Title: Endocannabinoids, anandamide and 2-AG, regulate mechanosensitivity of mucosal afferents in the guinea pig bladder.

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Running title: Endocannabinoids and mechanosensitivity of bladder afferents.

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Abstract

Bladder afferents play a crucial role in urine storage and voiding, and conscious sensations from the bladder. Endocannabinoids, anandamide (AEA) and 2-arachidonolylglycerol (2-AG), are endogenous ligands of G-protein coupled cannabinoid receptors 1 and 2 (CB1 and CB2) found in the CNS and peripheral organs. They also have off-target effects on some ligand- and voltage-gated channels. The aim of this study is to determine the role of AEA and 2-AG in regulation of mechanosensitivity of probable nociceptive neurons innervating the bladder - capsaicin-sensitive mucosal afferents. The activity of these afferents was determined by *ex vivo* single unit extracellular recordings in the guinea pig bladder. A stable analogue of anandamide, methanandamide (mAEA) evoked initial excitatory response of mucosal afferents followed by potentiation of their responses to mechanical stimulation. In the presence of TRPV1 antagonist (AMG9810), mAEA's effect on mechanosensitivity switched from excitatory to inhibitory. The inhibitory effect of mAEA is due to activation of both CB1 and CB2 cannabinoid receptors since it was abolished by combined application of selective CB1 (NESS0327) and CB2 (SR144528) antagonists. 2-AG application evoked a brief excitation of mucosal afferents, without potentiation of their mechanosensitivity, followed by the inhibition of their responses to mechanical stimulation. CB2 receptor antagonist, SR144528 abolished the inhibitory effect of 2-AG. Our data indicated that anandamide and 2-AG have opposite effects on mechanosensitivity of mucosal capsaicin-sensitive afferents in the guinea pig bladder; mAEA potentiated while 2-AG inhibited responses of mucosal afferents to mechanical stimulation. These findings are important for understanding of the role of endocannabinoids in regulating bladder sensation and function.

Key words

Endocannabinoids, anandamide, 2-AG, CB1 and CB2 cannabinoid receptors, bladder afferents.

Abbreviations

2-AG: 2-arachidonylglycerol; 4Q3C: 4-quinoloine 3-carboxyamide; AEA: anandamide; CB: cannabinoid; DRG: dorsal root ganglia; LUT: lower urinary tract; CGRP: calcitonin gene-related peptide; SP: substance P; mAEA: methanandamide; TRPV1: transient receptor potential vanilloid 1

Introduction

Endocannabinoids such as anandamide (AEA) and 2-arachidonolylglycerol (2-AG) affect bladder function acting via their G-protein coupled receptors, the cannabinoid 1 (CB1) and CB2 receptors (Cristino et al., 2020; Hedlund and Gratzke, 2016). Given that AEA and 2-AG have been detected in the bladder itself, it is likely that there is a local production. The likely cell type involved in the synthesis and release of AEA and 2-AG is urothelial cells given that the enzymes involved in their synthesis and breakdown have been demonstrated in the urothelium (Sultana et al., 2022). Endocannabinoids and CB1 and CB2 agonists can ameliorate bladder function in pathophysiological conditions as it was shown using animal models of overactive bladder and painful bladder syndromes (Bjorling and Wang, 2018; Hedlund and Gratzke, 2016). However, the exact mechanism by which endocannabinoids regulate bladder function is not known. Essential parts of endocannabinoid action on the lower urinary tract (LUT) organs could be mediated via their effects on spinal sensory nerves innervating the bladder (Christie et al., 2021). Bladder afferents are involved in reflexes controlling urine storage and voiding. They are also responsible for all conscious sensations from the bladder, ranging from physiological sensation of filling and fulness through to LUT symptoms such as excessive urgency and pain (de Groat and Yoshimura, 2009). 5 major types of bladder afferents have been discovered including stretch-insensitive mucosal (=urothelial) afferents, stretch-sensitive muscular-mucosal (=muscular-urothelial) and muscular afferents, high threshold vascular (=serosal) and silent afferents (Christie et al., 2021; Grundy et al., 2019; Xu and Gebhart, 2008; Zagorodnyuk et al., 2007).

In the bladder, CB1 and CB2 receptors are expressed on the urothelial cells, detrusor muscle and nerve fibres in the suburothelium and detrusor (Bakali et al., 2013; Gratzke et al., 2009; Hayn et al., 2008; Kim et al., 2017; Walczak et al., 2009). It has been previously demonstrated that a non-selective CB1/CB2 agonist reduced firing of high-threshold distension-sensitive bladder afferents via CB1 receptors (Walczak and Cervero, 2011; Walczak et al., 2009). Selective inhibition of fatty acid amide hydrolase (resulting in an increase in concentration of endocannabinoids in the tissue) decreased distension-induced activity of Aδ and C fibre bladder afferents via both CB1 and the CB2 receptors (Aizawa et al., 2014). CB2 receptors could be attractive therapeutic targets since CB2 agonists reduced bladder inflammation and pain (Bjorling and Wang, 2018; Liu et al., 2020). We recently reported that the selective synthetic CB2 agonist, 4-quinolone-3-carboxamide (4Q3C) inhibited the mechanosensitivity of mucosal, but not muscular-mucosal, bladder afferents via CB2 receptors (Christie and Zagorodnyuk,

2021). However, in addition to acting on CB1 and CB2 receptors, cannabinoids have off-target effects on some ligand- and voltage-gated channels. Endocannabinoids can directly act on transient receptor potential (TRP) channels, in particularly TRP vanilloid 1 (TRPV1) (Pertwee et al., 2010). TRPV1 is likely involved in nociception since their antagonists or desensitisation with resiniferatoxin can ameliorate painful sensation from the bladder (Charrua et al., 2007; Charrua et al., 2009; Dinis et al., 2004a; Guo et al., 2013). Among 5 types of bladder afferents, mucosal afferents are probable polymodal capsaicin-sensitive peptidergic (containing both CGRP and SP) nociceptors (Christie et al., 2021; Spencer et al., 2018; Zagorodnyuk et al., 2010). Bearing in mind the importance of cannabinoids as potential analgesics (Vuckovic et al., 2018), in this study we aim to determine the effects of endocannabinoids, AEA and 2-AG, on the excitability and mechanosensitivity of capsaicin-sensitive mucosal bladder afferents.

2. Materials and methods

2.1 Ethical Approval

This study was approved by the Animal Welfare Committee of Flinders University (AEM1574-5) and performed in accordance with the Australian code for the care and use of animals for scientific purposes, 8th edition 2013 and the ARRIVE guidelines (Lilley et al., 2020).

2.2 Ex vivo bladder afferent preparation

Adult female guinea pigs (N=30, weight 300-400g) were housed in a 12hr:12hr light dark cycle with ad libitum access to a standard diet and water. The guinea pigs were euthanised by isoflurane inhalation overdose and severing of the spinal cord at the cervical level. This was performed between 0700-0730hrs to minimise any possible circadian rhythm influence on the bladder afferents mechanosensitivity.

This *ex vivo* bladder afferent preparation has been described in detail previously (Christie and Zagorodnyuk, 2021; Ramsay and Zagorodnyuk, 2022). Briefly, the bladder and associated connective tissue containing the nerves was removed and opened into a flat sheet along the anterior bladder wall and placed in a modified Krebs solution consisting of (in mM): NaCl 118; KCl 4.74; NaH_2PO_4 1.0 $NaHCO_3$ 25; $MgCl_2$ 1.2; $CaCl_2$ 2.5; glucose 11 and nicardipine (3µM) bubbled with 95% oxygen in 5% carbon dioxide. A rectangular full thickness sheet (approximately 12mm by 15mm) was formed and pinned mucosa up along the edge containing the nerves in a 22ml organ bath containing warmed Krebs. The opposite side of the bladder was attached to a 12mm custom-made stainless steel "rake", also attached to a cantilever system with isotonic transducer (Harvard Bioscience 52-9511, S Natick, MA. U.S.A). Counterweights (1-40g) could be applied to the cantilever to distend the bladder while measuring the resulting changes in length. Fine nerve trunks entering the trigone were freed from the associated connective tissue, pinned, and placed in a paraffin oil bubble, sealed by paraffin wax, thus electrically isolating the nerves. Each nerve was then individually placed on a platinum electrode for recording. Electrical nerve signals were amplified using a DAM80 (World Precision Instruments, USA) filtered using a Band-Pass filter (10Hz-10kHz; BPF-932; CWE, USA) and recorded at 20kHz using a data acquisition system (Micro 1401-4; CED, UK). Single-unit analysis of bladder afferents was performed offline with Spike2 software (version 10, CED, Cambridge, UK).

2.3 Identification of mucosal bladder afferents

High-responding mucosal bladder afferents which respond to mucosal stroking with light von Frey hairs (10-100mg), but do not respond to stretch of the bladder wall (1-40g), were investigated in this study. They have a low threshold to stroking of their receptive fields, longer action potential duration, and majority are capsaicin-sensitive (Christie and Zagorodnyuk, 2021; Zagorodnyuk et al., 2009; Zagorodnyuk et al., 2007).

To facilitate agonists and antagonists' penetration to nerve endings, a small hole (~2x2mm) in the urothelium was made adjacent to their receptive field. Spontaneous activity and mechanosensitivity were measured before and after the creation of a small hole in the urothelium; no significant differences were found. After this, the mechanosensitivity of bladder afferents to mucosal stroking was determined using a calibrated von Frey hair (100mg) stroked across a hot spot area at a rate of 5mms-1 5 times with 10 second intervals. To identified whether recorded bladder afferents are stretch-sensitive or stretch-insensitive a weight (20g) was added to the cantilever for a period of ten seconds.

2.4 Effects of endocannabinoids and CB1 and CB2 receptor antagonists on bladder afferent mechanosensitivity

The metabolically stable analogue of anandamide, methanandamide (mAEA) (Abadji et al., 1994) was used instead of anandamide in this study since anandamide is quite an unstable molecule. In contrast to methanandamide, it is light-sensitive and could be enzymatically catalysed by FAAH (Deutsch and Chin, 1993) and oxidised by COX-2 (Yu et al., 1997). This also makes the present results compatible with our previous study (Christie and Zagorodnyuk, 2021). After determination of baseline mechanosensitivity, drugs were added to the organ bath over the receptive field area. Endocannabinoids, mAEA and 2-arachidonolylglycerol (2-AG), were allowed to equilibrate for 10 minutes and bladder afferent mechanosensitivity were re-determined. Antagonists of TRPV1 (AMG9810), and CB1 and CB2 receptors (NESS0327 and SR144528, respectively) were added to the organ bath and to the Krebs solution flow through for 20-minutes before bladder afferent spontaneous activity and mechanosensitivity were re-measured. To allow the rapid washout of drugs from the organ bath, the bath was half emptied, and the perfusion system was restored to normal Krebs. Initial excitatory response of mucosal afferents evoked by mAEA and 2-AG was calculated as mean firing rate averaged for a 10 second period after drug application minus the 10 second period immediately before drug application. Time controls were previously performed equal to the maximum time of experiments; no significant changes over time were observed. Concentrations used of mAEA,

2-AG, NESS0327 and SR114528 were based on available literature sources or our previous findings (Christie and Zagorodnyuk, 2021).

2.5 Drugs

Stock solutions of 2-AG, AMG9180, NESS0327and SR144528 were kept at 10mM in DMSO. Stock solutions of mAEA were kept at 10mM in ethanol. All drugs were stored at -20ºC and diluted prior to experiments in Krebs. DMSO and ethanol at highest concentrations used (0.1%) had no significant effects on excitability or mechanosensitivity of mucosal bladder afferents. mAEA, 2-AG, AMG9180 and NESS0327 were obtained from Sapphire Bioscience Australia, and SR144528 (SML1899) was obtained from Sigma Australia.

2.6 Data Analysis

All data is presented as the mean ± SEM, with n referring to the number of afferents and N to number of animals. Analysis was performed using GraphPad Prism 9 software. All data was analysed using a one-way ANOVA with Tukey's post hoc test. P-values were less than 0.05 were considered significant.

3. Results

3.1 Potentiating effect of methanandamide on mechanosensitivty of mucosal afferents switches to inhibitory by TRPV1 antagonist, AMG9810

The effects of mAEA (3μM) on bladder mucosal afferents alone or in combination with the selective TRPV1 antagonist AMG9810 (3μM) are illustrated in Fig.1.

Application of mAEA alone produced an immediate excitatory response of mucosal afferents which was abolished in the presence of AMG9810 (Fig. 1A and 1D; mAEA alone 6.1 ± 0.7Hz, mAEA + AMG9810 0.6 ± 0.04Hz; $P<0.0001$).

mAEA (3μM) alone significantly increased the response of mucosal afferents to stroking (100mg) compared to controls (Fig. 1B and 1E; +80.7 ± 5.6%, $P<0.0001$). AMG9810 (3μM) alone significantly decreased the response of mucosal afferents to stroking (100mg) compared to controls (Fig. 1B; -60.9 ± 2.6%, $P<0.0001$). In the presence of AMG9810, mAEA significantly decreased the response of mucosal afferents to stroking (100mg) compared to AMG9810 alone (Fig. 1B, 1C and 1F; -64.2 ± 5.4%, $P<0.001$). AMG9810 alone had no

significant effects on the basal spontaneous activity of mucosal afferents (0uM 0.6 ± 0.1 Hz, 3μM 0.4 ± 0.3Hz; NS).

3.2 The inhibitory effect of methanandamide is mediated via both CB1 and CB2 receptors

The effects of mAEA (3μM) in the presence of the TRPV1 antagonist AMG9810 (3μM) and the CB1 receptor antagonist NESS0327 (1μM), or the CB2 receptor antagonist SR144528 (10μM), or both are illustrated in Fig. 2.

AMG9810 alone significantly decreased the response of mucosal afferents to stroking compared to controls (Fig. 2Ai; -61.8 ± 3.8%, $P<0.001$). In the presence of AMG9810, mAEA (3μM) significantly reduced the response of mucosal afferents to stroking further compared to AMG9810 alone (Fig. 2Ai and 2Aii; -63.2 ± 6.1%, $P<0.05$). The CB1 receptor antagonist NESS0327 (1μM), in the presence of AMG9810, significantly increased the response of mucosal afferents to stroking compared to AMG9810 alone (Fig. 2Ai; +54.4 ± 6.3%, $P<0.001$). In the presence of both AMG9810 and NESS0327, mAEA still significantly reduced the response of mucosal afferents to stroking (Fig. 2Ai; -25.1 ± 1.4%, $P<0.05$), however to a significantly lesser extent than in the presence of AMG9180 only (Figure 2Aii, $P<0.01$).

In a separate set of experiments, AMG9810 significantly reduced mucosal afferent responses to stroking (100mg) compared to controls (Fig. 2Bi; -62.8 ± 1.8%; $P<0.01$). In the presence of AMG9810 and mAEA, the response of mucosal afferents to stroking were significantly reduced further (Fig. 2Bi; -65.1 ± 3.7%; $P<0.05$). The CB2 receptor antagonist SR144528 (10μM), in the presence of AMG9810, did not significantly affect the response of mucosal afferents to stroking compared to AMG9810 alone (Fig. 2Bi; +30.3 ± 9.5%, NS). In the presence of both AMG9810 and SR144528, mAEA still significantly reduced the response of mucosal afferents to stroking (Fig. 2Bi; -51.9 ± 4.3%; $P<0.05$), however to a significantly lesser extent than in the presence of AMG9180 only (Fig 2Bii; $P<0.05$).

In a final set of separate experiments, the response of mucosal afferents to stroking (100mg) was significantly reduced in the presence of AMG9810 (Fig. 2Ci; -58.1 ± 4.0%; $P<0.001$). In the presence of AMG9810, mAEA significantly reduced the response of mucosal afferents to stroking (100mg) further (Figure 2Ci; -60.2 ± 5.7%; $P<0.05$). The CB1 and CB2 receptor antagonists (NESS-0327 and SR144528), in the presence of AMG9810, significantly increased the response of mucosal afferents to stroking (100mg; Fig. 2Ci; +65.4 ± 10.5%; $P<0.01$). In the presence of AMG9810, NESS-0327, and SR144528, the inhibitory effect of mAEA was lost (Fig. 2Ci and Cii; -6.3 ± 2.6%, NS).

3.3 2-AG inhibits mechanosensitivity of mucosal afferents via CB2 receptors

The effect of 2-AG (3μM) on the mechanosensitivity of mucosal afferents alone or in combination with the TRPV1 antagonist AMG9810 (3μM) is illustrated in Fig. 3. Application of 2-AG alone produced a short excitatory response of mucosal afferent activity which was significantly reduced, but not lost, in the presence of the TRPV1 antagonist AMG9810 (Fig. 3A and 3AD; 2-AG alone 2.8 ± 0.58Hz, 2-AG + AMG9810 0.7 ± 0.36Hz, $P<0.001$).

2-AG alone significantly reduced the response of mucosal afferents to stroking (100mg) compared to controls (Fig. 3B; -53.7 ± 4.4%; $P<0.001$). AMG9810 alone also significantly decreased the response of mucosal afferents to stroking (100mg) compared to control (-56.6 ± 4.2%; $P<0.01$). In the presence of AMG9810, 2-AG significantly reduced the response of mucosal afferents to stroking further (Fig. 3B; -70.8 ± 7.7%; $P<0.05$), which was significantly greater than the reduction caused by 2-AG alone (Fig. 3C; $P<0.05$).

The effect of 2-AG (3μM) in the presence of the TRPV1 antagonist AMG9810 (3μM) and the CB2 antagonist SR144528 (10μM) is illustrated in Fig. 4. AMG9810 alone significantly reduced the response of mucosal afferents to stroking (100mg) compared to controls (Fig. 4A; -57.4 ± 5.8%; $P<0.001$). In the presence of AMG9810, 2-AG further reduced the response of mucosal afferents to stroking (100mg) compared to AMG9810 alone (Fig. 4A; -67.4 ± 2.1%; $P<0.05$). The CB2 receptor antagonist SR144528, in the presence of AMG9810, did not significantly affect the response of mucosal afferents to stroking. In the presence of AMG9810 and SR144528, the inhibitory effect of 2-AG was lost (Fig. 4A and 4B; -3.7 ± 3.4%, NS).

4. Discussion

The study reveals opposite effects of endocannabinoids on mechanosensitivity of mucosal capsaicin-sensitive afferents in the guinea pig bladder; mAEA potentiated while 2-AG inhibited responses of mucosal afferents to mechanical stimulation. Potentiation of mechanosensitivity by mAEA was due to activation of TRPV1 channels since selective antagonist, AMG9810 abolished potentiation revealing an inhibition. The inhibitory effect of mAEA on mechanosensitivity was due to activation of both CB1 and CB2 receptors. 2-AG inhibited mechanosensitivity of bladder mucosal afferents predominantly via activation of CB2 receptors.

Endocannabinoids such as anandamide and 2-AG are involved in the regulation of nociception (Bjorling and Wang, 2018; Clapper et al., 2010; Pacher et al., 2006), although their analgesic mechanisms are yet to be fully elucidated. Recently, cannabinoid research has moved, in some health clinics, into the frontline of treatment for many health conditions including chronic pain (Donvito et al., 2018; Vuckovic et al., 2018). It is well established that AEA is a high-affinity, partial agonist of CB1 receptors, while almost inactive at CB2 receptors (Di Marzo and De Petrocellis, 2012; Pertwee et al., 2010; Ruggieri, 2011). Cannabinoids mediate analgesia in the skin predominantly via peripheral CB1 receptors since mice with conditional knockout of CB1 receptors in the $Na_V1.8$ expressing dorsal root ganglia (DRG) neurons exhibit allodynia and hyperalgesia (Agarwal et al., 2007). However, in addition to acting on their G-protein coupled CB1 and CB2 receptors, cannabinoids are capable of directly activating some ligand- and voltage-gated ion channels, including TRPV1 channels (Pertwee et al., 2010; Ruggieri, 2011). Both CB1 and CB2 receptors are colocalised with TRPV1 in DRG neurons (Ahluwalia et al., 2000; Anand et al., 2008) and all three are up-regulated in DRG neurons in inflammation (Amaya et al., 2006; Anand et al., 2008). In the bladder, CB1 and CB2 are co-localised with TRPV1 and CGRP in the axons of sensory nerves in the detrusor and sub-urothelium (Gratzke et al., 2009; Walczak et al., 2009).

TRPV1 is a well-established sensor for noxious stimuli such as noxious heat and low pH and can be also activated by endogenous vanniloids and cannabinoids and exogenous compounds such as capsaicin and resiniferatoxin (Caterina et al., 1997; Nilius and Szallasi, 2014). TRPV1 channels are not mechano-gated channels, however, they contribute to the modulation of the general level of excitability of bladder afferents, thus contributing to mechanosensitivity indirectly (Daly et al., 2007). They powerfully modulate the excitability of sensory fibres in the normal tissue and in cystitis (Charrua et al., 2007; Daly et al., 2007; Yoshimura et al., 2014). This explains the reduction in the mechanosensitivity of mucosal afferents by the TRPV1 antagonist, AMG9810 in the guinea pig bladder. Activation and up-regulation of TRPV1 channels results in pain and hypersensitivity in many visceral organs including the bladder (Charrua et al., 2009; Nagy et al., 2004). Conceivably, it has been previously shown that AEA, via activation of TRPV1, contributed to the development of hyperreflexia and hyperalgesia revealed in cyclophosphamide-induced cystitis in rats (Dinis et al., 2004b). In the guinea pig bladder, high-responding capsaicin-sensitive mucosal afferents are not stretch-sensitive, peptidergic afferents (containing both SP and CGRP) that can be activated by light von Frey hair stimulation of their receptive filed in the mucosa (Spencer et al., 2018; Zagorodnyuk et

al., 2007, 2008, 2010). Although the role of capsaicin-sensitive mucosal bladder afferents in bladder function is not fully determined, they are likely involved in transmission of nociceptive stimuli to CNS (Christie et al., 2021; Dinis et al., 2004b; Zagorodnyuk et al., 2007, 2010). Therefore, to use AEA as a potential analgesic, it may be necessary to consider the potential excitatory effects and use in combination with a TRPV1 antagonist. It is worth mentioning that dual inhibitors of FAAH and TRPV1 or FAAH and COX have been synthesised and are currently undergoing preclinical studies for their efficacy in providing analgesia (Bjorling and Wang, 2018).

We have recently reported that the potentiating effect of mAEA on mucosal bladder afferents was convert to inhibitory when TRPV1 channels were blocked with capsazepine (Christie and Zagorodnyuk, 2021), which was confirmed in the present study by using a more selective and potent TRPV1 antagonist, AMG9810 (Gavva et al., 2005). The question that remained, however, was which cannabinoid receptors are responsible for the revealed inhibitory effect of AEA. The present data indicate that inhibition of mechanosensitivity of mucosal afferents, in the presence TRPV1 antagonist, is due to activation of both CB1 and CB2 receptors since selective CB1 antagonist, NESS0327 and selective CB2 antagonist, SR144528 were capable to reduce this effect of AEA. Simultaneous application of both CB1 and CB2 antagonist was necessary to abolish the inhibitory effect of AEA on mucosal afferent mechanosensitivity.

It is well known that TRPV1 can be desensitised by capsaicin and other vanilloids. This process contributes to the analgesic effects of these compounds and used in clinical trials to ameliorate pain in patients suffering from painful bladder syndrome (Guo et al., 2013; Payne et al., 2005; Touska et al., 2011). TRPV1 desensitisation could be also responsible for reducing hyperalgesia during acute inflammation and mechanical allodynia developed in the rat model of neuropathic pain by the phytocannabinoid, cannabidiol (Costa et al., 2004; De Gregorio et al., 2019). It is important to note, however, that AEA, in contrast to capsaicin, does not evoke desensitisation of TRPV1 (Nagy et al., 2004), so it is unlikely that this mechanism operates in analgesic effects of AEA in the bladder.

Significantly less studies were done to investigate possible role of 2-AG in the bladder function. 2-AG is a full agonist at both CB1 and CB2 cannabinoid receptors, however, it has moderate to low affinity (Di Marzo and De Petrocellis, 2012; Ruggieri, 2011). In addition to different efficacy at CB1 and CB2, tissue biosynthesis and breakdown of AEA and 2-AG are regulated independent of each other, thus allowing them to exert different function within the same organ

(Di Marzo and De Petrocellis, 2012). Recently, it has been suggested that CB2 receptors in the bladder could be an attractive therapeutic target for the treatment of bladder pain since they capable of reducing bladder inflammation and their activation devoid of the central psychotropic effects of CB1 ligands (Bjorling and Wang, 2018). Administration of selective CB2 receptor agonists (JWH015, GP1a, JWH-133) reduces bladder inflammation and pain in animal models of interstitial cystitis (Liu et al., 2020; Tambaro et al., 2014; Wang et al., 2014). However, it is likely that ameliorating effect of CB2 receptors agonists on cystitis-induced pain is due not only to a reduction in bladder inflammation but also by their direct activation of CB2 receptors on nociceptive fibres. This is supported by our recent findings indicating that activation of CB2 receptors by selective synthetic ligand, 4Q3C inhibited mechanosensitive responses of mucosal capsaicin-sensitive bladder afferents; the effect was abolished by SR144528 (Christie and Zagorodnyuk, 2021). The CB2 antagonist, SR144528 (which is inverse agonist of the CB2 receptors) has a 700-fold higher affinity for the CB2 receptors compared to the CB1 receptors (Rinaldi-Carmona et al., 1998). It has been previously shown that non-selective CB1/CB2 agonist reduced firing of high threshold distension-sensitive bladder afferents via CB1 receptors in mice (Walczak and Cervero, 2011; Walczak et al., 2009). In the rat bladder, an increase in endogenous cannabinoids, produced by periphery-restricted fatty acid amide hydrolase inhibitor, URB937 moderately decreased distension-induced activity of Aδ and C fibers via both CB1 and CB2 receptors (Aizawa et al., 2014). Further studies are warranted to investigate the effect of endocannabinoids and their selective CB1 and CB2 agonists on different classes of bladder afferents.

Both mAEA and 2-AG evoked initial transient activation of mucosal afferents. This effect of mAEA was abolished by TRPV1 antagonist and significantly reduced in the case of 2-AG. AMG9810 also significantly potentiated the inhibitory effect of 2-AG on bladder afferents in normal Krebs solution, indicating that 2-AG has ability to act on TRPV1 channels expressed on mucosal afferents. In contrast to anandamide, 2-AG has very low potency and efficacy at TRPV1 channels (Di Marzo and De Petrocellis, 2012). This likely explains its lack in potentiation of mechanosensitivity of bladder afferents. In contrast to mAEA, the inhibitory effect of 2-AG was abolished by SR1144528, indicating that this action of 2-AG on bladder afferents is predominantly via CB2 receptors. We have previously shown that in $0Ca^{2+}$ Krebs solution (which prevents Ca^{2+}-dependent release of the endogenous mediators) there was no change in responses of mucosal afferents to mucosal stroking (Zagorodnyuk et al., 2009). This suggests that mucosal stroking directly activates some type of mechano-gated channels

expressed by the nerve endings. In addition to the presence of CB1 and CB2 receptors on nerve fibres in the suburothelium and detrusor, they are also expressed on the urothelial cells (Bakali et al., 2013; Christie et al., 2021). We cannot completely exclude possible contribution of the urothelial CB1 and CB2 receptors to the effects of cannabinoid agonists and antagonists on the mechanosensitivity of mucosal afferents. This requires further investigation.

Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are major enzymes that primarily degrade AEA or 2-AG, respectively (Bjorling and Wang, 2018; Ruggieri, 2011). However, simply increasing levels of endogenous cannabinoids causes off-target effects on ligand- and voltage-gated ion channels (Pertwee et al., 2010; Watkins, 2019) and has low efficacy in reducing chronic pain (Vuckovic et al., 2018). Instead, the use of peripherally restricted synthetic CB1 and CB2 receptor ligands are more promising to eliminate LUT symptoms in animal models of bladder overactivity and interstitial cystitis (Christie et al., 2021).

One of the noteworthy properties of endogenous cannabinoid system in the bladder is tonic regulation of mechanosensitivity of bladder afferents by constitutively downregulating bladder sensory neurons' function (Hedlund and Gratzke, 2016). It has been previously demonstrated that CB1 antagonists (AM251 and rimonabant) and CB2 antagonist (SR144528) on their own significantly increased distension-induced firing of bladder afferents in both *ex vivo* and *in vivo* conditions in rats and mice (Aizawa et al., 2014; Walczak and Cervero, 2011). Present data indicated that in the guinea pig bladder, CB1 antagonist (NESS0327), but not CB2 antagonist (SR144528), significantly potentiated mechanosensitivity of mucosal afferents. Interestingly, in rats, SR144528 potentiated the distension-induced firing of Aδ fibres but not C fibre afferents (Aizawa et al., 2014). Capsaicin-sensitive mucosal afferents innervating the guinea pig bladder belong to C fibre sensory fibres (Zagorodnyuk et al., 2007). All of this suggests tonic downregulation of nociceptive C fibres mechanosensitivity by endogenous endocannabinoids, most likely anandamide, predominantly via CB1 receptors. Previous data (Christie and Zagorodnyuk, 2021) and present findings demonstrated that CB2 receptors are present on the same mucosal afferent fibres. However, there are significantly less, if any, contribution of endogenous cannabinoids which have ability to activate CB2 receptors, most likely 2-AG, to tonic control of bladder afferent mechanosensitivity of mucosal afferents. The mechanism of this differential control deserves further investigation.

In summary, our data indicated that that both endocannabinoids, anandamide and 2-AG, participate in controlling activity of probable nociceptors in the bladder, mucosal capsaicin-sensitive afferents, resulting in opposite regulation of their mechanosensitivity. Our data indicates that TRPV1 mediates the excitatory effects of mAEA and 2-AG on mucosal afferents, while CB1 and CB2 receptors mediate their inhibitory effects.

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Informed consent statement

Not applicable.

Credit authorship contribution statement

Stewart Ramsay: Data collection, formal analysis, performed experiments and analysed the data with. **Nick J. Spencer**: wrote the manuscript and approved its final version. **Vladimir Zagorodnyuk**: Writhing original draft, designed the experiments, wrote the manuscript and have approved its final version.

Declaration of competing interest

The authors of "Endocannabinoids, anandamide and 2-AG, regulate mechanosensitivity of mucosal afferents in guinea pig bladder", declare no conflicts of interest.

Data availability

Data will be made available on request.

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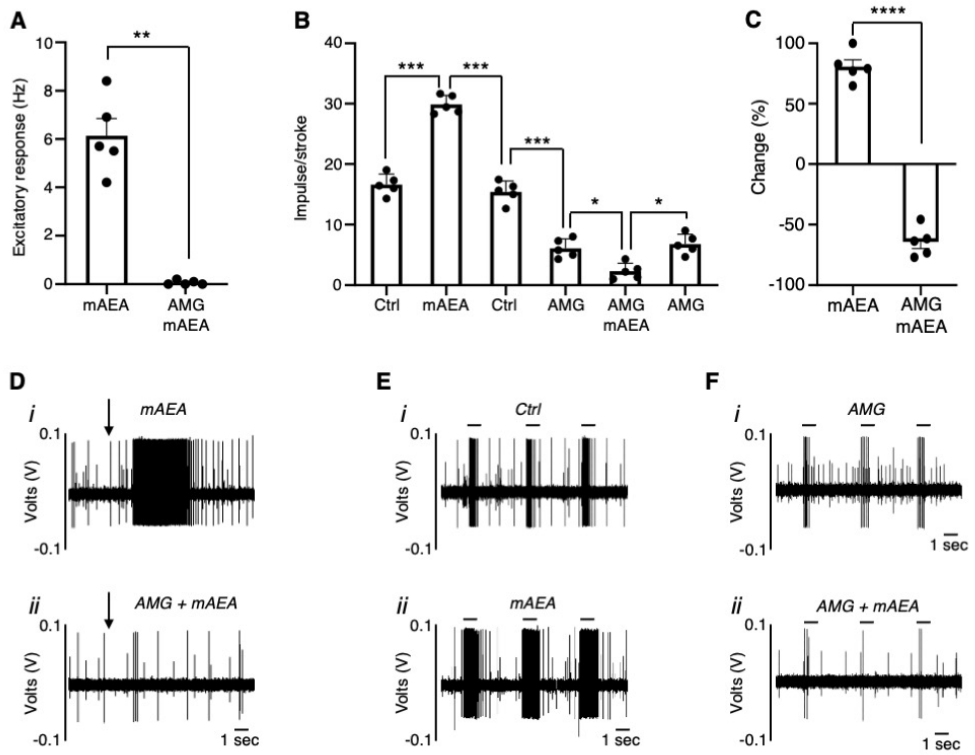
Figures



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554 **Fig. 1:** The effect of methanandamide is dependent on TRPV1 status. (A) The transient
555 excitatory effect of methanandamide (mAEA; 3μM) on mucosal afferents in the absence and
556 presence of the TRPV1 antagonist AMG9810 (3μM). (B) The effect of mAEA (3μM) on the
557 mechanosensitivity of bladder mucosal afferents in the absence and presence AMG9810
558 (3μM). (C) AMG9810 (3μM) converts potentiating effect of mAEA (3μM) on mucosal afferent
559 mechanosensitivity into inhibitory. (D) Representative trace of application of mAEA (3μM) in
560 the (i) absence or (ii) presence of AMG9810 (3μM). (E) Representative trace of the response
561 of bladder mucosal afferents to stroking (100mg) in the (i) absence and (ii) presence of mAEA
562 (3μM) alone. (F) Representative trace of the response of bladder mucosal afferents to stroking
563 (100mg) in the presence of (i) AMG9810 (3μM) or (ii) AMG9810 (3μM) plus mAEA (3μM).
564 Data is presented as the mean ± the SEM. N=5, n=5 per group. *P<0.05, **P<0.01,
565 ***P<0.001.

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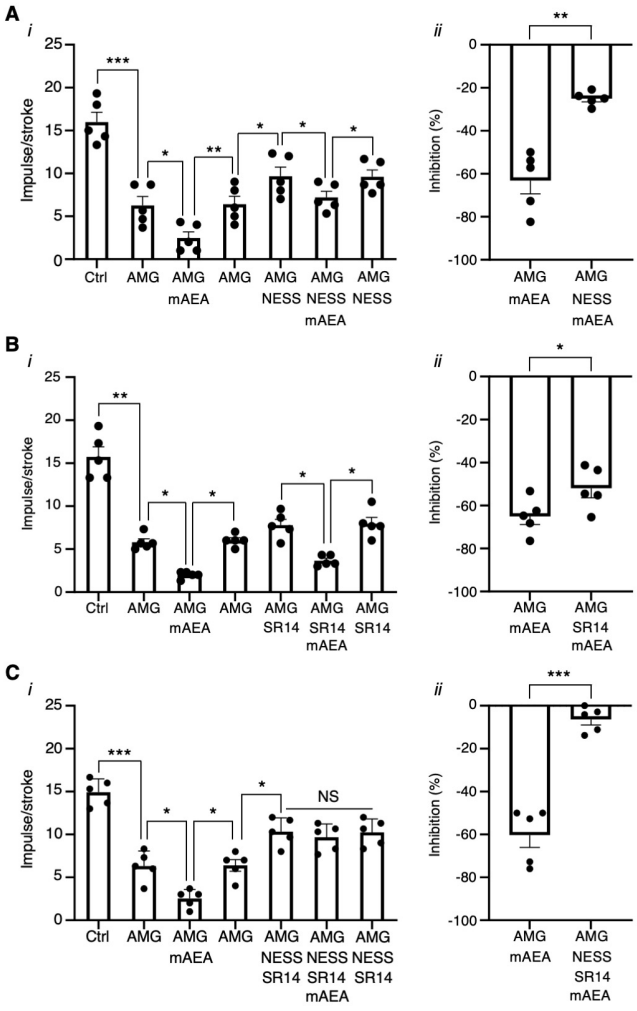


Fig. 2: The inhibitory effect of methanandamide is mediated via both CB1 and CB2 receptors. (Ai) The effect of methanandamide (mAEA) on response of bladder mucosal afferents to stroking (100mg) in the presence of the TRPV1 antagonist AMG9810 (3μM) alone and in combination with the CB1 receptor antagonist NESS-0327 (1μM). (Aii) The magnitude of inhibition (%) of mAEA in the presence of AMG9810 alone and in combination with NESS-0327. (Bi) The effect of mAEA (3μM) on response of bladder mucosal afferents to stroking (100mg) in the presence of AMG9810 (3μM) alone and in combination with the CB2 receptor inverse agonist SR144528 (10μM). (Bii) The magnitude of inhibition (%) of mAEA in the presence of AMG9810 alone and in combination with SR144528. (Ci) The effect of mAEA (3μM) on response of bladder mucosal afferents to stroking (100mg) in the presence of AMG9810 (3μM) alone and in combination with NESS-0327 (1μM) and SR144528 (10μM). (Cii) The magnitude of inhibition (%) of mAEA in the presence of AMG9810 alone and in combination with NESS-0327 and SR144528. Data is presented as the mean ± the SEM. N=5, n=5 per group. *P<0.05, **P<0.01, ***P<0.001.

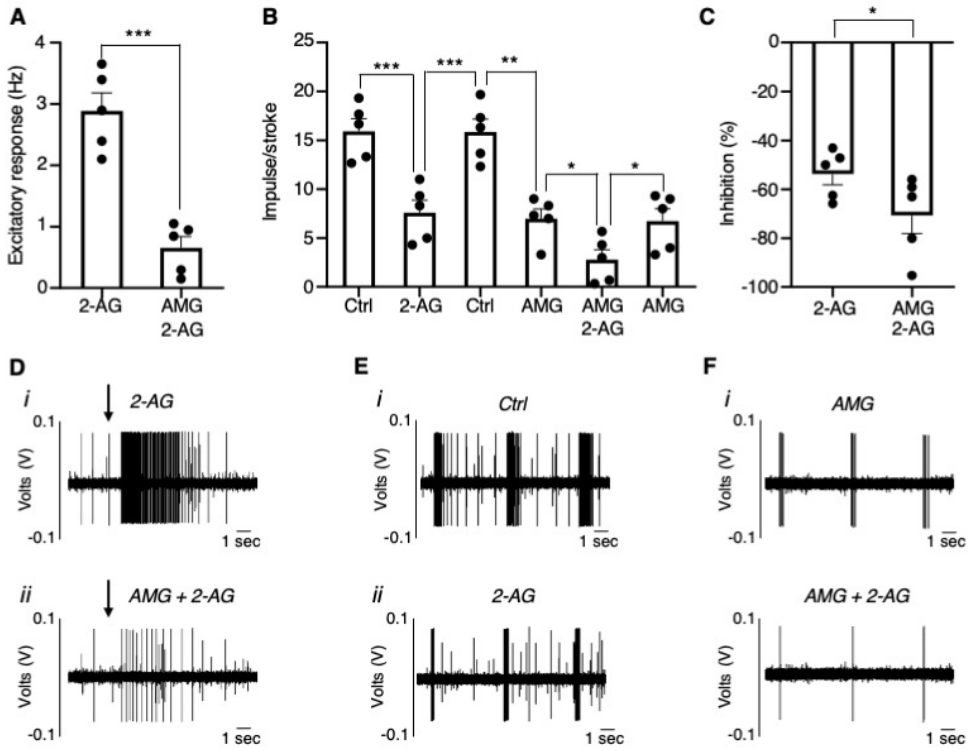
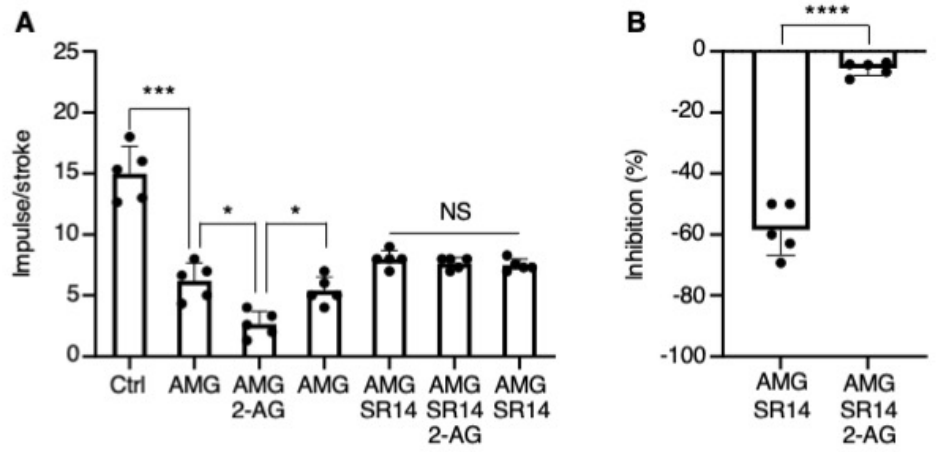


Fig. 3: The inhibitory effect of 2-AG on mechanosensitivity of mucosal afferents. (A) The effect of application of 2-AG (3μM) on mucosal afferents in the absence and presence of the TRPV1 antagonist AMG9810 (3μM). (B) The effect of 2-AG (3μM) on the mechanosensitivity of bladder mucosal afferents in the absence and presence AMG9810 (3μM). (C) The magnitude of effect of 2-AG on the mechanosensitivity of bladder mucosal afferents in the absence and presence of AMG9810. (D) Representative trace of application of 2-AG (3μM) in the (i) absence or (ii) presence of AMG9810 (3μM). (E) Representative trace of the response of bladder mucosal afferents to stroking (100mg) in the (i) absence and (ii) presence of 2-AG (3μM) alone. (F) Representative trace of the response of bladder mucosal afferents to stroking (100mg) in the presence of (i) AMG9810 (3μM) or (ii) AMG9810 (3μM) plus 2-AG (3μM). Data is presented as the mean ± the SEM. N=5, n=5 per group. *P<0.05, **P<0.01, ***P<0.001.



598

599 **Fig. 4:** The inhibitory effect of 2-AG is mediated via the CB2 receptor. (A) The effect of 2-AG
600 on response of bladder mucosal afferents to stroking (100mg) in the presence of the TRPV1
601 antagonist AMG9810 (3μM) alone and in combination with the CB2 receptor inverse agonist
602 SR144528 (10μM). (B) The magnitude of inhibition (%) of 2-AG in the presence of AMG9810
603 alone and in combination with SR144528. Data is presented as the mean ± the SEM. N=5, n=5
604 per group. *P<0.05, ***P<0.001, ****P<0.0001.

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