

CANNABINOID CB₁ RECEPTORS ARE EXPRESSED IN THE MOUSE URINARY BLADDER AND THEIR ACTIVATION MODULATES AFFERENT BLADDER ACTIVITY

J. S. WALCZAK,^{a*} T. J. PRICE^b AND F. CERVERO^a

^aMcGill University, Anesthesia Research Unit, Faculty of Medicine, Faculty of Dentistry and Alan Edwards Center for Research on Pain, 3655 Promenade Sir William Osler, Montréal, Québec, Canada H3G 1Y6

^bUniversity of Arizona, Department of Pharmacology, Tucson, AZ, USA

Abstract—Pharmacological studies have indirectly shown the possible presence of cannabinoid receptors in the urinary bladder and their potential role in reducing bladder inflammatory pain. However, the localization of cannabinoid receptors in the urinary bladder remains unknown and there are no published data on the effects of cannabinoids on the sensory system of the bladder. The present study was performed to evaluate the expression of the cannabinoid CB₁ receptors in the mouse urinary bladder and to assess their co-localization with the purinergic P2X₃ receptor, a major player in the transduction of sensory events in the bladder. Also, the effect of intravesical administration of a cannabinoid agonist on the electrical activity of bladder afferent fibers was studied. The expression of mRNA coding for CB₁ receptor was assessed by reverse transcriptase–polymerase chain reaction (RT-PCR). Immunofluorescence experiments were performed to study CB₁ and P2X₃ protein expression in the bladder. The electrical activity of bladder afferent fibers was recorded using an *ex vivo* bladder-nerve preparation. Mechanical stimulation of the bladder was performed by a controlled slow inflation with an external pump. A bolus of a cannabinoid agonist (AZ12646915) was administered intravesically prior to a second inflation. Afferent activity was measured before and after administration of the cannabinoid compound or its vehicle. The effects of CB₁ receptor antagonist (AM251) on the AZ12646915 response were also analyzed. Cannabinoid receptor CB₁ mRNA was detected in the urinary bladder of the mouse. The protein was found in the urothelium, as well as in nerve fibers. CB₁ and P2X₃ receptors were found to be co-expressed in urothelial cells and in some nerve fibers. In addition, intravesical administration of a cannabinoid receptor agonist reduced the mechanically-evoked activity of bladder afferents in the pelvic nerve. This effect was abolished by the previous administration of the CB₁ antagonist AM251. These data demonstrate the presence of cannabinoid CB₁ receptor mRNA and the protein in the mouse urinary bladder. CB₁ and P2X₃ protein co-localization supports the hypothesis of an interaction between the cannabinoid and the purinergic systems in the transduction of sensory information in the urinary bladder. Finally, the reduction of nerve activity induced by cannabinoid-receptor acti-

vation implicates CB₁ receptors in the peripheral modulation of bladder afferent information. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: CB₁ receptors, RT-PCR, immunofluorescence, *ex vivo*, electrophysiology.

Cannabinoid receptors include two types of G protein-coupled receptor. CB₁ receptors are mainly found in the CNS and peripheral tissue whereas CB₂ receptors are mainly expressed in immune tissues (Pertwee and Ross, 2002; Demuth and Molleman, 2006). The cannabinoid system is involved in the modulation of pain sensitivity either in the CNS or in the periphery (Walker and Hohmann, 2005; Manzanares et al., 2006; Lever and Rice, 2007). Previous studies have reported that cannabinoids administered systemically could reduce pain after inflammation of the urinary bladder (Jaggar et al., 1998a; Farquhar-Smith and Rice, 2001). In addition, cystometric studies have shown an increase of the micturition threshold in animals receiving systemic cannabinoid in normal or inflamed condition (Dmitrieva and Berkley, 2002; Farquhar-Smith et al., 2002; Hiragata et al., 2007). These effects were more intense when the cannabinoid solution was administered via a close-arterial route compared to a systemic administration (Dmitrieva and Berkley, 2002), supporting the hypothesis of a local regulatory role of the cannabinoid system in the micturition reflex.

The mechanisms by which cannabinoid receptors agonists could modulate this reflex locally may involve an action on the motor functions of the bladder. Indeed, some *in vitro* pharmacological experiments have shown that CB₁ receptor agonists can modulate bladder contractility in isolated bladder strips. Cannabinoids can act by prejunctional inhibition of contractile transmitter release (Pertwee and Fernando, 1996; Martin et al., 2000). Taken together, these data support the idea of the presence of cannabinoid receptors, especially CB₁ receptors, in the motor system of the urinary bladder.

A very recent study has described by Western blot the expression of the cannabinoid receptors in the urinary bladder of the rat (Merriam et al., 2008). However, despite the antinociceptive effects observed with cannabinoids after bladder inflammation, nothing is known about the local regulatory role of the cannabinoid system in the sensory system of the urinary bladder.

On the other hand, the purinergic system is reputed to mediate sensory transmission in the bladder (Andersson,

*Corresponding author. Tel: +1-514-398-4565; fax: +1-514-398-8241. E-mail address: jean.walczak@mcgill.ca (J. S. Walczak).

Abbreviations: cDNA, complementary DNA; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NGS, normal goat serum; PBS, phosphate-buffered saline; RT, reverse transcriptase; RT-PCR, reverse transcriptase–polymerase chain reaction.

2002; Cervero and Laird, 2004). ATP is released by urothelial cells after bladder distension and can activate purinergic P2X₂/P2X₃ receptors located on sensory fibers of the pelvic nerve (Ferguson et al., 1997). In addition, mice lacking the P2X₃ receptor gene show reduced inflammatory pain and urinary bladder hyporeflexia (Vlaskovska et al., 2001).

The purpose of the present study was first, to establish if cannabinoid receptor CB₁ mRNA was transcribed in the mouse urinary bladder and to determine the localization of the protein in the tissue. Co-expression with the purinergic P2X₃ receptor was assessed to evaluate the possibility of an interaction between the cannabinoid and the purinergic system in the bladder. A second objective was to characterize the afferent nerve responses to bladder distension and their modulation by the administration of a cannabinoid agonist, using an *ex vivo* mouse bladder–pelvic nerve preparation. The effects of a CB₁ receptor antagonist on this modulation were also studied.

EXPERIMENTAL PROCEDURES

Animals

Female C57BL/6 mice weighing 18–26 g (supplied by Charles River Canada, St Constant, QC, Canada) were used in this study. Animals were anesthetized with urethane (2 g/kg) *i.p.* in saline solution (0.9% NaCl) before cardiac perfusions or in order to dissect the bladder for electrophysiological studies. The procedures followed the guidelines of the committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983) and the project was approved by the Animal Care and Use Ethics Committee of McGill University. Care was taken to minimize the number of animals used and to avoid their suffering.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Mice were transcardially perfused with phosphate-buffered saline (PBS) 0.1 M+1 UI heparin/ml to remove blood cells. For each mouse, total RNA was extracted from the urinary bladder and the brain cortex tissues using RNAqueous isolation kit (Ambion, Inc., Austin, TX, USA). The extracts were then treated by DNase with the Turbo DNA-free kit (Ambion, Inc.) to remove any contamination by genomic DNA. For each sample, 0.2 μg of total RNA was converted into complementary DNA (cDNA) using Superscript II reverse transcriptase (RT) and nucleotide oligodeoxythymidine (Invitrogen Canada Inc., Burlington, ON, Canada). Aliquots of cDNA from the RT reaction were used for PCR amplification (35 cycles, annealing temperature 61 °C) using specific pairs of primers corresponding to the ubiquitous glyceraldehyde-3-phosphate dehydrogenase, GAPDH: fwd 5'-GTGGATGGCCCCTCTGGAAA-3', rev 5'-GGCCTCTCTTGTCTCAGTGTC-3' (expected size of 495 bp) and corresponding to CB₁ receptor: fwd 5'-GAATGATTGGGCTAAGG-3', rev 5'-AAGAAGGGTACTGCCCTGT-3' (expected size of 313 bp) (IDT, Coralville, IA, USA). In addition, amplification was assessed with the total RNA ex-

tracts corresponding to each mouse in order to verify the absence of contamination by genomic DNA. These reactions would therefore correspond to negative controls. Amplifications made on brain cortex samples were used as positive control for CB₁ primers. The reaction products were separated by electrophoresis on 1% agarose gel and visualized by staining with ethidium bromide under UV light.

Immunofluorescence

A vascular rinse was performed by transcardial perfusion with 50 ml of PBS 0.1 M+1 UI heparin/ml followed by 200 ml of PBS+4% paraformaldehyde as a fixative solution. The bladder was harvested and placed in the same fixative solution during 3 h at +4 °C then transferred in PBS 0.1 M+30% sucrose overnight at +4 °C. After permeabilization (PBS, 10% normal goat serum (NGS), 0.25% Triton, 0.05% NaN₃) and saturation with PBS+20% NGS, cryostat sections (12 μm) of the bladder were incubated with the primary antibodies solution (anti-CB₁ 1:100, Cayman Chemical, raised in rabbit and anti-P2X₃ 1:1000, Neuromics, raised in guinea pig) 48 h at +4 °C. After rinsing, the sections were incubated with goat antirabbit IgG (1:600) conjugated with fluorescein isothiocyanate (FITC, 488 nm) and with goat anti–guinea pig IgG (1:600) conjugated with Texas Red (568 nm) 1 h at room temperature. Mounted slides were observed under a Zeiss Axioplan 2 imaging fluorescence microscope and images were captured by a digital camera, AxioCam HRc (Zeiss, Toronto, ON, Canada). Microphotographs were obtained with ×20 and ×40 objectives. Negative controls were sections incubated without the primary antibodies. In addition, the specificity of the CB₁ receptor antibody was assessed by neutralization of the primary antibody solution with the specific synthetic peptide used to produce the antibody (Cayman Chemicals, Ann Arbor, MI, USA) with a ratio of 1:2 (v/v) for the antibody and blocking solutions respectively. The time of exposure of each image was constant during the acquisition of images.

Electrophysiology

Mouse bladder–nerve preparation. The mouse urinary bladder and surrounding tissues were dissected and placed in a chamber where they were continuously perfused with oxygenated (95% O₂, 5% CO₂) Tyrode solution pH 7.4 (content in mM: NaCl 136.9; KCl 2.7; CaCl₂ 1.8, MgCl₂ 1; NaH₂PO₄ 0.4; NaHCO₃ 11.9 and glucose 5.6). A fine triple-lumen canula (combined diameter 0.9 mm OD) was inserted into the bladder through the urethra. The canula was connected to: (i) a syringe pump (Harvard Apparatus, Holliston, MA, USA) to infuse Tyrode into the bladder, (ii) to a pressure transducer (P75, Hugo Sachs Elektronik–Harvard Apparatus, GmbH, March-Hugstetten, Germany) to record intravesical pressure changes and (iii) to an outlet tube equipped with a three way stopcock to block or release intravesical fluids from the bladder. An additional canula (MicroFil MF34G 0.1 mm ID, WPI, Sarasota, FL, USA, diameter 0.164 mm OD) was added along the triple-lumen canula and connected to a 1 ml syringe to allow bolus application of drugs or vehicle. The urethra and

the ureters were ligated to avoid leakage of fluid from the urinary bladder.

Under a microscope, a branch of the pelvic nerve arising from the urinary bladder was dissected. Nerve activity was recorded from very fine filaments dissected from the pelvic nerve and sucked inside a glass suction electrode connected to an Axon Instrument head stage (Al 402×50 ultra-low noise differential amplifier, Axon Instrument) and an AC/DC amplifier (CyberAmp 380, Axon Instruments). Signals were amplified (250×), filtered (band-pass 10–10,000 Hz), and relayed to a noise eliminator (Hum Bug, Quest Scientific, Vancouver, Canada). The electrical activity of the nerve and the intravesical pressure were digitized using a computer connected to a Micro 1401 MK II analog-to-digital interface controlled with Spike 2 (version 6.08) software (Cambridge Electronic Design, Cambridge, UK).

Mechanical stimulation of the bladder was performed by a slow infusion of Tyrode solution (0.1 ml/min) until the intravesical pressure reached 40 mm Hg, then the pressure was released by opening the outlet valve. The mean duration of each stimulus was of 81 ± 9 s (mean \pm SEM). Single unit discrimination was performed by using the spike sorting function of Spike 2 software.

Pharmacology. For the pharmacological assay a bolus of the non-selective (CB₁–CB₂) cannabinoid agonist AZ12646915 (100 μ M, 100 μ l) or of its vehicle (Tyrode+1% dimethyl sulfoxide (DMSO)) was administered intravesically 30 min after a first inflation and 20 min prior to a second inflation. The drugs were administered as bolus with the outlet valve opened in order to avoid a possible sensitization due to a prolonged distension. Therefore, the bladder was empty before the second inflation. The total number of action potentials evoked in each afferent fiber was counted from the beginning of the application of the pressure ramp to 1 min after the point when a pressure value of 40 mm Hg was reached. This was done to assess the total response of the afferents to the stimulus and to take into account any possible after discharge. Counts were taken of the responses to distension before and after the administration of the cannabinoid agonist or its vehicle. The results were normalized by dividing the number of spikes by the area under the curve of the intravesical pressure recording. Also, for each unit a percentage of change of activity was calculated using this formula:

$$\% \text{ of change} = \left(\frac{[\text{post-activity}]}{[\text{pre-activity}]} \right) \times 100 - 100$$

In addition, the volume of Tyrode infused in the bladder was measured together with the time required for the intravesical pressure to reach 40 mm Hg and the flow rate of the pump (0.1 ml/min). This was done before and after the administration of the cannabinoid agonist or its vehicle to assess that the bladder received the same distension stimuli before and after the cannabinoid tests.

The effects of a previous administration of a CB₁ receptor antagonist were studied in another series of experiments. After a test distension stimulus, a bolus of AZ12646915 (100 μ M, 100 μ l) was administered 20 min

prior to a second stimulus as described above. Recovery was assessed by a third stimulus following a washout period (infusion of Tyrode 0.1 ml/min during 10 min with the outlet valve opened) and 20 min of rest. Thirty minutes after the third inflation, a bolus of the cannabinoid CB₁ antagonist (AM251, 100 μ M, 100 μ l) was administered followed by another bolus of AZ12646915 (100 μ M, 100 μ l) 5 min later. A fourth stimulus 20 min later was then applied. Results are expressed as the percentage of each response in relation to the baseline activity of each fiber.

Chemicals

The cannabinoid compound used in this study, AZ12646915 (AstraZeneca R&D, Montreal, QC, Canada) has equal affinity for both human CB₁ and CB₂ receptor (K_i=17 and 16 nM respectively). It was selected for its good solubility (up to 150 μ M) in aqueous solution in order to prevent as much as possible precipitation in the tubing. The CB₁ receptor antagonist AM251 was purchased from Tocris BioScience (Ellisville, MO, USA). A 10 mM stock solution was prepared in DMSO and then diluted in Tyrode to obtain a 100 μ M solution containing 1% DMSO. Salts used for the solutions were all purchased from ACP chemicals (Montreal, QC, Canada).

Statistical analysis

Analysis of possible sub-populations of afferents according to their firing thresholds was performed using the D'Agostino and Pearson normality test. The effects of treatments before and after the cannabinoid agonist or the vehicle were analyzed with a paired *t*-test (Wilcoxon matched pairs test). Unpaired *t*-test was used to analyze the differences between the two groups (agonist vs. vehicle). Analysis of the mean percentage of change of activity versus a theoretical mean of zero (no change) was performed with a one-sample *t*-test. Fisher's exact test was used to analyze the statistical significance between the number of afferent fibers that had a reduced activity after the cannabinoid agonist and after vehicle. Repeated measures ANOVA (Friedman's test) followed by Dunn's post hoc test for multiple comparisons versus pretreatment was used to analyze the recovery of the electrophysiological responses and the effects of the antagonist AM251 on fibers responsive to AZ12646915. *P*<0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, CA, USA.

RESULTS

RT-PCR

The mRNA coding for CB₁ was found in extracts from the bladder as well as extracts from the brain cortex (positive controls for CB₁ primers). There was no PCR amplification from RNA extracts that did not get the reverse transcription. Therefore, no contamination from genomic DNA was observed in the samples. Amplification with primers corresponding to GAPDH (positive control for the experiment) showed the same pattern of results (Fig. 1).

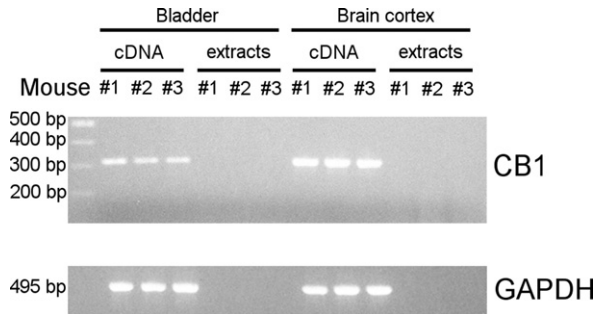


Fig. 1. Results of PCR amplification obtained with specific primers for CB₁ receptor on cDNA obtained after the reverse transcription RNA extracts from the urinary bladder and the brain cortex of mice. Experiments were done on tissues from three different mice. GAPDH was used as a positive control and RNA extracts were used as negative controls.

Immunofluorescence

As shown in Fig. 2 the preincubation of the antibody solution with the blocking peptide resulted in an absence of immunofluorescence, which demonstrates the specificity of the labeling for CB₁ in the mouse urinary bladder.

Low magnification of sections of the bladder wall showed immunoreactivity for CB₁ in the urothelium as well as in nerve fibers in the muscular (Fig. 3A) and the suburothelial layers of the bladder wall (Fig. 3A, B). Slides incubated without the primary antibodies solution did not show a significant non-specific fluorescence (Fig. 3C). With higher magnification, co-localization of CB₁ and P2X₃ receptor immunoreactivity was observed in urothelial cells especially in the umbrella cells (Fig. 3D). However, P2X₃ immunofluorescence observed in the zone of the basal membrane was not co-localized with CB₁-immunoreactivity (Fig. 3D asterisks). In addition, some nerve fibers displayed co-immunofluorescence for CB₁ and P2X₃. This was found mainly in the muscular layer of the bladder wall (Fig. 3E).

Electrophysiology

The responses of 132 afferents (from 45 animals) to distension of the bladder were analyzed. A wide range of mechanical thresholds was observed in these recordings. Fig. 4A shows an example of a single unit with a firing threshold of 7 mm Hg and Fig. 4B shows an example of a single unit with a higher threshold of 21 mm Hg. The frequency histogram for the whole population of the thresholds of activation of afferent fibers is shown in Fig. 4C. During stimuli going up to 40 mm Hg, the thresholds of firing had values comprised between 0.3 and 27 mm Hg. The majority of the afferents recorded had low thresholds to distension in the range 0–15 mm Hg. A smaller number of fibers showed mechanical thresholds above 15 mm Hg. An attempt was made to divide the distribution of thresholds into several populations (0–8, 8–17 and 17–28 mm Hg with a mean \pm SD of 3.8 ± 2.3 , 12.4 ± 2.5 and 20.9 ± 2.9 mm Hg respectively). However, only the 8–17 and 17–28 mm Hg groups were distributed according to a gaussian function.

Examples of afferent activity evoked by distension before and after administration of the cannabinoid agonist (100 μ M) are illustrated in Fig. 5A. In the recordings illustrated in the figure, two different spikes could be discriminated. One fiber (a, black) was not affected by the cannabinoid compound, while the activity of the other fiber (b, grey) is reduced after the intravesical administration of the cannabinoid agonist.

The responses to mechanical distension of the bladder of the afferents were quantified before and after administration of the vehicle ($n=15$ fibers, 6 animals) or the cannabinoid agonist ($n=39$ fibers, 9 animals). In the vehicle group, one afferent was unchanged, six showed a reduced response and nine showed increased responsiveness. On the other hand, in the group receiving the cannabinoid agonist, 31 out of 39 afferents showed reduced responsiveness and only 8 out of the 39 fibers displayed either no change (one afferent) or an increased response (seven afferents) (Table 1). The overall responsiveness of the afferent fibers after administration of the cannabinoid agonist was reduced by 40% (Fig. 5C) and the analysis of the two populations (vehicle and cannabinoid) showed a statistically significant difference in responsiveness (Fig. 5C). The mean percentage of change of activity after cannabinoid agonist administration was significantly different from the vehicle group ($P=0.011$).

Interestingly, the population of fibers that responded to cannabinoid with reduced activity had a significantly higher threshold than the population that did not reduce their responsiveness (Table 1). On the contrary, no difference could be observed in the mechanical thresholds of the populations of fibers which had an increased and the ones with a decreased activity after the vehicle treatment. Therefore, taking into account the overall spectrum of thresholds, the afferents with higher thresholds were more likely to be inhibited by the cannabinoid agonist (Table 1).

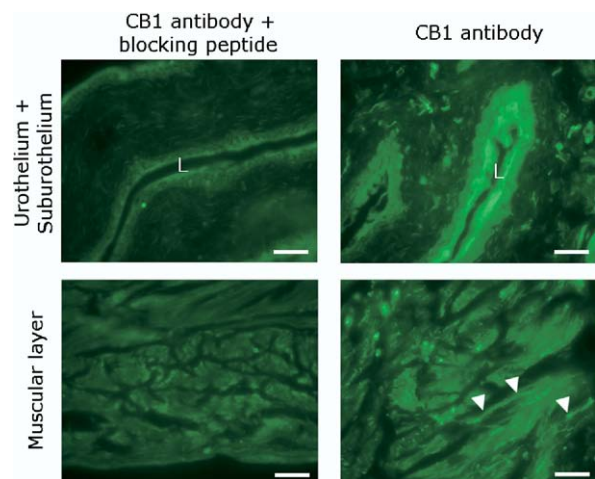


Fig. 2. Control for CB₁ antibody specificity. The left panels show sections incubated with a mixture of the antibody solution and the blocking peptide. The right panels show the immunofluorescence observed after incubation with the antibody solution only. The urothelium and suburothelium layers are shown in the upper part and the muscular layer in the lower part. L=lumen. Scale bars=20 μ m.

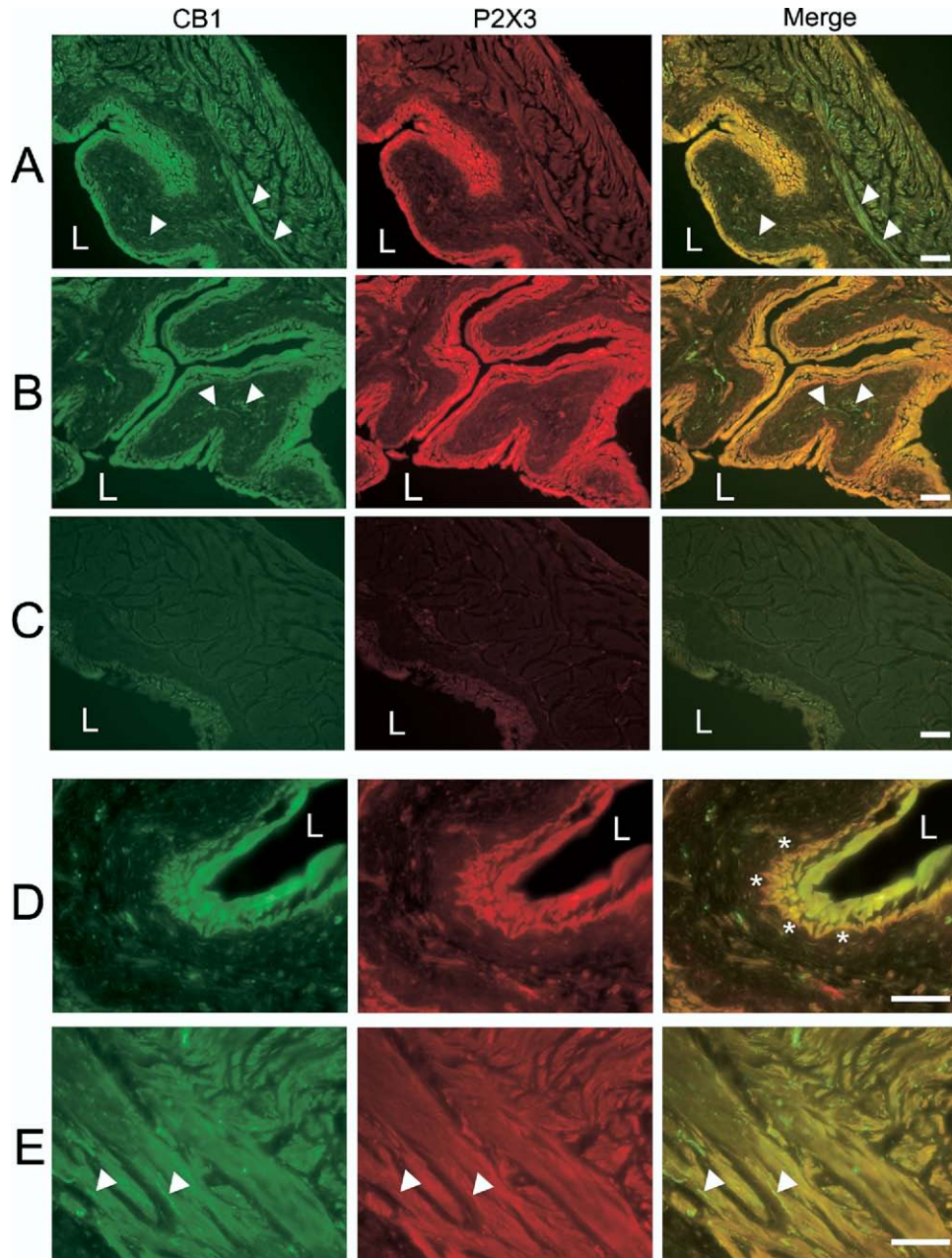


Fig. 3. Double staining for CB₁ and P2X₃ in the mouse urinary bladder. (A–C) Low magnification sections of the bladder with all layers. (C) The sections incubated without the primary antibodies solution. (D, E) Higher magnification of bladder sections showing the urothelial and suburothelial layers (D) and the muscular layer (E). Immunofluorescence is green for CB₁, red for P2X₃ and the merged pictures show co-localization in yellow. Arrowheads indicate nerve fiber structures. The absence of co-localization in the zone of the basal membrane (D, merge) is noted by asterisks. L=lumen. Scale bars=20 μ m.

The volume of infused Tyrode necessary to reach 40 mm Hg was not significantly different after the cannabinoid agonist or its vehicle administration ($P=0.59$ and $P=0.86$ respectively) and therefore the compound had no obvious effect on the compliance of the bladder tissue.

Fig. 6A shows an example of the activity of one fiber during the protocol used to assess the recovery and the effects of the CB₁ antagonist AM251. In this series of experiments 19 fibers could be discriminated in four animals. Fifteen of them had their activity reduced by

AZ12646915 administration and their responses returned to baseline levels after washout (Fig. 6B). Administration of AM251 prior to AZ12646915 blocked the effects of the cannabinoid agonist.

DISCUSSION

Two main observations have been made in this study: (i) we have demonstrated for the first time the presence of the CB₁ gene transcript and the location of the protein in the

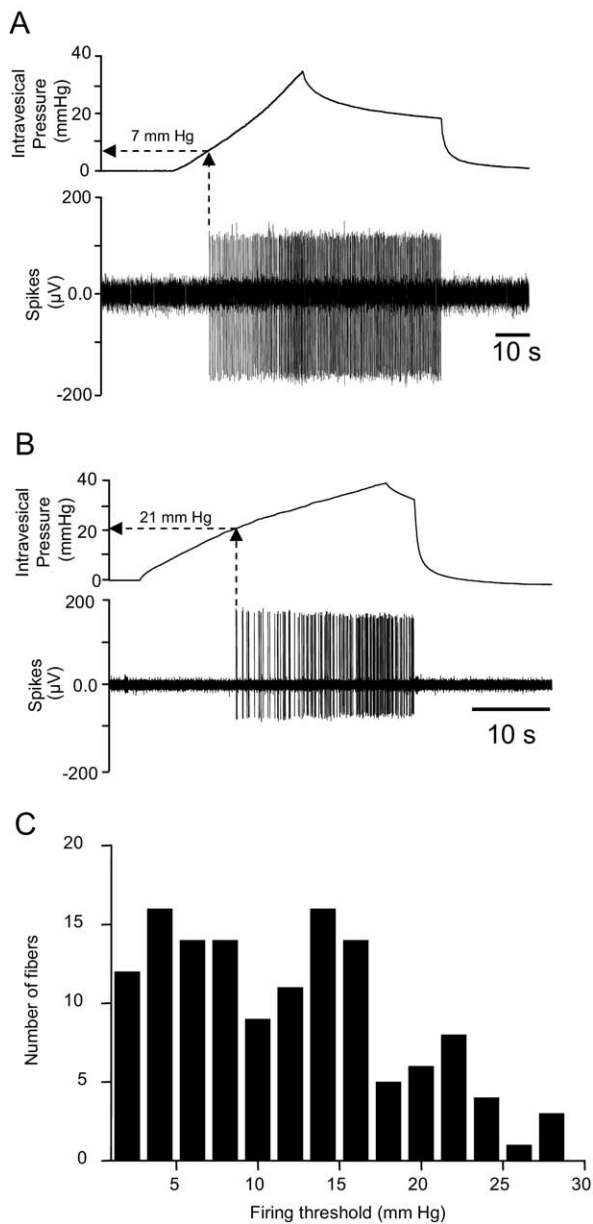


Fig. 4. Representative recordings of low and high threshold fibers evoked by mechanical distension of the bladder. (A) A single unit recording with a lower threshold of firing (7 mm Hg). (B) A single unit recording of a fiber having a higher threshold of firing (21 mm Hg). (C) Frequency histogram of firing thresholds of 132 discriminated pelvic nerve fibers recorded from 45 animals.

mouse urinary bladder tissue and (ii) we have also shown that intraluminal administration of a cannabinoid agonist reduces mechanically-evoked afferent bladder activity, preferentially of those afferents with a high mechanical threshold to distension. This reduction of activity was blocked by a CB₁ receptor antagonist.

Expression of the cannabinoid receptor in the mouse urinary bladder

The presence of CB₁ receptors in nerve fibers of the muscular layer is in line with reports showing that admin-

istration of cannabinoids in *in vitro* preparations of bladder strips decreased the contractility of the bladder (Pertwee and Fernando, 1996; Martin et al., 2000). Pertwee and Fernando (1996) determined that cannabinoids were effective in reducing electrically evoked stimulations of the bladder strips whereas they were unable to reduce the contractions induced by acetylcholine or an analog of ATP. These observations supported the idea of cannabinoids acting on prejunctional nerve endings to reduce the release of contractile molecules. In the present study, we observed a clear labeling with CB₁ immunofluorescence in nerve fiber structures located in the muscular layer. Also, the fact that we did not observe any change in the volume necessary to reach 40 mm Hg before and after a cannabinoid agonist administration in our preparation also suggests that the action of cannabinoids was limited to the nerve endings. This would therefore support the hypothesis of a prejunctional localization of CB₁ receptors in the muscular layer of the bladder wall.

CB₁ receptor mRNA and protein are expressed in cell bodies of neurons in the DRG (Bridges et al., 2003). Afferent fiber endings are located adjacent to the urothelium, in the suburothelial layer (Yoshimura, 2007). The localization of CB₁ receptors in nerve fibers located in the suburothelial layer of the bladder wall, and therefore putative afferent fibers, could be the substrate for the effects observed in the electrophysiological experiments as it is known that activation of CB₁ receptors can reduce neuronal activity (Di Marzo et al., 2004).

In addition to a direct action on nerve fibers, another explanation for the reduction of nerve activity after cannabinoid agonist administration could be through the urothelial cells. We have observed that CB₁ receptors are expressed in those cells, especially the umbrella cells. Therefore, the finding of mRNA coding for CB₁ in bladder tissue is presumably due to transcription of the gene in the urothelial cells. These results are in line with a recent report, showing the presence of CB₁ and CB₂ mRNA in the urinary bladder of the rat (Merriam et al., 2008). We have also observed a co-localization of CB₁ receptor with the purinergic P2X₃ receptor. This co-localization was present mostly in urothelial cells, including the umbrella cells. The urothelium is thought to be a critical player in the sensory process of the bladder. During the filling of the bladder, the urothelium is stretched which stimulates the release of ATP from those cells (Wang et al., 2005). Once released, ATP can act via P2X₂–P2X₃ receptors present on urothelial cells to stimulate stretched induced exocytosis from the urothelium (Wang et al., 2005; Apodaca et al., 2007). Also, ATP can directly depolarize and initiate firing in sensory nerves by activating P2X receptors (Vlaskovska et al., 2001; Cockayne et al., 2005). We found some co-localization of CB₁ and P2X₃ receptors in nerve fibers, these fibers were found in the muscular layer, also the majority of CB₁ positive fibers did not express P2X₃ receptors. Therefore any interaction between the purinergic and the cannabinoid systems in the sensory processes would be mostly through an action on urothelial cells possibly by reducing the release of ATP from those cells.

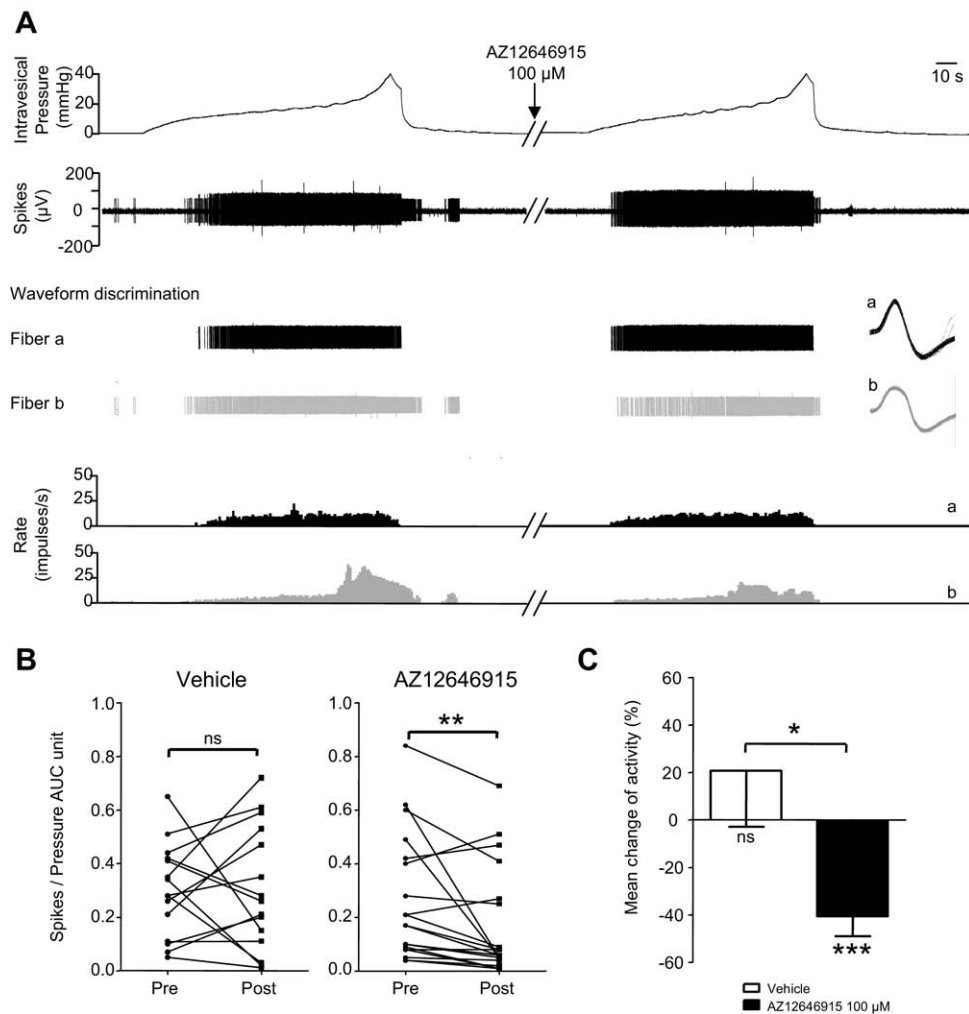


Fig. 5. Nerve activity evoked by mechanical distension recorded from the ex vivo bladder-nerve preparation. (A) An example of the recording during a first stimulus and a second stimulus following the administration of a bolus of the cannabinoid agonist AZ12646915 (100 μM). The spike analysis displays two fibers (a) and (b) with their respective rate of firing. (B) The normalized activity against treatment before and after AZ12646915 (20 fibers, 5 animals) or vehicle (15 fibers, 6 animals) administrations, paired *t*-test. (C) The mean change of neuronal activity after the treatment with vehicle or the cannabinoid agonist (CB ago), *t*-test. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: non-significant.

Mechanosensitivity of bladder afferent fibers

The presence of low and high threshold afferent fibers in the urinary bladder has been reported in rodents (Sengupta and Gebhart, 1994; Shea et al., 2000; Rong et al., 2002). Low threshold fibers are presumed to have a role in

the sensation of innocuous filling and represent the majority of the afferent fibers of the pelvic nerve. High threshold fibers respond to intense mechanical stimuli and are likely candidates to mediate painful sensations from the bladder. As in these previous studies we have also observed a

Table 1. Classification of fibers responsive to the cannabinoid agonist AZ12646915 or its vehicle according to a decreased activity or not

	Vehicle		AZ12646915 100 μM	
	Decreased activity	Increase or no change	Decreased activity	Increase or no change
Number of fibers	6	9	31**	8
Range of firing thresholds (mm Hg)	5–19	4–20	2–21	3–16
Median (mm Hg)	9.5	13	12	5.5
Mean threshold (mm Hg)	11 \pm 2.2	13 \pm 1.4	11.3 \pm 0.9	7.5 \pm 1.8*

The number of fibers with a decreased activity is significantly higher after the administration of AZ12646915 than after the vehicle, Fisher's exact test ($P = 0.009$). The range, median and the mean firing threshold of each category are indicated. *t*-Test (decreased activity vs. increase or no change). Statistical significance: * $P < 0.05$; ** $P < 0.01$.

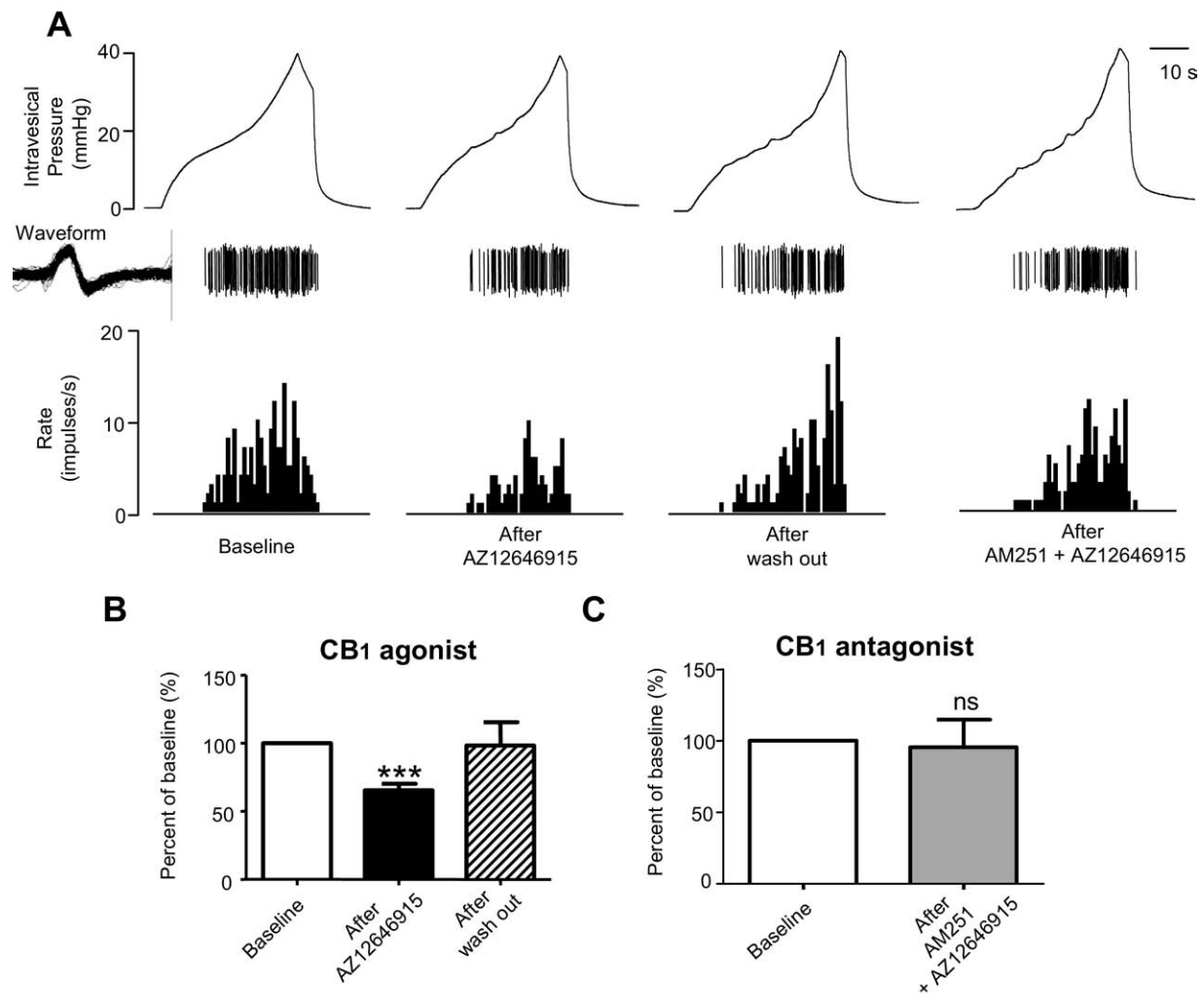


Fig. 6. Effects of a CB₁ receptor antagonist (AM251) on the activity of afferent fibers responsive to the cannabinoid agonist AZ12646915. (A) An example of the activity of a pelvic afferent fiber in response to mechanical distention in pretreatment (baseline), after AZ12646915 (100 μ M), after washout (recovery) and after administration of AZ12646915 (100 μ M) pretreated with AM251 (100 μ M). (B) Activity of fibers responsive to the cannabinoid agonist ($n=15$). Results are expressed in percent of baseline activity. (C) Activity of fibers responsive to the cannabinoid agonist after preadministration of AM251 before AZ12646915 ($n=15$). Results are expressed in percent of baseline activity, repeated measures ANOVA. *** $P<0.001$, ns: non-significant.

majority of afferent fibers with low mechanical thresholds but we have failed to appreciate two distinct peaks of mechanosensitivity. In previous studies, 15 mm Hg has been proposed as the threshold above which a fiber was classified as high threshold (Sengupta and Gebhart, 1994; Rong et al., 2002). In the present study no clear gap in the distribution of the mechanical thresholds allowed us to make a distinction between low and high threshold fibers. We observed instead a continuous distribution of thresholds similar to that previously reported in the rat (Shea et al., 2000). In this study, Shea et al. (2000) observed three mean thresholds of firing according to the location of the receptive fields of afferents determined by gentle probing of the bladder. The average thresholds were of 3.2 mm Hg for afferent receptive fields in the dome of the bladder, 8.4–10.0 mm Hg for the ureterovesical junction and the base respectively, and 25.2 mm Hg for unprobed fibers.

Although we did not establish the location of the receptive fields of our afferents it is possible that the distribution of thresholds in our study also reflects differences in the location of the afferent being recorded.

Activity of pelvic nerve afferent in response to distension and cannabinoid agonist

The most consistent effect of the administration of the cannabinoid agonist was a reduction in the afferent activity evoked by distension. However, while every fiber with a medium to higher threshold of activation was inhibited by the cannabinoid agonist, some lower threshold fibers did not. Therefore the administration of a cannabinoid agonist would be more efficient in reducing the activity of fibers with a high threshold than those with a lower one. The dose we used is high but might not reflect the exact con-

centration of drug that reaches the cannabinoid receptors in this particular *ex vivo* model after an intravesical administration since the bladder wall is highly impermeable. This dose is in line with previous report showing a reduction of evoked nerve activity with the same *ex vivo* preparation after intravesical administration of 300 μ M of the purinergic antagonist PPADS (Vlaskovska et al., 2001) while 30 μ M was sufficient after an application on the serosal side (Yu and de Groat, 2008). The compound that we used is an agonist for both CB₁ and CB₂ receptors and therefore the reduction of afferent activity could be mediated by either or both of these receptors. Administration of a CB₁ receptor antagonist blocked the effects of the agonist. Therefore, the modulation of bladder mechanosensitivity by AZ12646915 appears to be mediated only through CB₁ receptors. Behavioral studies with systemic administration of endocannabinoid have shown a reduction of referred hyperalgesia and visceral hyper-reflexia associated with inflammation of the urinary bladder. However, these signs of hypersensitivity could not be prevented by activation of CB₂ receptor, suggesting a possible role of this receptor only after the inflammation is established (Jaggur et al., 1998a, b; Farquhar-Smith and Rice, 2001). Since there was no inflammation in the present study, our results are in line with previous studies. As confirmed by immunofluorescence, the action of the cannabinoid agonist would be through a direct action on sensory afferents and/or an indirect action by interacting with the purinergic system mostly on urothelial cells.

CONCLUSION

This study reports, for the first time, the presence of the cannabinoid receptor CB₁ mRNA and the protein localization in the mouse urinary bladder. Topical activation of the cannabinoid system through CB₁ receptors leads to a decrease in the firing activity evoked by urinary bladder distension, mainly on high threshold afferents. The data also support the hypothesis of a possible interaction between the cannabinoid and the purinergic system in the urinary bladder.

Acknowledgments—This study was supported by a grant from the Canadian Institutes of Health Research. J.S.W. is supported by a Postdoctoral Fellowship from the Fonds de Recherche en Santé du Québec. The cannabinoid agonist AZ12646915 was kindly provided by AstraZeneca R&D Montreal, QC, Canada.

REFERENCES

- Andersson KE (2002) Bladder activation: afferent mechanisms. *Urology* 59:43–50.
- Apodaca G, Balestreire E, Birder LA (2007) The uroepithelial-associated sensory web. *Kidney Int* 72:1057–1064.
- Bridges D, Rice AS, Egertova M, Elphick MR, Winter J, Michael GJ (2003) Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using *in situ* hybridisation and immunohistochemistry. *Neuroscience* 119:803–812.
- Cervero F, Laird JM (2004) Understanding the signaling and transmission of visceral nociceptive events. *J Neurobiol* 61:45–54.
- Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE, Ruan HZ, Ma B, Yip P, Nunn P, McMahon SB, Burnstock G, Ford AP (2005) P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. *J Physiol* 567:621–639.
- Demuth DG, Molleman A (2006) Cannabinoid signalling. *Life Sci* 78:549–563.
- Di Marzo V, Bifulco M, De Petrocellis L (2004) The endocannabinoid system and its therapeutic exploitation. *Nat Rev Drug Discov* 3:771–784.
- Dmitrieva N, Berkley KJ (2002) Contrasting effects of WIN 55,212-2 on motility of the rat bladder and uterus. *J Neurosci* 22:7147–7153.
- Farquhar-Smith WP, Jaggur SI, Rice AS (2002) Attenuation of nerve growth factor-induced visceral hyperalgesia via cannabinoid CB(1) and CB(2)-like receptors. *Pain* 97:11–21.
- Farquhar-Smith WP, Rice AS (2001) Administration of endocannabinoids prevents a referred hyperalgesia associated with inflammation of the urinary bladder. *Anesthesiology* 94:507–513; discussion 506A.
- Ferguson DR, Kennedy I, Burton TJ (1997) ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes—a possible sensory mechanism? *J Physiol* 505 (Pt 2):503–511.
- Hiragata S, Ogawa T, Hayashi Y, Tyagi P, Seki S, Nishizawa O, de Miguel F, Chancellor MB, Yoshimura N (2007) Effects of IP-751, ajulemic acid, on bladder overactivity induced by bladder irritation in rats. *Urology* 70:202–208.
- Jaggur SI, Hasnie FS, Sellaturay S, Rice AS (1998a) The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. *Pain* 76:189–199.
- Jaggur SI, Sellaturay S, Rice AS (1998b) The endogenous cannabinoid anandamide, but not the CB2 ligand palmitoylethanolamide, prevents the viscerovisceral hyper-reflexia associated with inflammation of the rat urinary bladder. *Neurosci Lett* 253:123–126.
- Lever IJ, Rice AS (2007) Cannabinoids and pain. *Handb Exp Pharmacol* 265–306.
- Manzanares J, Julian M, Carrascosa A (2006) Role of the cannabinoid system in pain control and therapeutic implications for the management of acute and chronic pain episodes. *Curr Neuropharmacol* 4:239–257.
- Martin RS, Luong LA, Welsh NJ, Eglen RM, Martin GR, MacLennan SJ (2000) Effects of cannabinoid receptor agonists on neuronally-evoked contractions of urinary bladder tissues isolated from rat, mouse, pig, dog, monkey and human. *Br J Pharmacol* 129:1707–1715.
- Merriam FV, Wang ZY, Guerios SD, Bjorling DE (2008) Cannabinoid receptor 2 is increased in acutely and chronically inflamed bladder of rats. *Neurosci Lett* 445:130–134.
- Pertwee RG, Fernando SR (1996) Evidence for the presence of cannabinoid CB1 receptors in mouse urinary bladder. *Br J Pharmacol* 118:2053–2058.
- Pertwee RG, Ross RA (2002) Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids* 66:101–121.
- Rong W, Spyer KM, Burnstock G (2002) Activation and sensitisation of low and high threshold afferent fibres mediated by P2X2 receptors in the mouse urinary bladder. *J Physiol* 541:591–600.
- Sengupta JN, Gebhart GF (1994) Mechanosensitive properties of pelvic nerve afferent fibers innervating the urinary bladder of the rat. *J Neurophysiol* 72:2420–2430.
- Shea VK, Cai R, Crepps B, Mason JL, Perl ER (2000) Sensory fibers of the pelvic nerve innervating the rat's urinary bladder. *J Neurophysiol* 84:1924–1933.
- Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford AP, Burnstock G (2001) P2X3 knock-out mice reveal a major sensory role for urothelially released ATP. *J Neurosci* 21:5670–5677.
- Walker JM, Hohmann AG (2005) Cannabinoid mechanisms of pain suppression. *Handb Exp Pharmacol* 509–554.

Wang EC, Lee JM, Ruiz WG, Balestreire EM, von Bodungen M, Barrick S, Cockayne DA, Birder LA, Apodaca G (2005) ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *J Clin Invest* 115:2412–2422.

Yoshimura N (2007) Lower urinary tract symptoms (LUTS) and bladder afferent activity. *NeuroUrol Urodyn* 26:908–913.

Yu Y, de Groat WC (2008) Sensitization of pelvic afferent nerves in the in vitro rat urinary bladder-pelvic nerve preparation by purinergic agonists and cyclophosphamide pretreatment. *Am J Physiol Ren Physiol* 294:F1146–F1156.

Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 6:109–110.

(Accepted 23 January 2009)
(Available online 3 February 2009)