

1 **ABSTRACT**

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3 **Aims**

4 To evaluate effects of CP55,940 on normal bladder function *in vivo* and to
5 examine whether it suppresses urinary frequency induced by nociceptive stimuli
6 in the bladder. Cannabinoid receptor (CBR) activity may be involved in the
7 regulation of bladder function. However, the role of CBR subtypes in micturition
8 has yet to be established. CP55,940 is a synthetic analogue of
9 tetrahydrocannabinol, which is a psychoactive ingredient of the *Cannabis* plant.

10 **Methods**

11 Cystometry under urethane anaesthesia was performed to evaluate the effect
12 of intravesical CP55,940 with or without administration of CB1 antagonist-
13 AM251 or CB2 antagonist-AM630 on bladder function in female rats. The
14 effects of CP55,940 were also examined in rats with urinary irritation induced by
15 intravesical infusion with acetic acid.

16 **Results**

17 Infusion of CP55,940 significantly ($p < 0.05$) increased the micturition interval
18 (MI) and bladder capacity (BC) by 52% and decreased maximal voiding
19 pressure (MP) by 25%. Pre-treatment with AM251 or AM630 before CP55,940
20 administration, prevented CP55,940-induced increases in MI, BC and reduction
21 in MP. Acetic acid-induced urinary frequency as evidenced by a reduction in MI,
22 and was suppressed by CP55,940.

23 **Conclusions**

24 CP55,940 decreases bladder activity and urinary frequency induced by
25 nociceptive stimuli, probably by suppression of bladder afferent activity. Effects

26 of CP55,940 were abolished by both CBR antagonists. This data implicates a
27 role for the endocannabinoid system in bladder mechanoafferent function in
28 rats. In addition, our results show that CP55,940 reverses urinary frequency
29 exemplified in an overactive bladder model suggesting it could be an effective
30 treatment for patients with lower urinary tract symptoms.

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32 **Keywords**

33 Cannabinoid receptors, cystometry, urinary bladder, overactive bladder

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35 **Brief Summary**

36 Intravesical CP55,940 increased micturition interval and bladder capacity and
37 decreased maximal pressure suggesting a role for the endocannabinoid system
38 in bladder mechanoafferent function in rats.

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51 **Introduction**

52 The modulation of urge incontinence by cannabis, in patients with Multiple
53 Sclerosis (MS) presenting with neurogenic detrusor overactivity (DO), has
54 sparked interest in the functional involvement of the endocannabinoid system in
55 the lower urinary tract (1). The endocannabinoid system (ECS) consists of the
56 cannabinoid receptors, their endogenous ligands, and enzymes that regulate
57 the biosynthesis and degradation of the endocannabinoids (2). The presence of
58 the ECS in the urinary bladder has led to speculation that endocannabinoid
59 signalling is involved in signal transduction pathways regulating bladder
60 relaxation and may be involved in pathophysiological processes of the bladder
61 (1). On the basis of this evidence, it was postulated that activation of
62 cannabinoid receptors (CB1 and CB2) might promote relaxation of the urinary
63 bladder during the filling phase. Dysregulation of the ECS in human bladder
64 may be responsible for the pathogenesis of overactive bladder syndrome (OAB)
65 and DO. We have previously localised the components of the endocannabinoid
66 system in rat and human bladders where we showed expression of cannabinoid
67 receptors and the modulating enzymes. Our functional *in vitro* studies showed
68 that CB1 receptors have both pre- and post-synaptic inhibitory effects on rat
69 bladder contraction, whereas CB2 acts only post-synaptically (3).

70

71 Although the processes involved in the pathogenesis of overactive bladder are
72 better understood, the molecular and signal transduction mechanisms involved
73 have not been fully evaluated. The ECS has been suggested as one of these
74 mechanisms (4). Cystometric studies have shown an increase of the micturition
75 threshold in rats receiving systemic cannabinoids in an overactive bladder

76 model induced by acetic acid, cyclophosphamide (5) or turpentine oil (6). These
77 effects were stronger when the cannabinoids were administered through a
78 close-arterial route rather than systemically through the tail vein of the rat,
79 supporting the hypothesis of a local regulatory role of the cannabinoid system in
80 the micturition reflex (6). Intravesical infusion of acetic acid has been shown to
81 alter the micturition reflex in rats (7) by increasing the frequency of contractions,
82 and decreasing bladder capacity, with the micturition pressure unaffected or
83 increased (8). We therefore used acetic acid to induce an overactive rat bladder
84 model (6, 9, 10), assessing the effects of intravesical 3-(2-hydroxy-4-(1,1-
85 dimethylheptyl)phenyl)-4-(3-hydroxypropyl)cyclo-hexanol (CP55,940) in a
86 pathophysiological state, in order to determine its potential use as a therapeutic
87 agent. CP55,940 displays similar affinity for both central and peripheral
88 cannabinoid receptors.

89

90 The aim of the current study was to characterise the receptor subtype (CB1 or
91 CB2) responsible for mediating normal rat bladder function following
92 intravesical administration of CP55,940. Additionally, the effects of CP55,940
93 on a pathophysiological overactive model in the rat bladder were also
94 investigated.

95

96 **Methods**

97 *Animals*

98 27 in total, adult female Sprague-Dawley rats (n=5-6 per group weighing 250-
99 300 g, Charles River, UK) were group housed in an environmentally controlled
100 room with a 12-hour light/dark cycle and unlimited access to food and water. All

101 experiments were carried out in accordance with the UK Home office
102 regulations and the Animals (Scientific Procedures) Act 1986. The study had
103 Home Office Licence Approval (Project Licence 40/3647 Protocol 19B-3)

104

105 *General Preparation*

106 Anaesthesia was induced and maintained with isoflurane (induction dose - 4%;
107 maintenance dose 1.5-3%) until intravenous (i.v.) access was obtained. This
108 took an average of 5-10 min. Once the femoral vein was cannulated with an i.v.
109 infusion set containing an injection port and safety device, urethane (1.2 g / kg
110 i.v.) was slowly administered until a suitable depth of anaesthesia (assessed by
111 an absence of hind limb withdrawal in response to paw pinch and absence of a
112 corneal reflex) was achieved. Supplementary doses of urethane (0.1 g / kg i.v.)
113 were administered as required. Rats were under urethane anaesthesia for the
114 duration of the protocol which on average lasted for 2-2.5 hrs. Body
115 temperature was maintained at 37 °C by placing the animal on a homeothermic
116 blanket system (Harvard, USA) and monitoring changes in temperature using a
117 rectal temperature probe.

118

119 *Cystometry*

120 The ureters were exposed by retroperitoneal incisions and the distal ends were
121 tied and cut to ensure no backflow and hence no leakage of saline from the
122 bladder. The abdomen was opened through a midline incision and a single
123 cuffed polyethylene catheter (PE-90 1.02 mm, Portex, Kent UK) was inserted
124 through a small incision into the bladder dome. The catheter was secured with
125 a suture around the top of the bladder dome and this was followed by closure of

126 the abdominal incision. The catheter was connected via a three-way stopcock
127 to a pressure transducer (DTX-plus transducer, Digitimer, Singapore) to record
128 bladder pressure and a syringe pump (Pump33, Harvard apparatus) for
129 continuous infusion (0.15 ml / min) to evoke the micturition reflex.

130

131 During cystometry, data was captured using the data acquisition software
132 (LabChart v7.3, AD Instruments, UK). The cystometric parameters, such as
133 micturition interval (MI), baseline pressure (BP), maximum voiding pressure
134 (MP) and bladder capacity (BC, calculated as MI x rate of infusion), were
135 measured.

136

137 *Experimental protocol*

138 A stabilisation period of 30-45 min, with continuous intravesical infusion of 0.9%
139 w/v sterile saline (0.15 ml / min) into the bladder was undertaken after
140 completion of surgery. Following the acquisition of stable contractile responses,
141 saline was infused for a further one hour to evaluate bladder activity and this
142 was designated as the 'control period. This step was repeated for each
143 individual rat for every experimental group ensuring there was a control bladder
144 activity trace for each rat.

145

146 Step 1

147 The first part of the study was to assess the effect of vehicle (0.2% DMSO) on
148 cystometric parameters and 6 rats were used for this experiment. Each rat
149 underwent a stabilization period with saline described above. Each rat served
150 its own control with the administration of intravesical infusion of saline for 1 hr

151 and then was administered intravesical vehicle for 1 hour. After completion of
152 the experiments, rats were administered 5% isoflurane as a schedule 1 method.

153

154 Step 2

155 In order to explore the effects of non-selective CB agonist CP55,940 on
156 cystometric parameters, 5 rats were administered intravesical CP55,940 (0.005
157 mg / kg) for 1 hr after the saline stabilization and control period described above.
158 At completion, rats underwent Schedule 1.

159

160 Step 3

161 In experiments examining effects of the CB1 receptor antagonist AM251, saline
162 was administered intravesically to a total of 5 rats as a control for 1 hr as
163 described above. After the 1 hr, AM251 (1 mg / kg), was administered
164 intravenously while the saline intravesical infusion was running. Five minutes
165 after the i.v. antagonist was given CP55,940 was administered by intravesical
166 infusion for 1 hr. At the end of the experiment the 5 rats underwent Schedule 1.

167

168 Step 4

169 This experiment investigated the effects of the CB2 receptor antagonist AM630
170 in a total of 5 rats. The experimental procedure described in step 3 was
171 followed using AM630 (3 mg / kg).

172

173 Step 5

174 In the studies examining the effect of CP55,940 on irritated bladders (the acetic
175 acid-treated group) 6 rats were used and underwent a saline stabilization and

176 control period described above. To induce bladder hyperactivity in the rats,
177 0.25% acetic acid (9, 10) was infused intravesically for 30 min to induce bladder
178 irritation as previously described by Hiragata *et al.* (5). In the same group of rats,
179 intravesical CP55,940 (0.005 mg / kg) infusion was then commenced for 1 hr to
180 evaluate the effects on acetic acid-induced bladder irritation. Rats underwent
181 Schedule 1 after the experiments.

182

183 *Drugs and solutions*

184 CP55,940 was purchased from Tocris, UK, and was dissolved in DMSO. The
185 dose of 0.005 mg / kg of CP55,940 was based on the evidence from a previous
186 study by Gratzke *et al.* (11), who demonstrated a significant increase in the
187 micturition interval following intravesical infusion of the drug. AM251, the
188 selective CB1 receptor antagonist, and AM630, the selective CB2 antagonist,
189 were purchased from Tocris, UK and dissolved in DMSO. The dose of 1 mg / kg
190 used for AM251 was selected as an acceptable dose based on current
191 knowledge that AM251 'loses' CB1 receptor selectivity at higher concentrations
192 through actions on PPARs, GPR18 and GPR55 receptors. The dose for AM630
193 (3 mg / kg) and the timescale for administration (5 min) was chosen based on
194 previous published studies (5, 12).

195

196 *Statistical Analysis*

197 For data comparison, the cystometric parameters were measured for 1 hr from
198 the application of the drug. In a 1 hr trace there were an average of 42
199 micturition cycles that were analysed. A software macro was designed to
200 analyse all the micturition cycles in the control 1 hr trace and in the 1 hr

201 experimental trace. All data values are expressed as mean \pm standard error of
202 the mean. Student's t-test was used to compare the cystometric parameters
203 between control with saline and after application of CP55,940 in the same
204 group and also used to compare CP55,940 and acetic acid-exposed rats.
205 Kruskal-Wallis with Dunn's multiple comparisons test was used to compare
206 cystometric parameters between CP55,940 and CB receptor antagonists.
207 $p < 0.05$ was considered significant. GraphPad Prism v6.0 was used for
208 statistical analysis and for the generation of graphs.

209

210 **Results**

211 *Rats with normal bladders*

212 Representative cystometric traces taken from Sprague-Dawley rats are
213 presented in **Figure 1 (A-D)** showing the effects of vehicle, CP55,940, CB
214 antagonists and acetic acid and saline control. **Figure 1A** illustrates the
215 cystometric parameters measured (MI, MP, BP).

216

217 CP55,940 (0.005 mg / kg) increased MI and BC by $52 \pm 5.5\%$ (each $p < 0.05$,
218 **Figure 2, Table 1**) compared to the saline treated control. Although there was a
219 $25 \pm 2.9\%$ decrease in MP recorded after intravesical CP55,940 administration
220 ($p < 0.05$, **Table 1**), there was no reproducible change in baseline pressure
221 compared to intravesical saline infusion. Addition of the DMSO (vehicle) had no
222 effect on any of the cystometric parameters when compared to the saline-
223 treated control (Table 1).

224

225 Intravenous pre-treatment with AM251 or AM630 5 min prior to intravesical

226 infusion of CP55,940, inhibited the previously observed increases in MI and BC,
227 as well as the decreases in MP (**Figure 3, Table 1**). Representative cystometry
228 traces showing the effects of the addition of either AM251 or AM630 prior to
229 infusion of CP55,940 are displayed in Figure 1B and C, respectively. This
230 postulates a functional role of CB1 and CB2 receptors mediating bladder
231 function.

232

233 *Acetic acid induced hyperactivity*

234 Changes in cystometric parameters were compared to the saline control to
235 ensure that acetic acid had indeed induced bladder hyperactivity. The effects of
236 CP55,940 on cystometric parameters were compared to changes induced by
237 acetic acid. Intravesical infusion of acetic acid induced bladder irritation (**Figure**
238 **1D**), as shown by a significant $41 \pm 6.3\%$ reduction in MI ($p < 0.01$) compared
239 with values obtained from the saline control. For the other cystometric
240 parameters, acetic acid did not cause any significant changes in MP, BP or BC
241 when compared to saline control. Addition of intravesical CP55,940 in the same
242 rats increased MI by $78 \pm 16.7\%$ ($p < 0.05$) and reduced MP by $22 \pm 5.8\%$
243 ($p < 0.05$, **Table 2**).

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245

246 **Discussion**

247 The results of this study demonstrate that intravesical infusion of CP55,940
248 suppressed bladder activity, as well as urinary frequency induced by bladder
249 irritation. The inhibitory effects of CP55,940 appeared to be mediated by both
250 CB1 and CB2 receptor subtypes. Since, MI and BC cystometric parameters can

251 be considered indicators for afferent function of the micturition reflex and MP
252 and BP are indicators of motor activity (11), then the main function of CP55,940
253 seemed to be mediated by modulation of both afferent and motor activity as it
254 not only induced an increase in MI and BC but also affected MP. In a similar
255 study, which utilised intravesical bolus dosing of CP55,940, an increase in MI
256 and BC was reported, with no changes in MP (11). Furthermore, the authors
257 did not report the CB receptor subtype involved in mediating these responses
258 (11). One reason for the difference between the results presented in our study
259 and those reported previously could be differences in methodologies and/or
260 anaesthetic agents used. In this study, isoflurane and i.v. urethane were used
261 to anaesthetise the rats, whereas Gratzke *et al.* (2009) used intraperitoneal
262 ketamine and xylazine and the cystometry was performed on conscious rats. It
263 has been reported that ketamine inhibits the micturition reflex (13,14).
264 Castroman and Ness (2002) reported that ketamine inhibits the reflexive
265 responses of the bladder to the acute stimulus of distension in a dose-
266 dependent manner (13). In addition to the effects of CP55,940 on afferent
267 activity, its effect on motor activity, is in accordance with our previously
268 published *in vitro* studies, where activation of both CB receptors reduced
269 contractions of the detrusor muscle (3). Taken together, it may be speculated
270 that CP55,940 activates CB receptors directly on the muscle resulting to
271 inhibition of contractility.

272

273 The observed effects of CP55,940 on bladder function, demonstrated in this
274 study, are in agreement with the effects of cannabinoid agonists reported in
275 human clinical trials. In a large randomised controlled trial, patients with

276 neurogenic DO who were randomised to the THC and cannabidiol (CBD)
277 treatment groups, reported a significant reduction in urge incontinence episodes
278 compared to placebo (1). Similarly, in another study, patients with neurogenic
279 DO given a cannabis extract containing THC and CBD, demonstrated a
280 reduction in urinary urgency, number of incontinence episodes, frequency and
281 nocturia. In addition, there was an increase in the maximum cystometric
282 capacity in these patients (15), which is in accordance with the cystometric
283 observations described in this study, where CP55,940 increased MI and BC.

284

285 With the knowledge that cannabinoid agonists are negatively coupled to
286 calcium channels, and their inhibition of adenylate cyclase, the mixed picture of
287 cystometric effects described in this study and those reported in the literature is
288 understandable. The increased micturition interval and the decrease in maximal
289 pressure of the contractions could be due to inhibition of adenylate cyclase,
290 which may be involved in relaxation of detrusor smooth muscle during urine
291 collection. The alteration of calcium fluxes may decrease neurotransmitter
292 release required for detrusor contractions, and that decrease could be
293 manifested as an increase in the volume requirement (demonstrated as an
294 increase in bladder capacity). More experiments must be performed to clarify
295 these preliminary results.

296

297 It appears from our study, that the activation of both CB1 and CB2 receptors
298 have inhibitory effects on the bladder, as demonstrated by both CB antagonists
299 blocking the actions of CP55,940. This is supported by a study that suggested a
300 CB2-mediated effect in the rat urinary bladder (15), where cannabior (a

301 selective high-affinity CB2 receptor full agonist) was used and resulted in a 44%
302 increase in MI. In another study, using the non-selective CB receptor agonist
303 WIN55212, an increase in the threshold for micturition was reported in
304 anaesthetised rats, and this effect was counteracted by the CB1 receptor
305 antagonist SR141716A, suggesting a CB1-mediated effect (6). Similarly, yet
306 another study using a synthetic analogue of THC, IP-751 at 10 mg / kg,
307 reported an increase in MI by 63% and threshold pressure by 53%. These
308 effects were blocked by the CB1 antagonist AM251 but not the CB2 antagonist
309 AM630 (5). In the present study both AM251 and AM630 inhibited the effects of
310 CP55,940 suggesting a role for both CB receptors in urinary bladder function. A
311 reason for this discrepancy between the results presented in this study and that
312 of Hiragata, could be that in the present study CP55,940 was administered
313 intravesically while Hiragata administered IP-751 systemically. The intravesical
314 route allows the instillation of the cannabinoid drug directly to the bladder
315 therefore bypassing any systemic effects. Therefore the effects of IP-751 on the
316 central nervous system cannot be excluded. Another possibility for these
317 differences could be related to the receptor subtype that is activated when
318 using different agonists suggesting differences in receptor activation between
319 the urothelium and detrusor muscle. It has been reported that CB1 and CB2
320 receptor expression is different in the urothelium and detrusor (16) and so
321 different agonists may differentially activate the endocannabinoid receptor
322 response in each of these specific tissues resulting in different downstream
323 events. In addition, there may be an element of CB receptor turnover that
324 depends on the type of agonist used, which could explain this discrepancy. This

325 can be assumed since full reversal of CP55,940 effects seen on some
326 cystometric parameters occurred with both CB1 and CB2 antagonists.

327

328 The mechanism by which cannabinoid receptors modulate the micturition reflex
329 is by their presence in the afferent nerve fibre endings located in the
330 suburothelial layer, which is supported by *in vitro* studies using CB1 or CB2
331 receptor agonists. *In vitro* studies using CB1 receptor agonists have been
332 shown to reduce neuronal activity (17) and attenuate bladder contractility as a
333 result of electrical field stimulation in isolated mouse bladder strips (18). In rats,
334 anandamide, WIN 55212-2 (a synthetic CB non-selective agonist), and IP-751
335 (also known as Ajulemic acid), suppressed normal bladder activity and urinary
336 frequency induced by bladder irritation, suggesting that the inhibitory effects are
337 in part mediated by CB1 receptors (5, 6, 19). In conscious rats, cannabior, a
338 selective CB2 receptor agonist, increased the micturition intervals and volumes
339 by 52% ($p<0.05$) and 96% ($p<0.01$), respectively, and increased the threshold
340 and flow pressures by 73% ($p<0.01$) and 49% ($p<0.001$), respectively (15). To
341 support the findings described and the results presented in this study, ATP-
342 evoked calcitonin gene-related peptide release (CGRP; a neurotransmitter
343 released by sensory nerve endings) was reported to be inhibited by the
344 activation of both CB1 and CB2 receptors after an alteration in the afferent
345 cystometric parameters from the infusion of a CB receptor agonist (20).
346 However, it is possible that some of the effect could be centrally mediated and
347 whether these actions are related to CB receptors in the central nervous system,
348 at peripheral sites in the lower urinary tract, or both needs to be clarified.

349 Furthermore, it is not known which of the two CB receptor subtypes is primarily
350 responsible for the regulation of micturition in different species.

351

352 Cannabinoid receptor expression appears to be altered in pathological
353 conditions, as has been shown in a clinical study where CB1 receptor
354 immunoreactive nerve fibres were increased in the suburothelium of patients
355 with painful bladder syndrome and detrusor overactivity (4). In addition, CB1
356 mRNA transcripts were increased in patients with chronic bladder pain
357 syndromes (21) and CB2 transcripts and protein expression was increased in
358 rats with bladder inflammation induced by acrolein (22). *In vivo* studies have
359 shown an increase in micturition interval by cannabinoid agonists in rat
360 bladders with induced bladder hyperactivity (5,19,23,24).

361

362 The results in this study and those in the literature support a regulatory role for
363 the CB1 and CB2 receptor subtypes in both normal bladder function and
364 bladder dysfunction. Furthermore, it is not known which of the two CB receptor
365 isoforms is of primary importance in the regulation of micturition. In this study,
366 both CB1 and CB2 antagonists inhibited the effects of CP55,940 equally. As
367 AM251 and AM630 are known to cross the blood-brain barrier (25,26), it can be
368 speculated that CB1 and CB2-receptor-related changes in micturition could
369 result from CNS or peripheral nervous site of action (or both). It should be kept
370 in mind that genes can compensate for each other, especially as CB1 and CB2
371 share intracellular pathways ($G_{i/o}$ protein). Therefore, discrepancies in reports of
372 CB receptor subtype involvement in regulation of micturition may very well

373 suggest their multiple functions in different regulatory units of lower urinary tract
374 control, reached *via* systemic or local administration of drugs.

375

376 **Conclusions**

377 CP55,940 decreased rat bladder activity and urinary frequency induced by
378 bladder nociceptive stimuli, probably by suppression of bladder afferent activity.

379 These effects are modulated by activation of both CB1 and CB2 receptors and

380 these data implicate a role for the endocannabinoid system in bladder

381 mechanoafferent functions in rats. In addition, the results of this study show that

382 intravesical CP55,940 reverses urinary frequency exemplified in an overactive

383 bladder model, suggesting it could potentially be an effective treatment for

384 patients with lower urinary tract symptoms.

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389 awarded to Dr E Bakali.

390

391 **Conflicts of Interest**

392 The authors declare no conflicts of interest

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523 **Figure Legends**

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525 **Figure 1.** Representative cystometry pressure recordings obtained from female
526 Sprague-Dawley rats. Scale bars indicate 60 second intervals. **A.** Shows a section
527 of the trace from infusion with intravesical saline as control and a section showing
528 the addition of intravesical CP55,940 (0.005 mg / Kg) infusion. The trace is
529 annotated to demonstrate maximal voiding pressure (MP), micturition interval (MI),
530 and basal pressure (BP). **B.** representative trace showing control and then the
531 addition of DMSO (vehicle). **C.** A cystometry trace showing infusion with saline
532 (control) and then CP55,940 infusion after administration of intravenous AM251 (1
533 mg / Kg). **D.** A cystometry trace showing infusion with saline (control) and then
534 intravesical infusion of then CP55,940 infusion after administration of intravenous
535 AM630 (3 mg / Kg). **E.** Representative sections of a cystometry recording showing
536 saline (control), the effects of intravesical 0.25% acetic acid infusion and the effects
537 of intravesical CP55,940 on the irritated bladder.

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539 **Figure 2.** Results of cystometry using female Sprague-Dawley rats (n=5). Panels **A**
540 and **B** show a significant increase in MI and BC, respectively, on addition of
541 CP55,940. BC was calculated by rate of infusion x MI. Panel **C** shows that
542 CP55,940 significantly reduced MP but did not affect BP. Control was performed
543 with a saline infusion (0.15 ml/hr) for 1 hour and then CP55,940 (0.005mg/Kg)
544 infusion for an additional hour. Data are presented as the mean \pm SEM for 5
545 separate experiments; **p<0.01 Student's paired t-test.

546 **MI**, Micturition interval; **BC**, Bladder capacity; **MP**, Maximal voiding pressure; **BP**,
547 basal pressure.

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549 **Figure 3.** The cystometry results using Sprague-Dawley rats are shown as a
550 percentage of the saline control. Micturition interval (**MI**), bladder capacity (**BC**) and
551 maximum voiding pressure (**MP**) for vehicle, CP55,940 with or without addition of
552 AM251 or AM630 (n=5 for each group). Values are given as mean \pm SEM. For
553 comparisons between CP55,940 and AM251 and AM630, Kruskal-Wallis with
554 Dunn's multiple comparisons test was used. * $p < 0.05$, ** $p < 0.01$.

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