BJP British Journal of Pharmacology

DOI:10.1111/bph.12809
www.brjpharmacol.org

Themed Section: Opioids: New Pathways to Functional Selectivity

RESEARCH PAPER

Repeated morphine treatment alters cannabinoid modulation of GABAergic synaptic transmission within the rat periaqueductal grey

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Received
17 November 2013
Revised
29 May 2014
Accepted
5 June 2014

BACKGROUND AND PURPOSE
Cannabinoids and opioids produce antinociception by modulating GABAergic synaptic transmission in a descending analgesic pathway from the midbrain periaqueductal grey (PAG). While chronic opioid treatment produces opioid tolerance, it has recently been shown to enhance cannabinoid-induced antinociception within the PAG. This study examined the effect of repeated opioid treatment on opioid and cannabinoid presynaptic modulation of GABAergic synaptic transmission in PAG.

EXPERIMENTAL APPROACH
Midbrain PAG slices were prepared from untreated rats, and rats that had undergone repeated morphine or saline pretreatment. Whole-cell voltage-clamp recordings were made from neurons within the ventrolateral PAG.

KEY RESULTS
In slices from untreated animals, the cannabinoid receptor agonist WIN55212 and the μ receptor agonist DAMGO inhibited electrically evoked $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) IPSCs in PAG neurons, with IC_{50}s of 30 and 100 nM respectively. The inhibition of evoked IPSCs produced by WIN55212 (30 nM) and DAMGO (100 nM) was similar in PAG neurons from morphine- and saline-treated animals. The cannabinoid CB_1 receptor antagonist AM251 increased the frequency of spontaneous miniature IPSCs in PAG neurons from repeated morphine-, but not saline-treated animals. DAMGO inhibition of evoked IPSCs was enhanced in the presence of AM251 in morphine-, but not saline-treated animals.

CONCLUSIONS AND IMPLICATIONS
These results indicate that the efficiency of agonist-induced inhibition of GABAergic synaptic transmission is enhanced by morphine treatment, although this is dampened by endocannabinoid-mediated tonic inhibition. Thus, endocannabinoid modulation of synaptic transmission could provide an alternative analgesic approach in a morphine-tolerant state.

LINKED ARTICLES
This article is part of a themed section on Opioids: New Pathways to Functional Selectivity. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-2

Abbreviations
ACSF, artificial CSF; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DAMGO, Ala^2-$MePhe^4$-$Glyol^5$-enkephalin; GIRK, G-protein coupled inwardly rectifying K^+; IPSC, inhibitory postsynaptic current; PAG, periaqueductal grey; TTX, tetrodotoxin; WIN55212, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

Table of Links

TARGETS	LIGANDS
Cannabinoid CB_1 receptor	AM251
$GABA_A$ receptor	Baclofen
$GABA_B$ receptor	CGP55845
GIRK, $K_{ir}3.x$ channels	DAMGO
μ opioid receptor	Gabazine
TRPV1 channels	Naloxone
	WIN55212

This Table lists the protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, b, c).

Introduction

While opioids are well known for their antinociceptive properties, they induce tolerance following chronic administration (Morgan and Christie, 2011). There is evidence, however, that the antinociception produced by cannabinoids such as tetrahydrocannabinol is enhanced by acute and chronic morphine treatment (Rubino *et al.*, 1997; Cichewicz *et al.*, 1999; Cichewicz and Welch, 2003). Opioids and cannabinoids produce antinociception, in part, by activating a descending analgesic system that originates in the midbrain periaqueductal grey (PAG) and projects via the medulla to the spinal dorsal horn (Fields *et al.*, 2006). The ventrolateral column of the midbrain PAG plays a crucial role in tolerance to the antinociceptive effects of opioids (Lane *et al.*, 2005). Interestingly, antinociception produced by microinjection of a cannabinoid agonist into the ventrolateral PAG is enhanced in morphine-tolerant animals (Wilson-Poe *et al.*, 2013).

Activation of this endogenous analgesic system is thought to occur by a reduction in GABAergic inhibition (disinhibition) of neurons in the descending PAG-medullary pathway. Within the PAG, opioids reduce the release of GABA via both pre- and postsynaptic mechanisms, while cannabinoids are thought to act solely via presynaptic mechanisms. Opioids directly inhibit PAG neurons by activating a G-protein coupled inwardly rectifying K^+ (GIRK) conductance (Chieng and Christie, 1994a). Both opioids and cannabinoids inhibit GABAergic synaptic transmission by presynaptically reducing the probability of neurotransmitter release from nerve terminals (Chieng and Christie, 1994b; Vaughan and Christie, 1997; Vaughan *et al.*, 2000).

Chronic morphine treatment alters the pre- and postsynaptic modulation of PAG neurons by opioids. Repeated morphine treatment produces a reduction in postsynaptic μ receptor modulation of a GIRK and voltage-gated Ca^{2+} conductances, although enhanced coupling to other targets has also been reported (Bagley *et al.*, 2005a,b; Ingram *et al.*, 2007). An increased potency of opioid-induced postsynaptic GIRK activation has also been reported following chronic morphine treatment (Ingram *et al.*, 2008). Similarly, both reduced and increased presynaptic opioid inhibition of GABAergic synaptic transmission within PAG had been reported following repeated morphine treatment (Ingram *et al.*, 1998; Fyfe *et al.*, 2010). The effects of repeated morphine treatment on the cellular actions of cannabinoids within the PAG are unknown. In the present study, we examined the effect of repeated morphine treatment on cannabinoid and opioid presynaptic modulation of GABAergic synaptic transmission in the PAG.

Methods

All animal care and experimental protocols followed the guidelines of the 'NH&MRC Code of Practice for the Care and Use of Animals in Research in Australia' and were approved by the Royal North Shore Hospital Institutional Animal Care and Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 156 animals were used in the experiments described here.

Repeated morphine treatment

Our aim was to assess cellular adaptations during morphine tolerance without the confounding adaptations that occur during opioid withdrawal. Therefore, we chose previously utilized drug administration paradigms that have been shown to produce behavioural antinociceptive tolerance, without eliciting a spontaneous withdrawal syndrome in rats (Animal Resources Centre, Canning Vale, Australia) (Morgan *et al.*, 2006; Fyfe *et al.*, 2010). Male and female Sprague-Dawley rats (16–29 days old) were used in all experiments. Subcutaneous injections of morphine or saline were administered twice daily (0900 and 1600 h) for 2 days (days 1 and 2). Animals were returned to their home cage after each injection. Two repeated morphine treatment protocols were used. One cohort of animals received a static dosing schedule of morphine (5 $mg \cdot kg^{-1}$), which has been shown to produce morphine tolerance (Ingram *et al.*, 2008; Loyd *et al.*, 2008; Fyfe *et al.*, 2010). In a second cohort of animals, we utilized an escalating dose paradigm for morphine treatment (3.2, 5.6, 10.0 and 18.0 $mg \cdot kg^{-1}$). Longer or higher dosing regimen

produce both tolerance and withdrawal (Bagley *et al.*, 2005a,b). Separate groups of animals were used for behavioural and *in vitro* electrophysiological experiments to avoid potential crossover effects from cumulative morphine dosing analgesia experiments into the electrophysiological experiments.

Behavioural testing

Behavioural tolerance to morphine was assessed in a subset of animals as follows. Animals were assessed on the hot plate 20 m after their first injection on day 1. Animals were not tested again until day 3, when cumulative doses of morphine were injected at 20 min intervals and nociception was assessed 15 min after each injection. This cumulative dosing procedure allows for reliable within-subject dose–response analysis (Morgan *et al.*, 2006; Wilson *et al.*, 2008; Fyfe *et al.*, 2010). For the morphine cumulative dosing procedure, actual doses injected were 1.8, 1.4, 2.4, 4.4, 5.6 and 8.0 $mg{\cdot}kg^{-1}$, resulting in cumulative quarter log doses of 1.8, 3.2, 5.6, 10.0 and 18.0 $mg{\cdot}kg^{-1}$. Nociception was assessed using the hot plate test (50.5°C). The rat was immediately removed from the hot plate following a response, or after 50 s if no response occurred. The experimenter conducting the behavioural tests was unaware of the treatment condition. For analysis, dose–response curves were constructed by fitting data to a sigmoidal function with variable slope (GraphPad Prism, Graphad Software Inc., La Jolla, CA, USA).

Brain slice preparation

In vitro experiments on brain slices were conducted 18–24 h after the last morphine or saline injection. Animals were deeply anaesthetized with isoflurane and decapitated. Brains were quickly removed and submerged in ice-cold artificial CSF (ACSF, in mM; 126 NaCl, 2.5 KCl, 1.4 NaH_2PO_4, 1.2$MgCl_2$, 2.4$CaCl_2$, 11 glucose, and 25 $NaHCO_3$). Coronal midbrain sections (300 µm) that contained the PAG were cut using a vibratome (VT1000S, Leica Microsystems, North Ryde, Australia) in ice-cold ACSF. PAG slices were maintained at 34°C, submerged in ACSF and equilibrated with 95% O_2 and 5% CO_2. Slices were then individually transferred to the recording chamber (volume 0.8 mL) and superfused continuously (2.2 $mL{\cdot}min^{-1}$) with 34°C ACSF.

Electrophysiology

Ventrolateral PAG neurons were visualized using infrared Dodt-tube optics on an upright microscope (BX51WI, Olympus, Sydney, Australia). Whole-cell patch clamp recordings of PAG neurons were obtained using an Axopatch 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). For experiments on postsynaptic opioid actions, the internal solution contained (in mM): 95 K-gluconate, 30 KCl, 10 HEPES, 11 EGTA, 15 NaCl, 1 $MgCl_2$, 2 Mg-ATP, 0.3 Na-GTP. For experiments on inhibitory postsynaptic currents (IPSCs), the internal solution contained (in mM): 140 CsCl, 10 EGTA, 5 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 2 MgATP and 3 QX-314. Both internal solutions had pH 7.3 and osmolality, 270–290 $mOsm{\cdot}L^{-1}$. Series resistance (<20 MΩ) was compensated by 80% and continuously monitored during experiments.

Postsynaptic opioid actions were examined in neurons voltage-clamped at –60 mV. In these experiments Ala^2-$MePhe^4$-$Glyol^5$-enkephalin (DAMGO) (3 µM) was applied for a 2 min period before addition of naloxone (1 µM). The $GABA_B$ agonist baclofen (10 µM) was added next, followed by addition the $GABA_B$ antagonist CGP55845 (1 µM). Neurons were only included for analysis if they responded to baclofen with an outward current, which was twice that of the SD of the baseline noise.

The presynaptic effects of opioid and cannabinoid agonists on evoked IPSCs were examined in PAG neurons, voltage-clamped at –70 mV, in the presence of the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 µM) and the glycine receptor antagonist strychnine (5 µM). Evoked IPSCs were elicited every 12 s using a monopolar glass stimulating electrode (tip width 20–40 µm) placed 50–200 µm lateral to the recording electrode. The stimulation strength (1–70 V, 40–400 µs) was adjusted until consistent evoked IPSCs were obtained (between 500 and 2000 pA). In these experiments, a stable 10–15 min recording was obtained before addition of an opioid/cannabinoid agonist, then addition of an appropriate antagonist and then the $GABA_A$ antagonist gabazine. Cannabinoids (12–15 min) and opioids (5–6 min) were applied for periods long enough to produce equilibrium effects. The effect of one cannabinoid or opioid was tested in one cell per slice.

The presynaptic effect of the cannabinoid antagonist on spontaneous miniature IPSCs was examined in PAG neurons in the presence of CNQX (5 µM), strychnine (5 µM) and the voltage-gated sodium channel blocker tetrodotoxin (TTX, 300 nM), and in the absence of electrical stimulation. Unlike the agonist experiments mentioned earlier, these antagonist experiments did not have an internal control for changes over the recording period, thus, miniature IPSCs were examined because of their longer-term stability (Vaughan *et al.*, 2000). In these experiments, a stable 10–15 min recording was obtained before addition of 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide (AM251) for a 12–15 min period.

All recordings were filtered (2 kHz low-pass filter) and sampled (10 kHz) for on-line and later off-line analysis (Axograph X, Axograph Scientific Software, Sydney, Australia). For analysis, evoked and miniature IPSCs were examined over the last 2–5 min before and during application of each agent. The amplitude of averaged evoked IPSCs was measured over corresponding 0.5 ms periods at the peak of the IPSC before and during application of the agonist [DAMGO or (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (WIN55212)], then after addition of the antagonist (naloxone, or AM251) and gabazine. Neurons in which the antagonist did not reverse the agonist-induced inhibition because of rundown were excluded. Miniature IPSCs above a preset threshold (3.5 SDs above baseline noise) were automatically detected by a sliding template algorithm, and then manually checked off-line. Normalized cumulative distribution plots of IPSC inter-event interval and amplitude were constructed.

Data analysis

All numerical data are expressed as mean ± SEM. Statistical comparisons of drug effects were made using Student's *t*-test and one-way ANOVA (GraphPad Prism), and differences were considered significant if $P < 0.05$.

Materials

Morphine sulfate was obtained from the National Measurement Institute (Sydney Australia). Baclofen, DAMGO and strychnine hydrochloride were obtained from Sigma (Sydney, Australia); 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1 H-pyrazole-3-carboxamide (AM251), CGP55845, CNQX, gabazine (SR95531), naloxone hydrochloride, QX-314 bromide, TTX and WIN55212 mesylate from Abcam Biochemicals (Cambridge, UK). For *in vitro* experiments, stock solutions of drugs were made in distilled water, except AM251, CGP55845 and WIN55212 (in DMSO), then diluted to working concentrations using ACSF immediately before use and applied by superfusion to the slice chamber. For *in vivo* experiments, morphine was dissolved in saline and injected s.c. (0.1 mL·kg^{-1}).

Results

Repeated morphine administration causes antinociceptive tolerance

We first examined the effect of the two repeated morphine treatment protocols on the antinociceptive actions of morphine. In the first cohort, animals received static doses of morphine (5 mg·kg^{-1}) or saline twice daily, for 2 days (days 1–2: n = 10 each). On day 1, 5 mg·kg^{-1} morphine produced significant behavioural antinociception (mean hot plate latency = 44.3 ± 3.8 s and 14.3 ± 2.2 s following injection of morphine and saline respectively). After 2 days of treatment injections, baseline hot plate latencies on day 3 were similar in morphine- (12.0 ± 1.3 s) and saline- (12.7 ± 1.3 s) treated animals. There was a significant rightward shift in the dose–response curve of morphine-treated animals compared with those treated with saline, as expected with the development of antinociceptive tolerance to morphine [Figure 1, $F(1,84)$ = 40.9, P < 0.0001]. In a second cohort, animals received escalating doses of morphine in (days 1–2: 3.2, 5.6, 10 and 18 mg·kg^{-1} morphine s.c., n = 4). When assessed on day 3, the ED_{50} for morphine was similar for animals that received static and escalating morphine dose pretreatment [Figure 1, $F(1, 59)$ = 1.8, P = 0.2]. The ED_{50} for morphine was 2.6 (2.3–2.9) mg·kg^{-1}, 5.2 (4.9–5.5) mg·kg^{-1} and 5.5 (5.2–5.8) mg·kg^{-1}, following saline, static morphine dose and escalating morphine dose treatment (mean and 95% confidence interval). Neither of the morphine pretreatment protocols elicited spontaneous withdrawal symptoms.

Effect of repeated morphine treatment on postsynaptic opioid inhibition

Previous studies have demonstrated that opioid, but not cannabinoid receptors couple to postsynaptic GIRK channels within the ventrolateral PAG of untreated naïve animals (Chieng and Christie, 1994a; Vaughan *et al.*, 2000), and that opioid-induced GIRK currents are reduced following repeated morphine treatment (Bagley *et al.*, 2005a). We therefore compared the postsynaptic effect of a maximal concentration of the μ receptor agonist DAMGO on neurons in the ventrolateral PAG from animals that underwent saline and morphine

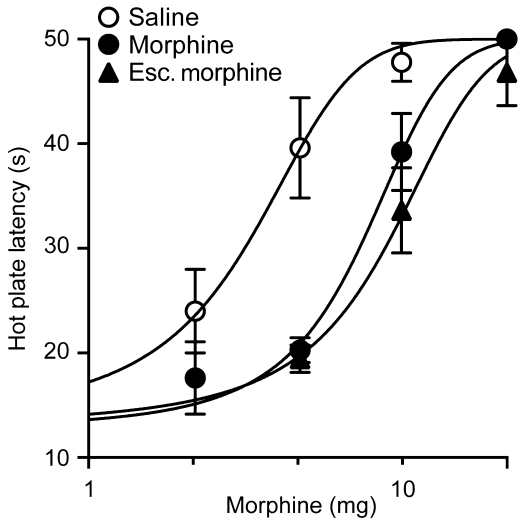

Figure 1

Repeated morphine treatment induces antinociceptive tolerance. Dose–response curves for morphine-induced antinociception, assessed with the hot plate test. Animals were treated twice daily for 2 days with saline, a static morphine dose (morphine, 5 mg·kg^{-1}), or escalating morphine doses (Esc. morphine, 3.2–18 mg·kg^{-1}). The lines represent sigmoidal curve fits to the data.

treatment. DAMGO (3 μM) produced an outward current, which was reversed by the addition of naloxone (1 μM) in PAG neurons from animals that underwent repeated saline and morphine pretreatment (5 mg·kg^{-1} morphine on days 1 and 2; Figure 2A and B). The DAMGO-induced current was 31 ± 5 pA and 20 ± 4 pA in saline- and morphine-treated animals, respectively (n = 16, 10). Subsequent addition of the $GABA_B$ agonist baclofen produced outward currents of 34 ± 5 pA and 29 ± 3 pA [$t(24)$ = 0.67, P > 0.05] in PAG neurons from saline- and morphine-treated animals, respectively, which were reversed the $GABA_B$ antagonist CGP55845 (1 μM) (Figure 2A and B). The DAMGO-induced current, measured as a percentage of the baclofen current, was less in PAG neurons from morphine-treated animals compared with saline-treated animals [Figure 2C, $t(24)$ = 2.38, P = 0.03]. These results indicate that repeated morphine treatment using the static dosing schedule produces tolerance to opioids at the cellular level.

Effect of repeated morphine treatment on cannabinoid and opioid inhibition of GABAergic transmission

We first determined the potency of DAMGO and the pan-cannabinoid receptor agonist WIN55212 induced inhibition of evoked IPSCs in PAG neurons from untreated naïve animals. In these experiments, evoked IPSCs were observed in the presence of CNQX (5 μM) and strychnine (5 μM), and were abolished by the $GABA_A$ receptor antagonist gabazine (10 μM) (Figures 3A and B). Superfusion of WIN55212 reduced the amplitude of evoked IPSCs in a concentration-dependent manner, and this inhibition was reversed by adding the CB_1 receptor antagonist AM251 (3 μM) (Figure 3A–C). While WIN55212 and AM251 have off-target

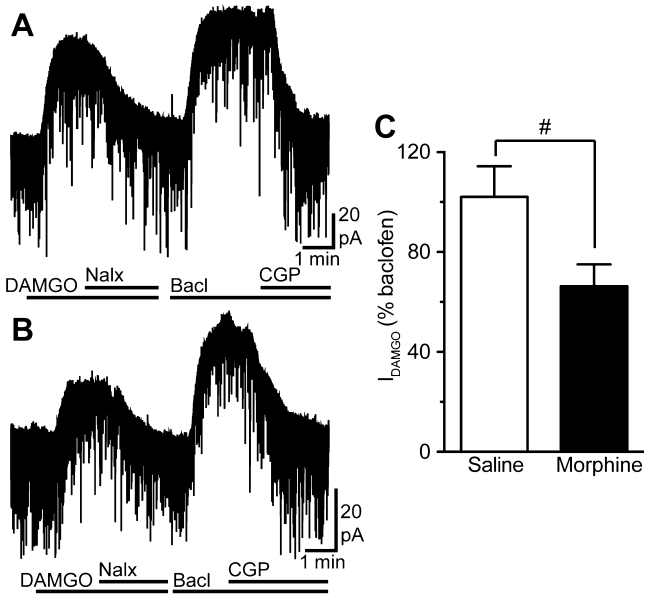

Figure 2

Repeated morphine treatment induces tolerance to postsynaptic μ-opioid receptor induced currents. Records of membrane currents from two PAG neurons from (A) saline- and (B) morphine- (5 mg·kg^{-1} static dose) treated animals, during superfusion of DAMGO (3 μM), naloxone (Nalx; 1 μM), baclofen (Bacl; 10 μM) and CGP55845 (CGP; 1 μM). (C) Bar chart of the current produced by DAMGO in PAG neurons from saline- and morphine-treated animals; the DAMGO current is expressed as a percentage of the baclofen induced current in each neuron. In (C), #$P < 0.05$, significant difference between saline and morphine.

effects, this suggests that WIN55212 acted via cannabinoid CB_1 receptors to presynaptically inhibit evoked IPSCs. The WIN55212-induced inhibition of evoked IPSCs had an IC_{50} of 29 nM (95% confidence interval = 12–63 nM) (Figure 3C). DAMGO also reduced the amplitude of evoked IPSCs in a concentration-dependent manner, and had an IC_{50} of 113 nM (95% confidence interval = 48–268 nM) (Figure 3D).

We next examined whether repeated morphine treatment alters the presynaptic coupling efficiency of cannabinoid and opioid agonists. To do this, we used probe IC_{50} concentrations of WIN55212 and DAMGO (obtained earlier from naïve animals) to detect an overall difference in potency/efficacy between saline and morphine (static 5 mg·kg^{-1} dose) pretreated animals. A near IC_{50} concentration of WIN55212 (30 nM) reduced the amplitude of evoked IPSCs in PAG neurons from both saline-treated ($P = 0.0004$, $n = 6$) and morphine-treated ($P = 0.001$, $n = 7$) animals (Figure 4A and B). The WIN55212-induced inhibition of evoked IPSCs, however, did not differ between saline- and morphine-treated animals ($P = 0.3$). Likewise, a near IC_{50} concentration of DAMGO (100 nM) reduced the amplitude of evoked IPSCs in PAG neurons from both saline-treated ($P = 0.005$, $n = 8$) and morphine-treated ($P = 0.0003$, $n = 11$) animals (Figure 4C and D). As observed for WIN55212, the DAMGO-induced inhibition of evoked IPSCs did not differ between saline- and morphine-treated animals ($P = 0.4$). The WIN55212- and DAMGO-induced inhibition of evoked IPSCs in morphine-treated animals was reversed by adding AM251 (3 μM) and naloxone (1 μM), respectively (Figure 4A and C).

The lack of effect of repeated morphine treatment may have been due to the use of a low-static dose schedule. The DAMGO- (100 nM) induced inhibition of evoked IPSCs, however, did not differ between saline-treated animals and animals that underwent the static dosing schedule, or the higher escalating dosing schedule [Figure 4D, $F(2, 17) = 0.4$, $P = 0.7$]. Thus, presynaptic opioid and cannabinoid agonist-induced inhibition of GABAergic synaptic transmission within the PAG is unaffected by repeated morphine treatment, despite the presence of postsynaptic opioid tolerance.

Effect of repeated morphine treatment on endogenous cannabinoid tone

We have recently shown that activation of G_q-coupled GPCRs induces endocannabinoid modulation of GABAergic synaptic transmission in PAG slices (Drew *et al.*, 2008). We therefore examined if there is a basal endocannabinoid inhibition of GABAergic synaptic transmission and whether this is affected by repeated morphine treatment. To examine this, we compared the effect of AM251 on miniature IPSCs in PAG neurons from morphine- and saline-treated animals.

In the presence of CNQX, strychnine and TTX (300 nM), spontaneous miniature IPSCs were readily observed (Figure 5A). Superfusion of AM251 (3 μM) produced an increase in the frequency of miniature IPSCs in PAG neurons from morphine-treated, but not in saline-treated animals (Figure 5A, D, E, $P = 0.04$ and 0.40 for morphine- and saline-treated animals, $n = 11$ each). The increase in miniature IPSC frequency was associated with a leftward shift in the cumulative probability distribution of the miniature IPSC interevent intervals (Figure 5B). By contrast, AM251 did not affect the amplitude of miniature IPSCs in morphine- and saline-treated animals (Figure 5E, $P = 0.9$, 0.8). AM251 had no significant effect on the cumulative probability distribution of the miniature IPSC amplitudes (Figure 5C), nor did it affect miniature IPSC kinetics in saline- or morphine-treated animals (Figure 5A inset).

Endogenous cannabinoid tone alters presynaptic agonist-induced inhibition

The enhanced endocannabinoid tone in morphine-treated animals could potentially affect the inhibition of evoked IPSCs produced by the agonists DAMGO and WIN55212. We therefore examined the effect of blocking the endocannabinoid tone on the DAMGO-induced inhibition of evoked IPSCs in both saline- and morphine-treated animals. In the presence of AM251 (3 μM), DAMGO (100 nM) produced a reduction in the amplitude of evoked IPSCs in both saline- ($P < 0.0001$, $n = 8$) and morphine- ($P = 0.002$, $n = 6$) treated animals (Figure 4D). In saline-treated animals, the DAMGO-induced inhibition of evoked IPSCs was similar in the presence and absence of AM251. (Figure 4D, $P = 0.98$). However in morphine-treated animals, the inhibition of evoked IPSCs produced by DAMGO in the presence of AM251 was greater than that observed in its absence (Figure 4D, $P = 0.03$).

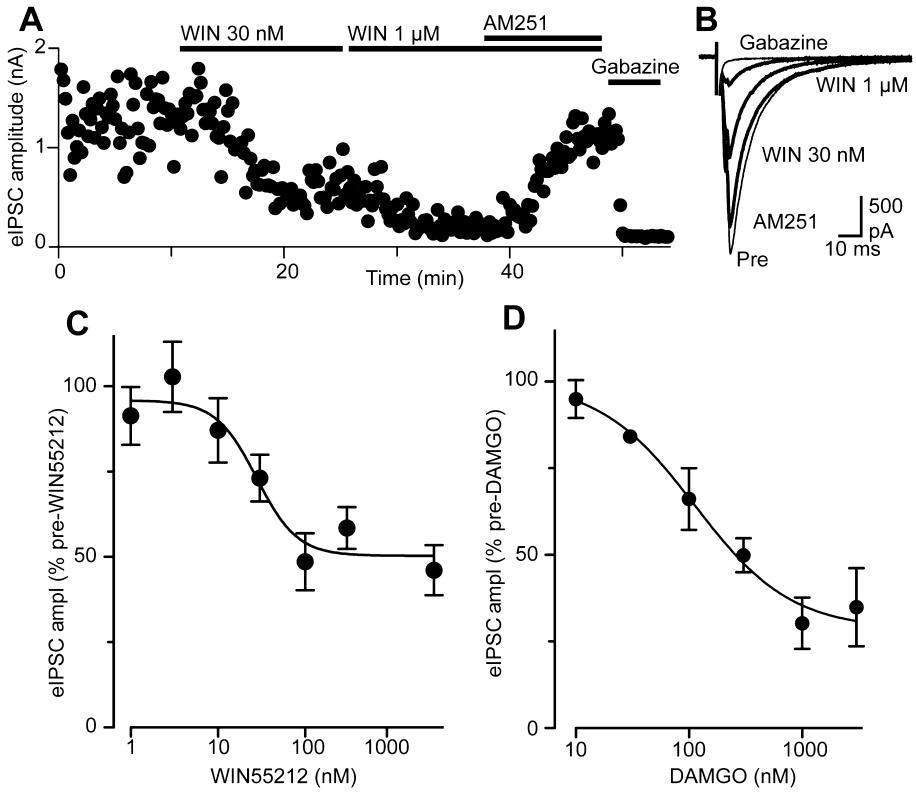

Figure 3

Cannabinoids and opioids inhibit evoked IPSCs in a concentration-dependent manner. (A) Time course of evoked IPSC (eIPSC) amplitude during superfusion of 30 nM and 1 μM WIN55212 (WIN), AM251 (3 μM) and then gabazine (10 μM). (B) Average records of evoked IPSCs from the time plot in (A) (averaged across last 3 min of each drug). Concentration–response curves for the reduction in evoked IPSC amplitude produced by (C) WIN55212 and (D) DAMGO, expressed as a percentage of the pre-agonist amplitude (n = 3–6 neurons per concentration). The lines in (C) and (D) represent sigmoidal curve fits to the data. (A) and (B) are from the same neuron.

Discussion

The present study has demonstrated that morphine treatment has differential effects on the pre- and postsynaptic cellular actions of opioids and cannabinoids within the rat midbrain PAG. Morphine treatment produced a reduction in post-synaptic opioid inhibition of PAG neurons. By contrast, morphine treatment enhanced the opioid receptor agonist-induced presynaptic inhibition of GABAergic synaptic transmission. This enhanced opioid inhibition was, however, masked by an elevation in tonic endocannabinoid inhibition, which partly occluded the effect of exogenously applied agonists.

In the present study, the postsynaptic current induced by a maximal concentration of the μ receptor agonist DAMGO in PAG neurons was reduced following repeated morphine treatment. This postsynaptic opioid tolerance is consistent with earlier studies that have used higher dose, sustained release morphine treatment (100–300 mg·kg^{-1} over 5 days) (Bagley *et al.*, 2005a,b), but differs to others that have used the treatment paradigm of the present study (Ingram *et al.*, 2007; 2008). The differences between these studies might be due to a number of factors. Firstly, mixed agonists, such met-enkephalin and morphine, have been used in some studies (Ingram *et al.*, 2007; 2008) and there is an up-regulation of δ opioid receptor actions in opioid-treated animals (Hack *et al.*, 2005). Secondly, morphine treatment induces an opioid-coupled transporter current, which counteracts the reduction in GIRK activation (Bagley *et al.*, 2005b; Ingram *et al.*, 2007). The present study did not differentiate between these competing currents because the postsynaptic opioid current–voltage relationship was not examined. Finally, these differences might be due to alterations in desensitization, which occur following morphine treatment (Ingram *et al.*, 2008). Nonetheless, the present findings indicate that the morphine pretreatment protocol used in the present study is sufficient to produce tolerance at both the behavioural and cellular (postsynaptic) levels. It should also be noted that while cannabinoids do not have postsynaptic CB_1 receptor-mediated actions in PAG neurons from naïve animals (Vaughan *et al.*, 2000), we cannot rule out the possibility of postsynaptic CB_1 receptor mediated actions following morphine treatment.

In contrast to the postsynaptic tolerance, we found that the presynaptic inhibition of GABAergic synaptic transmission produced by an IC_{50} concentration of DAMGO was similar in PAG neurons from saline- and morphine-treated animals, at least under normal *in vitro* conditions. Like DAMGO, the presynaptic effect of an IC_{50} concentration of WIN55212 on GABA synaptic transmission in PAG neurons

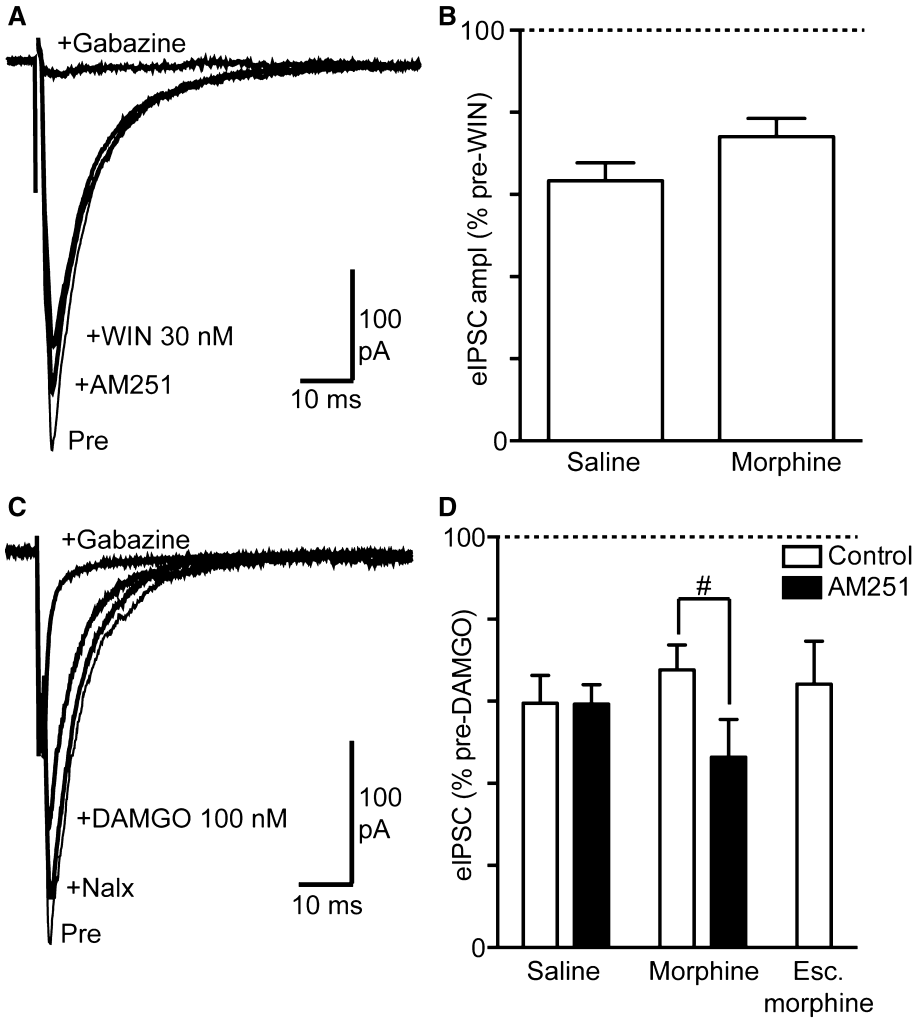

Figure 4

Effect of morphine treatment on cannabinoid and opioid-mediated inhibition of evoked IPSCs. Averaged records of evoked IPSCs (eIPSC) in two PAG neurons from morphine-treated animals showing the effect of IC_{50} concentrations of (A) WIN55212 and (C) DAMGO. In (A), traces are shown before (Pre), then after addition of WIN55212 (WIN 30 nM), AM251 (3 μM) and then gabazine (10 μM). In (C), traces are shown before (Pre), then after addition of DAMGO (100 nM), naloxone (Nalx, 1 μM) and then gabazine (10 μM). Summary bar charts of the mean effect of (B) WIN55212 (30 nM) and (D) DAMGO (100 nM) on the amplitude of eIPSC, expressed as a percentage of the pre-WIN55212 or DAMGO value. In (B) and (D), data are shown for saline-treated animals, and animals that underwent static (5 mg·kg^{-1}) and escalating morphine treatment (D only). In (D) data are shown for control slices (open bars) and slices superfused with AM251 (3 μM) for at least 15 min prior to the recording, and #denotes $P < 0.05$ for control versus AM251. In (B and D), all post-drug responses were significantly less than Pre-WIN55212 or DAMGO ($P < 0.05$–0.001).

was similar following saline and morphine treatment. Thus, repeated morphine treatment had no effect on the coupling efficiency of μ receptor- or CB_1 receptor-mediated inhibition of GABAergic synaptic transmission in the ventrolateral PAG. While the actions of cannabinoids in PAG have not been examined in morphine-treated animals previously, a decrease in the potency of DAMGO-induced presynaptic inhibition of GABAergic synaptic transmission has been observed in an earlier study that used the same morphine treatment paradigm (Fyfe *et al.*, 2010).

In the present study, the CB_1 receptor antagonist AM251 increased the frequency of spontaneous miniature IPSCs in PAG neurons from morphine, but not saline-treated animals, without affecting their amplitude or kinetics. This indicates that morphine treatment induces an endogenous cannabinoid tone, which acts via presynaptic CB_1 receptors to inhibit basal GABA release. This observation parallels that in mice where high-dose sustained release morphine treatment induced a presynaptic adenosine tone in PAG neurons (Hack *et al.*, 2003). It is unlikely that the enhanced tonic endocannabinoid inhibition was due to CB_1 receptor up-regulation within the PAG because expression of this receptor was unaffected by chronic morphine treatment (Wilson-Poe *et al.*, 2012). The effect of AM251 was also unlikely to be due to inverse CB_1 receptor agonism because it only affected IPSCs from morphine- (and not saline-) treated animals, indicating

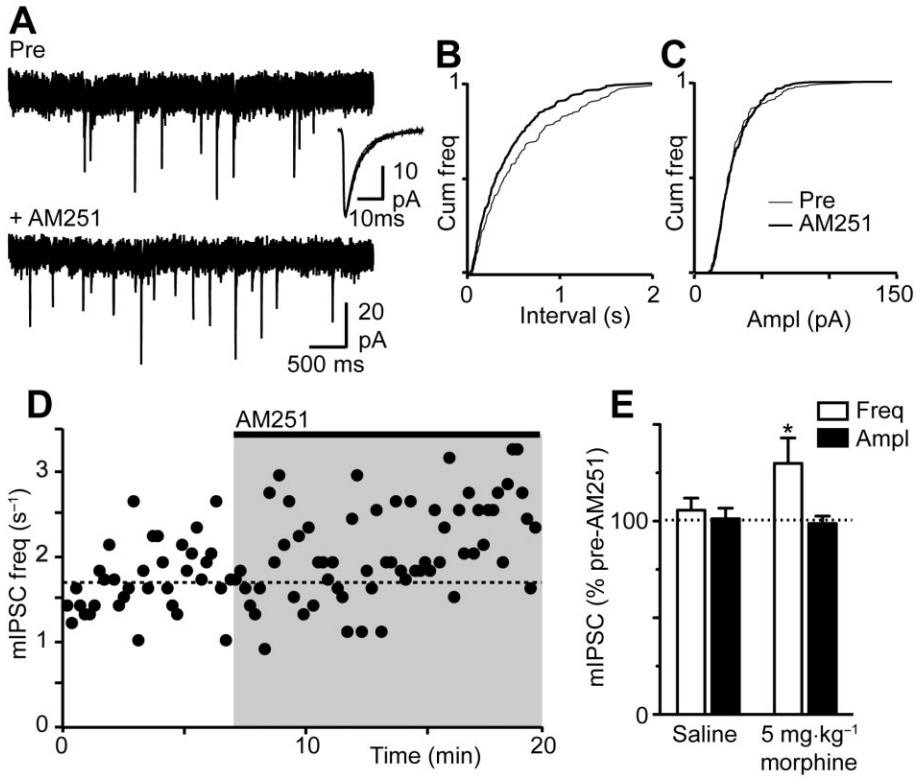

Figure 5

Morphine treatment enhances tonic endocannabinoid inhibition of miniature IPSCs. (A) Records of spontaneous miniature inhibitory post-synaptic currents miniature IPSCs (mIPSCs) before (Pre) and after addition of AM251 (3 μM) in a PAG neuron from a morphine-treated animal. Inset in (A): averaged mIPSCs obtained over 2 min epochs before and during AM251. (B) Inter-event interval and (C) amplitude cumulative distribution plots of mIPSCs before and during AM251 ($P < 0.03$ and >0.5, Kolmogorov–Smirnov statistic for Pre versus AM251; for inter-event interval and amplitude cumulative distributions). (D) Time course of the frequency of occurrence of mIPSCs during application of AM251, for the neuron depicted in (A–C). (E) Bar chart of the mean effect of AM251 on mIPSCs frequency and amplitude in PAG neurons from saline- and morphine- (static dosing, 5 mg·kg^{-1}) treated animals, expressed as a percentage of the pre-AM251 level, and *$P < 0.05$; significant difference between Pre and AM251.

that the effect was due to morphine pretreatment and not the ligand *per se*. Therefore, it is likely that the AM251-induced presynaptic facilitation of GABA release was due to an increase in endocannabinoid levels, as observed in other brain structures following chronic morphine treatment (Vigano *et al.*, 2003). While we did not identify the endocannabinoid(s) involved, both anandamide and 2-arachidonyl glycerol are present in the PAG (Hohmann *et al.*, 2005; Maione *et al.*, 2006) and their levels are elevated in other pathological states, such as neuropathic pain (Petrosino *et al.*, 2007).

The enhanced endocannabinoid-mediated tonic presynaptic inhibition was likely to influence the actions of exogenously applied agonists. In morphine-, but not saline-treated animals, it was observed that the DAMGO-induced inhibition of evoked IPSCs was enhanced when the endocannabinoid tone was blocked by the CB_1 receptor antagonist AM251. This enhancement of opioid agonist-induced presynaptic inhibition is similar to that observed in earlier studies using high dose, sustained release morphine treatment (Ingram *et al.*, 1998; Hack *et al.*, 2003). While an enhanced endocannabinoid tone increased basal presynaptic inhibition it occluded inhibition produced by higher concentrations of exogenously administered agonists. This occlusion might also account for the reduced opioid presynaptic inhibition observed in Fyfe *et al.* (2010, see earlier). These findings also suggest that cannabinoid presynaptic inhibition is likely to be enhanced following morphine treatment, although this could not be examined because both agonist and endocannabinoid inhibition would be abolished by AM251.

The PAG is a major site of opioid and cannabinoid analgesic actions within the CNS. While they act via partly different mechanisms, opioids and cannabinoids produce analgesia via GABAergic disinhibition within this brain region (Vaughan and Christie, 1997; Vaughan *et al.*, 2000). Both pre- and postsynaptic mechanisms contribute to opioid actions. This suggests that opioid tolerance within the PAG is largely determined by postsynaptic mechanisms, in spite of maintained (or even enhanced) opioid presynaptic disinhibition. By contrast, cannabinoids only have presynaptic actions within the PAG and the present results indicate that cannabinoid presynaptic inhibition is maintained following morphine treatment, and might even be enhanced when the elevated endocannabinoid tone is taken into account. These

findings are at least partly consistent with the finding that cannabinoid analgesia from within the PAG is enhanced in morphine-tolerant animals (Wilson *et al.*, 2008).

Besides the GABAergic neurotransmitter system, there are other potential sites of interaction between the opioid and cannabinoid systems that could account for alterations in cannabinoid-induced analgesia. Cannabinoid CB_1 receptor and opioid μ receptor activation also presynaptically inhibit excitatory glutamatergic synaptic transmission within the PAG (Vaughan and Christie, 1997; Vaughan *et al.*, 2000). While less is known about the effect of chronic morphine treatment on glutamatergic synaptic transmission within the PAG (Ingram *et al.*, 1998), the enhanced endocannabinoid tone could also influence glutamatergic synapses in this state. This action is, however, likely to be more complex because endocannabinoids modulate glutamatergic synaptic transmission within PAG via competing inhibitory CB_1 receptors and excitatory TRPV1 mechanisms (Kawahara *et al.*, 2011). In this regard, both CB_1 receptors and TRPV1 channels have been shown to have a role in endocannabinoid-induced analgesia within this brain structure (Maione *et al.*, 2006). Modulation of the endocannabinoid system might therefore represent a significant analgesic target to overcome morphine tolerance, as endocannabinoid degradation inhibitors also reduce the expression of opioid withdrawal (Shahidi and Hasanein, 2011; Ramesh *et al.*, 2013).

The present findings indicate that while morphine treatment produces tolerance at the behavioural and post-synaptic levels, opioid and cannabinoid presynaptic inhibition of GABAergic synaptic transmission is enhanced within the PAG. Furthermore, repeated morphine produces an elevation in basal endocannabinoid inhibition of GABAergic synaptic transmission. This compensatory change in endocannabinoid tone could be exploited in the design and development of novel analgesics for use in a morphine-tolerant state.

Acknowledgements

This work was supported by the National Institute on Drug Abuse of the National Institutes of Health under Award Number hrF32DA034464. We would like to thank Vanessa Mitchell for her technical assistance during data collection.

Author contributions

Study design, data analysis, figure and manuscript preparation were done by A. R. W.-P. and C. W. V. Data collection was performed by A. R. W.-P. and B. K. L.

Conflict of interest

The authors declare that there are no conflicts of interests.

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