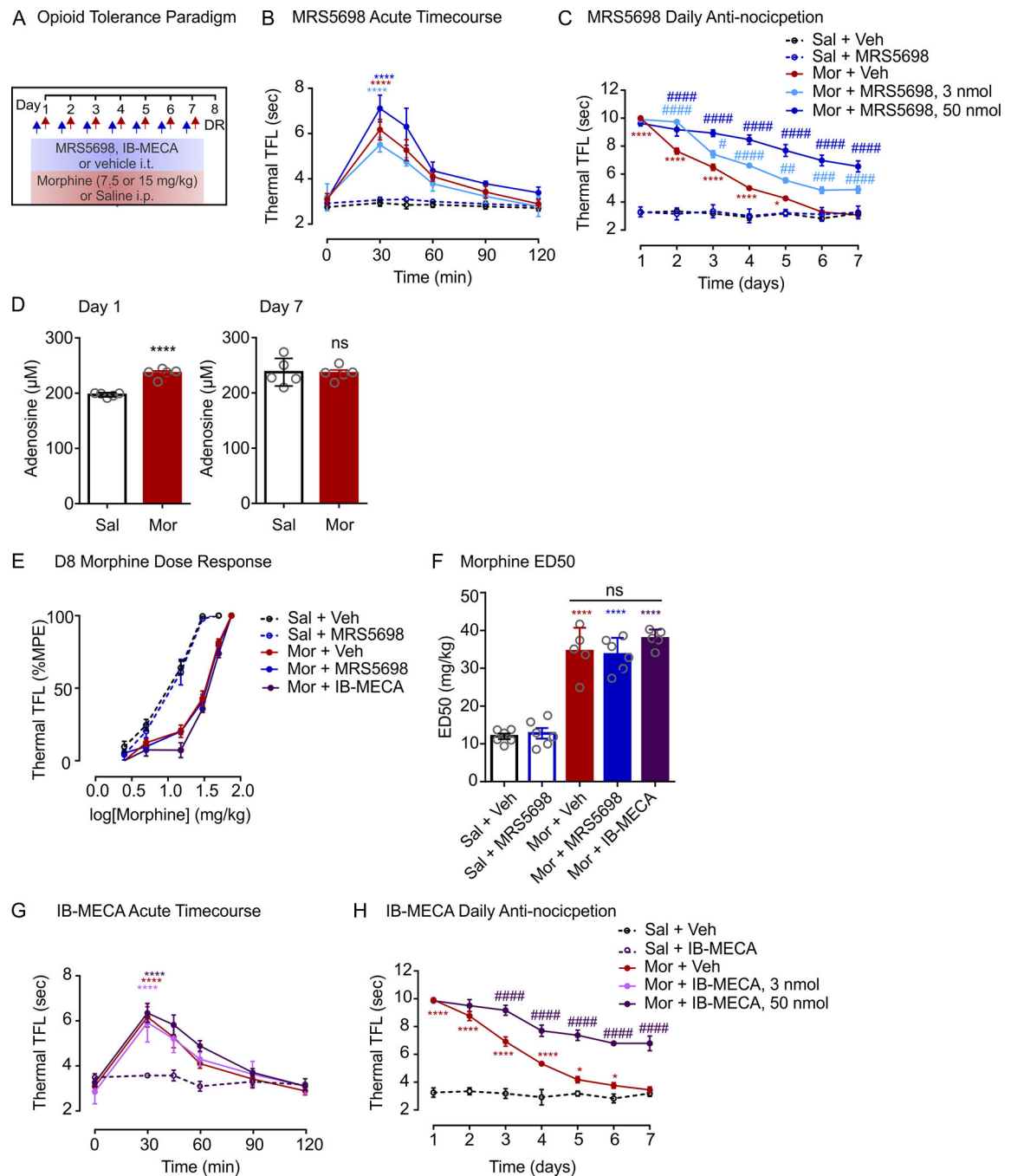
Tolerance to the pain-relieving effects of opioids leaves patients struggling to manage their pain. This limits the effectiveness of opioid pain therapy and can lead to dose escalation, which increases the risk of adverse effects. Here, we show that activating the adenosine A₃ receptor (A₃AR) acutely restores morphine antinociception in opioid tolerant rats. The findings have implications for developing A₃AR-targeted therapies as adjuvants to restore or augment opioid response in tolerant individuals.

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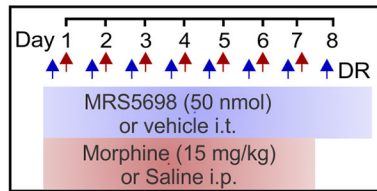
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**FIGURE 1.**

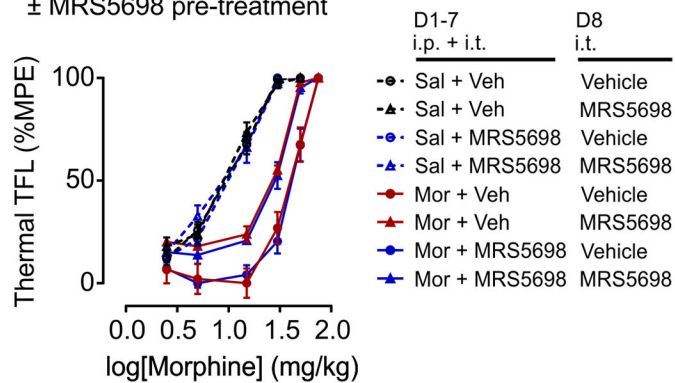
Spinal A_3AR activation potentiates morphine antinociception. (a) Schematic of drug administration paradigm. (b) Timecourse of antinociceptive response to an acute injection of morphine (7.5 mg/kg) or saline. Sal + Veh $n = 6$, Sal + MRS5698 $n = 6$, Mor + Veh $n = 6$, Mor + MRS5698 3 nmol $n = 6$, Mor + MRS5698 50 nmol $n = 5$. Two-way ANOVA, Tukey's post hoc test (Interaction $F(20,120) = 11.94$, $p < 0.0001$; Time $F(5,120) = 81.35$, $p < 0.0001$; Treatment $F(4,24) = 19.55$, $p < 0.0001$). *Compared to Sal + MRS5698 at 30 min (Mor + MRS5698 50 nmol $p < 0.0001$, Mor + MRS5698 3 nmol $p < 0.0001$, Mor + Veh $p <$

0.0001). Compared to Mor + Sal at 30 min (Mor + MRS5698 3 nmol $p = 0.345$, Mor + MRS5698 50 nmol $p = 0.1038$). (c) Daily morphine (15 mg/kg, i.p.) antinociception. Sal + Veh $n = 6$, Sal + MRS5698 $n = 6$, Mor + Veh $n = 10$, Mor + MRS5698 3 nmol $n = 6$, Mor + MRS5698 50 nmol $n = 10$. Two-way ANOVA, Dunnett's post hoc test (Interaction $F(24,198) = 22.87$, $p < 0.0001$; Time $F(6,198) = 113.5$, $p < 0.0001$; Treatment $F(4,33) = 278.1$, $p < 0.0001$). *Represents Mor + Veh compared to Sal + Veh (D1–4 $p < 0.0001$, D5 $p = 0.0127$). #Represents Mor + MRS5698 50 nmol (D2–7 $p < 0.0001$) and Mor + MRS5698 3 nmol (D3 $p = 0.0370$, D4 $p < 0.0001$, D5 $p = 0.0021$, D6 $p < 0.0001$, D7 $p < 0.0001$) compared to Sal + MRS5698. (d) ATP (μM) levels in CSF 30 min after saline or morphine injection on day 1 (left panel; Unpaired two-tailed t test, $n = 5$ $p < 0.0001$, $t = 3.937$, $df = 8$) or day 7 (right panel; Unpaired two-tailed t test, $n = 5$ $p = 0.8976$, $t = 0.1329$, $df = 8$). NS, not significant. (e) Morphine dose-response curves and (f) median effective dose (ED50) calculated on day 8. No pre-treatment with MRS5698 or IB-MECA. Sal + Veh $n = 6$, Sal + MRS5698 $n = 6$, Mor + Veh $n = 5$, Mor + MRS5698 50 nmol $n = 6$, Mor + IB-MECA 50 nmol $n = 5$. One-way ANOVA, Sidak's post hoc test $F(4,23) = 60.69$, $p < 0.0001$ *Comparison to saline controls (Mor + Veh $p < 0.0001$, Mor + MRS5698 $p < 0.0001$, Mor + IB-MECA $p < 0.0001$). (g) Timecourse of antinociceptive response to an acute injection of morphine or saline. For all groups $n = 6$. Two-way ANOVA, Dunnett's post hoc test (Interaction $F(15,100) = 7.595$, $p < 0.0001$; Time $F(5,020) = 80.37$, $p < 0.0001$; Treatment $F(3,20) = 7.421$, $p = 0.0016$). *Compared to Sal + IB-MECA at 30 min (Mor + IB-MECA 50 nmol $p < 0.0001$, Mor + IB-MECA 3 nmol $p < 0.0001$, Mor + Veh $p < 0.0001$). (h) Daily morphine antinociception. Sal + IB-MECA $n = 6$, Mor + Veh $n = 6$, Mor + IB-MECA 50 nmol $n = 5$. Two-way ANOVA, Tukey's post hoc test (Interaction $F(12,84) = 29.78$, $p < 0.0001$; Time $F(6,84) = 96.08$, $p < 0.0001$; Treatment $F(2,14) = 392.3$, $p < 0.0001$). *Represents Mor + Veh compared to Sal + IB-MECA (D1–4 $p < 0.0001$, D5 $p = 0.0121$, D6 $p = 0.0208$, D7 $p = 0.7373$). #Represents Mor + IB-MECA 50 nmol (D2–7 $p < 0.0001$) compared to Mor + Veh. All data are presented as mean \pm SEM

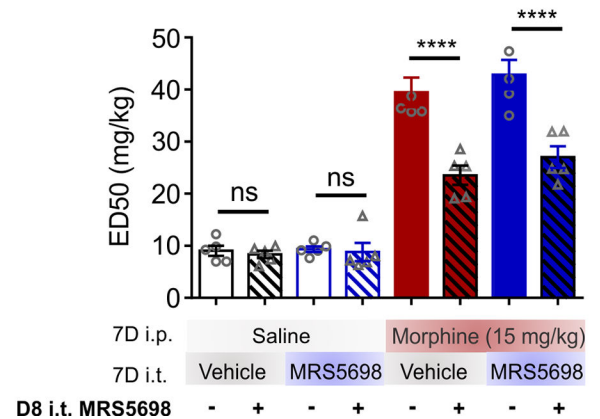
A Opioid Tolerance Paradigm with Intervention prior to Dose Response



B D8 Morphine Dose Response ± MRS5698 pre-treatment

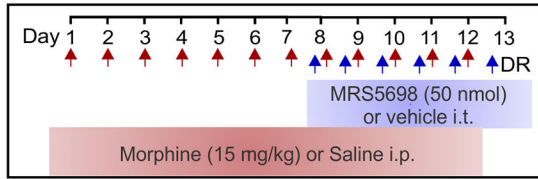


C D8 Morphine ED50 ± MRS5698 pre-treatment

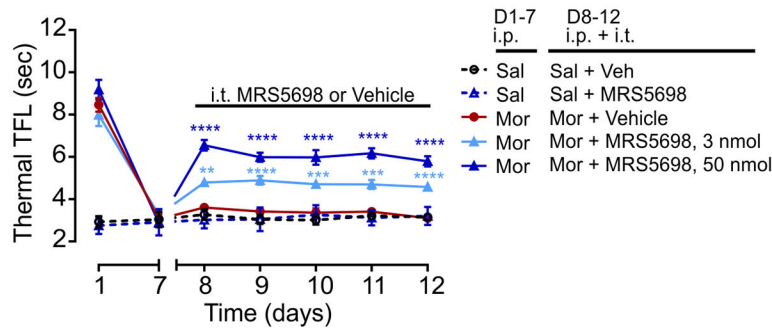
**FIGURE 2.**

Spinal A₃AR activation potentiates morphine antinociception in tolerant animals. (a) Schematic of drug administration paradigm. (b,c) Morphine dose-response was performed on day 8. MRS5698 (50 nmol; i.t) or vehicle (i.t) was administered with the first morphine injection. (b) Morphine dose-response curves and (c) median effective dose (ED50). Data are presented as mean ± SEM. *N* = 5 for all groups. One-way ANOVA, Sidak's post hoc test $F(7,32) = 58.11$, $p < 0.0001$ *Comparisons as indicated **** $p < 0.0001$, NS, not significant

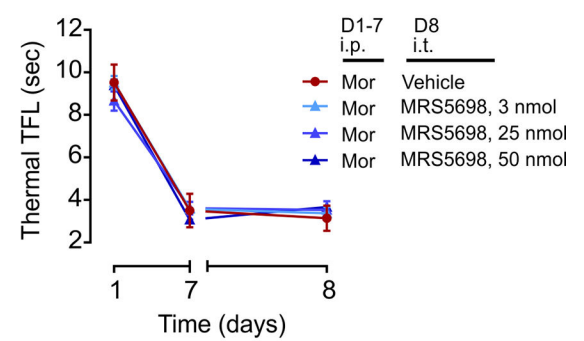
A Established Tolerance Reversal Paradigm



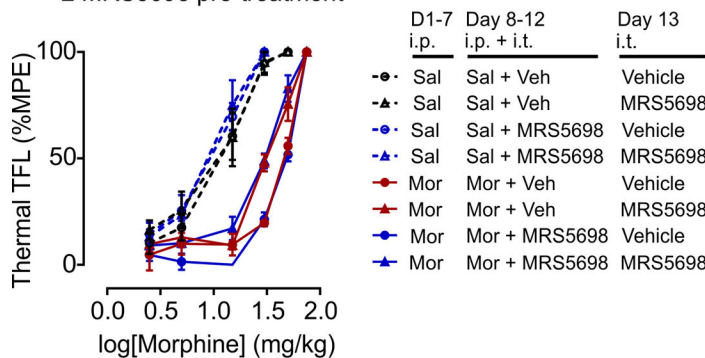
B Established Tolerance MRS5698 Reversal



C Established Tolerance



D D13 Morphine Dose Response ± MRS5698 pre-treatment



E D13 Morphine ED50 ± MRS5698 pre-treatment

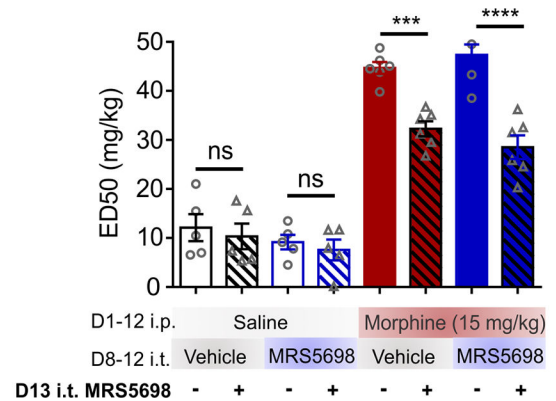


FIGURE 3.

Spinal A₃AR activation partially restores morphine antinociception in morphine-tolerant rats. (a) Schematic of drug administration paradigm. (b,c) Effect of intrathecal injection of MRS5698 in rats with established morphine tolerance. (b) Co-treatment of intrathecal MRS5698 or vehicle injection with morphine (15 mg/kg; i.p) or saline (i.p) on days 8–12. Sal + Veh $n = 6$, Sal + MRS5698 $n = 6$, Mor + Veh $n = 8$, Mor + MRS5698 3 nmol $n = 7$, Mor + MRS5698 50 nmol $n = 7$. Two-way ANOVA, Dunnett's post hoc test (Interaction $F(24,174) = 23.34$, $p < 0.0001$; Time $F(6,174) = 97.94$, $p < 0.0001$; Treatment $F(4,29) = 90.15$, $p < 0.0001$). *Significance compared to Sal + MRS5698 (Mor + MRS5698 50 nmol (D8–12 $p < 0.0001$) and Mor + MRS5698 3 nmol (D8 $p = 0.0014$, D9 $p < 0.0001$, D10 $p = 0.0002$, D11 $p = 0.0005$, D12 $p < 0.0001$). (c) Day 8 intrathecal MRS5698 injection in the absence of morphine. Mor + Veh $n = 5$, Mor + MRS5698 3 nmol $n = 6$, Mor + MRS5698 25 nmol $n = 5$, Mor + MRS5698 50 nmol $n = 6$. Two-way ANOVA, Dunnett's post hoc test (Interaction $F(6,36) = 1.404$, $p = 0.2398$; Time $F(2,36) = 503.9$, $p < 0.0001$; Treatment $F(3,18) = 0.2733$, $p = 0.8438$). No significance at each timepoint. (d) Morphine

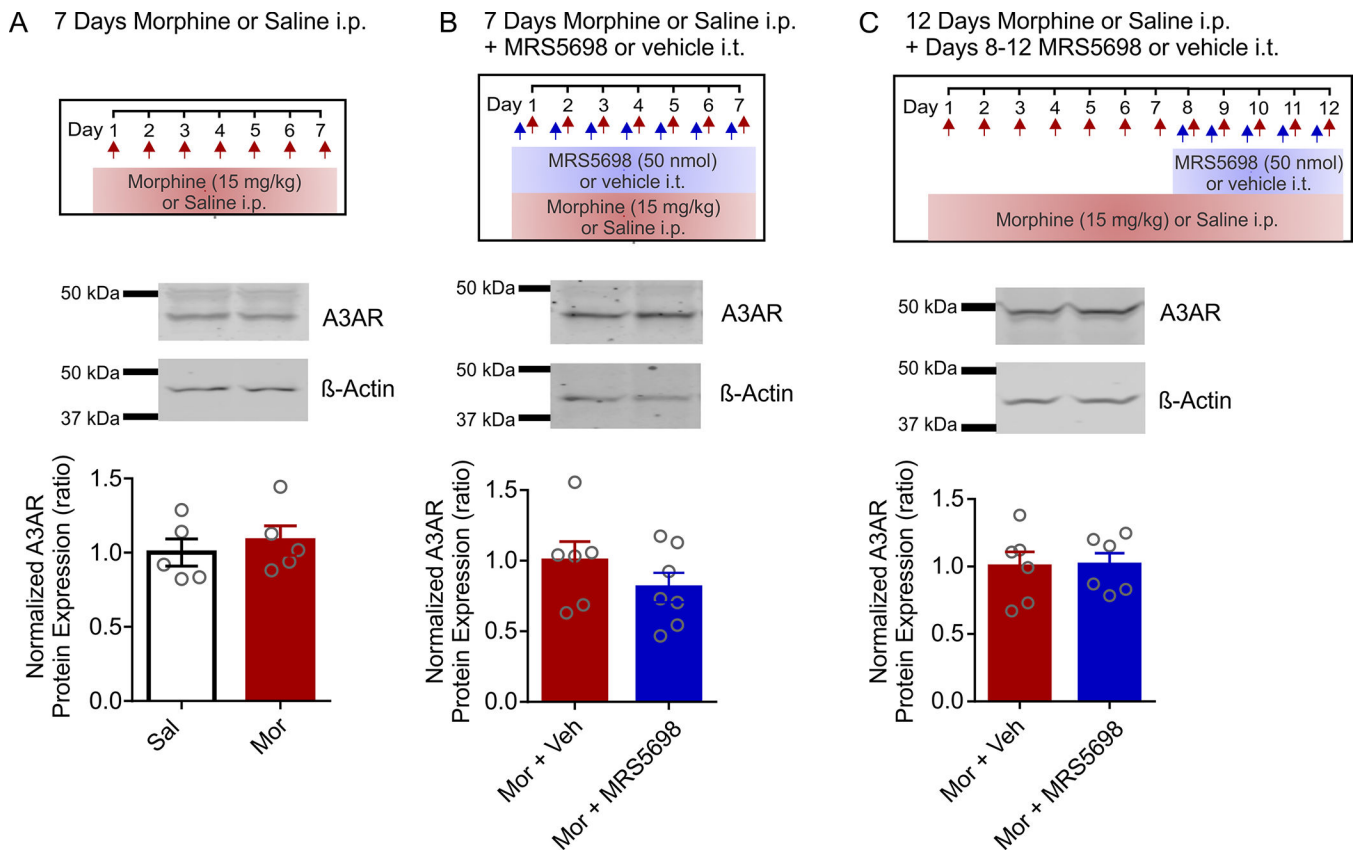
dose-response curves and (e) median effective dose (ED50) calculated on day 13. Intrathecal MRS5698 (50 nmol) or vehicle was administered with the first morphine injection. D1–12 ip Saline $n = 5$ for all groups, D1–12 ip Morphine $n = 6$ for all groups. One-way ANOVA, Sidak's post hoc test $F(7,36) = 63.13$, $p < 0.0001$. *Comparisons as indicated (Mor + Vehicle + D13 MRS5698 i.t. vs. Mor + Vehicle + D13 Vehicle i.t. $p = 0.0003$, Mor + MRS5698 + D13 MRS5698 i.t. vs. Mor + MRS5698 + D13 Vehicle i.t. $p < 0.0001$). NS, not significant. All data are presented as mean \pm SEM

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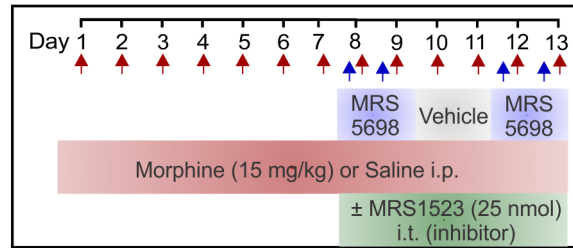
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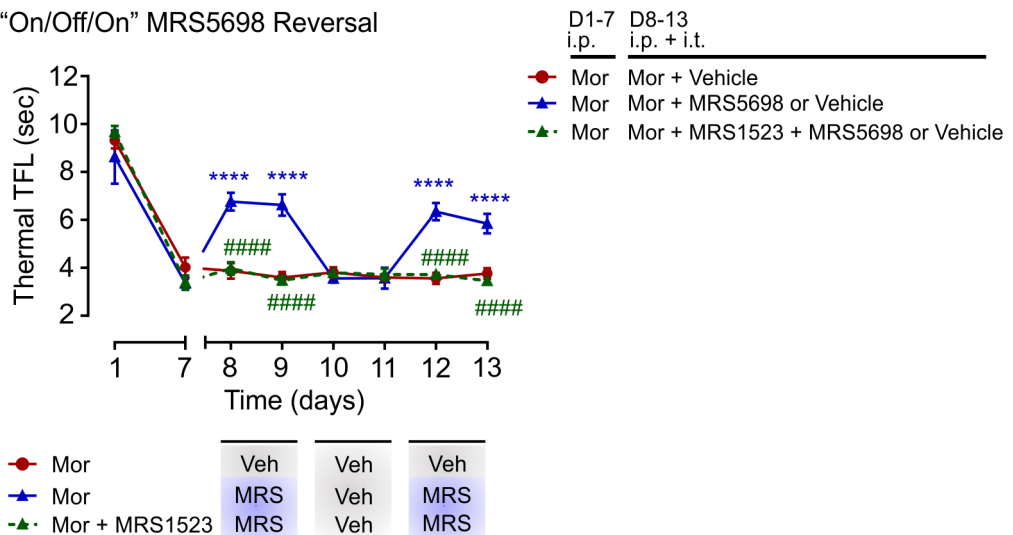
**FIGURE 4.**

Spinal A₃AR protein expression is not affected by morphine or MRS5698. Western blot analysis of A₃AR protein expression in rat spinal cord homogenates. Rat spinal cord was collected and processed for western blotting on D7 or D12 of treatment. Band intensity has been normalized to β-Actin as a loading control and expressed relative to Sal (a) or Mor + Veh (b,c) controls. All quantification was done on full-length unmodified images (Figure S1). (a) Day 7 saline versus morphine treatment Unpaired two-tailed *t* test; *n* = 5, *p* = 0.5650, *t* = 0.6002, *df* = 8; (b) Day 7 morphine and intrathecal vehicle versus MRS5698 Unpaired two-tailed *t* test; Mor + Veh *n* = 6, Mor + MRS5698 *n* = 7, *p* = 0.2814, *t* = 1.133, *df* = 11; (c) Day 12 morphine and intervention on days 8–12 with intrathecal vehicle versus MRS5698 Unpaired two-tailed *t* test, *n* = 6, *p* = 0.9224, *t* = 0.09987, *df* = 10

A MRS5698 "On/Off/On" Paradigm



B "On/Off/On" MRS5698 Reversal

**FIGURE 5.**

MRS5698 potentiates morphine antinociception in morphine-tolerant rats. (a) Schematic of drug administration paradigm. (b) Effect of intrathecal MRS5698 on morphine (15 mg/kg; i.p) antinociception in rats with established morphine tolerance. On days 8–9 and 12–13, intrathecal MRS5698 (50 nmol) was administered with morphine. On days 10–11, intrathecal vehicle was administered with morphine. Effects of MRS5698 were blocked by intrathecal treatment with the A₃AR antagonist MRS1523 (25 nmol). $N = 6$ for all groups. Two-way ANOVA, Sidak's post hoc test (Interaction $F(14,105) = 19.60$, $p < 0.0001$; Time $F(7,105) = 203.9$, $p < 0.0001$; Treatment $F(2,15) = 36.29$, $p < 0.0001$). *Significance compared to Mor + Vehicle control (D8,9,12,13 $p < 0.0001$). #Significance compared to Mor + MRS5698/vehicle (D8,9,12,13 $p < 0.0001$). All data are presented as mean \pm SEM

Primary antibodies

TABLE 1

	RRID	Company	Cat no.	Host	Reactivity	Target
A ₃ AR(IHC)	AB_10751536	Biorbyt, St. Louis, MO, USA	orb10053	Rabbit	Human, Mouse, Rat	KLH conjugated synthetic peptide derived between 95 and170 amino acids of human Adenosine Receptor A ₃
a ₃ ar (WB)	AB_2039711	Alomone Labs Ltd, Jerusalem, Israel	AAR-004	Rabbit	Rat, Human	Polyclonal antibody–Peptide (C) KETGAFYGREFFKTAK, corresponding to amino acid residues 216–230 of human A ₃ AR
β-Actin (WB)	AB_476743	Sigma-Aldrich, St. Louis, MO, USA	A5316	Mouse	Human, Mouse, Rat and others	Monoclonal antibody recognizes an epitope located on the N-terminal end of the β-isoform of actin, peptide Ac-DDDIAALVINGSGK
GFAP(IHC)	AB_449329	Abeam, Cambridge, UK	ab4648	Mouse	Human, Mouse, Rat, Pig	Monoclonal [2A5] to full-length native protein (purified) corresponding to Pig GFAP
Iba1(IHC)	AB_521594	Novus Biological, Centennial, CO, USA	NB100–1028	Goat	Human, Mouse, Rat and others	Polyclonal antibody recognizes peptide C-TGPPAKKAISELP, from the C Terminus of the protein sequence
NeuN (IHC)	AB_10711040	Abeam, Cambridge, UK	ab104224	Mouse	Human, Mouse, Rat	Monoclonal [1B7] recombinant fragment corresponding to Human NeuN aa 1–100 (N terminal)