BJP British Journal of Pharmacology

DOI:10.1111/bph.12839
www.brjpharmacol.org

RESEARCH PAPER

Endocannabinoid modulation by FAAH and monoacylglycerol lipase within the analgesic circuitry of the periaqueductal grey

Benjamin K Lau, Geoffrey M Drew, Vanessa A Mitchell and Christopher W Vaughan

Pain Management Research Institute, Kolling Institute of Medical Research, Northern Clinical School, The University of Sydney at Royal North Shore Hospital, St Leonards, NSW, Australia

Correspondence
Dr Benjamin Lau, Pain Management Research Institute, Kolling Institute of Medical Research, Level 13, Kolling Building, Royal North Shore Hospital, St. Leonards, NSW 2065, Australia. E-mail: benjamin.lau@sydney.edu.au

Received
21 January 2014
Revised
19 June 2014
Accepted
27 June 2014

BACKGROUND AND PURPOSE

Endogenous cannabinoids (endocannabinoids) in the periaqueductal grey (PAG) play a vital role in mediating stress-induced analgesia. This analgesic effect of endocannabinoids is enhanced by pharmacological inhibition of their degradative enzymes. However, the specific effects of endocannabinoids and the inhibitors of their degradation are largely unknown within this pain-modulating region.

EXPERIMENTAL APPROACH

In vitro electrophysiological recordings were conducted from PAG neurons in rat midbrain slices. The effects of the major endocannabinoids and their degradation inhibitors on inhibitory GABAergic synaptic transmission were examined.

KEY RESULTS

Exogenous application of the endocannabinoid, anandamide (AEA), but not 2-arachidonoylglycerol (2-AG), produced a reduction in inhibitory GABAergic transmission in PAG neurons. This AEA-induced suppression of inhibition was enhanced by the fatty acid amide hydrolase (FAAH) inhibitor, URB597, whereas a 2-AG-induced suppression of inhibition was unmasked by the monoacylglycerol lipase (MGL) inhibitor, JZL184. In addition, application of the CB_1 receptor antagonist, AM251, facilitated the basal GABAergic transmission in the presence of URB597 and JZL184, which was further enhanced by the dual FAAH/MGL inhibitor, JZL195.

CONCLUSIONS AND IMPLICATIONS

Our results indicate that AEA and 2-AG act via disinhibition within the PAG, a cellular action consistent with analgesia. These actions of AEA and 2-AG are tightly regulated by their respective degradative enzymes, FAAH and MGL. Furthermore, individual or combined inhibition of FAAH and/or MGL enhanced tonic disinhibition within the PAG. Therefore, the current findings support the therapeutic potential of FAAH and MGL inhibitors as a novel pharmacotherapy for pain.

Abbreviations

2-AG, 2-arachidonoylglycerol; ABHD6/ABHD12, abhydrolase domain-containing protein 6/12; ACSF, artificial CSF; AEA/anandamide, arachidonoyl ethanolamide; FAAH, fatty acid amide hydrolase; JZL195, 4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate; MGL, monoacylglycerol lipase; O-2050, (6a*R*,10a*R*)-1-hydroxy-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b,d*]pyran; PAG, periaqueductal grey; RVM, rostral ventromedial medulla

Table of Links

TARGETS	LIGANDS
CB_1 receptor	AM251
FAAH	Anandamide (AEA)
$GABA_A$ receptor	CNQX
Glycine receptor	JZL184
M_1 receptor	Tetrahydrolipstatin (orlistat)
M_3 receptor	URB597
MGL	WWL70
mGlu receptor	2-AG
TRPV1 channel	Tetrodotoxin
Voltage-dependent sodium channel	

This Table lists key protein targets and ligands in this document, 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 are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b,c,d).

Introduction

The midbrain periaqueductal grey (PAG) serves an important function in modulating pain and analgesia (Mansour *et al.*, 1995; Lichtman *et al.*, 1996; Finn *et al.*, 2003). It is a major site of analgesic action by exogenous cannabinoid agonists. Endogenous ligands of the cannabinoid system, whose actions are mimicked by exogenous agonists, also play a vital role in controlling nociception in this brain region. This is evidenced by elevated levels of the major endogenous cannabinoids (endocannabinoids), 2-arachidonoylglycerol (2-AG) and anandamide (AEA), observed in the PAG following acute noxious stimuli, or in chronic pain models (Walker *et al.*, 1999; Hohmann *et al.*, 2005; Petrosino *et al.*, 2007). The physiological significance of endocannabinoids in the PAG was previously highlighted in a study by Hohmann *et al.* (2005), who showed that the non-opioid component of stress-induced analgesia is mediated by endocannabinoids. Thus, endocannabinoids function in parallel with endogenous opioids in the PAG to mediate an animal's analgesic response to physical and psychological stressors.

Both exogenous and endogenous cannabinoids are thought to act predominantly via cannabinoid receptor type 1 (CB_1 receptor) (channel nomenclature follows Alexander *et al.*, 2013) to produce their antinociceptive effects in pain-modulating regions of the CNS (Rice, 2006). In particular, the brainstem circuit comprising the PAG and rostral ventromedial medulla (RVM) is rich in CB_1 receptors (Tsou *et al.*, 1998). Microinjection of cannabinoid agonists directly into the PAG or RVM elicits analgesia (Lichtman *et al.*, 1996; Martin *et al.*, 1998; 1999; Finn *et al.*, 2003), which is blocked in the presence of a CB_1 receptor antagonist (Martin *et al.*, 1998; Finn *et al.*, 2003). Conversely, systemic administration of a CB_1 receptor antagonist elicits hyperalgesia (Richardson *et al.*, 1997; 1998; Meng *et al.*, 1998; Strangman *et al.*, 1998).

Like opioids, cannabinoids produce antinociception by activating a descending analgesic pathway, which projects to the spinal cord via the PAG and RVM. At the cellular level, activation of this descending PAG–RVM system is thought to occur via a process of disinhibition, that is, suppression of inhibitory GABAergic synaptic transmission in the PAG and RVM (Basbaum and Fields, 1984; Meng *et al.*, 1998). In support of this hypothesis, *in vitro* electrophysiological studies have shown that exogenously applied cannabinoid agonists presynaptically inhibit GABAergic inputs in the PAG, RVM and spinal cord dorsal horn (Vaughan *et al.*, 1999; 2000; Jennings *et al.*, 2001). Although endocannabinoids are assumed to produce antinociception in a similar fashion, their direct cellular actions have yet to be confirmed in these pain-modulating regions. Nevertheless, we have recently shown that endocannabinoids induced by activation of the G_q-coupled group 1 metabotropic glutamate receptors (mGlu receptors) and M_1/M_3 muscarinic acetylcholine receptors inhibit GABAergic transmission in the PAG (Drew *et al.*, 2008; 2009; Lau and Vaughan, 2008; Mitchell *et al.*, 2011).

Endocannabinoid signalling is under the strict control of specific degradative enzymes. This includes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL), which predominantly metabolize AEA and 2-AG respectively (Di Marzo, 2008). There is also recent evidence showing that the serine hydrolases, abhydrolase domain-containing protein 6 and 12 (ABHD6 and ABHD12), metabolize 2-AG within the brain (Blankman *et al.*, 2007; Marrs *et al.*, 2010; Savinainen *et al.*, 2012). In recent years, the development of novel agents targeting the endocannabinoid system has been the focus of considerable clinical research. Specific inhibitors of FAAH, MGL and ABDH6/12 have been developed, which block the degradation of these endogenous transmitters (Boger *et al.*, 2000; Kathuria *et al.*, 2003; Long *et al.*, 2009a). In particular, both FAAH and MGL inhibitors have been explored as potential pharmacotherapies to treat pain with reduced side effects (Di Marzo, 2008). The rationale is that inhibition of FAAH and MGL selectively enhances endocannabinoids where they are produced on demand, thus resulting in more localized

receptor activation. Indeed, a number of studies have demonstrated that these agents produce antinociception with fewer side effects compared with globally acting exogenous cannabinoids (Kathuria *et al.*, 2003; Holt *et al.*, 2005; Jayamanne *et al.*, 2006; Russo *et al.*, 2007; Long *et al.*, 2009a). Although numerous functional studies have investigated the analgesic efficacy of FAAH/MGL inhibitors in animal models of pain (Kathuria *et al.*, 2003; Holt *et al.*, 2005; Jayamanne *et al.*, 2006; Russo *et al.*, 2007; Long *et al.*, 2009a), the cellular mechanisms by which these agents enhance endocannabinoid signalling have not been examined in pain pathways. Given the crucial role of the inhibitory neurotransmitter system in controlling nociception, the present study investigated the actions of endocannabinoids on GABAergic synaptic transmission within the PAG, and their modulation by degradation inhibitors.

Methods

Slice preparation

All experiments were performed on male/female Sprague–Dawley rat pups (16–24 days old), under a protocol approved by the Royal North Shore Hospital Animal Care and Ethics Committee. Animals were anaesthetized with isoflurane, decapitated, the brain rapidly removed and then placed into ice-cold artificial CSF (ACSF) of the following composition: 126 mM NaCl, 2.5 mM KCl, 1.4 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 11 mM glucose and 25 mM $NaHCO_3$, equilibrated with 95% O_2 and 5% CO_2. Coronal midbrain slices (300 μM) containing PAG tissue were then cut using a Vibratome (VT1000S; Leica Microsystems, Nussbloch, Germany) in ice-cold ACSF, as described previously (Vaughan *et al.*, 2000). The slices were maintained at 34°C in a submerged chamber containing ACSF. Before the recordings, each slice was individually transferred to a recording chamber, where it was continually superfused (1.6–1.8 mL·min^{-1}) with ACSF (34°C).

Electrophysiology

PAG neurons were visualized using infrared Dodt gradient contrast optics on an upright microscope (Olympus BX50; Olympus, Sydney, Australia). Whole-cell voltage clamp recordings (holding potential: −65 mV) were conducted via an Axopatch 700B patch clamp amplifier (Molecular Devices, Sunnyvale, CA, USA), using an internal solution of the following composition: 140 mM CsCl, 10 mM HEPES, 0.2 mM EGTA, 1 mM $MgCl_2$, 2 mM MgATP and 0.3 mM NaGTP (pH = 7.3; osmolarity ~280–285 mOsM). Series resistance (<30 MΩ) was compensated by 80% and continuously monitored during experiments. Liquid junction potentials of −4 mV were corrected. Recordings were mainly restricted to the ventrolateral PAG.

Data analysis

Miniature inhibitory postsynaptic currents (IPSCs) were filtered (2–5 kHz low-pass filter) and sampled (5–10 kHz) for online and later offline analysis (Axograph X; Axograph Scientific Software, Sydney, Australia). Miniature IPSCs were sampled in 5 s epochs every 6 s for analysis, and IPSCs above a preset threshold (5.0 SD above baseline noise) were automatically detected by a sliding template algorithm and then manually checked offline. Plots of detected event frequency versus time and cumulative probability distributions of event amplitudes and inter-event intervals were constructed.

Statistical differences from a theoretical mean within a group were calculated using a one-sampled *t*-test. Statistical comparisons between two groups were made using Student's paired *t*-tests, whereas those between more than two groups were made using a one-way ANOVA, followed by *post hoc* comparisons using the Dunnett correction for multiple comparisons (Prism; GraphPad Software Inc., San Diego, CA, USA). When data did not conform to a normal Gaussian distribution (via D'Agostino–Pearson omnibus test), they were logarithmically (log_{10}) transformed for analysis. Differences were considered statistically significant when $P < 0.05$. All pooled data are expressed as means ± SEM.

Drugs

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and strychnine hydrochloride were obtained from Sigma (Sydney, Australia). Tetrodotoxin (TTX) was obtained from Ascent Scientific (Bristol, UK). 2-AG, arachidonoylethanolamide (AEA), 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (AM251), 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate (JZL184), 4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate (JZL195) and (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate (URB597) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). N-methyl-N-[[3-(4-pyridinyl)phenyl] methyl]-4′-(aminocarbonyl)[1,1′-biphenyl]-4-yl ester, carbamic acid (WWL70) was obtained from Tocris Bioscience (Bristol, UK). Stock solutions of all drugs were diluted to working concentrations in ACSF (1000–1 000 000 times dilution) prior to use. In a number of experiments, inhibitors of endocannabinoid enzymatic degradation were utilized. In such instances, slices were pre-incubated in URB597, JZL184, JZl195 or WWL70 for at least 60 min before the recordings were performed.

Results

To first determine which endocannabinoids modulate GABAergic synaptic transmission in the PAG, the actions of exogenously applied AEA and 2-AG were examined on spontaneous miniature IPSCs. In the presence of the non-NMDA receptor antagonist, CNQX (5 μM), the glycine receptor antagonist, strychnine (5 μM) and the voltage-dependent sodium channel blocker, TTX (300 nM), miniature IPSCs were readily observed (Figure 1D). These events were $GABA_A$ receptor mediated, as they were abolished following addition of the $GABA_A$ receptor antagonist, SR95531 (10 μM).

Actions of exogenously applied anandamide

In untreated control slices, superfusion of AEA (30 μM) produced a minor reduction in the rate of miniature IPSCs, but had no effect on their amplitude or kinetics. On average, AEA decreased miniature IPSC rate to 79 ± 4% of the pre-AEA level

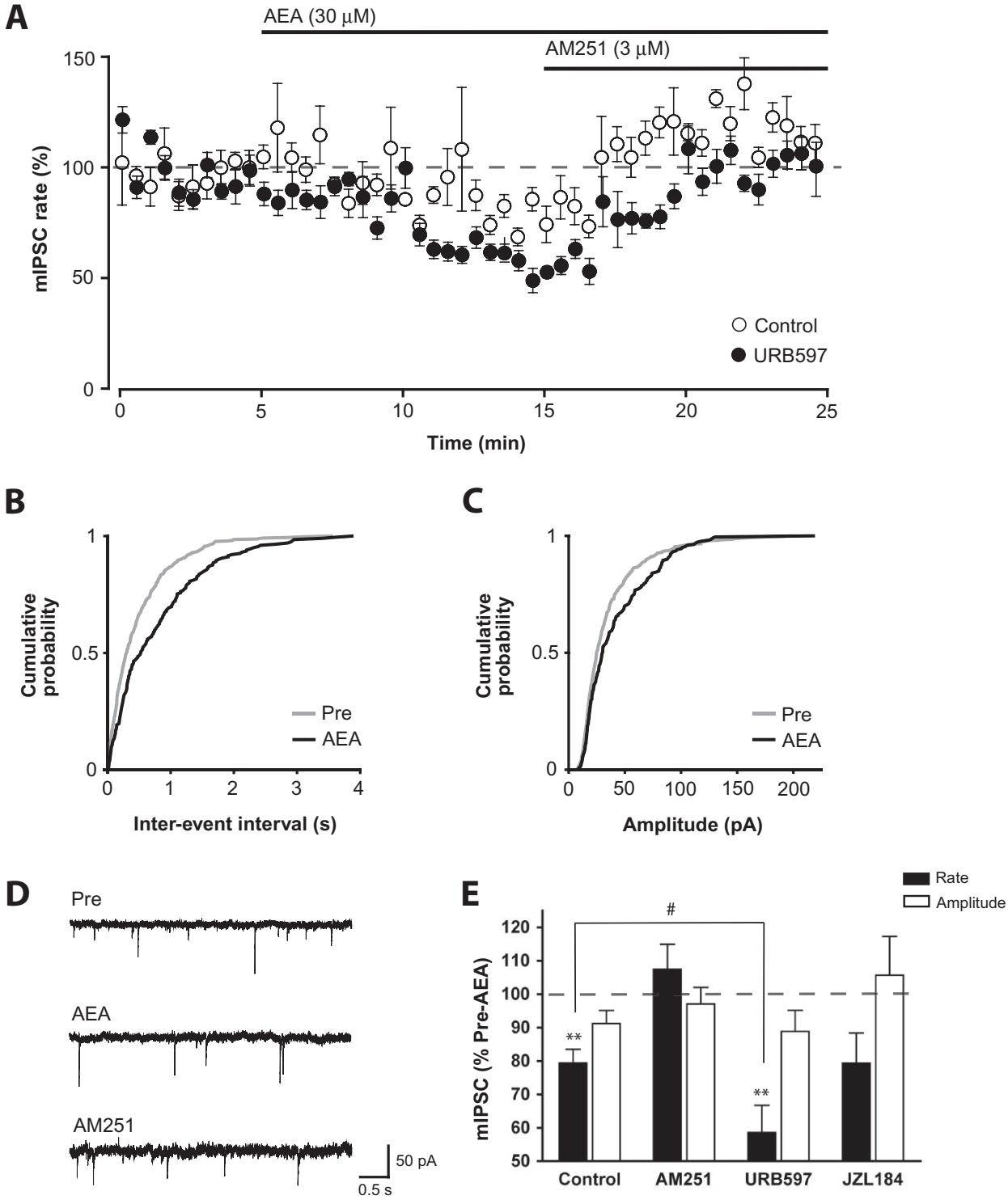

Figure 1

AEA decreases the rate but not the amplitude of miniature IPSCs and this is enhanced by a FAAH inhibitor. (A) Averaged time course of miniature IPSC rate during superfusion of AEA (30 μM), and then subsequent addition of AM251 (3 μM) in the absence or presence of the FAAH inhibitor, URB597 (1 μM). Miniature IPSC rate is expressed as % of the normalized pre-drug value. (B–C) Cumulative distribution plots of miniature IPSC (B) inter-event interval and (C) amplitude, before and during AEA application in the presence of URB597. (D) Raw current traces of miniature IPSCs before (pre) and during superfusion of AEA, and following addition of AM251. (E) Bar chart of the mean rate and amplitude of miniature IPSCs during superfusion of AEA, expressed as % of the pre-AEA level in untreated slices (Control), or slices pretreated with AM251 (3 μM), URB597 (1 μM) or JZL184 (1 μM). ** Denotes a significant difference compared with the pre-AEA value ($P < 0.01$), while # denotes a significant difference compared with Control ($P < 0.05$).

(range = 68–92%, $P < 0.01$), while miniature IPSC amplitude was 91 ± 4% ($P > 0.05$, $n = 6$; Figure 1A,E, Control). The inhibitory effect of AEA on miniature IPSC rate was reversed following addition of the CB_1 receptor antagonist, AM251 (3 μM) ($n = 4$; Figure 1A). Similarly, in slices pretreated with AM251, superfusion of AEA had no effect on miniature IPSC rate or amplitude (108 ± 7 and 97 ± 5% of the pre-AEA level, respectively, $P > 0.05$, $n = 4$; Figure 1E, AM251).

The effect of blocking the main AEA-hydrolysing enzyme, FAAH was subsequently examined on the AEA-induced suppression of inhibitory transmission. In slices pretreated with a supramaximal concentration of the FAAH inhibitor, URB597 (1 μM) (Kathuria *et al.*, 2003), AEA produced a significant reduction in the rate, but not amplitude of miniature IPSCs, which was reversed following addition of AM251 (Figure 1A,D). Under these conditions, miniature IPSC rate was reduced to 59 ± 8% of the pre-AEA level (range = 22–79%, $P < 0.01$), while miniature IPSC amplitude remained at 89 ± 6% ($P > 0.05$; Figure 1E, URB597). Importantly, the reduction of miniature IPSC rate by AEA was significantly greater in the presence of URB597 compared with that observed in untreated control slices ($P < 0.05$; Figure 1E, Control vs. URB597). The change in rate, but not amplitude of miniature IPSCs, was also reflected by a rightward shift in the cumulative inter-event interval distribution, and a lack of change in cumulative amplitude distribution (Figure 1B,C). The observed change in rate of miniature IPSCs suggests a presynaptic locus of action, while the negligible effect on their amplitude indicates the absence of a postsynaptic change in $GABA_A$ receptors, or other membrane conductances.

The above actions of AEA were specifically modulated by FAAH, as blockade of the 2-AG-hydrolysing enzyme MGL produced no further enhancement of the AEA-induced suppression of inhibition. In the presence of a supramaximal concentration of the MGL inhibitor, JZL184 (1 μM) (Long *et al.*, 2009a), AEA reduced miniature IPSC rate to a similar extent as that observed under control conditions ($P > 0.05$; Figure 1E, Control vs. JZL184). On average, miniature IPSC rate and amplitude was 81 ± 8 and 105 ± 11% of the pre-AEA level respectively ($n = 6$; Figure 1E, JZL184). Thus, exogenously applied AEA suppresses GABAergic synaptic transmission in the PAG, and this is specifically enhanced by inhibition of FAAH, but not by MGL. A similar action by AEA has previously been observed on excitatory glutamatergic synaptic transmission in the PAG (Kawahara *et al.*, 2011).

Actions of exogenously applied 2-AG

In contrast to AEA, superfusion of 2-AG (30 μM) had no effect on miniature IPSC rate or amplitude in untreated control slices (Figure 2A). On average, the rate and amplitude of miniature IPSCs was 97 ± 6 (range = 85–122%) and 96 ± 5% of the pre-2-AG level respectively ($P > 0.05$, $n = 7$; Figure 2E, Control). By contrast, in slices pre-incubated in the MGL inhibitor, JZL184 (1 μM), 2-AG produced a significant suppression of miniature IPSC rate, but not amplitude, which was reversed by addition of AM251 (3 μM) ($n = 5$; Figure 2A). On average, 2-AG reduced the rate of miniature IPSCs to 77 ± 7% of the pre-2-AG level (range = 43–101%, $P < 0.05$), while miniature IPSC amplitude was 91 ± 5% in the presence of JZL184 ($P > 0.05$, $n = 11$; Figure 2E, JZL184). The change in rate, but not amplitude of miniature IPSCs, was reflected by a rightward shift in the cumulative inter-event interval distribution, and a lack of change in cumulative amplitude distribution (Figure 2B,C). This is indicative of a presynaptic locus of action by 2-AG.

The actions of 2-AG were specifically modulated by MGL as inhibition of FAAH did not unmask a 2-AG-induced suppression of inhibition. Specifically, in slices pretreated with URB597 (1 μM), 2-AG application had no significant effect on miniature IPSC rate or amplitude (91 ± 14 and 95 ± 12% of the pre-2-AG level, $P > 0.05$, $n = 5$; Figure 2E, URB597). Thus, the above results suggest that exogenously applied 2-AG has a negligible effect on GABAergic transmission, which may be enhanced following inhibition of MGL, but not FAAH. It should be noted that the minimal 2-AG effect observed in this study contrasts the moderate-to-high 2-AG-induced suppression of inhibitory transmission reported in the hippocampus (Kim and Alger, 2004; Hashimotodani *et al.*, 2007).

Endocannabinoid tone in the PAG

The previous experiments indicate that, under certain conditions, exogenous application of AEA and 2-AG presynaptically inhibits GABAergic synaptic transmission. Microdialysis studies have indicated the presence of both AEA and 2-AG in the PAG under basal conditions (Hohmann *et al.*, 2005; Maione *et al.*, 2006). The presence of endogenous cannabinoid tone in this region was investigated by examining the action of a CB_1 receptor antagonist on spontaneous miniature IPSCs. In untreated control slices, superfusion of AM251 (3 μM) did not significantly alter the rate of miniature IPSCs (118 ± 10% of pre-AM251 level, $P = 0.07$, $n = 10$; Figure 3A,F, Control); however, a trend towards increased facilitation was observed. By contrast, in slices pre-incubated with a supramaximal concentration of UBR597 (1 μM) or JZL184 (1 μM), AM251 application significantly increased miniature IPSC rate (146 ± 13 and 160 ± 18% of pre-AM251 level, $P < 0.05$, $n = 11$, 11; Figure 3B,C,F). Although the magnitude of this AM251-induced facilitation appeared greater in the presence of FAAH or MGL inhibition, it was not significantly different from that observed in their absence ($P > 0.05$, Kruskal–Wallis test, Control vs. URB597 vs. JZL184). With the recent development of a dual inhibitor for both FAAH and MGL, we examined whether concurrent inhibition of both enzymes would further increase endocannabinoid tone. In slices pre-incubated in a supramaximal concentration of the dual FAAH/MGL inhibitor, JZL195 (1 μM) (Long *et al.*, 2009b), AM251 produced a significant increase in miniature IPSC rate (162 ± 23% of pre-AM251 level, $P < 0.05$, $n = 16$; Figure 3D,F), which was significantly different to that produced in untreated control slices, or those pre-incubated in URB597 or JZL184 ($P > 0.05$).

Recently, the serine hydrolases ABHD6 and ABHD12 were shown to participate in the hydrolysis of 2-AG in a number of brain regions (Blankman *et al.*, 2007). However, the presence of these enzymes within the PAG has yet to be determined. Therefore, the effect of ABHD6/ABHD12 inhibition was investigated. In slices pre-incubated in a supramaximal concentration of the ABHD6 inhibitor, WWL70 (10 μM) (Blankman *et al.*, 2007; Marrs *et al.*, 2010), AM251 application did not significantly alter miniature IPSC rate (122 ± 15% of pre-AM251 level, $P > 0.05$, $n = 8$; Figure 3E,F, WWL70), indicating an absence or lack of basal modulation by ABHD6.

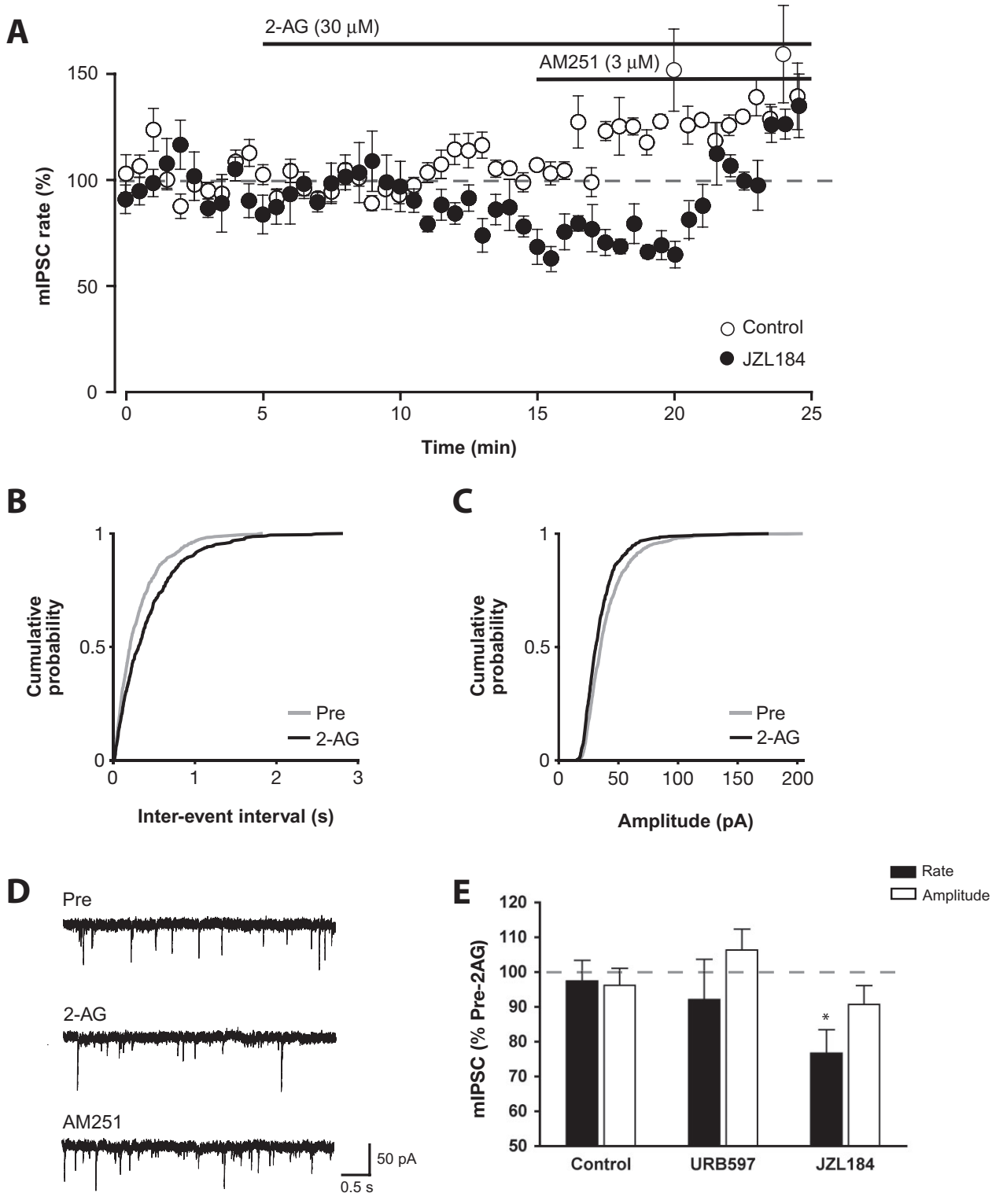

Figure 2

2-AG decreases the rate but not the amplitude of miniature IPSCs in the presence of a MGL inhibitor, but has no effect alone. (A) Averaged time course of miniature IPSC rate during superfusion of 2-AG (30 μM), and then subsequent addition of AM251 (3 μM) in the absence or presence of the MGL inhibitor, JZL184 (1 μM). Miniature IPSC rate is expressed as % of the normalized pre-drug value. (B–C) Cumulative distribution plots of miniature IPSC (B) inter-event interval and (C) amplitude, before and during 2-AG application in the presence of JZL184. (D) Raw current traces of miniature IPSCs before (pre) and during superfusion of 2-AG, and following addition of AM251. (E) Bar chart of the mean rate and amplitude of miniature IPSCs during superfusion of 2-AG, expressed as % of the pre-2-AG level in untreated slices (Control) and slices pretreated with URB597 (1 μM) or JZL184 (1 μM). In (E), *denotes a significant difference compared with the pre-2-AG value ($P < 0.05$).

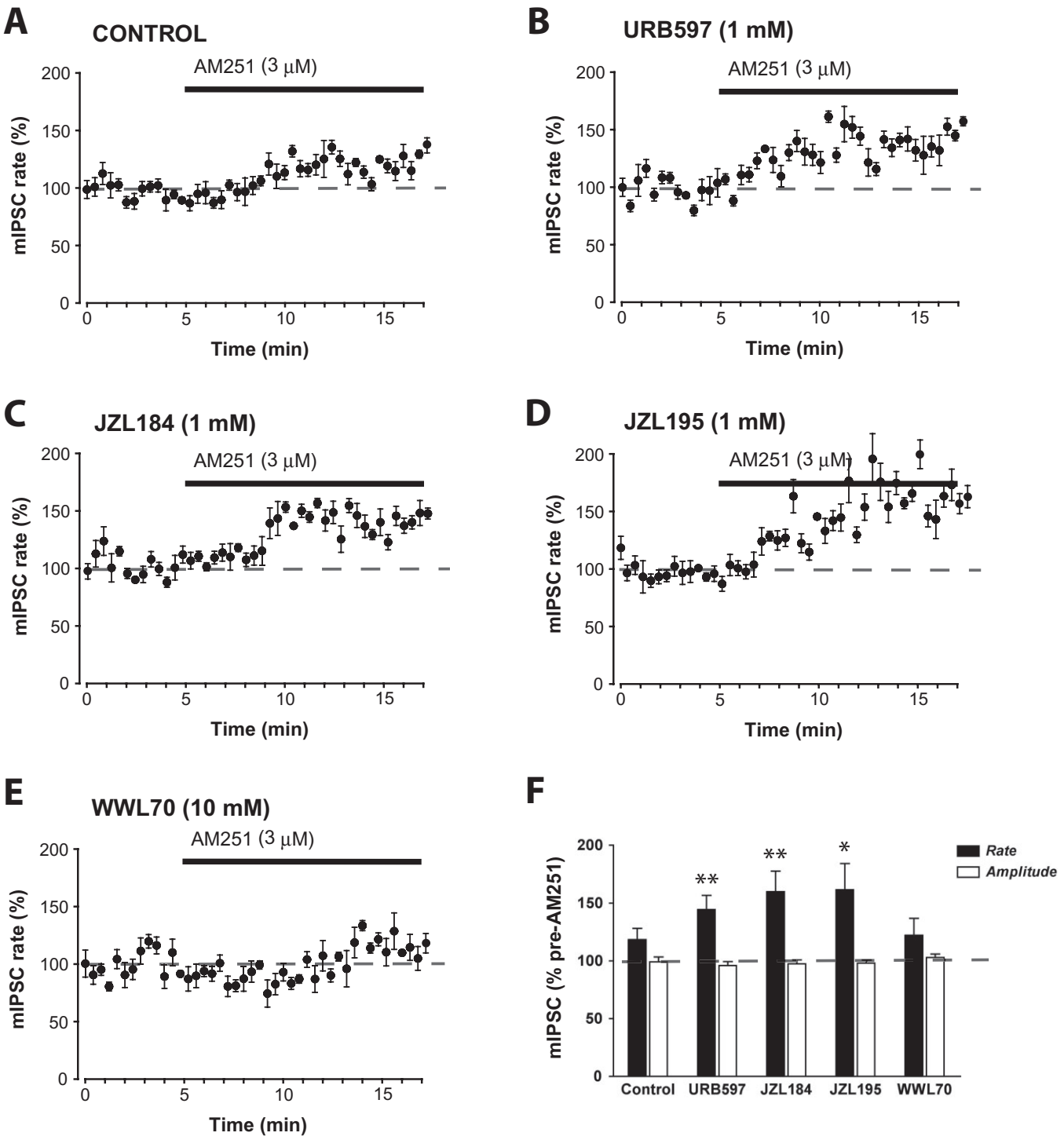

Figure 3

The endocannabinoids, AEA and 2-AG tonically mediate spontaneous inhibitory synaptic transmission. (A–E) Normalized average time courses of miniature IPSC rate during superfusion of AM251 (3 μM) in the presence of (A) Control, (B) URB597 (1 μM), (C) JZL184 (1 μM), (D) JZL195 (1 μM) and (E) WWL70 (10 μM). (F) Bar chart showing the mean rate and amplitude of miniature IPSCs during application of AM251. In (A–E), data were normalized in each recording over the 5 min preceding AM251 application and averaged across all neurons. In (F), rate and amplitude values were calculated by measuring the 7–12 min period following application of AM251. This was expressed as % of the pre-AM251 value. A significant difference compared with the pre-AM251 level is denoted as *$P < 0.05$ and **$P < 0.01$.

In all the above experiments, AM251 did not affect miniature IPSC amplitude in the absence or presence of URB597, JZL184, JZL195 or WWL70 (99 ± 15, 98 ± 18, 97 ± 12, 98 ± 11 and 103 ± 8% of pre-AM251 level, $P > 0.05$; Figure 3F). This indicated that the degradation inhibitors examined do not have direct postsynaptic effects, nor do the endocannabinoids they enhance.

In addition to spontaneous miniature IPSCs, we also examined endocannabinoid modulation of synchronous-evoked IPSCs elicited via electrical stimulation. In the presence of CNQX (5 μM) and strychnine (5 μM), electrically evoked IPSCs were elicited in PAG neurons via unipolar glass or bipolar tungsten-stimulating electrodes, placed ~20–100 μm away from the recording electrode (rate: 0.083 Hz, stimuli: 5–40 V, 25–200 ms). In untreated control slices, superfusion of AM251 (3 uM) had no effect on evoked IPSC amplitude (98 ± 3%, $P > 0.05$, $n = 6$; Figure 4A,E). By contrast, in slices pretreated with URB597, JZL184 and JZL195, superfusion of AM251 significantly enhanced evoked IPSC amplitude (132 ± 6, 138 ± 10, 182 ± 18%, $P < 0.01$, 0.05, 0.01, n = 7, 6, 6; Figure 4B–E). Unlike our observations on miniature IPSCs, the AM251-induced facilitation of evoked IPSCs was significantly greater in the presence of JZL195 than URB597 or JZL184 ($P < 0.05$, Newman–Keuls multiple comparisons test; Figure 4E, JZL195 vs. URB597/JZL184). Together, the above results suggest the presence of an endocannabinoid tone modulating GABAergic transmission, which is strictly regulated by FAAH and MGL, but not ABHD6.

Constitutive activity does not regulate the tonic action at CB_1 receptors

In the preceding experiments, the facilitatory effect of AM251 on inhibitory transmission may have been mediated by either blockade of basal endocannabinoid tone or inhibition of constitutive activity at the CB_1 receptor. This latter possibility could not be excluded as AM251 acts as an inverse agonist. To address this issue, we additionally examined the effect of a neutral antagonist.

Similar to AM251, the neutral CB_1 receptor antagonist, O-2050 (1 μM) did not significantly increase miniature IPSC rate under these conditions (118 ± 13%, $P > 0.05$, $n = 7$; Figure 5A,B, control), but did in the presence of JZL195 (1 μM) (165 ± 12%, $P < 0.01$, $n = 8$; Figure 5A,B, JZL195). This O-2050-induced facilitation observed in the presence of the dual FAAH/MGL inhibitor was significantly greater than that in its absence ($P < 0.05$, unpaired t-test; Figure 5D, Control vs. JZL195). Thus, it appears that an endocannabinoid tone, rather constitutive activity, accounts for the AM251-induced facilitation of GABAergic transmission.

We finally examined whether blockade of endocannabinoid production disrupted the AM251-induced facilitation of GABAergic transmission. In the presence of the DAG lipase inhibitor, tetrahydrolipstatin (THL, 10 uM), AM251 (3 uM) had no effect on miniature IPSC rate when applied alone, or in the presence of JZL184 (111 ± 12 and 107 ± 5% of pre-AM251 level, respectively; $P > 0.05$; Figure 5C,D, Control, JZL184), indicating that the endocannabinoid 2-AG was likely to mediate the tonic CB_1 receptor-mediated inhibition of miniature IPSCs. By contrast, AM251 still significantly increased miniature IPSC rate in the presence of JZL195 (160 ± 13% of pre-AM251 level; $P < 0.01$; Figure 5C,D, JZL195),

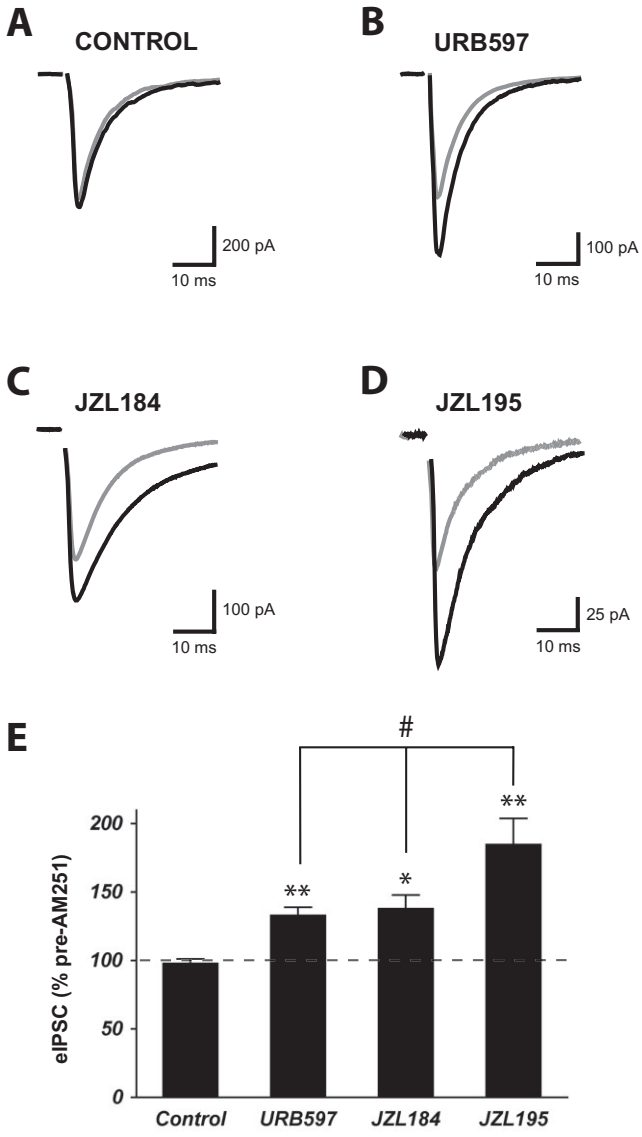

Figure 4

AEA and 2-AG tonically mediate evoked inhibitory synaptic transmission. (A–D) Averaged traces of evoked IPSC amplitude before and during superfusion of AM251 (3 μM) in the presence of (A) Control, (B) URB597 (1 μM), (C) JZL184 (1 μM), (D) JZL195 (1 μM). (E) Bar chart of evoked IPSC amplitude during superfusion of AM251 in the presence of control, URB597, JZL184 or JZL195. A significant difference compared with the pre-AM251 level is shown as *$P < 0.05$ and **0.01, while # denotes a significant difference compared with Control ($P < 0.05$).

consistent with the preserved increased level of AEA expected in the presence of a dual enzyme inhibitor.

Discussion

Although the role of endocannabinoids in modulating pain has been well established, the cellular mechanisms underly-

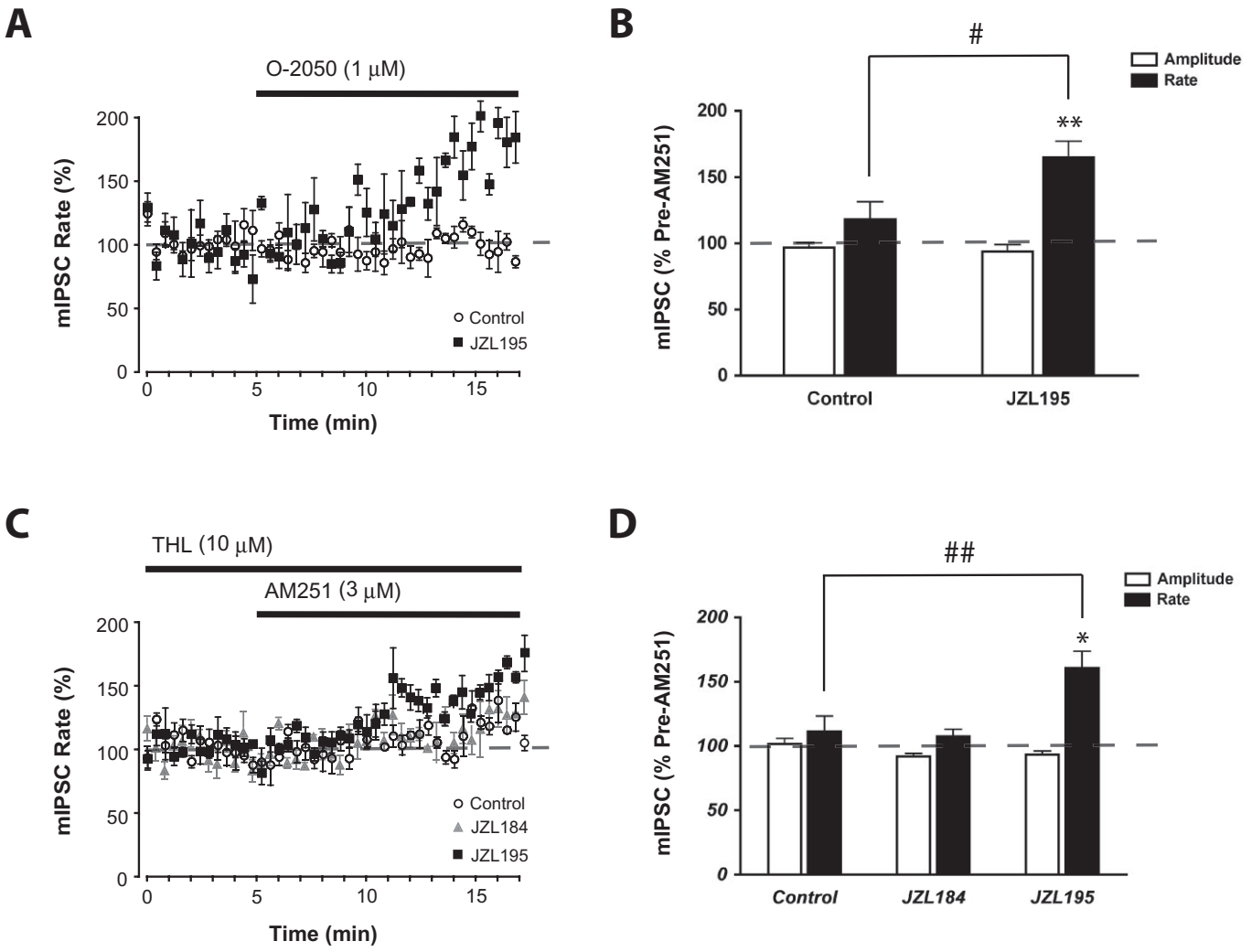

Figure 5

Endocannabinoids rather than constitutive activity mediate the inhibitory tone at CB_1 receptors. (A) Averaged time course of miniature IPSC rate during superfusion of the neutral CB_1 receptor antagonist, O-2050 (1 μM) in the absence or presence of JZL195 (1 μM). (B) Bar chart of the mean miniature IPSC rate and amplitude during superfusion of O-2050 in the presence of Control or JZL195. (C) Averaged time course of miniature IPSC rate during superfusion of AM251 (3 μM) in the presence of the DAG lipase inhibitor, THL + Control/JZL184/JZL195. (D) Bar chart of the mean miniature IPSC rate and amplitude during superfusion of AM251 in the presence of THL + Control/JZL184/JZL195. In (B) and (D), a significant difference compared with the pre-AM251 level is shown as *$P < 0.05$ and **0.01, while # and ## denote a significant difference compared with Control ($P < 0.05$ and 0.01 respectively).

ing their analgesic action are largely unconfirmed. Here we have demonstrated that the major endocannabinoids, AEA and 2-AG, act presynaptically via CB_1 receptors to suppress inhibitory GABAergic transmission in the PAG, a region that plays a pivotal role in the analgesic actions endocannabinoids. The inhibition of GABAergic transmission produced by AEA and 2-AG was selectively enhanced by inhibitors of the endocannabinoid degradation enzymes, FAAH and MGL respectively. Furthermore, inhibition of FAAH and MGL unmasked a tonic GABAergic disinhibition. Together, these results suggest that under basal conditions, GABAergic transmission in the PAG is under the influence of endocannabinoids, but this is strictly regulated by enzymatic degradation, at least *in vitro*. These findings provide a potential basis for the use of FAAH and MGL inhibitors as analgesic targets.

The present experiments on miniature IPSCs demonstrated that endocannabinoids act via a presynaptic locus of action to produce disinhibition in the PAG. The CB_1 receptor-mediated suppression of GABAergic transmission elicited by AEA and 2-AG is similar to that previously reported for synthetic cannabinoid agonists in the PAG–RVM system (Vaughan *et al.*, 1999; 2000), and is consistent with the presynaptic anatomical localization of CB_1 receptors on inhibitory nerve terminals throughout this region (Tsou *et al.*, 1998). Ultimately, such an action is concordant with the disinhibition hypothesis of analgesia (Meng *et al.*, 1998). It should be noted that the observed endocannabinoid actions on inhibitory transmission slightly differ to those on excitatory transmission, where AEA has been reported to act via both CB_1 and TRPV1 receptors to respectively suppress and

enhance non-NMDA-mediated glutamatergic transmission (Maione *et al.*, 2006; Kawahara *et al.*, 2011). Together, CB_1 receptor-mediated disinhibition and TRPV1 receptor-mediated excitation would be expected to activate the descending analgesic system. Although it remains to be determined if endocannabinoids modulate inhibitory transmission onto PAG output neurons specifically involved in nociception, we have recently demonstrated that indirect activation of the endocannabinoid system elicits CB_1 receptor-mediated disinhibition of PAG output neurons projecting to the RVM (Drew *et al.*, 2009).

In the present study, URB597 and JZL184 respectively enhanced the inhibition of miniature IPSC rate produced by exogenously applied AEA and 2-AG, suggesting that FAAH and MGL selectively regulate the activity of these endocannabinoids. Our results indicated that in the absence of these enzyme inhibitors, AEA suppressed miniature IPSC rate to a greater degree than 2-AG. This result was surprising as the PAG contains higher levels of 2-AG than AEA under basal conditions (Hohmann *et al.*, 2005; Hohmann and Suplita, 2006; Maione *et al.*, 2006; Petrosino *et al.*, 2007). Furthermore, 2-AG has higher efficacy as a full CB_1 receptor agonist, despite a lower receptor affinity compared with AEA (Hillard, 2000; Luk *et al.*, 2004). The difference between AEA and 2-AG might be due to the distinct anatomical distribution of their degradative enzymes. Although it has yet to be examined in the PAG, hippocampal studies suggest that MGL is localized in presynaptic terminals, whereas FAAH is generally localized at a postsynaptic site (Hashimotodani *et al.*, 2007). This suggests that the degradative enzyme for 2-AG is in closer proximity to the target CB_1 receptor than AEA. Hence, the lower efficacy of 2-AG observed might be due to lower levels of 2-AG accessing presynaptic CB_1 receptors, in spite of its higher global levels within the PAG. Another potential explanation is that 2-AG has a lower critical micelle concentration than AEA, thereby limiting the effective concentration of 2-AG to a greater extent than AEA (Raduner *et al.*, 2007).

Functional *in vivo* studies have previously demonstrated that administration of a CB_1 receptor antagonist causes hyperalgesia (Richardson *et al.*, 1997; Meng *et al.*, 1998), implying that endocannabinoids tonically regulate nociception. In the present *in vitro* study, the CB_1 receptor antagonist AM251 had little or no effect under control conditions, but produced a significant facilitation of miniature and evoked IPSCs in the presence of URB597, or JZL184. Although this result suggests that endogenously released AEA and 2-AG lack functional effects on GABAergic synaptic transmission under basal conditions, it highlights the utility of enzyme degradation inhibitors in unmasking a functional 'endocannabinoid tone'. Given the crucial role of GABAergic disinhibition in PAG-mediated analgesia, these findings support the growing number of *in vivo* studies demonstrating analgesic efficacy by FAAH and MGL inhibitors (Blankman and Cravatt, 2013; Starowicz and Di Marzo, 2013). Like URB597 and JZL184, the dual FAAH/MGL inhibitor JZL195 also enhanced the AM251-induced facilitation of miniature/evoked IPSCs. Interestingly, JZL195 had a greater effect than URB597 or JZL184 on evoked IPSCs, but not on miniature IPSCs. This indicates a differing CB_1 receptor number, or receptor saturation point between spontaneous and evoked GABAergic transmission. Overall, these findings suggest that dual inhibition of FAAH and MGL has a potentially greater analgesic efficacy than individual inhibition of FAAH or MGL, although this remains to be examined.

It has recently become established that the CB_1 receptor is constitutively active in numerous intact biological systems. This has confounded the interpretation of studies utilizing inverse agonists, which are unable to distinguish between endogenous ligand activity and constitutive activity of a receptor (Pertwee, 2005). In this study, a number of observations suggested that the tonic effects observed were not mediated by constitutive activity at CB_1 receptors, but rather their activation by endogenously released cannabinoids. Firstly, the inverse agonist AM251 significantly enhanced miniature and evoked IPSCs only in the presence of a FAAH and/or MGL inhibitor. Secondly, the neutral antagonist O-2050 similarly enhanced miniature IPSCs in the presence, but not the absence of a FAAH/MGL inhibitor. Thirdly, following blockade of 2-AG synthesis with THL, the AM251-induced facilitation of miniature IPSCs observed in the presence of a MGL inhibitor was abolished. Together, these results suggest that tonic inhibition is not mediated by spontaneous, agonist-independent activity at the CB_1 receptor, but by a continuous, on-demand synthesis of an endocannabinoid ligand. Such agonist-induced, rather than receptor-induced tone, implies a more specific and localized modulation of inhibitory transmission within the PAG. This is concordant with the action of FAAH and MGL inhibitors, which are hypothesized to elicit antinociception by selectively enhancing levels of AEA and 2-AG in regions where they are produced on demand (Roques *et al.*, 2012). Consistent with this mechanism of action, URB597, JZL184 and JZL195 not only enhanced disinhibition of exogenously applied AEA and 2-AG, but unmasked a tonic disinhibition mediated by endogenous release of these neurotransmitters. Thus, FAAH and MGL inhibitors function as indirect agonists on the endocannabinoid system.

In conclusion, there has recently been much interest in targeting the endogenous cannabinoid system to treat chronic pain. Although numerous functional studies have established the analgesic efficacy of endocannabinoids and their degradation inhibitors, the present study has identified their mode of action at the cellular level, in a brain region that plays a pivotal role in their analgesic effects. Although our findings suggest that AEA and 2-AG produce cellular disinhibition consistent with analgesia, their actions are tightly restricted by FAAH and MGL degradation under basal conditions. Therefore, modulation of FAAH and MGL, particularly in combination, provides a potential therapeutic target for pain relief.

Acknowledgements

This work was supported by National Health & Medical Research Council Project Grant 1003097.

Author contributions

B. K. L. and C. W. V. designed the research. B. K. L., G. M. D. and V. A. M. performed the research. B. K. L., G. M. D.,

V. A. M. and C. W. V analysed the data. B. K. L. and C. W. V. wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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