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Acetaminophen Relieves Inflammatory Pain Through CB1 Cannabinoid Receptors in the Rostral Ventromedial Medulla

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1 **Acetaminophen Relieves Inflammatory Pain Through CB₁ Cannabinoid**
2 **Receptors in the Rostral Ventromedial Medulla**

3
4 abbreviated title:

5 RVM cannabinoid signaling in acetaminophen analgesia

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40

41 **Abstract**

42 Acetaminophen (paracetamol) is a widely used analgesic and antipyretic drug with only
43 incompletely understood mechanisms of action. Previous work, using models of acute
44 nociceptive pain, indicated that analgesia by acetaminophen involves an indirect activation of
45 CB₁ receptors by the acetaminophen metabolite and endocannabinoid re-uptake inhibitor
46 AM 404. However, the contribution of the cannabinoid system to anti-hyperalgesia against
47 inflammatory pain, the main indication of acetaminophen, and the precise site of the relevant
48 CB₁ receptors have remained elusive. Here, we analyzed acetaminophen analgesia in mice of
49 either sex with inflammatory pain and found that acetaminophen exerted a dose-dependent anti-
50 hyperalgesic action, which was mimicked by intrathecally injected AM 404. Both compounds lost
51 their anti-hyperalgesic activity in CB₁^{-/-} mice confirming the involvement of the cannabinoid
52 system. Consistent with a mechanism down-stream of pro-inflammatory prostaglandin formation,
53 acetaminophen also reversed hyperalgesia induced by intrathecal prostaglandin E₂ (PGE₂). To
54 distinguish between a peripheral/spinal and a supraspinal action, we administered
55 acetaminophen and AM 404 to *hoxB8*-CB₁^{-/-} mice, which lack CB₁ receptors from the peripheral
56 nervous system and the spinal cord. These mice exhibited unchanged anti-hyperalgesia
57 indicating a supraspinal site of action. Accordingly, local injection of the CB₁ receptor antagonist
58 rimonabant into the rostral ventromedial medulla (RVM) blocked acetaminophen-induced anti-
59 hyperalgesia, while local RVM injection of AM 404 reduced hyperalgesia in wild-type mice but
60 not in CB₁^{-/-} mice. Our results indicate that the cannabinoid system contributes not only to
61 acetaminophen analgesia against acute pain but also against inflammatory pain, and suggest
62 that the relevant CB₁ receptors reside in the RVM.

63

64

65 **Significance statement**

66 Acetaminophen is a widely used analgesic drug with multiple but only incompletely understood
67 mechanisms of action including a facilitation of endogenous cannabinoid signaling via one of its
68 metabolites. Our present data indicate that enhanced cannabinoid signaling is also responsible
69 for the analgesic effects of acetaminophen against inflammatory pain. Local injections of the
70 acetaminophen metabolite AM 404 and of cannabinoid receptor antagonists as well as data from
71 tissue specific CB₁ receptor deficient mice suggest the rostral ventromedial medulla as an
72 important site of the cannabinoid-mediated analgesia by acetaminophen.

73

74 **Introduction**

75 In the past decades, several potential molecular mechanisms have been proposed that may
76 explain how acetaminophen exerts its analgesic action. These include the inhibition of
77 cyclooxygenases (COXs) (Flower and Vane, 1972; Hanel and Lands, 1982; Graham and Scott,
78 2005), the activation of spinal serotonergic descending projections (Tjolsen et al., 1991; Pini et
79 al., 1996), an involvement of the brain opioid system (Tjolsen et al., 1991; Herrero and Headley,
80 1996; Pini et al., 1996; Sandrini et al., 2001), inhibition of nitric oxide generation (Bjorkman et al.,
81 1994; Bujalska, 2004), and activation of spinal TRPA1 channels by the acetaminophen
82 metabolites N-acetyl-p-benzoquinoneimine (NAPQI) and p-benzoquinone (Andersson et al.,
83 2011). In addition, the generation of N-arachidonoylphenolamin (AM 404) from acetaminophen
84 through deacetylation to p-aminophenol and the subsequent conjugation with arachidonic acid
85 by central nervous system fatty amide hydrolase (FAAH) (Höggestatt et al., 2005) has drawn the
86 attention to a possible involvement of the endocannabinoid system. AM 404 increases tissue
87 concentrations of the endocannabinoid arachidonoyl ethanolamide (AEA), also known as
88 anandamide, through an inhibition of anandamide reuptake into neurons and astrocytes
89 (Beltramo et al., 1997; Fegley et al., 2004). After spinal or systemic application, AM 404 exerts
90 analgesic activity against acute pain, evoked by noxious chemical stimuli, as well as against
91 inflammatory and neuropathic pain (Gühring et al., 2002; La Rana et al., 2006). In line with an
92 important contribution of the endocannabinoid system, acetaminophen-mediated antinociception
93 was lost in CB₁ receptor-deficient (CB₁^{-/-}) mice (Mallet et al., 2008) as well as in mice lacking
94 FAAH (FAAH^{-/-} mice) (Mallet et al., 2010). Accordingly, acetaminophen-induced analgesia was
95 also reduced by the FAAH inhibitor URB 597 (Mallet et al., 2008) and by the CB₁ receptor
96 antagonists AM 251 and rimonabant (Ottani et al., 2006; Dani et al., 2007; Mallet et al., 2008).

97 The studies discussed above support a contribution of the endocannabinoid system to
98 acetaminophen-mediated analgesia. However, most of these studies (Ottani et al., 2006; Mallet
99 et al., 2008; Mallet et al., 2010) tested acetaminophen in models of acute nociceptive pain, i.e.
100 pain evoked by acute noxious thermal, mechanical, or chemical stimuli applied to naïve animals
101 in the absence of nociceptive sensitization by inflammation or neuropathy. These acute pain
102 models only poorly reflect the clinical indications for acetaminophen, which is primarily used to
103 treat mild inflammatory pain (Bradley et al., 1991). In fact, acute antinociceptive effects of
104 acetaminophen in humans are rather vague or do not exist at all (Olesen et al., 2012; Tiippana
105 et al., 2013). In the present study, we have analyzed the anti-hyperalgesic properties of
106 acetaminophen in mice with inflammatory hyperalgesia and demonstrate a critical contribution of
107 CB₁ receptors to the effects of acetaminophen against inflammatory hyperalgesia. Additional

108 experiments with tissue-specific $CB_1^{-/-}$ mice and local injections of AM 404 or the CB_1 receptor
109 antagonist rimonabant suggest that the CB_1 receptors relevant for inflammatory anti-
110 hyperalgesia reside in the RVM which is a well-known site for endogenous pain control.

111

112 **Methods**

113 *Mice.* Experiments were performed in wild-type mice (C57BL/6J; www.jax.org/strain/000664),
114 $CB_1^{-/-}$ mice (genetic background C57BL/6N; (Marsicano et al., 2002);
115 www.informatics.jax.org/allele/MGI:2182924), and $hoxb8-CB_1^{-/-}$ mice (genetic background
116 C57BL/6; (Witschi et al., 2010); <http://www.informatics.jax.org/allele/MGI:4881836>). $hoxb8-CB_1^{-/-}$
117 mice were obtained by crossing mice carrying floxed CB_1 receptor alleles ($CB_1^{fl/fl}$ mice;
118 www.informatics.jax.org/allele/MGI:3045419; Marsicano et al., 2003) with mice expressing in
119 addition the cre recombinase in spinal cord neurons and glial cells as well as in neurons of the
120 dorsal root ganglia ($hoxb8$ -cre mice; Witschi et al., 2010). Behavioral experiments on $hoxb8-CB_1^{-/-}$
121 $^{-/-}$ mice were performed with $hoxb8$ -cre-negative $CB_1^{fl/fl}$ littermates as “wild-type” controls.
122 Animals were housed under controlled environmental conditions (22°C, 12/12 light/dark cycle)
123 and were allowed to take food and water *ad libitum*.

124

125 *Behavioral testing.* Experiments were performed in adult (7-9 week old) female and male mice.
126 Mice were randomly assigned to treatment groups. On the first day of the experiments, each
127 mouse was tested several times to obtain baseline paw withdrawal thresholds (PWTs). Animals
128 were placed in Plexiglas boxes on a metal grid and allowed to accommodate to the test
129 confinement for at least 1 hour prior to starting behavioral experiments. Mechanical sensitivity
130 was measured using electronically controlled von Frey filaments (IITC, Woodland Hills, USA). At
131 least 3 measurements were made for each time point. The experimenter was blind to the
132 genotype or to the type of treatment (vehicle or drug) in all experiments. Permission for animal
133 experiments was obtained from the Veterinäramt des Kantons Zürich (license 92/2007 and
134 126/2012/16).

135 Inflammatory hyperalgesia was induced using the yeast extract zymosan A (Meller and Gebhart,
136 1997). Zymosan A (Fluka) was suspended in 0.9% NaCl and injected subcutaneously (0.06 mg /
137 20 μ l) into the plantar side of the left hind paw 24 hours prior to the administration of
138 acetaminophen or AM 404. Spinal PGE₂-induced hyperalgesia was evoked through intrathecal
139 injection of PGE₂ (Sigma; 0.4 nmoles / 4 μ l, dissolved in 1% ethanol and 99% artificial

140 cerebrospinal fluid (aCSF)). Intrathecal injections were made 1 hour before application of
141 acetaminophen. For details, see ref. (Reinold et al., 2005).

142

143 *Drug administration, intrathecal and intraRVM injections.* Acetaminophen (Sigma) was dissolved
144 in 0.9% NaCl. The acetaminophen-containing solution or vehicle (0.9% NaCl, 400 μ l) was given
145 per os (p.o.) through stainless steel tubes (Delvo SA, Switzerland). Rimonabant (SR141716A;
146 Tocris) (Rinaldi-Carmona et al., 1994) was dissolved in a mixture of 43% (vol/vol) DMSO, 43%
147 aCSF and 14% ethanol. Injection volumes were 5 and 4 μ l for AM 404 (Tocris) and PGE₂,
148 respectively. AM 404 (Tocris) was dissolved in 40% DMSO and 60% 0.9% NaCl. Intrathecal (i.t.)
149 injections were performed under isoflurane anesthesia at the level of the lumbar spine using a
150 Hamilton syringe (Ahmadi et al., 2001). A small amount of black ink (1% v/v) was added to
151 permit post-hoc verification of proper i.t. injections. Injections into the rostral ventromedial
152 medulla (RVM) were performed with stainless steel cannulas. Fully anaesthetized mice were
153 placed in a Kopf stereotaxic frame and implanted with a cannula using the following coordinates
154 which were calibrated to the cranial Bregma points: x= -5.7; y= 0; Z_{cranium}= +4.2. The cannula was
155 fixed with dental cement and the cement was secured at the skull with 2 - 3 screws. The fixed
156 cannula was used to insert a 30G needle attached to a Hamilton syringe 5.8 mm deep. A
157 volume of 300 nl was injected. For post hoc verification of correct targeting of the RVM 1 % v/v
158 Evans blue was included in the injection solution.

159

160 *Hepatotoxicity assays.* Mice were treated p.o. with vehicle (0.9 % NaCl), 200, 300 or 400 mg/kg
161 acetaminophen. Twenty four hours later, blood was collected after decapitation, and the liver
162 was dissected. To quantify liver damage we determined the blood levels of three enzymes,
163 alanine aminotransferase (ALT), aspartic aminotransferase (AST) and lactate dehydrogenase
164 (LDH), that are released upon acute liver damage from hepatocytes into the blood stream using
165 the UniCel DxC 800 Synchron Clinical Systems (Beckman Coulter, USA). Livers were put in 4%
166 formalin overnight and subsequently embedded in paraffin. Tissue sections (3 μ m) were cut and
167 stained with hematoxylin-eosin following standard procedures (Fischer et al., 2008). Liver
168 degeneration was defined by the presence of vacuolar degeneration and pink-red tissue
169 discoloration due to sinusoidal congestion and apoptotic cell body formation, as described
170 previously (Zhao et al., 2016). For quantification of liver degeneration, the ratio of venules
171 surrounded by healthy or discoloured tissue was calculated.

172

173 *Immunohistochemistry and in situ hybridization.* For immunohistochemistry, three mice of each
174 genotype were deeply anesthetized with a mixture of 25 mg/ml ketamine, 5 mg/ml xylazine, and

175 0.1 w/w% promethazine in H₂O (1 ml/100 g, intraperitoneal [i.p.]) and subsequently perfused
176 transcardially through the ascending aorta with 0.9% NaCl for 2 min, followed by 100 ml of a
177 fixative containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for
178 another 20 min. After perfusion, spinal cords and brains were immediately isolated and postfixed
179 in 4% PFA for 2 hours and washed in 0.1 M PB. Transverse sections of the spinal cord at a
180 lumbar level as well as coronal sections of the cerebral hemispheres and the cerebellum (all 50
181 µm thick) were cut using a vibratome (Leica, VTS-1000). Free-floating sections were collected in
182 0.1 M PB. For immunoperoxidase staining, the sections were first extensively washed in 0.1 M
183 PB. To block endogenous peroxidase activity, sections were afterwards incubated in 1% H₂O₂ in
184 0.1 M PB for 10 min and again washed in 0.1 M PB. Following washing in 0.05 M Tris-buffered
185 saline (TBS; pH 7.4) conditioning Triton X-100 (TBST), the sections were blocked in 10% normal
186 donkey serum (Vector Laboratories, Burlingame, USA) for 45 min. Sections were then incubated
187 with polyclonal affinity-purified guinea pig anti-CB₁ antibodies (1 : 250; ~1 µg/ml; Fukudome et
188 al., 2004) at 4°C for 48 hours. The antibodies were dissolved in 0.05 M TBS. After multiple
189 washings, the sections were treated in TBS with biotinylated goat anti-guinea pig IgG (1 : 300;
190 Vector Laboratories) for 2 hours and after further washing in TBS incubated with avidin-
191 biotinylated horseradish peroxidase complex (1 : 500; Elite-ABC, Vector Laboratories) for 1.5
192 hours. Development of the immunoperoxidase reaction was done with 3,3'-diaminobenzidine
193 (DAB) as chromogen and 0.01% H₂O₂ dissolved in TB (pH 7.6). Sections were briefly
194 submerged in chrome gelatin (0.05% chromium potassium sulfate dodecahydrate, 0.5% gelatin
195 and 0.05% NaN₃ in DW), dried, soaked in xylene (2 x 15 min), and covered in DePeX (SERVA).
196 Sections containing the RVM were treated with 0.5% OsO₄ in PB for 20 min at 4°C, dehydrated
197 in an ascending series of ethanol and propylene oxide, and embedded in Durcupan (ACM,
198 Fluka, Buchs, Switzerland) following DAB development. During dehydration, sections were
199 treated with 1% uranyl acetate in 70% ethanol for 15 min at 4°C. Light microscopic analysis of
200 immunostaining was carried out with a Nikon Eclipse 80i upright microscope. Micrographs were
201 taken with a Nikon DS-Fi1 digital camera.

202

203 *Statistical analyses.* Data are presented as mean ± SEM and *n* indicates the number of animals
204 tested. For dose response curves, PWTs were transformed into % maximum possible effects (%
205 MPE), with 0% and 100% being the inflamed pre-drug value and the full return to pre-
206 inflammation value, respectively. Data from the dose response relationship of acetaminophen
207 and AM 404 were fitted to the Hill equation $y = y_{\max} - [(y_{\max} - y_{\min}) / (1 + (ED_{50}/D)^{nH})]$; with y_{\max} ,
208 maximum %MPE reached with saturating doses; $y_{\min} = 0$; D , actual dose; ED_{50} half-maximum
209 effective dose; and nH , Hill coefficient. To compare the magnitude of antihyperalgesic effects of

210 acetaminophen or AM 404 in wild-type and $CB_1^{-/-}$ mice or in the presence or absence of
211 antagonists, areas under the curve (AUCs) were calculated for the changes of PWTs from pre-
212 drug baseline over 150 min or 80 min, following application of acetaminophen or AM 404,
213 respectively. When more than two groups were compared, statistical analyses were done by
214 one-way ANOVA followed by Bonferroni or Dunnett's post hoc tests or two-way ANOVA, when
215 two factors were analyzed. In all other experiments, statistical analyses were performed using
216 the unpaired Student's t-test (two-tailed). Statistical significance was accepted for $P \leq 0.05$.

217

218 **Results**

219 *Anti-hyperalgesic actions of acetaminophen and AM 404 in inflammatory pain*

220 Because acetaminophen is an antipyretic analgesic whose main indication is mild inflammatory
221 pain, we analyzed its analgesic effects in the zymosan A model of inflammatory pain (Meller and
222 Gebhart, 1997; Reinold et al., 2005). Subcutaneous (sct) zymosan A injection (0.06 mg in 20 μ l
223 0.9% NaCl) into one hindpaw decreased mechanical PWT from 4.11 ± 0.06 g (mean \pm SEM, $n =$
224 30 mice) to 1.10 ± 0.06 g within 24 hours after injection. For first experiments we chose a dose
225 of 200 mg/kg, p.o., because this dose has successfully been used in studies by others (e.g.
226 Högestätt et al., 2005; Mallet et al., 2010; Dalmann et al., 2015; Gentry et al., 2015).
227 Acetaminophen caused a time-dependent partial reversal of zymosan A-induced decreases in
228 PWT. Acetaminophen reached a maximum effect at 60 to 80 min after administration (Fig. 1A).
229 PWT in the contralateral non-inflamed paws were not affected. Accordingly, acetaminophen had
230 no effects on PWT in naïve mice (Fig. 1B). Testing the effects of different doses of
231 acetaminophen revealed significant anti-hyperalgesic effects at doses ≥ 30 mg/kg. Dose-
232 response curves (Fig. 1D) display % maximum possible analgesia determined for the time
233 interval between 60 and 80 min after drug application. Data were fitted to the Hill equation
234 revealing an ED_{50} of 30.1 ± 4.9 mg/kg and a maximal effect (E_{max}) of 44.3 ± 3.4 %.

235 We next tested whether this anti-hyperalgesia would be mimicked by CNS injection of the
236 acetaminophen metabolite AM 404. Different doses of AM 404 were injected directly into the
237 mouse spinal canal 24 hours after zymosan A injection (Fig. 1E,F). Mechanical sensitivities were
238 measured for 100 min at 20 min intervals. Similar to acetaminophen, AM 404 caused a
239 significant dose-dependent increase in PWTs (Fig. 1E). Dose-response curves (Fig. 1F) reveal
240 an ED_{50} was 2.55 ± 0.04 nmol and E_{max} of 46.2 ± 0.2 %. These experiments demonstrate that
241 acetaminophen and its metabolite AM 404 exert potent dose-dependent anti-hyperalgesic
242 actions against inflammatory pain.

243 We also examined whether acetaminophen exerted behavioral effects that might interfere with
244 the read-outs of pain tests (Fig. 1G,H). To this end, we assessed effects of acetaminophen on
245 motor coordination and sedation in the rotarod test and on muscle strength in the horizontal wire
246 test. At doses of 200 and 300 mg/kg (p.o.) acetaminophen did not impair performance in these
247 two tests (for statistics see figure legends).

248

249 *Liver toxicity of acute treatment with acetaminophen*

250 Compared to clinically used doses in humans (1 g in a 70 kg person is equivalent to 15 mg/kg),
251 the acetaminophen doses required in the present study to achieve at least 40% reduction in
252 hyperalgesia (≥ 200 mg/kg) appear rather high. In humans, doses higher than 150 - 250 mg/kg
253 may induce hepatotoxicity (Brunton et al., 2011). On the other hand, a 10 to 15-fold difference
254 between effective doses in humans and rodents is not unusual given the much higher metabolic
255 rate of mice (Sharma and McNeill, 2009). However, because this ratio provides only an estimate
256 and may differ between drugs, we tested whether the doses employed here would cause acute
257 liver toxicity in mice (Fig 2). We measured blood levels of alanine aminotransferase (ALT),
258 aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) 24 hours after
259 administration of different doses of acetaminophen (figure 2A-C). For all three enzymes,
260 increases in enzyme activities were minor at a dose of 200 mg/kg and did not reach significance
261 (ALT [IU/l]: 63 ± 10 , 214 ± 104 , 3624 ± 2010 , for vehicle, 200 mg/kg and 300 mg/kg,
262 respectively; AST [IU/l]: 281 ± 42 , 457 ± 48.6 , and 1349 ± 730 ; LDH [IU/l]: 1072 ± 170 , $1674 \pm$
263 147 , 7498 ± 4663 ; for statistics see figure 2). At a dose of 300 mg/kg, blood levels of all three
264 enzymes increased several-fold and increases became statistically significant for ALT. We also
265 investigated potential changes in liver histology caused by acetaminophen (Fig. 2D). Tissue
266 damage was quantified by counting the number of venules surrounded by healthy or discolored
267 liver tissue per field of view. No detectable liver degeneration was observed after 200 mg/kg. At
268 300 mg/kg, the number of venules in degenerating tissue was increased but this increase did not
269 reach statistical significance. Statistically significant tissue damage was however found after 400
270 mg/kg. Based on these results, we decided to perform all subsequent experiments with an
271 acetaminophen dose of 200 mg/kg.

272

273 *Contribution of CB₁ receptors to anti-hyperalgesia by acetaminophen*

274 In order to test for a possible contribution of the cannabinoid systems to acetaminophen and
275 AM 404-mediated analgesia in inflammatory pain conditions, we tested the effects of
276 acetaminophen and AM 404 in global CB₁ receptor deficient (CB₁^{-/-}) mice with an inflamed
277 hindpaw. Wild-type and CB₁^{-/-} mice did not differ in their baseline mechanical sensitivities (PWTs

278 were 3.9 ± 0.1 g, $n = 15$ and 4.0 ± 0.09 g, $n = 13$), for naïve wild-type and $CB_1^{-/-}$, respectively)
279 and developed similar inflammatory hyperalgesia (PWTs were 0.93 ± 0.10 g, $n = 15$, and $1.00 \pm$
280 0.05 g, $n = 13$, for zymosan A injected wild-type and $CB_1^{-/-}$ mice, respectively). Anti-hyperalgesic
281 effects of acetaminophen were virtually absent in the $CB_1^{-/-}$ mice. For statistical analyses, we
282 calculated the area under the curve over time (AUC [g·h]) for the difference between post-drug
283 PWTs and the pre-drug PWT baseline. AUCs were 0.30 ± 0.34 g·h, $n = 6$, versus 1.23 ± 0.16
284 g·h, $n = 8$, in wild-type mice ($P = 0.012$, unpaired Student's *t*-test) (Fig. 3A). We next assessed
285 whether the anti-hyperalgesic action of the acetaminophen metabolite AM 404 would also be
286 lost in $CB_1^{-/-}$ mice (Fig. 3B). To this end, we injected 10 nmoles of AM 404 intrathecally. AM 404
287 again reversed mechanical hyperalgesia in wild-type mice (AUC: 1.07 ± 0.14 g·h; $n = 7$) but
288 completely failed to reduce hyperalgesia in $CB_1^{-/-}$ mice (AUC: -0.22 ± 0.03 g·h, $n = 6$, $P < 0.001$,
289 unpaired Student's *t*-test). The lack of a pain-relieving action of acetaminophen and AM 404 in
290 $CB_1^{-/-}$ mice corresponds well with the reversal of acetaminophen- and AM 404-mediated
291 analgesia by the CB_1 receptor antagonists (inverse agonists) AM 251 and rimonabant described
292 previously by others in different pain models (La Rana et al., 2006; Ottani et al., 2006; Dani et
293 al., 2007; Mallet et al., 2008). It strongly suggests that anti-hyperalgesia by systemic
294 acetaminophen requires activation of CB_1 receptors. A lack of CB_1 receptors during development
295 may cause changes in neuronal circuits (Berghuis et al., 2007) that could potentially interfere
296 with the actions of acetaminophen. In order to exclude this possibility, we tested whether
297 systemic antagonism of CB_1 receptors with rimonabant would recapitulate the effect of genetic
298 ablation of CB_1 receptors. Rimonabant (5 mg/kg, i.p.) administered immediately before
299 acetaminophen indeed completely prevented the anti-hyperalgesic action of acetaminophen
300 (Fig. 3C).

301

302 *Analgesic effect of acetaminophen in PGE₂-induced inflammatory pain*

303 It has previously been suggested that acetaminophen might act through an inhibition of COX-
304 dependent prostaglandin formation in the central nervous system (Flower and Vane, 1972;
305 Hanel and Lands, 1982; Chandrasekharan et al., 2002; Graham and Scott, 2005). To test
306 whether acetaminophen reduces inflammatory hyperalgesia through a mechanism downstream
307 of central prostaglandin production, we induced hyperalgesia through intrathecal PGE₂ injection
308 (Taiwo and Levine, 1986; Uda et al., 1990; Reinold et al., 2005). One hour after PGE₂ injection
309 (0.4 nmol), PWTs decreased from a baseline value of 3.50 ± 0.08 g to 0.90 ± 0.06 g ($n = 13$)
310 (Fig. 4A). Acetaminophen (p.o., 200 mg/kg) but not vehicle (p.o. 0.9% NaCl) administered 1 hour
311 after PGE₂ injection partially reversed PGE₂-induced hyperalgesia. The AUCs ([g·h]) were
312 calculated between the post-drug PWTs and a straight line between the PWT at 1.5 and 4.0

313 hours after PGE₂ injection. In wild-type mice, the average AUC (anti-hyperalgesia) in
314 acetaminophen-treated mice (AUC: 1.51 ± 0.14 g·h, *n* = 7) was significantly higher than that of
315 the vehicle treated group (AUC: 0.073 ± 0.073 g·h, *n* = 6 mice, *P* < 0.001, unpaired Student's *t*-
316 test) (Fig. 4B). We also assessed the hyperalgesic effect of intrathecal PGE₂ in CB₁^{-/-} mice and
317 the potential reversal of PGE₂-induced hyperalgesia by acetaminophen in these mice. PGE₂
318 induced the same level of hyperalgesia, but acetaminophen was again completely devoid of
319 anti-hyperalgesic effects in CB₁^{-/-} mice. Average AUCs in acetaminophen-treated CB₁^{-/-} mice
320 (AUC: 0.20 ± 0.58 g·h, *n* = 6) were virtually identical to those in vehicle-treated CB₁^{-/-} mice (AUC:
321 0.064 ± 0.46 g·h, *n* = 6, *P* = 0.95, unpaired Student's *t*-test). Two-way ANOVA yielded a
322 significant genotype x treatment interaction *F*(1,25) = 5.46, *P* = 0.03. These results suggest that
323 acetaminophen alleviates inflammatory hyperalgesia through a mechanism independent of
324 prostaglandin formation.

325

326 *Ablation of CB₁ receptors from the periphery and the spinal cord does not block anti-*
327 *hyperalgesia by systemic acetaminophen*

328 We next aimed at identifying the anatomical origin of acetaminophen-induced anti-hyperalgesia.
329 Our first analyses concentrated on CB₁ receptors in the spinal cord for two reasons. First,
330 intrathecal injection of AM 404 mimicked the anti-hyperalgesia induced by systemic treatment
331 with acetaminophen in several respects and, second, activation of spinal CB₁ receptors inhibits
332 transmission for nociceptive signals between primary nociceptors and second order dorsal horn
333 neurons *in vitro* (Liang et al., 2004; Kato et al., 2012). The latter action might be considered a
334 prime candidate mechanism for acetaminophen-induced anti-hyperalgesia. To distinguish a
335 peripheral/spinal from a supraspinal site of action, we made use of *hoxb8*-CB₁^{-/-} mice, which
336 were generated by crossing *hoxb8*-cre mice with CB₁^{fl/fl} mice. During development, *hoxb8*-cre is
337 expressed in all DRG neurons and in all neurons and astrocytes of the spinal cord up to level
338 C4. *hoxb8*-cre is however virtually absent from the brain (Witschi et al., 2010). We verified the
339 specific ablation of CB₁ receptors from the spinal cord by comparing CB₁ receptor expression in
340 the spinal dorsal horn and in the periaqueductal grey (PAG), a midbrain area rich in CB₁
341 receptors (Fig. 5). In wild-type (CB₁^{fl/fl}) mice, intense CB₁ receptor staining was observed in the
342 grey matter of the superficial dorsal horn and in the dorsolateral funiculus as well as around the
343 cerebral aqueduct in the PAG (Fig. 5A,D,D',G). This staining was completely absent in spinal
344 cord and PAG sections obtained from global CB₁^{-/-} mice (Fig. 5B,E,E',H) indicating the specificity
345 of the CB₁ receptor antibody (see also Nyilas et al., 2009). As expected, *hoxb8*-CB₁^{-/-} mice
346 exhibited a drastic reduction in CB₁ receptor expression in the spinal dorsal horn (Fig. 5C,F,F'),
347 but not in the PAG (Fig. 5I). A side-by-side comparison of global CB₁^{-/-} and conditional *hoxb8*-

348 CB₁^{-/-} mice showed some remaining CB₁ immunoreactivity in the dorsal horn of the *hoxb8*-CB₁^{-/-}
349 mice, especially in the most superficial layers of the dorsal horn, which might result from
350 terminals of axons descending from supraspinal sites to the dorsal horn.

351 In behavioral experiments, *hoxb8*-CB₁^{-/-} mice and wild-type (*hoxB8*-cre negative CB₁^{fl/fl})
352 littermates did not differ in their baseline sensitivity to mechanical stimulation (PWT were 4.21 ±
353 0.10 g (*n* = 15) and 4.39 ± 0.07 g (*n* = 12) in naïve *hoxb8*-CB₁^{-/-} mice and CB₁^{fl/fl} littermates) and
354 developed virtually identical inflammatory hyperalgesia with PWTs of 0.79 ± 0.07 g and 0.73 ±
355 0.08 g in *hoxb8*-CB₁^{-/-} mice and CB₁^{fl/fl} littermates. Both genotypes also exhibited virtually
356 identical anti-hyperalgesic responses to systemic acetaminophen treatment. AUC were 2.15 ±
357 0.08 g·h (*n* = 6) and 1.59 ± 0.27 g·h (*n* = 6) for *hoxB8*-CB₁^{-/-} and cre-negative wild-type (CB₁^{fl/fl})
358 mice, respectively (Fig. 5J). Very similar results were obtained with AM 404. AUCs were 1.41 ±
359 0.12 g·h (*n* = 9) and 1.38 ± 0.11 g·h (*n* = 6), for *hoxB8*-CB₁^{-/-} and cre-negative littermates (Fig.
360 5K). Together with the complete lack of anti-hyperalgesia by acetaminophen and AM 404 in CB₁^{-/-}
361 mice, these results suggest that acetaminophen acted through CB₁ expressed at supraspinal
362 sites. Alternatively, acetaminophen might act via CB₁ receptors expressed in the spinal cord on
363 the terminals of neurons descending from supraspinal sites, which are not targeted by the
364 *hoxB8*-cre (compare Fig. 5C,F,F'). To distinguish between these two possibilities we continued
365 with local injections of AM 404 and of the CB₁ receptor antagonist rimonabant.

366

367 *Local injection of rimonabant and AM 404 suggest a critical role of the RVM in anti-hyperalgesia*
368 *by systemic acetaminophen.*

369 The RVM serves well-established roles in endogenous pain control (Heinricher and Fields, 2013)
370 and as a site of action of centrally acting analgesic drugs including cannabinoid ligands (Meng et
371 al., 1998; Suplita et al., 2005). We therefore tested whether the RVM was also involved in the
372 anti-hyperalgesic actions of acetaminophen. To this end, we analyzed whether local injection
373 into the RVM of the CB₁ receptor antagonist rimonabant would interfere with anti-hyperalgesia
374 by systemic acetaminophen (Fig. 6). Rimonabant (and vehicle) injections were made via chronic
375 cannulas that had been pre-implanted into the RVM one week before the experiment. Proper
376 RVM injections were verified by addition of a small amount of Evans Blue to the injection
377 solution and post-hoc anatomical analysis of mouse brain sections (Fig. 6A,B). Injection of
378 rimonabant (0.67 µg in 300 nl) completely prevented the anti-hyperalgesic action of systemic
379 acetaminophen (200 mg/kg) (Fig. 6C,D). The AUCs were 4.89 ± 1.35 g·h (*n* = 5) versus 0.67 ±
380 0.54 g·h (*n* = 6), in aCSF and rimonabant pretreated mice, respectively (*P* = 0.013, unpaired
381 Student's t-test). RVM injection of rimonabant *per se* did not affect inflammatory hyperalgesia
382 and RVM injection of vehicle did neither affect the inflammatory hyperalgesia nor change the

383 anti-hyperalgesic response of acetaminophen. Injection of rimonabant or vehicle or cannula
384 implantation into the RVM of naïve mice was tested in 5 - 7 mice per group. These interventions
385 had no effect on mechanical pain response threshold (data not shown). We next tested whether
386 the effect of acetaminophen would be mimicked by local RVM injection of AM 404. As expected,
387 AM 404 (1 μ g, equivalent to 2.5 nmoles) significantly alleviated inflammatory hyperalgesia in
388 wild-type mice but not in $CB_1^{-/-}$ mice (Fig. 6E,F). In naïve mice, RVM injection of AM 404 did not
389 significantly change PWTs (4.65 ± 0.56 g versus 4.23 ± 0.36 g, for AM 404 and vehicle, $P =$
390 0.54 , $n = 4$ mice per group). In this series of experiments, we finally tested whether injection of
391 acetaminophen into the RVM would reduce hyperalgesia (Fig. 6 G). Consistent with an only very
392 low conversion of acetaminophen in AM 404 in the brain (Högstätt et al., 2005), acetaminophen
393 (1 μ g in 300 nl) failed to significantly change PWTs ($n = 6$).

394

395 *Distribution of CB_1 receptor mRNA and protein in the RVM.*

396 In many parts of the CNS, cannabinoid receptors are located on presynaptic axon terminal
397 where they control neuronal activity through the inhibition of neurotransmitter release. The
398 experiments described above suggest that acetaminophen exerts its anti-hyperalgesia action
399 through a perhaps indirect activation of antinociceptive fiber tracts descending from the RVM. To
400 gain insights into the distribution of CB_1 receptors at this site, we performed
401 immunohistochemistry and *in situ* hybridization experiments in wild-type and global $CB_1^{-/-}$ mice
402 (Fig. 7). The immunohistochemical experiments revealed that CB_1 receptors at the protein level
403 were abundantly distributed throughout the RVM (Fig. 7A-D), which is consistent with a central
404 role of the RVM in the CB_1 -mediated anti-hyperalgesic action of acetaminophen. In contrast, CB_1
405 receptor mRNA was only detected in a few selected cells in the RVM close to the midline (Fig.
406 7E). No such cells were detected in tissue from $CB_1^{-/-}$ mice (Fig. 7F). The low density CB_1 -
407 immunolabelling found in the dorsal horn of the spinal cord of *hoxB8*- $CB_1^{-/-}$ mice (Fig. 5F,F')
408 likely reflects those descending fibers, which originate from the few RVM CB_1 mRNA-expressing
409 cells.

410

411 *Local ablation of CB_1 receptors in the RVM does not prevent the anti-hyperalgesic actions of*
412 *acetaminophen.*

413 The results obtained with local injection into the RVM of rimonabant and AM 404 suggest a
414 critical role of the RVM in the anti-hyperalgesic actions of acetaminophen. The relevant CB_1
415 receptors in the RVM may either reside on RVM neurons themselves or may be located on axon
416 terminals of neurons innervating the RVM. To distinguish between these possibilities, we
417 selectively ablated receptors on intrinsic RVM neurons by local injection of $CB_1^{fl/m}$ mice with

418 adeno-associated virus (AAV) carrying a cre recombinase expression cassette. AAV-cre virus
419 injections were performed one week before acetaminophen treatment. Successful cre-mediated
420 ablation of the CB₁ receptor gene was verified with real time RT-PCR. The number of CB₁
421 receptor transcripts in the RVM was reduced to about 25% (Fig. 8A). However, despite this
422 significant down-regulation of CB₁ receptors, acetaminophen-induced anti-hyperalgesia
423 remained largely unaffected (Fig. 8B,C). These results suggest that the relevant CB₁ receptors
424 reside on axon terminals of neurons projecting to the RVM rather than on intrinsic RVM neurons.
425 Figure 9 illustrates a possible scenario: AM 404 in the RVM would increase the concentration of
426 endocannabinoids (anandamide and 2-AG) and thereby indirectly activate CB₁ receptors on
427 inhibitory neurons that project to the RVM to tonically inhibit antinociceptive fiber tracts
428 descending to the spinal cord. Increased activation of CB₁ receptors on these neurons will
429 reduce GABA release and dis-inhibit endogenous descending pain control units. Since many of
430 the descending fibers release serotonin (Heinricher and Fields, 2013), this scenario is consistent
431 with previous reports proposing not only a central site of action of acetaminophen but also a
432 contribution of spinal serotonin receptors (Pelissier et al., 1995; Bonnefont et al., 2005).
433

434 Discussion

435 Our study demonstrates that acetaminophen exerts anti-hyperalgesic actions in a mouse model
436 of inflammatory pain consistent with previous experimental (Vinegar et al., 1976; McQueen et
437 al., 1991; Abbadie and Besson, 1994) and clinical studies (Skjelbred et al., 1977; Bradley et al.,
438 1991; Bjornsson et al., 2003; Brandt et al., 2006). These previous data have shown analgesia in
439 adjuvant-induced monoarthritis or postoperative swelling and against secondary pain in oral
440 surgery or osteoarthritic knee pain. Activity against inflammatory hyperalgesia and the well-
441 known antipyretic effect of acetaminophen have led researchers to speculate about an inhibitory
442 action of acetaminophen on prostaglandin formation, e.g. through COX inhibition. However,
443 acetaminophen is largely devoid of anti-inflammatory activity (Clissold, 1986; Bertolini et al.,
444 2006; Brunton et al., 2011), which is a hallmark effect of classical COX inhibitors. Significant
445 activity against inflammatory hyperalgesia in the absence of general anti-inflammatory efficacy
446 could be due to a specific inhibition of prostaglandin production in the CNS or to an analgesic
447 mechanism independent of the inhibition of prostaglandin formation. Several studies have
448 support a contribution of the endocannabinoid system. However, most of these studies used
449 models of acute nociceptive pain, which do not necessarily permit conclusions about the
450 mechanisms of anti-hyperalgesic actions.

451 As shown in a previous study from our group, zymosan A-induced hyperalgesia strongly
452 depends on spinally produced PGE₂ (Reinold et al., 2005). This model is therefore well-suited to
453 investigate mechanisms of drugs with anti-hyperalgesic actions in inflammatory conditions and
454 should permit a straightforward detection of prostaglandin-dependent drug actions. The reversal
455 of inflammatory hyperalgesia by acetaminophen observed in our study would hence be
456 consistent with a block of PGE₂ production by acetaminophen. However, acetaminophen was
457 still active when hyperalgesia was induced by local spinal injection of PGE₂ favoring a
458 mechanism different from inhibition of prostaglandin formation. Several results of the present
459 study support instead the involvement of central CB₁ receptors: the reversal of PGE₂-induced
460 hyperalgesia by acetaminophen was absent in CB₁^{-/-} mice, and both AM 404 and
461 acetaminophen failed to reverse zymosan A-induced hyperalgesia in CB₁^{-/-} mice. Furthermore,
462 the congruent pattern of efficacy of acetaminophen and of AM 404 in different (global and spinal
463 cord-specific) CB₁ receptor-deficient mouse lines supports the contribution of AM 404 to the anti-
464 hyperalgesic actions of acetaminophen. These results also correspond well with previous
465 findings demonstrating that acetaminophen-induced analgesia was lost in FAAH^{-/-} mice, which
466 do not convert acetaminophen into AM 404 (Högestätt et al., 2005; Dalmann et al., 2015).
467 However, neither the present nor previously published results (Ottani et al., 2006; Dani et al.,
468 2007; Mallet et al., 2008) exclude an involvement of COX-1 or COX-2 (Flower and Vane, 1972;
469 Hanel and Lands, 1982; Muth-Selbach et al., 1999; Boutaud et al., 2002; Graham and Scott,
470 2005). An *ex vivo* study performed in human volunteers demonstrated inhibition of COX-1 and
471 COX-2 following the oral administration of acetaminophen (Hinz et al., 2008), and AM 404 has
472 also been shown to block COX-1 and COX-2 in lipopolysaccharide-stimulated macrophages
473 (Högestätt et al., 2005). In this context, it is important to note that COX-2 contributes to the
474 metabolism of endocannabinoids (Yu et al., 1997; Kozak et al., 2000). The extent to which
475 inhibition of COX-dependent endocannabinoid degradation or blockade of endocannabinoid
476 transporters contribute to acetaminophen-induced analgesia remains to be determined.

477 Our results can also be reconciled with a report by (Mallet et al., 2010), who have proposed a
478 role of supraspinal TRPV1 receptors as additional targets in acetaminophen and AM 404-
479 induced analgesia. AM 404 is not only an inhibitor of anandamide reuptake but also an agonist
480 at TRPV1 receptors (De Petrocellis et al., 2000). The observation that AM 404-induced
481 analgesia was absent in TRPV1^{-/-} mice and abolished by intracerebroventricular injection of the
482 TRPV1 receptor antagonist capsazepine may suggest functional interactions of CB₁ and TRPV1
483 receptors in the CNS (Fioravanti et al., 2008). More difficult to reconcile with our findings is the
484 report by (Andersson et al., 2011). These authors ascribe the analgesic action of acetaminophen
485 to the activation of TRPA1 channels on the spinal terminals of nociceptive fibers by the

486 acetaminophen metabolites NPQI and p-benzoquinone, and a subsequent inhibition of
487 transmitter release via primary afferent depolarization. Since anti-hyperalgesia by
488 acetaminophen was retained in *hoxb8*-CB₁^{-/-} mice, an interaction of TRPA1 channels with CB₁
489 receptors cannot explain these findings. It is likely that distinct mechanisms underlie the acute
490 analgesic and the anti-hyperalgesic actions of acetaminophen.

491 Comparing the effects of classical cannabinoids with those of acetaminophen reveals similarities
492 and differences. Classical cannabinoids exert a tetrad of actions in rodents, which includes
493 analgesia, hypothermia, sedation (reduced locomotor activity), and catalepsy (Little et al., 1988).
494 Analgesia, sedation and hypothermia do also occur in mice in response to acetaminophen
495 (Mallet et al., 2010). While our data provide strong support for the involvement of cannabinoid
496 signaling in acetaminophen-induced anti-hyperalgesia, cannabinoid independent actions are
497 likely more relevant for the hypothermic and antipyretic effects of acetaminophen (Gentry et al.,
498 2015). Such CB₁ receptor-independent mechanisms include the inhibition of hypothalamic COX
499 by AM 404 (Högestätt et al., 2005) and the activation of TRPA1 via the acetaminophen
500 metabolite NAPQI (Gentry et al., 2015). The mechanisms of acetaminophen-induced sedation in
501 mice have not been identified so far and catalepsy is not seen in mice. Furthermore, the
502 psychotropic actions seen with classical CB₁ receptor agonists in humans do not occur with
503 acetaminophen. Local differences in the conversion of the acetaminophen metabolite p-
504 aminophenol into pharmacologically active AM 404, caused for example by varying FAAH
505 activity in different CNS regions, or differences in the local activity of endocannabinoid system
506 may explain these discrepancies. Such differences may also account for another discrepancy.
507 While a previous report has suggested that CB₁ receptor agonists exert most of their analgesic
508 action through CB₁ receptors on peripheral nociceptors (Agarwal et al., 2007), our experiments
509 in *hoxB8*-CB₁^{-/-}, which lack CB₁ receptors also from these cells, suggest that this is not the case
510 for acetaminophen (see also Dalmann et al., 2015).

511 In our experiments, we also aimed at a better definition of the site of acetaminophen's action. To
512 this end, we used *hoxb8*-CB₁^{-/-} mice, which lack CB₁ receptors specifically from the spinal cord
513 and peripheral sensory neurons. Because CB₁ receptors are densely expressed on different
514 types of intrinsic spinal dorsal horn neurons and on sensory fiber terminals (Tsou et al., 1998;
515 Farquhar-Smith et al., 2000; Bridges et al., 2003; Hegyi et al., 2009; Nyilas et al., 2009),
516 experiments first focused on a possible spinal site of action. However, the anti-hyperalgesia by
517 acetaminophen were completely preserved in *hoxb8*-CB₁^{-/-} mice.

518 At least two explanations may account for these findings. The CB₁ receptors responsible for
519 acetaminophen analgesia might reside on the spinal terminals of fibers descending from
520 supraspinal sites which are spared from *hoxb8*-cre mediated gene deletion. This scenario is

521 consistent with the presence of CB₁ receptors in the termination area of descending fiber tracts
522 in spinal cords of *hoxb8*-CB₁^{-/-} mice, and with the efficacy of AM 404 after intrathecal injection.
523 However, AM 404 might have diffused to supraspinal sites after lumbar intrathecal injection.
524 Such diffusion has been demonstrated earlier for radioactively labeled morphine (Gustafsson et
525 al., 1985). Alternatively, acetaminophen might act via CB₁ receptors at supraspinal sites located
526 e.g. in the brainstem, where the somata of descending antinociceptive fiber tracts are located.
527 Our experiments with local injection of rimonabant and AM 404 into the RVM provide strong
528 support for this scenario (see also Högestätt et al., 2005; Mallet et al., 2008; Mallet et al., 2010;
529 Dalmann et al., 2015). According to these previous studies, acetaminophen acts through a CB₁
530 receptor-mediated reinforcement of descending serotonergic bulbospinal pathways originating
531 from the RVM (Mallet et al., 2008) with subsequent activation of pain-suppressing serotonin
532 receptors in the spinal cord (Tjolsen et al., 1991; Pelissier et al., 1995; Pini et al., 1996;
533 Bonnefont et al., 2005). Our results are thus in line with the important role of supraspinal CB₁
534 receptors in stress-induced analgesia (Hohmann et al., 2005; Suplita et al., 2006).

535 Strong CB₁ receptor immune reactivity but weak *in situ* hybridization signals in the RVM suggest
536 that the relevant CB₁ receptors reside on processes of neurons that project to the RVM from
537 other brain areas. In this scenario, it is likely that the acetaminophen metabolite AM 404
538 promotes the activation of CB₁ receptors on GABAergic axon terminals that tonically inhibit
539 serotonergic antinociceptive fiber tracts descending from the RVM to the spinal cord. Since the
540 periaqueductal grey (PAG) controls RVM activity via descending axons (Heinricher and Fields,
541 2013), it is conceivable that the CB₁ receptors relevant for the analgesic action of
542 acetaminophen reside on the terminals of fibers reaching the RVM from the PAG.
543 Acetaminophen would thus indirectly reduce GABA release from these projections and dis-inhibit
544 descending serotonergic fibers to facilitate endogenous pain control.

545 In summary, our results shed new light on the mechanisms and sites of action of the anti-
546 hyperalgesic action of the widely used analgesic acetaminophen. They support the involvement
547 of the endocannabinoid system in the analgesic action of acetaminophen against inflammatory
548 pain and identify the RVM and descending antinociceptive fiber tracts as a likely site and
549 mechanism of action.

550

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- 730

731 **Figure legends**

732

733 **Fig. 1** Anti-hyperalgesic actions of acetaminophen (p.o.) and AM 404 (i.t.) in the zymosan A
734 model of inflammatory hyperalgesia. **(A)** Partial reversal of reduction in PWT (g) by
735 acetaminophen 200 mg/kg. $n = 6$ mice. **(B)** The same dose of acetaminophen had no significant
736 effect on PWT in naïve mice. Unpaired Student's t-test, $P = 0.66$, $n = 5$ and 7 , for
737 acetaminophen and vehicle, respectively. Horizontal line indicates the time interval used to
738 determine the maximal effects. **(C)** Effects of different doses of systemic acetaminophen
739 administered 24 hours following s.c. injection of zymosan A ($n = 6$ mice per dose) on mechanical
740 PWTs quantified as percent maximal possible effect (% maximum possible analgesia; mean \pm
741 SEM). **(D)** Dose response curve. Average % maximum possible analgesia determined for the
742 intervals 60 and 80 min after drug administration was calculated for each group and fitted to the
743 Hill equation. $*P \leq 0.05$, $***P < 0.001$, ANOVA followed by Dunnett's *post-hoc* test, $F(4,25) =$
744 10.11 with $F_{crit} = 2.76$. **(E,F)** Same as (C,D) but intrathecal AM 404 ($n = 6$ mice per group).
745 Average % maximum possible analgesia was determined for the time interval between 20 and
746 40 min after drug injection. $*P \leq 0.05$, $***P < 0.001$, ANOVA followed by Dunnett's *post-hoc* test,
747 $F(4,25) = 25.15$. **(G,H)** Impact of systemic acetaminophen on muscle strength (percent
748 successful attempts in the horizontal wire test) **(G)** and on motor coordination (time on rotarod)
749 **(H)** at 60 – 90 min after oral acetaminophen administration. No statistically significant effects
750 were found in the two tests. (G) ANOVA followed by Dunnett's *post hoc* test. $F(2,22) = 1.46$. $P =$
751 0.33 and 0.92 , for 200 and 300 mg/kg, $n = 7-8$ mice. (H) $F(2,22) = 1.43$. $P = 0.33$ and 0.97 , for
752 200 and 300 mg/kg, $n = 7-8$ mice.

753

754

755 **Fig. 2** Acute liver toxicity of acetaminophen. **(A-C)** Plasma levels of enzymatic markers of liver
756 damage were quantified in mice 24 hours after p.o. treatment with vehicle, 200 mg/kg or 300
757 mg/kg acetaminophen. Statistical comparisons were made with ANOVA followed by Dunnett's
758 post-hoc test. **(A)** ALT: $F(2,21) = 2.55$, $P = 0.99$ and 0.02 , for 200 and 300 mg/kg, $n = 6 - 8$
759 mice. **(B)** AST: $F(2,21) = 2.67$, $P = 0.91$ and 0.08 , for 200 and 300 mg/kg, $n = 7 - 8$ mice. **(C)**
760 LDH: $F(2,20) = 5.28$, $P = 0.97$ and 0.09 , for 200 and 300 mg/kg, $n = 7 - 8$ mice. **(D)** Histological
761 changes caused by acetaminophen treatment were assessed 24 hours after drug administration.
762 The percent venules surrounded by discolored tissue was calculated. No significant changes
763 were observed after 200 and 300 mg/kg, however 400 mg/kg caused statistically significant liver
764 damage. $F(3,20) = 6.05$, $P = 0.69$, 0.78 , and 0.014 , for 200, 300 and 400 mg/kg, $n = 6$ mice for
765 all four groups. Right micrographs show magnifications of the indicated areas with healthy tissue
766 surrounding a venule in the section taken from a vehicle treated mouse (veh) and damaged
767 tissue around a venue in the section prepared from a mouse treated with 400 mg/kg. Dotted line
768 in the top left micrograph indicates the damage area around the venule in the center.

769

770 **Fig. 3** Effect of CB₁ receptor ablation on the antihyperalgesic actions of by acetaminophen and
771 AM 404. **(A)** Acetaminophen (200 mg/kg, p.o.). Time course of changes in PWT. Acetaminophen
772 was given 24 hours after injection of zymosan A to wild-type mice ($n = 6$) and to CB₁^{-/-} mice ($n =$
773 8). Bar chart: AUCs (g·h, mean \pm SEM). *, $P \leq 0.05$, unpaired Student's t-test. **(B)** AM 404 (10
774 nmol, i.t.) was administered 24 hours after injection of zymosan A in wild-type and CB₁^{-/-} mice (n
775 $= 7$ each). *** $P < 0.001$, unpaired Student's t-test. **(C)** Systemic pretreatment with rimonabant
776 (rim, 5 mg/kg, i.p.) completely blocked anti-hyperalgesia by acetaminophen. Two-way ANOVA
777 $F(1,22) = 9.08$, $P = 0.007$ for pretreatment x treatment interaction, $n = 4 - 8$ per group. **, $P <$
778 0.01 , $n = 6$ and 8 mice for vehicle and rimonabant pretreated mice (unpaired Student's t-test).

779

780 **Fig. 4** Effect of acetaminophen (200 mg/kg, p.o.) on mechanical hyperalgesia evoked by
781 intrathecal PGE₂ (0.4 nmol) in wild-type and CB₁^{-/-} mice. **(A)** Change in PWTs (mean \pm SEM).
782 PGE₂ was injected i.t. at time 0. Acetaminophen or vehicle were given p.o. (1 hour after PGE₂
783 injection. $n = 7$ and 6 for acetaminophen and vehicle, respectively. **(B)** AUC (mean \pm SEM).
784 Two-way ANOVA yielded a significant genotype x treatment interaction $F(1,25) = 5.46$, $P = 0.03$.
785 $n = 6 - 7$ mice per group.

786

787 **Fig. 5** Morphological and behavioral analysis of *hoxb8*-CB₁^{-/-} mice. **(A-I)** CB₁ receptor expression
 788 in the spinal dorsal horn and PAG of wild-type, CB₁^{-/-} and *hoxb8*-CB₁^{-/-} mice. **(A)** High density of
 789 CB₁ receptor-immunostaining is found in the superficial layers in the dorsal horn of wild type
 790 (CB₁^{fl/fl}) mouse spinal cord. **(D,D')** At higher magnification, an abundant punctate staining pattern
 791 corresponding mostly to axon terminals is observed. **(B,E,E')** The specificity of this staining
 792 pattern is validated by the complete lack of immunostaining on spinal cord sections derived from
 793 global CB₁^{-/-} animals. **(C,F,F')** Deletion of CB₁ receptors from DRG and spinal neurons as well as
 794 from astrocytes in *hoxb8*-CB₁^{-/-} animals did not fully eliminate CB₁ receptor immunostaining. A
 795 remaining weak staining pattern was found in lamina I and II, where most descending
 796 monoaminergic fibers terminate. **(G)** Immunostaining for CB₁ receptors in the midbrain
 797 periaqueductal grey nucleus (PAG) is concentrated around the dorsal and central part of the
 798 PAG. **(H)** This staining pattern is completely eliminated in the global CB₁^{-/-} animals, but remains
 799 fully intact in *hoxb8*-CB₁^{-/-} mice. Similar results were obtained in three mice of both genotypes.
 800 Scale bars are: (C valid also for A,B) 100 μm; (F applies also for D,E) 20 μm; (F' applies also for
 801 D',E') 10 μm; and (I valid also for G,H) 200 μm. **(J,K)** Behavioral analysis. Changes in PWTs
 802 induced by the acetaminophen (200 mg/kg, p.o., **J**) in *hoxb8*-CB₁^{-/-} (*n* = 6) and wild-type (CB₁^{fl/fl})
 803 mice (*n* = 6), and by AM 404 (10 nmol, i.t., **K**) in *hoxb8*-CB₁^{-/-} (*n* = 6) and wild-type (CB₁^{fl/fl}) mice
 804 (*n* = 9). Acetaminophen and AM 404 were administered 24 hours after zymosan A injection.
 805 Differences in AUCs were statistically insignificant (unpaired Student's t-test).

806

807 **Fig. 6** Local RVM injection of rimonabant blocks and local RVM injection of AM 404 mimics the
 808 anti-hyperalgesic action of systemic acetaminophen.

809 **(A)** Sagittal brain section taken from a mouse after RVM injection verifies proper local RVM
 810 injection procedures. Red, Evans Blue; blue, DAPI **(B)** Respective brain regions (sagittal section
 811 at -0.04 mm) redrawn and simplified from Paxinos and Franklin (2001) for comparison. **(C,D)**
 812 Local injection of rimonabant (0.67 μg in 300 nl) prevented anti-hyperalgesia by systemic
 813 acetaminophen. Cannulation of the RVM, and injection of vehicle or rimonabant were *per se*
 814 without effect on mechanical pain thresholds. **(C)** Time course. **(D)** Two-way ANOVA revealed a
 815 significant pretreatment x treatment interaction. ($F(1,23) = 10.8$, *n* = 5 – 7 mice per group $P <$
 816 0.004). *, $P < 0.05$, unpaired Student's t-test, acetaminophen in aCSF (*n* = 5) or rimonabant (*n* =
 817 6) pretreated mice. **(E,F)** Local injection of AM 404 (1 μg in 300 nl) into the RVM mimicked
 818 acetaminophen-induced anti-hyperalgesia. **(E)** Time course. **(F)** Statistics. ANOVA followed by
 819 Bonferroni post hoc test. $F(2,17) = 13.4$. ***, $P \leq 0.001$, *n* = 6 mice per group. **(G)** Local injection
 820 of acetaminophen (1 μg in 300 nl) into the RVM had no effect on paw withdrawal threshold.

821

822 **Fig. 7** CB₁ receptor immunoreactivity and in situ hybridization in the RVM.

823 **(A,B)** CB₁ receptor immunoreactivity in coronal sections through the brainstem of wild-type (A)
824 and CB₁^{-/-} mice (B). The lack of brownish color of the DAB precipitate in the CB₁^{-/-} tissue (B)
825 confirms the specificity of CB₁ immunolabeling. Squares indicate the area of the RVM shown at
826 high magnification in **(C-F)**. **(C)** CB₁ protein is present in high density within the RVM of wild-
827 type mice. Note the dense DAB puncta around the cell bodies, which are always devoid of
828 labeling. **(D)** No CB₁ immunostaining can be found in control sections from CB₁^{-/-} mice, which
829 were processed together with the wild-type sections throughout the whole immunostaining
830 procedure. The dark yellow color of the white matter bundles is due to an osmification step of
831 tissue dehydration. **(E)** CB₁ in situ hybridization signal in the RVM. Only a few scattered neurons
832 (blue) express CB₁ receptor mRNA at rather low levels. **(F)** No labelled cells are present in
833 control sections prepared from CB₁ receptor-deficient mice. Scale bars are 250 μm in A,B; 50
834 μm in C-F.

835

836 **Fig. 8** Local knock-down of CB₁ receptor expression in intrinsic RVM neurons fails to prevent
837 acetaminophen-induced anti-hyperalgesia.

838 **(A)** Changes in CB₁ receptor mRNA levels seven days after AAV-cre injection in CB₁^{fl/fl} mice.
839 mRNA levels have been normalized to β-actin mRNA copy numbers. **, $P < 0.01$. $n = 19$ and 14,
840 for AAV-Cre and AAV-GFP, respectively. Unpaired Student's t-test. **(B)** Anti-hyperalgesia by
841 acetaminophen (200 mg/kg). RVM cannula implantation and AAV-cre injections were made 7
842 days before acetaminophen treatment. Zymosan A was injected 1 day, before acetaminophen
843 treatment. Mechanical PWTs were determined before AAV-cre injection, after zymosan A
844 injection, and after acetaminophen or vehicle administration. **(C)** Statistical analyses.
845 Comparisons of acetaminophen effects in the three treatment groups (AAV-cre, AAV-eGFP,
846 sham operated mice) revealed significant acetaminophen versus vehicle effects (*, $P < 0.05$, $n =$
847 $6 - 8$ / group) but no significant treatment x pretreatment interaction (two-way ANOVA $F(2,39) =$
848 0.41 , $P = 0.67$).

849

850

851 **Fig. 9** Hypothetical scheme of the central site of action of acetaminophen in inflammatory pain
852 conditions.

853 AM 404 produced from systemically administered acetaminophen increases the concentration of
854 endocannabinoids (AEA and 2-AG) in the RVM by inhibiting their uptake or degradation. This
855 increase activates CB₁ receptors on axon terminals of neurons projecting to the RVM from
856 upstream brain regions such as the PAG. These terminals normally release GABA to tonically
857 inhibit serotonergic antinociceptive fiber tracts, which descend from the RVM to the spinal cord.
858 Increased activation of CB₁ receptors in the RVM would then reduce GABA release in the RVM
859 and dis-inhibit descending pain control units. For a detailed discussion on the role of
860 serotonergic neurons in the RVM, see (Heinricher and Fields, 2013).

861

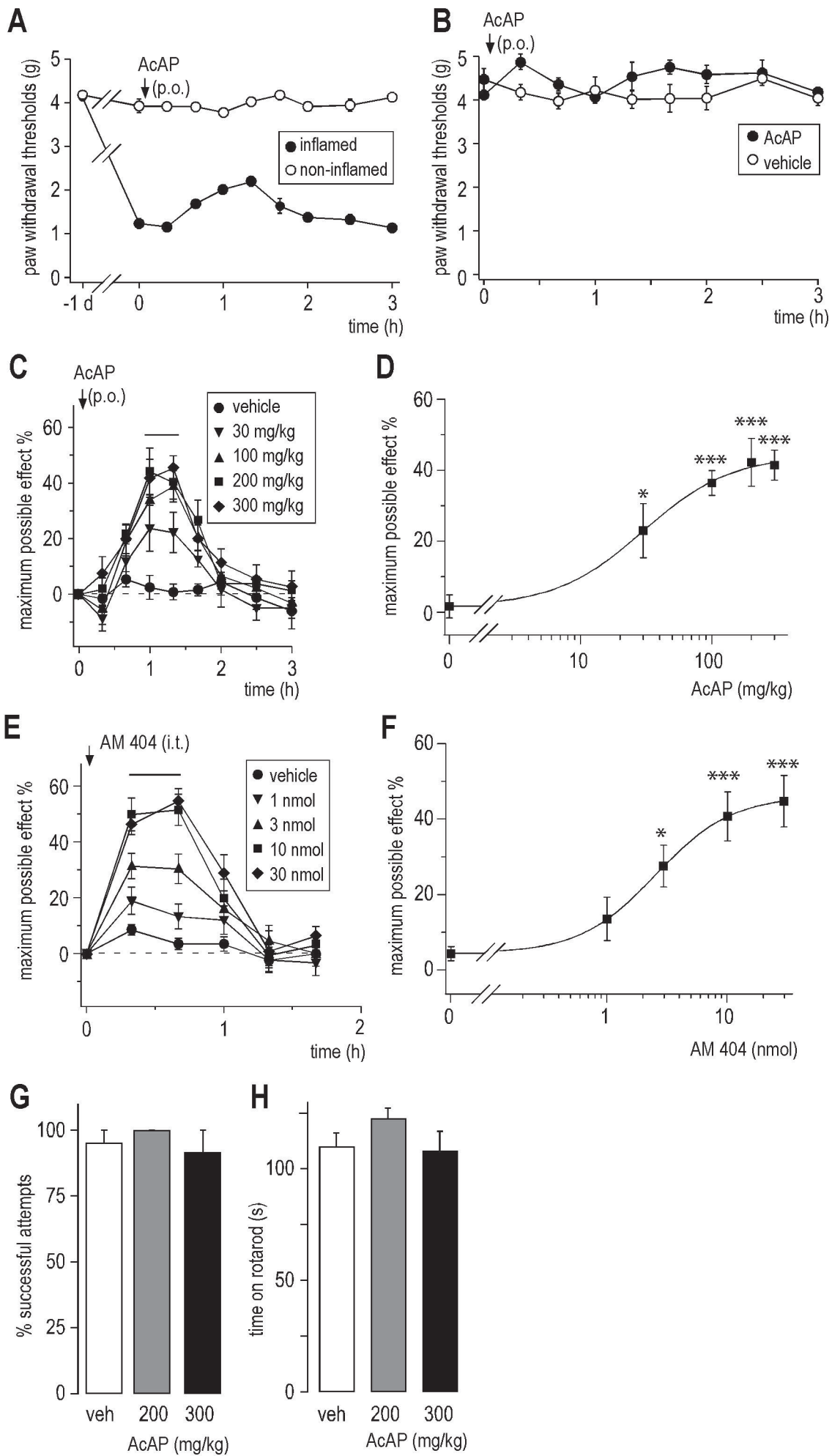


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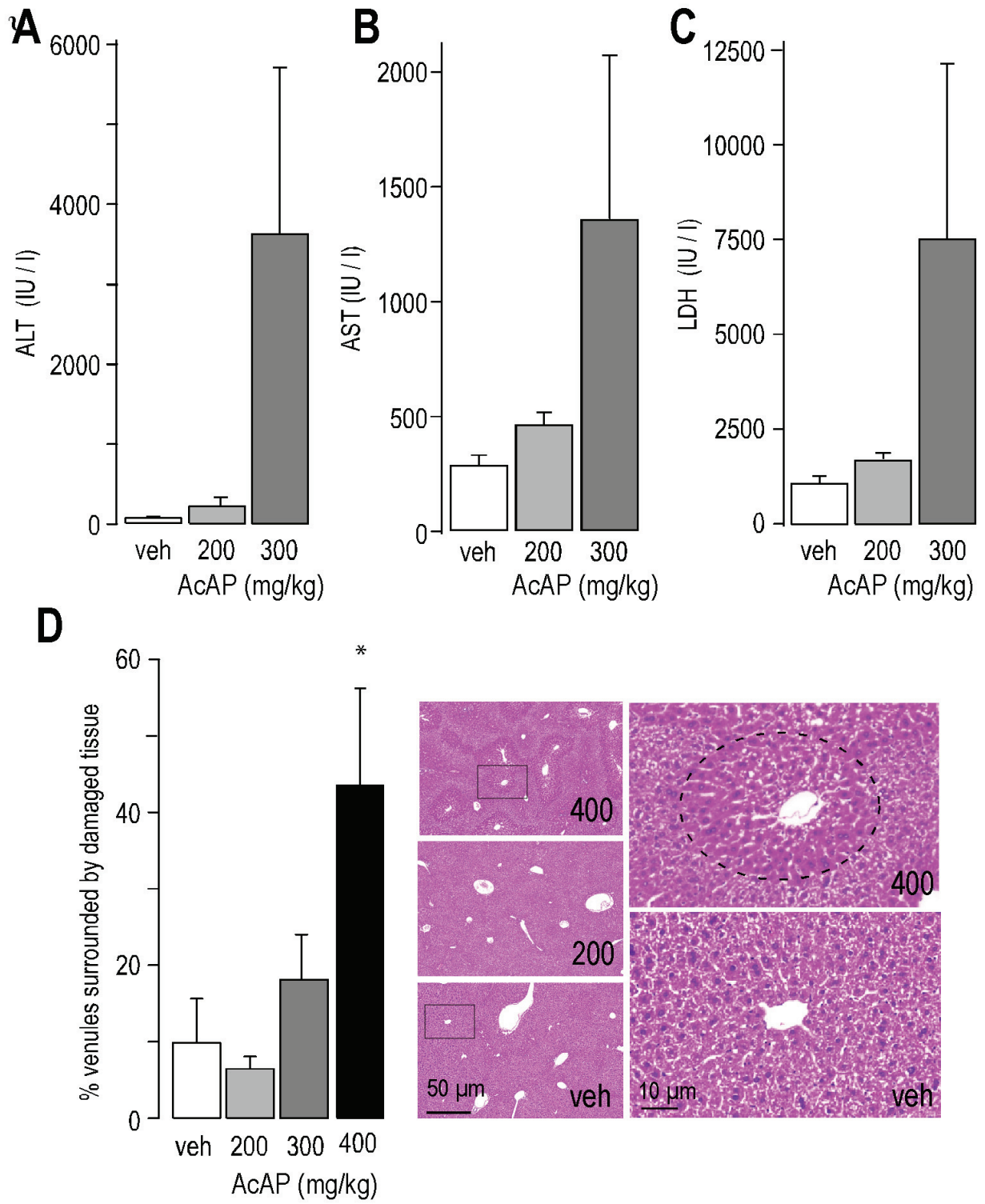


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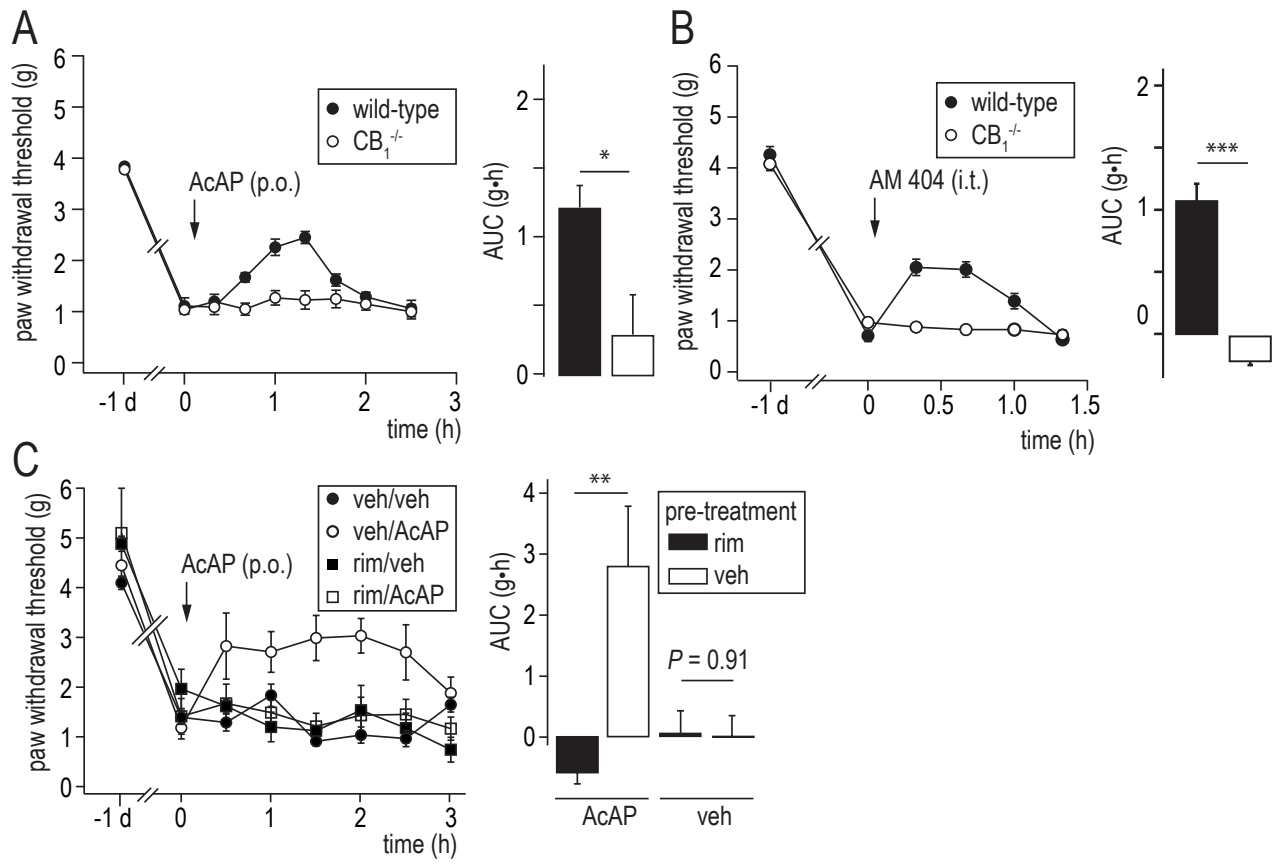


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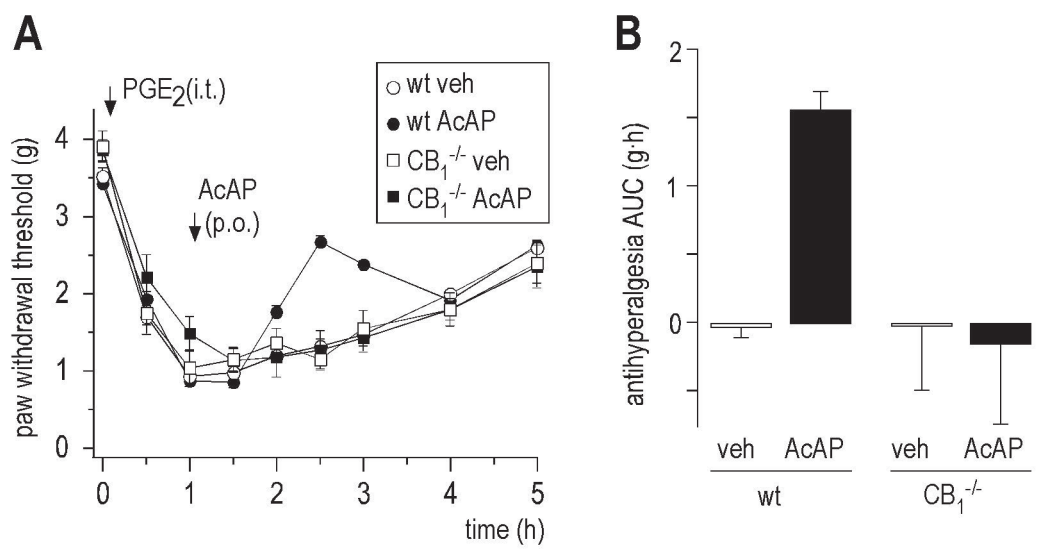


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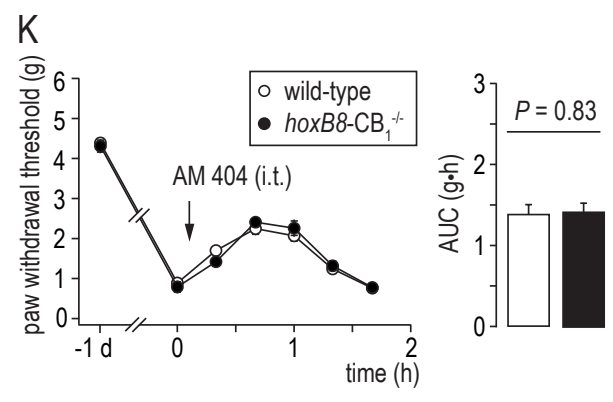
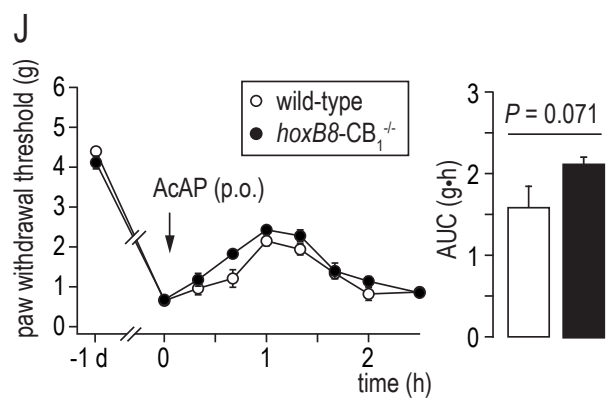
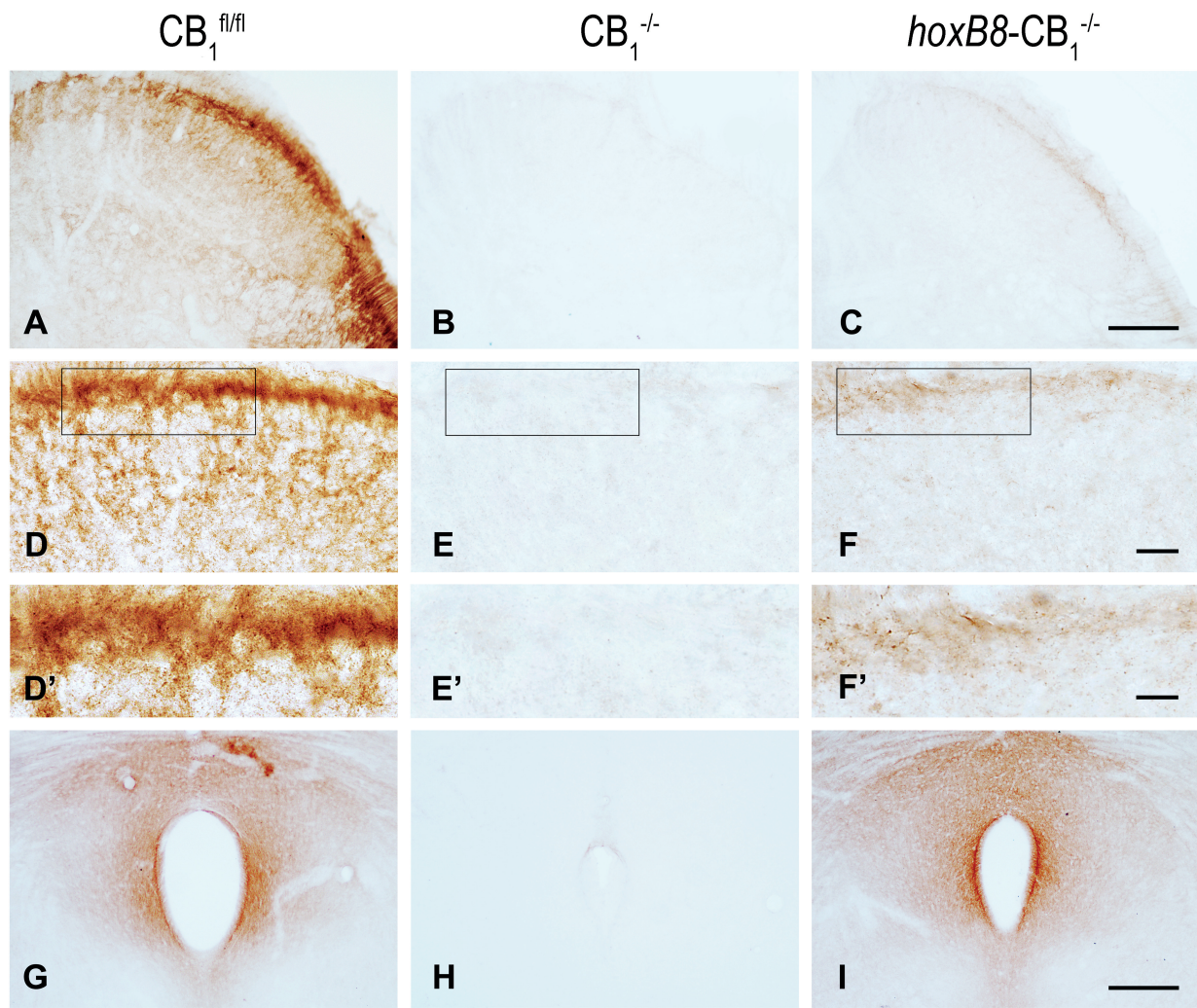


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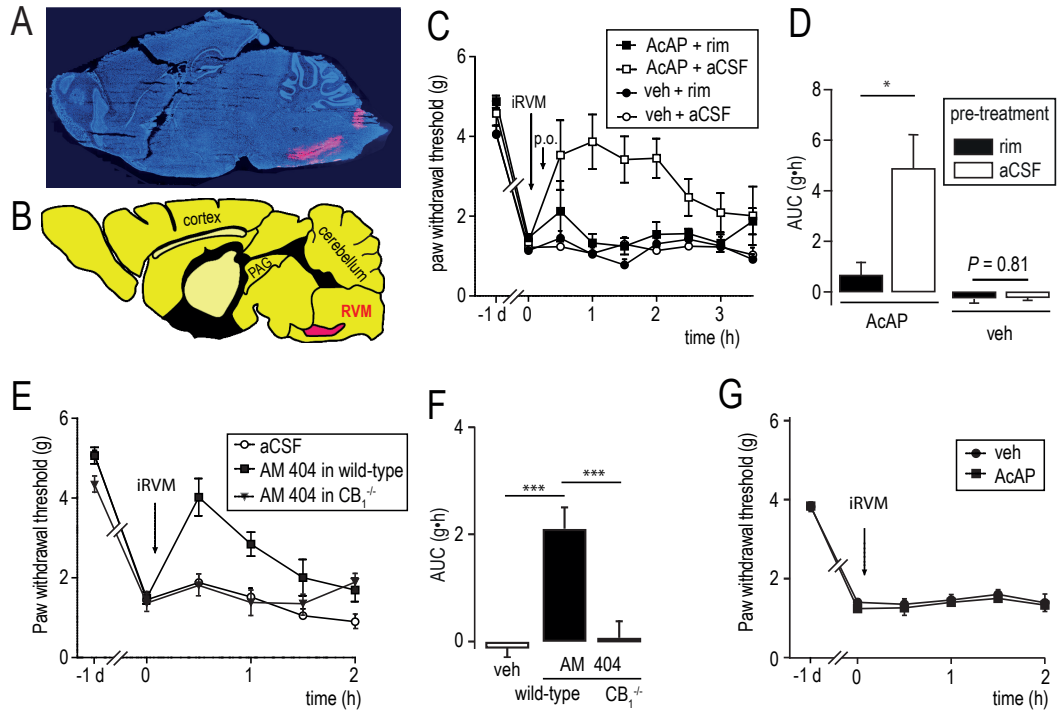


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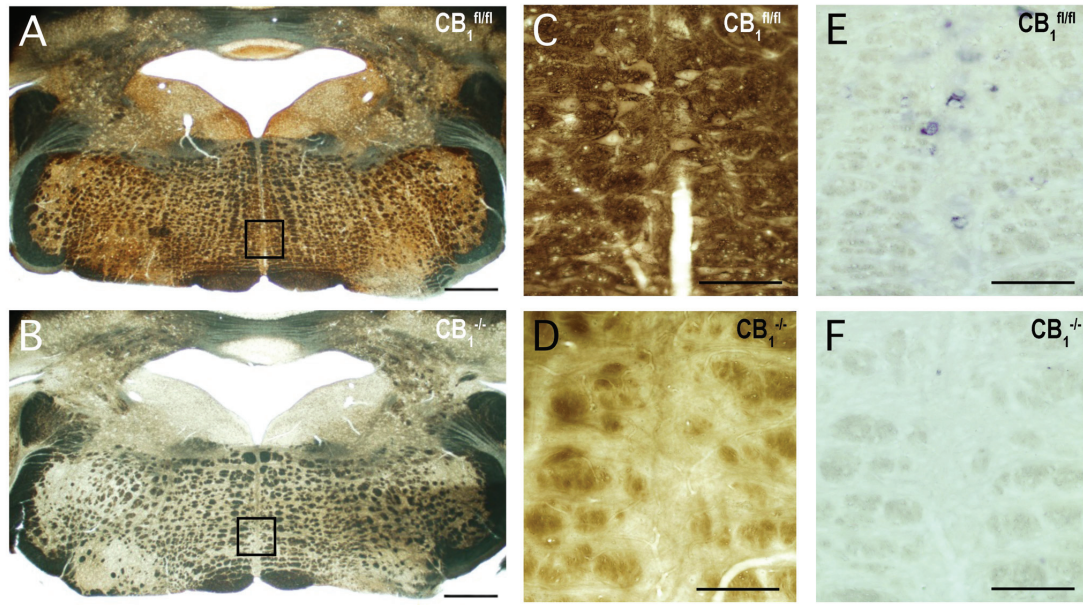


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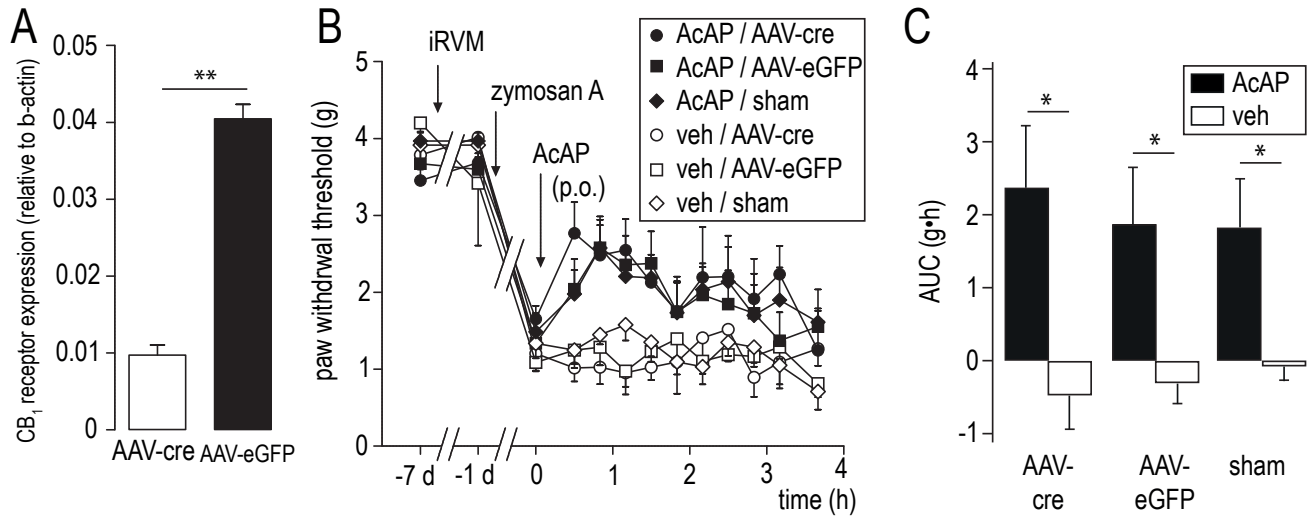


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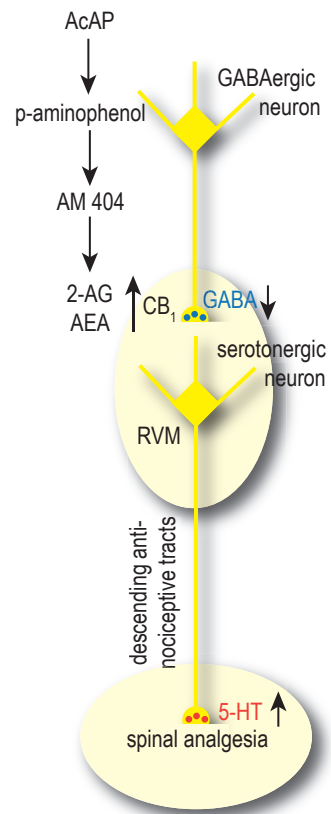


figure 9