American Journal of Physiology Renal Physiology

Title: CB2 cannabinoid receptor agonist selectively inhibits the mechanosensitivity of mucosal afferents in the guinea pig bladder.

Authors: Stewart Christie, Vladimir Zagorodnyuk.

Affiliation: Discipline of Human Physiology, Flinders Health & Medical Research Institute, College of Medicine and Public Health, Flinders University, South Australia, Australia.

Running title: CB2 receptor agonists and mechanosensitivity of bladder afferents

Keywords: Endocannabinoids, TRPV1 channels, CB2 receptor agonists, bladder afferents

Correspondence: Dr Vladimir Zagorodnyuk, Discipline of Human Physiology, College of Medicine and Public Health, Flinders University, GPO Box 2100, Adelaide, South Australia, Australia.

Email: vladimir.zagorodnyuk@flinders.edu.au

Abstract

Bladder afferents play a pivotal role in bladder function such as urine storage and micturition, and conscious sensations such as urgency and pain. Endocannabinoids are ligands of cannabinoid receptors 1 and 2 (CB1 and CB2) but can influence activity of a variety of G-protein coupled receptors, and ligand- and voltage-gated channels. It is still not known which classes of bladder afferents are influenced by the CB1 and CB2 receptor agonists. This study aimed to determine the role of the CB2 receptors in two major classes of afferents in the guinea pig bladder, mucosal and muscular-mucosal.

The mechanosensitivity of these two classes was determined by an *ex vivo* extracellular electrophysiological recording technique. A stable analogue of endocannabinoid anandamide, methanandamide (mAEA) potentiated the mechanosensitivity of mucosal bladder afferents in response to stroking. In the presence of TRPV1 antagonist (capsazepine), the effect of mAEA switched from excitatory to inhibitory. The selective CB2 receptor agonist, 4-quinolone-3-carboxyamide (4Q3C) significantly inhibited the mechanosensitivity of mucosal bladder afferents to stroking. In the presence of a CB2 receptor antagonist, the inhibitory effect of 4C3F was lost. mAEA and 4Q3C did not affect responses to stretch and/or mucosal stroking of muscular-mucosal afferents.

Our findings revealed that agonists of the CB2 receptors selectively inhibited the mechanosensitivity of capsaicin-sensitive mucosal bladder afferents, but not muscular-mucosal afferents. This may have important implications for understanding of the role of endocannabinoids in modulating bladder function and sensation in health and diseases.

Introduction

Endocannabinoids such as anandamide (AEA) and 2-arachidonolylglycerol (2-AG) can modulate pain and inflammation via their receptors, the cannabinoid-1 (CB1) and CB2 receptors [1]. It is known that endocannabinoids can ameliorate bladder function and sensation, particularly during disorders such as overactive and painful bladder syndromes [2]. However, the mechanisms by which they modulate bladder function are largely elusive, particularly if they act on bladder sensory neurons.

Bladder afferents are involved in urine storage and micturition and their activation may cause conscious sensations from the bladder, ranging from physiological sensation of filling and fulness through to lower urinary tract symptoms such as urgency and pain [3]. To date, numerous classes of bladder afferents have been discovered including mucosal (urothelial), muscular-mucosal (muscular-urothelial), muscular, high threshold (serosal) and silent afferents [4-9]. However, the exact function of each of these classes in health and diseases is not known.

Currently it is well known that endocannabinoids can modulate bladder contractile activity via bladder efferent nerves [10, 11], however, there is little information available regarding the role of endocannabinoids in modulating the activity of different classes of bladder afferents. Previous studies have demonstrated that a non-selective CB1/CB2 agonist reduces firing of high-threshold distension-sensitive bladder afferents in the mouse bladder *ex vivo*, an effect was blocked by a CB1 receptor antagonist (AM251) but not a CB2 receptor antagonist (AM630) [12, 13]. Further, the CB1 receptors are co-expressed with transient receptor potential vanilloid 1 (TPRV1) in the suburothelium [13]. TRPV1 can bind some endocannabinoids such as AEA and is involved in processes such as pain and inflammation [14, 15]. Selective fatty acid amide hydrolase inhibitor (URB937), which increases

concentrations of endogenous cannabinoids, decreases distension-induced activity of Aδ and C fibers in the rat bladder in vivo by 20-30%. Both the CB1 antagonist (rimonabant) or the CB2 antagonist (SR144528) abolished its inhibitory effect [16].

It should be noted that a limitation to these studies [16] is that they are not able to directly measure the activity of other bladder afferents such as mucosal and muscular-mucosal afferents. In addition to CB1 receptors, recent studies have shown that CB2 receptors are attractive therapeutic targets for the treatment of bladder inflammation and pain [2, 17-19] and their activation is devoid of the normal psychotropic effects of CB1 agonists. Therefore, using an *ex vivo* open sheet bladder preparations [5], the current study aims to determine the role of the endocannabinoid AEA and the selective CB2 receptor agonist 4-quinolone-3-carboxyamide (4Q3C) [20] in modulating the activity of two major classes of bladder afferents, mucosal and muscular-mucosal.

Methods

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 [21].

Ex vivo bladder afferent preparation

Adult female guinea pigs (N=22; 297.7 ± 9.31g) were humanely killed via isoflurane inhalation overdose followed by severing the cervical spinal cord. The method of extracellular recordings has been described previously [5, 22]. Briefly, the bladder was dissected out and opened into a flat sheet 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 full thickness sheet (approximately 12mm by 15mm) was created and pinned along one edge mucosal side up in a 22ml organ bath containing warmed Krebs. The other edge of the preparation was attached to a 12mm custom-made stainless steel "rake" which was attached via a cantilever system to an isotonic transducer (Harvard Bioscience 52-9511, S Natick, MA. U.S.A). Increasing counterweights (10g) could be applied to the bladder to distend the preparation isotonically while measuring the resulting changes in length. Fine nerve trunks entering the trigone were dissected out and pinned via a small piece of attached connective tissue and placed in a paraffin oil bubble, sealed by paraffin wax, isolating the nerves. The dissected nerves were individually placed on a platinum electrode for extracellular recording. Electrical signals were amplified (DAM 80, WPI, USA) and recorded at 20 kHz with a Maclab/8s data acquisition system with Chart 7 software (AD Instruments, Castle Hill, NSW, Australia) using an iMac computer running OSX 10.8.5 (Apple, Cupertino, CA). Single units were

discriminated by amplitude and duration using Spike Histogram software (AD Instruments, Sydney, Australia).

Bladder afferent classes

Mucosal high-responding bladder afferents respond to light mucosal stroking, but not to stretch. They generally have a long duration of their action potentials and are activated by capsaicin [5, 22]. Muscular-mucosal bladder afferents respond to both light mucosal stroking and low intensity stretch and generally have shorter duration of action potentials [5, 22]. For both classes of bladder afferents, a tiny hole was made in the mucosa adjacent to their receptive field (hot spot) area to facilitate penetration of drugs to the lamina propria layer. 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 30 second intervals. The mechanosensitivity of bladder afferents to stretch was determined by adding weights (10g) to the cantilever for a period of ten seconds followed by a two-minute rest period and another 10 seconds of weight.

Effect of cannabinoid receptor agonists and antagonists on the mechanosensitivity of bladder afferents.

After initial recordings, either meth-AEA (mAEA, 1µM) or 4Q3C (1µM) were added to the Krebs organ bath over the afferent hot spot area and allowed to equilibrate for 5 minutes. The mechanosensitivity of the bladder afferents was then redetermined. This was repeated for increasing concentrations of both drugs (3µM, 10µM, 30µM).

In different experiments mAEA and 4Q3C were combined with antagonists of TRPV1 (capsazepine, 10µM) or CB2 receptors (SR144528, 10µM). The initial effect of agonists on the mechanosensitivity of bladder afferents was re-determined, washed out, followed by

application of the antagonist alone, and the effect of agonists when combined with antagonists.

Drugs

Stock solutions of mAEA, 4Q3C, capsazepine, and SR144528 (10mM) were made using absolute ethanol and stored at -20C. All drugs were diluted prior to experiments in Krebs. Preliminary ethanol (0.1%) control studies were carried out and no significant effects were found on the mechanosensitivity of the studied bladder afferents. mAEA (90070) and 4Q3C (11094) were obtained from Sapphire Bioscience Australia, and capsazepine (C191) and SR144528 (SML1899) were obtained from Sigma Australia.

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 8 software. All data was analyzed using a one-way ANOVA with Tukey's *post hoc* test. P-values were less than 0.05 were considered significant.

Results

Methanandamide selectively potentiated mucosal bladder afferents.

The effects of mAEA on the mechanosensitivity of mucosal and muscular-mucosal bladder afferents are illustrated in Figure 1. At all concentrations mAEA had no significant effect on the mechanosensitivity of muscular-mucosal bladders afferents to stroking or stretch (Figure 1Ai and 1Aii). mAEA did not affect bladder wall compliance measured as a change in length during imposed load (10g) to the bladder preparations (3.26 ± 0.14 mm in control, N=6 and 3.31 ± 0.18mm after 30µM mAEA application, N=6, NS, t-test paired). Application of capsaicin (1 µM) did not activate muscular-mucosal afferents (n=5, N=5).

At 1µM, mAEA had no significant effect on the mechanosensitivity of mucosal bladder afferents to stroking (100mg). However, at 3µM, 10µM, and 30µM, mAEA significantly potentiated the mechanosensitivity of bladder mucosal afferents to 100mg stroking (Figure 1Bi), with the greatest potentiation seen at 30µM, which was significantly greater than 10µM ($P<0.05$) (Figure 1Bii) (at 3µM: +39.63 ± 3.81%, $P<0.001$; at 10µM: +66.26 ± 4.88%, $P<0.0001$ and at 30µM: +95.72 ± 6.72%, $P<0.0001$; n=10, N=6, $F(5,54)=37.69$; mAEA effect, One-way ANOVA, Tukey's *post hoc* test). Further, there was a trend towards increased basal firing rate of mucosal afferents at 30µM of mAEA (0µM: 2.82 ± 0.21 Hz; 30µM: 3.72 ± 0.40, P=0.062, One-way ANOVA, Tukey's *post hoc* test).

Capsaicin (1µM0, applied at the end of experiments, activated mucosal afferents with mean maximum firing rate 11.63 ± 2.57 Hz (n=5, N=5).

Capsazepine switched the potentiating effect of methanandamide on mucosal bladder afferents to inhibitory.

The effects of mAEA and capsazapine alone or in combination are illustrated in Figure 2. Capsazepine alone (10µM) significantly inhibited the mechanosensitivity of mucosal bladder

afferents to 100mg stroking (P<0.0001). In the presence of capsazepine, the excitatory effect of mAEA (3μM) was lost and an inhibitory effect was revealed. This inhibitory effect of mAEA in the presence of capsazepine was significantly different compared to capsazepine alone (Figure 2; P<0.05) (n=6, N=5, F(5,30)=110.7, P<0.0001, mAEA and capsazepine effect, One-way ANOVA, Tukey's *post hoc* test).

CB2 receptor selective agonist inhibits the mechanosensitivity of mucosal bladder afferents.

The effects of 4Q3C on the mechanosensitivity of mucosal and muscular-mucosal bladder afferents are illustrated in Figure 3. At all concentrations tested 4Q3C had no significant effect on the mechanosensitivity of muscular-mucosal bladders afferents either to stroking or stretch (Figure 3Ai and 3Aii). 4Q3C did not affect bladder wall compliance (3.47 ± 0.21 mm in control, N=6 and 3.29 ± 0.25 mm after 30μM mAEA application, N=6, NS, t-test paired).

At 1μM, 4Q3C had no significant effect on the mechanosensitivity of mucosal bladder afferents to stroking. At 3μM, 10μM, and 30μM, 4Q3C significantly inhibited the mechanosensitivity of mucosal bladder afferents to stroking (Figure 3Bi), with the greatest inhibition at 10μM and 30μM (Figure 2Bii) (at 3μM: -23.40 ± 1.62%, P<0.01; at 10μM: -46.05 ± 2.55%, P<0.0001; at 30μM: -49.46 ± 2.92%, P<0.0001; n=10, N=6, F(5,54)=16.04, P<0.0001, 4Q3C effect, One-way ANOVA, Tukey's *post hoc* test). Further, at 10μM and 30μM 4Q3C significantly decreased the spontaneous firing rate of mucosal afferents (0μM: 2.25 ± 0.19 Hz, 10μM: 1.25 ± 0.11 Hz, P=0.0018; 30μM: 1.27 ± 0.11 Hz, P=0.0023, n=10, N=6, F(5,54)=6.61, P<0.0001 One-way ANOVA, Tukey's *post hoc* test).

SR144528 prevents the inhibitory effect of CB2 receptor agonist on the mechanosensitivity of mucosal bladder afferents.

The effects of 4Q3C or SR144528 alone or in combination are illustrated in Figure 4. SR144528 alone (10μM) had no significant effect on the mechanosensitivity of mucosal bladder afferents to stroking (100mg) (Figure 4). When combined with SR144528 the inhibitory effect of 4Q3C was lost ($P<0.001$) and not significantly different from either control or SR144528 alone (Figure 4) ($n=6$, $N=5$, $F(4,25)=15.95$, $P<0.0001$, 4Q3C and SR144528 effect, One-way ANOVA, Tukey's *post hoc* test).

Discussion

This study demonstrates that mAEA selectively potentiated the mechanosensitivity of mucosal bladder afferents to stimuli via TRVP1 activation, an effect switched to inhibitory upon TRPV1 antagonism. Further, this study also demonstrated that the selective CB2 receptor agonist 4Q3C inhibited the mechanosensitivity of mucosal bladder afferents to stimuli via the CB2 receptors. Neither mAEA nor 4Q3C had significant effects on the mechanosensitivity of muscular-mucosal afferents.

In the guinea pig bladder, two subclasses of mucosal mechanoreceptors were previously distinguished: the first were capsaicin-sensitive "mucosal high-responding mechanoreceptors", which are high responders to light mucosal stroking and activated by a variety of noxious stimuli including capsaicin. There are also "mucosal low-responding mechanoreceptors" responding only weakly to mucosal stroking, but small proportions are activated by hypertonic stimuli [5, 22]. The role of mucosal bladder afferents in bladder function is not well understood. However, given their location and expressed receptors associated with potentially harmful stimuli, they are likely involved in the detection and transmission of nociceptive related stimuli [22, 23]. TRPV1 is involved in nociception and inflammation [24, 25] and also present on mucosal high-responding mechanoreceptors [22]. The current study demonstrated that mAEA, via TRPV1, concentration-dependently potentiates mucosal bladder afferents. It is likely that mAEA could be involved in nociception and/or inflammation in the bladder. In experimental cystitis, levels of AEA are increased and AEA via activation of TRPV1 contributes to the development of hyperreflexia and hyperalgesia during cystitis [26]. However, present data indicates that when TRPV1 was blocked the effect of mAEA switched to inhibitory. This suggests that in the absence of TPRV1, AEA has an inhibitory effect which may reduce pain. AEA has previously been

implicated in analgesia [27], however, there are differing reports regarding which CB receptor mediates this since AEA can bind both CB1 and CB2 receptors [28, 29].

The CB2 receptors is also involved in inflammation and pain [30], with previous studies demonstrating that administration of a CB2 receptor agonist reduces bladder inflammation and pain [18]. However, whether the reduction in pain is directly due to activation of CB2 receptors on bladder afferents or due to a reduction in bladder inflammation is unclear. The current study clearly demonstrated that the activation of the CB2 receptors inhibits mucosal capsaicin-sensitive bladder afferents. Given the possible role of mucosal bladder afferents in pain [5], activation of the CB2 receptor on bladder afferents may cause an analgesic effect, particularly in bladder disorders such as painful bladder syndrome.

In the current study, despite the receptive fields of mucosal and muscular-mucosal afferents in the vicinity of the urothelium, neither mAEA nor 4Q3C significantly affected the mechanosensitivity of muscular-mucosal afferents to stimuli. In contrast to mucosal high-responding afferents, low-threshold muscular-mucosal afferents are usually not activated by capsaicin in the guinea pigs [5, 22]. Whilst their exact physiological role is not known, hey may detect bladder volume and stretch during distension [6, 22]. Neither mAEA nor 4Q3C altered muscular-mucosal mechanosensitivity, suggesting that either there are no CB2 receptors on this class of afferents or that the presence of TRPV1 channels is necessary for cannabinoids modulate the mechanosensitivity of bladder afferents. However, the only two studies that assess the effects of cannabinoids on bladder afferents in mice demonstrated that it inhibits high-threshold stretch-sensitive bladder afferents via CB1 [12, 13], a class of afferents not studied in the current report. It is possible that endocannabinoids may directly modulate activity of high threshold afferents in the guinea pigs. In the rat bladder, an increase in endogenous cannabinoids, produced by periphery-restricted fatty acid amide hydrolase inhibitor, URB937 decreased (by 20-30%) distension-induced activity of Aδ and C fibers via

both CB1 and CB2 receptors [16]. This implies a necessity to further investigate the possible effecst of CB1 and CB2 receptor agonists on all known classes of bladder afferents.

It should be noted that some of the drugs used in this study may also act on other targets. For example, SR144528, whilst an inverse agonist of the CB2 receptor, can also bind to the CB1 receptors. However, SR144528 has a higher affinity for the CB2 receptors compared to the CB1 receptors [31]. Further, capsazepine, can also bind and influence some sodium channels [32]. The extent of binding to other targets must be analysed in future studies.

In conclusion, this study demonstrates that mAEA and 4Q3C selectively modulate the mechanosensitivity of mucosal bladder afferents, but not muscular-mucosal afferents. Given the role of endocannabinoids in bladder function, it is possible that this CB2 receptor-mediated inhibitory effect on mucosal afferents may play an important role in controlling nociception in the bladder. This may have an important implication for treating lower urinary tract symptoms such as urgency and pain in patients with overactive bladder and painful bladder syndromes.

Grants

This study has been supported by the National Health and Medical Research Council of Australia grant #1184546 to V.Z.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

V.Z. designed the experiments. S.C. performed experiments, analysed the data and wrote the manuscript. Both authors have approved a final version of manuscript.

References

1. Cristino, L., T. Bisogno, and V. Di Marzo, *Cannabinoids and the expanded endocannabinoid system in neurological disorders.* Nat Rev Neurol, 2020. **16**(1): p. 9-29.
2. Bjorling, D.E. and Z.Y. Wang, *Potential of Endocannabinoids to Control Bladder Pain.* Front Syst Neurosci, 2018. **12**: p. 17.
3. de Groat, W.C. and N. Yoshimura, *Afferent nerve regulation of bladder function in health and disease.* Handb Exp Pharmacol, 2009(194): p. 91-138.
4. McMahon, S.B., N. Dmitrieva, and M. Koltzenburg, *Visceral pain.* Br J Anaesth, 1995. **75**(2): p. 132-44.
5. Zagorodnyuk, V.P., et al., *Properties of the major classes of mechanoreceptors in the guinea pig bladder.* J Physiol, 2007. **585**(Pt 1): p. 147-63.
6. Grundy, L., A. Erickson, and S.M. Brierley, *Visceral Pain.* Annu Rev Physiol, 2019. **81**: p. 261-284.
7. Song, X., et al., *Identification of medium/high-threshold extrinsic mechanosensitive afferent nerves to the gastrointestinal tract.* Gastroenterology, 2009. **137**(1): p. 274-84, 284 e1.
8. Xu, L. and G.F. Gebhart, *Characterization of mouse lumbar splanchnic and pelvic nerve urinary bladder mechanosensory afferents.* J Neurophysiol, 2008. **99**(1): p. 244-53.
9. Zagorodnyuk, V.P., S.J. Brookes, and N.J. Spencer, *Structure-function relationship of sensory endings in the gut and bladder.* Auton Neurosci, 2010. **153**(1-2): p. 3-11.
10. Hedlund, P. and C. Gratzke, *The endocannabinoid system - a target for the treatment of LUTS?* Nat Rev Urol, 2016. **13**(8): p. 463-70.
11. Izzo, A.A., et al., *Endocannabinoids and the Digestive Tract and Bladder in Health and Disease.* Handb Exp Pharmacol, 2015. **231**: p. 423-47.
12. Walczak, J.S. and F. Cervero, *Local activation of cannabinoid CB(1) receptors in the urinary bladder reduces the inflammation-induced sensitization of bladder afferents.* Mol Pain, 2011. **7**: p. 31.
13. Walczak, J.S., T.J. Price, and F. Cervero, *Cannabinoid CB1 receptors are expressed in the mouse urinary bladder and their activation modulates afferent bladder activity.* Neuroscience, 2009. **159**(3): p. 1154-63.
14. Holzer, P., *TRPV1 and the gut: from a tasty receptor for a painful vanilloid to a key player in hyperalgesia.* Eur J Pharmacol, 2004. **500**(1-3): p. 231-41.
15. Pertwee, R.G., *Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists.* Curr Med Chem, 2010. **17**(14): p. 1360-81.
16. Aizawa, N., et al., *Inhibition of peripheral FAAH depresses activities of bladder mechanosensitive nerve fibers of the rat.* J Urol, 2014. **192**(3): p. 956-63.
17. Liu, Q., et al., *Cannabinoid receptor 2 activation decreases severity of cyclophosphamide-induced cystitis via regulating autophagy.* Neurourol Urodyn, 2020. **39**(1): p. 158-169.
18. Wang, Z.Y., P. Wang, and D.E. Bjorling, *Activation of cannabinoid receptor 2 inhibits experimental cystitis.* Am J Physiol Regul Integr Comp Physiol, 2013. **304**(10): p. R846-53.
19. Wang, Z.Y., P. Wang, and D.E. Bjorling, *Treatment with a cannabinoid receptor 2 agonist decreases severity of established cystitis.* J Urol, 2014. **191**(4): p. 1153-8.
20. Pasquini, S., et al., *Investigations on the 4-quinolone-3-carboxylic acid motif. 4. Identification of new potent and selective ligands for the cannabinoid type 2 receptor with diverse substitution patterns and antihyperalgesic effects in mice.* J Med Chem, 2011. **54**(15): p. 5444-53.
21. Kilkenny, C., et al., *Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research.* Osteoarthritis Cartilage, 2012. **20**(4): p. 256-60.
22. Zagorodnyuk, V.P., et al., *Mechanotransduction and chemosensitivity of two major classes of bladder afferents with endings in the vicinity to the urothelium.* J Physiol, 2009. **587**(Pt 14): p. 3523-38.

23. Fry, C.H. and B. Vahabi, *The Role of the Mucosa in Normal and Abnormal Bladder Function.* Basic Clin Pharmacol Toxicol, 2016. **119 Suppl 3**: p. 57-62.
24. Bujak, J.K., et al., *Inflammation, Cancer and Immunity-Implication of TRPV1 Channel.* Front Oncol, 2019. **9**: p. 1087.
25. Charrua, A., et al., *Transient receptor potential vanilloid subfamily 1 is essential for the generation of noxious bladder input and bladder overactivity in cystitis.* J Urol, 2007. **177**(4): p. 1537-41.
26. Dinis, P., et al., *Anandamide-evoked activation of vanilloid receptor 1 contributes to the development of bladder hyperreflexia and nociceptive transmission to spinal dorsal horn neurons in cystitis.* J Neurosci, 2004. **24**(50): p. 11253-63.
27. Clapper, J.R., et al., *Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism.* Nat Neurosci, 2010. **13**(10): p. 1265-70.
28. Brusberg, M., et al., *CB1 receptors mediate the analgesic effects of cannabinoids on colorectal distension-induced visceral pain in rodents.* J Neurosci, 2009. **29**(5): p. 1554-64.
29. Chen, L., et al., *Endogenous anandamide and cannabinoid receptor-2 contribute to electroacupuncture analgesia in rats.* J Pain, 2009. **10**(7): p. 732-9.
30. Vuckovic, S., et al., *Cannabinoids and Pain: New Insights From Old Molecules.* Front Pharmacol, 2018. **9**: p. 1259.
31. Rinaldi-Carmona, M., et al., *SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor.* J Pharmacol Exp Ther, 1998. **284**(2): p. 644-50.
32. Lundbaek, J.A., et al., *Capsaicin regulates voltage-dependent sodium channels by altering lipid bilayer elasticity.* Mol Pharmacol, 2005. **68**(3): p. 680-9.

Figures

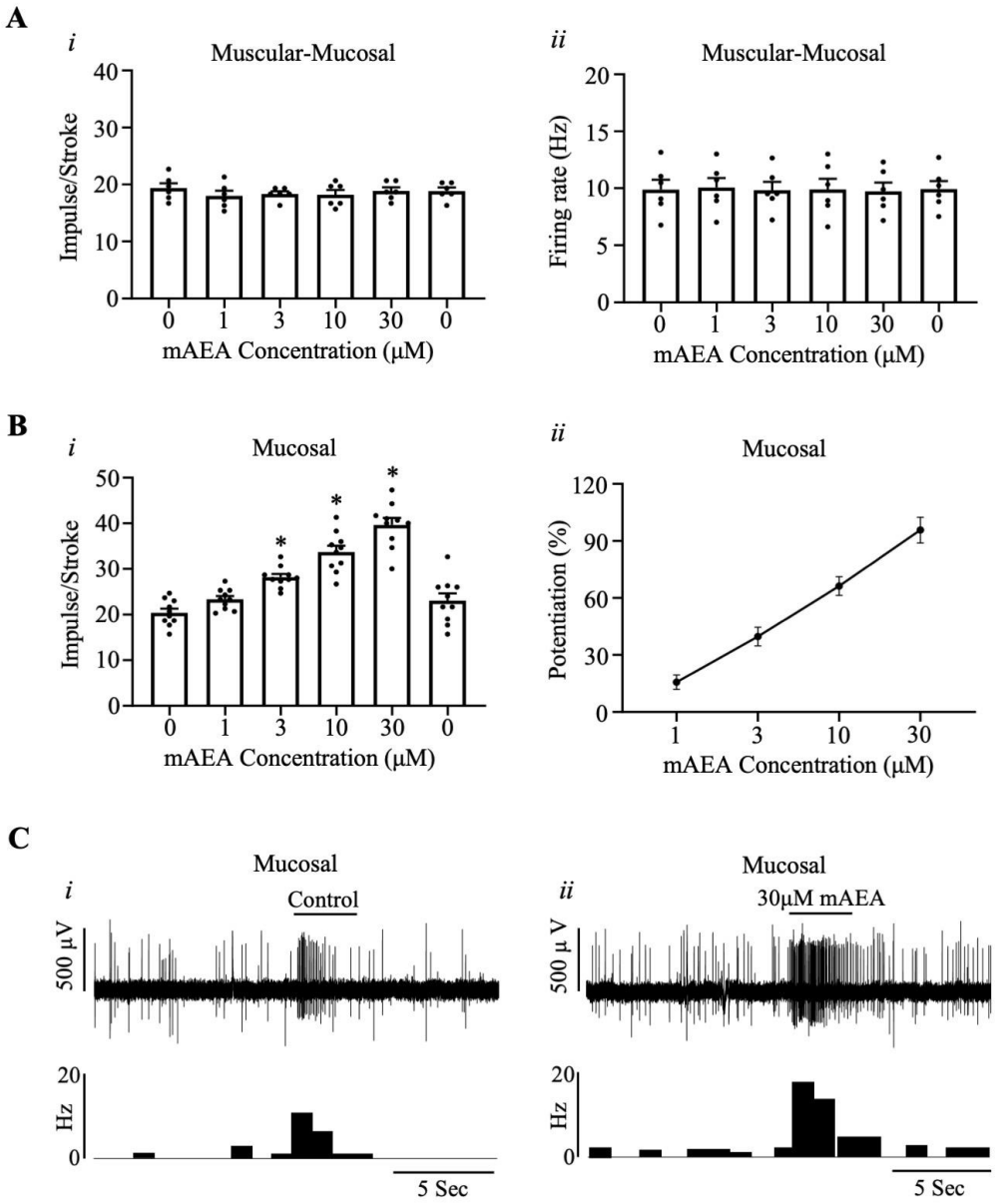
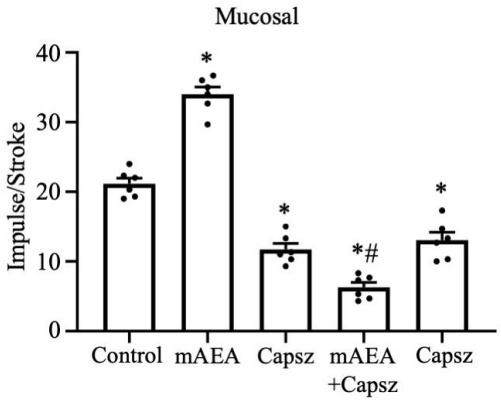


Figure 1: Methanandamide selectively potentiates the mechanosensitivity of bladder mucosal afferents. A) The sensitivity of muscular-mucosal afferents to 100mg mucosal stroking (*i*) or 10g stretch (*ii*) in the absence and presence of methanandamide (mAEA; 0-30μM). N=6, n=6 per concentration. B) The sensitivity of mucosal afferents to 100mg mucosal stroking in the absence and presence of mAEA (0-30μM) (*i & ii)*. N=6, n=10 afferents per concentration. C) Typical traces of the responses of mucosal afferents to stroking in the absence (i) and presence of 30μM mAEA (*ii*). * P<0.05 vs 0μM mAEA.



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380 **Figure 2: Capsazepine switches the effect of methanandamide from excitatory to**
381 **inhibitory.** The sensitivity of mucosal afferents to 100mg mucosal stroking in the absence
382 and presence of methanandamide (mAEA; 3μM), the transient receptor potential 1 (TRPV1)
383 antagonist capsazepine (Capsz; 10μM), and mAEA and capsazepine combined. The last bar
384 shows washing out of mAEA in the presence of capsazepine. N=5, n=6. * $P<0.05$ vs control,
385 # $P<0.05$ vs capsazepine alone.

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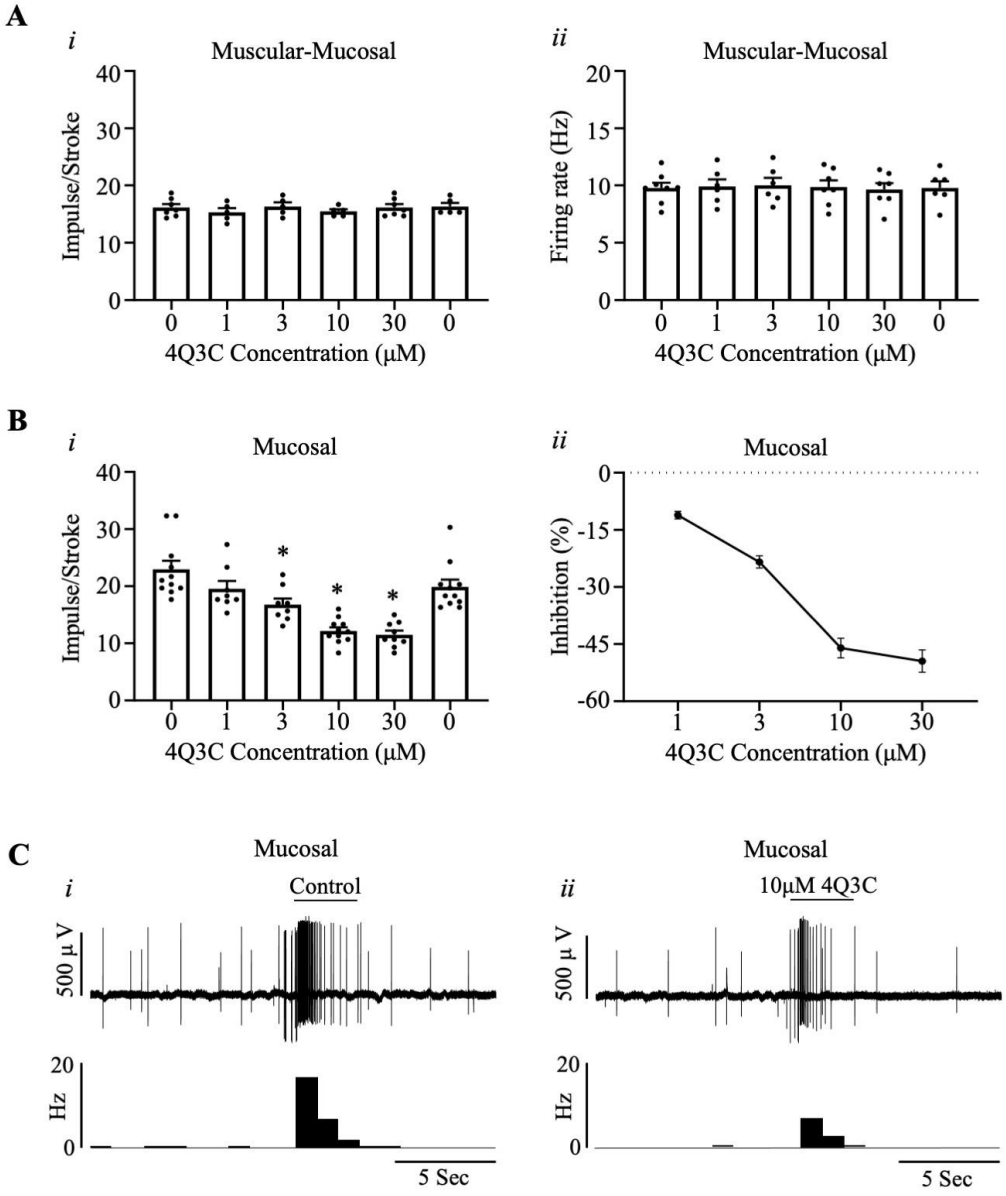
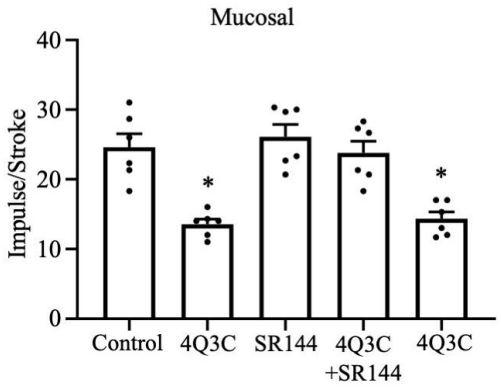


Figure 3: CB2 agonist selectively inhibits the mechanosensitivity of bladder mucosal afferents. A) The sensitivity of muscular-mucosal afferents to 100mg mucosal stroking (*i*) or 10g stretch (*ii*) in the absence and presence of the cannabinoid 2 (CB2) receptor agonist 4-Quinolone-3-Carboxyamide Furan (4Q3C). N=6, n=6 afferents per concentration. B) The sensitivity of mucosal afferents to 100mg mucosal stroking (*i & ii*) in the absence and presence of 4Q3C. N=6, n=10 per concentration. C) Typical traces of the responses of mucosal afferents to stroking in the absence (*i*) and presence of 10μM 4Q3C (*ii*). * P<0.05 vs 0μM 4Q3C.



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404 **Figure 4: SR144528 prevents the inhibitory effect of CB2 agonist.** The responses of
405 mucosal afferents to 100mg mucosal stroking in the absence and presence of the CB2
406 receptor agonist 4-Quinolone-3-Carboxyamide Furan (4Q3C; 10μM), the CB2 antagonist
407 SR144528 (SR144; 10μM), 4Q3C and SR144528 combined, and washing out of SR144528.
408 N=5, n=6. * P<0.05 vs control.

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