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Acetaminophen Relieves Inflammatory Pain Through CB_1 Cannabinoid Receptors in the Rostral Ventromedial Medulla

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RVM cannabinoid signaling in acetaminophen analgesia

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Abstract

Acetaminophen (paracetamol) is a widely used analgesic and antipyretic drug with only incompletely understood mechanisms of action. Previous work, using models of acute nociceptive pain, indicated that analgesia by acetaminophen involves an indirect activation of CB_1 receptors by the acetaminophen metabolite and endocannabinoid re-uptake inhibitor AM 404. However, the contribution of the cannabinoid system to anti-hyperalgesia against inflammatory pain, the main indication of acetaminophen, and the precise site of the relevant CB_1 receptors have remained elusive. Here, we analyzed acetaminophen analgesia in mice of either sex with inflammatory pain and found that acetaminophen exerted a dose-dependent anti-hyperalgesic action, which was mimicked by intrathecally injected AM 404. Both compounds lost their anti-hyperalgesic activity in $CB_1^{-/-}$ mice confirming the involvement of the cannabinoid system. Consistent with a mechanism down-stream of pro-inflammatory prostaglandin formation, acetaminophen also reversed hyperalgesia induced by intrathecal prostaglandin E_2 (PGE_2). To distinguish between a peripheral/spinal and a supraspinal action, we administered acetaminophen and AM 404 to *hoxB8*-$CB_1^{-/-}$ mice, which lack CB_1 receptors from the peripheral nervous system and the spinal cord. These mice exhibited unchanged anti-hyperalgesia indicating a supraspinal site of action. Accordingly, local injection of the CB_1 receptor antagonist rimonabant into the rostral ventromedial medulla (RVM) blocked acetaminophen-induced anti-hyperalgesia, while local RVM injection of AM 404 reduced hyperalgesia in wild-type mice but not in $CB_1^{-/-}$ mice. Our results indicate that the cannabinoid system contributes not only to acetaminophen analgesia against acute pain but also against inflammatory pain, and suggest that the relevant CB_1 receptors reside in the RVM.

Significance statement

Acetaminophen is a widely used analgesic drug with multiple but only incompletely understood mechanisms of action including a facilitation of endogenous cannabinoid signaling via one of its metabolites. Our present data indicate that enhanced cannabinoid signaling is also responsible for the analgesic effects of acetaminophen against inflammatory pain. Local injections of the acetaminophen metabolite AM 404 and of cannabinoid receptor antagonists as well as data from tissue specific CB_1 receptor deficient mice suggest the rostral ventromedial medulla as an important site of the cannabinoid-mediated analgesia by acetaminophen.

Introduction

In the past decades, several potential molecular mechanisms have been proposed that may explain how acetaminophen exerts its analgesic action. These include the inhibition of cyclooxygenases (COXs) (Flower and Vane, 1972; Hanel and Lands, 1982; Graham and Scott, 2005), the activation of spinal serotonergic descending projections (Tjolsen et al., 1991; Pini et al., 1996), an involvement of the brain opioid system (Tjolsen et al., 1991; Herrero and Headley, 1996; Pini et al., 1996; Sandrini et al., 2001), inhibition of nitric oxide generation (Bjorkman et al., 1994; Bujalska, 2004), and activation of spinal TRPA1 channels by the acetaminophen metabolites N-acetyl-p-benzoquinoneimine (NAPQI) and p-benzoquinone (Andersson et al., 2011). In addition, the generation of N-arachidonoylphenolamin (AM 404) from acetaminophen through deacetylation to p-aminophenol and the subsequent conjugation with arachidonic acid by central nervous system fatty amide hydrolase (FAAH) (Högestatt et al., 2005) has drawn the attention to a possible involvement of the endocannabinoid system. AM 404 increases tissue concentrations of the endocannabinoid arachidonoyl ethanolamide (AEA), also known as anandamide, through an inhibition of anandamide reuptake into neurons and astrocytes (Beltramo et al., 1997; Fegley et al., 2004). After spinal or systemic application, AM 404 exerts analgesic activity against acute pain, evoked by noxious chemical stimuli, as well as against inflammatory and neuropathic pain (Gühring et al., 2002; La Rana et al., 2006). In line with an important contribution of the endocannabinoid system, acetaminophen-mediated antinociception was lost in CB_1 receptor-deficient ($CB_1^{-/-}$) mice (Mallet et al., 2008) as well as in mice lacking FAAH ($FAAH^{-/-}$ mice) (Mallet et al., 2010). Accordingly, acetaminophen-induced analgesia was also reduced by the FAAH inhibitor URB 597 (Mallet et al., 2008) and by the CB_1 receptor antagonists AM 251 and rimonabant (Ottani et al., 2006; Dani et al., 2007; Mallet et al., 2008).

The studies discussed above support a contribution of the endocannabinoid system to acetaminophen-mediated analgesia. However, most of these studies (Ottani et al., 2006; Mallet et al., 2008; Mallet et al., 2010) tested acetaminophen in models of acute nociceptive pain, i.e. pain evoked by acute noxious thermal, mechanical, or chemical stimuli applied to naïve animals in the absence of nociceptive sensitization by inflammation or neuropathy. These acute pain models only poorly reflect the clinical indications for acetaminophen, which is primarily used to treat mild inflammatory pain (Bradley et al., 1991). In fact, acute antinociceptive effects of acetaminophen in humans are rather vague or do not exist at all (Olesen et al., 2012; Tiippana et al., 2013). In the present study, we have analyzed the anti-hyperalgesic properties of acetaminophen in mice with inflammatory hyperalgesia and demonstrate a critical contribution of CB_1 receptors to the effects of acetaminophen against inflammatory hyperalgesia. Additional

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

Methods

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

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

Inflammatory hyperalgesia was induced using the yeast extract zymosan A (Meller and Gebhart, 1997). Zymosan A (Fluka) was suspended in 0.9% NaCl and injected subcutaneously (0.06 mg / 20 µl) into the plantar side of the left hind paw 24 hours prior to the administration of acetaminophen or AM 404. Spinal PGE_2-induced hyperalgesia was evoked through intrathecal injection of PGE_2 (Sigma; 0.4 nmoles / 4 µl, dissolved in 1% ethanol and 99% artificial

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

Drug administration, intrathecal and intraRVM injections. Acetaminophen (Sigma) was dissolved in 0.9% NaCl. The acetaminophen-containing solution or vehicle (0.9% NaCl, 400 µl) was given per os (p.o.) through stainless steel tubes (Delvo SA, Switzerland). Rimonabant (SR141716A; Tocris) (Rinaldi-Carmona et al., 1994) was dissolved in a mixture of 43% (vol/vol) DMSO, 43% aCSF and 14% ethanol. Injection volumes were 5 and 4 µl for AM 404 (Tocris) and PGE_2, respectively. AM 404 (Tocris) was dissolved in 40% DMSO and 60% 0.9% NaCl. Intrathecal (i.t.) injections were performed under isoflurane anesthesia at the level of the lumbar spine using a Hamilton syringe (Ahmadi et al., 2001). A small amount of black ink (1% v/v) was added to permit post-hoc verification of proper i.t. injections. Injections into the rostral ventromedial medulla (RVM) were performed with stainless steel cannulas. Fully anaesthetized mice were placed in a Kopf stereotaxic frame and implanted with a cannula using the following coordinates which were calibrated to the cranial Bregma points: x= -5.7; y= 0; $z_{cranium}$= +4.2. The cannula was fixed with dental cement and the cement was secured at the skull with 2 - 3 screws. The fixed cannula was used to insert a 30G needle attached to a Hamilton syringe 5.8 mm deep. A volume of 300 nl was injected. For post hoc verification of correct targeting of the RVM 1 % v/v Evans blue was included in the injection solution.

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

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

0.1 w/w% promethazine in H_2O (1 ml/100 g, intraperitoneal [i.p.]) and subsequently perfused transcardially through the ascending aorta with 0.9% NaCl for 2 min, followed by 100 ml of a fixative containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for another 20 min. After perfusion, spinal cords and brains were immediately isolated and postfixed in 4% PFA for 2 hours and washed in 0.1 M PB. Transverse sections of the spinal cord at a lumbar level as well as coronal sections of the cerebral hemispheres and the cerebellum (all 50 µm thick) were cut using a vibratome (Leica, VTS-1000). Free-floating sections were collected in 0.1 M PB. For immunoperoxidase staining, the sections were first extensively washed in 0.1 M PB. To block endogenous peroxidase activity, sections were afterwards incubated in 1% H_2O_2 in 0.1 M PB for 10 min and again washed in 0.1 M PB. Following washing in 0.05 M Tris-buffered saline (TBS; pH 7.4) conditioning Triton X-100 (TBST), the sections were blocked in 10% normal donkey serum (Vector Laboratories, Burlingame, USA) for 45 min. Sections were then incubated with polyclonal affinity-purified guinea pig anti-CB_1 antibodies (1 : 250; ~1 µg/ml; Fukudome et al., 2004) at 4°C for 48 hours. The antibodies were dissolved in 0.05 M TBS. After multiple washings, the sections were treated in TBS with biotinylated goat anti-guinea pig IgG (1 : 300; Vector Laboratories) for 2 hours and after further washing in TBS incubated with avidin-biotinylated horseradish peroxidase complex (1 : 500; Elite-ABC, Vector Laboratories) for 1.5 hours. Development of the immunoperoxidase reaction was done with 3,3'-diaminobenzidine (DAB) as chromogen and 0.01% H_2O_2 dissolved in TB (pH 7.6). Sections were briefly submerged in chrome gelatin (0.05% chromium potassium sulfate dodecahydrate, 0.5% gelatin and 0.05% NaN_3 in DW), dried, soaked in xylene (2 x 15 min), and covered in DePeX (SERVA). Sections containing the RVM were treated with 0.5% OsO4 in PB for 20 min at 4°C, dehydrated in an ascending series of ethanol and propylene oxide, and embedded in Durcupan (ACM, Fluka, Buchs, Switzerland) following DAB development. During dehydration, sections were treated with 1% uranyl acetate in 70% ethanol for 15 min at 4°C. Light microscopic analysis of immunostaining was carried out with a Nikon Eclipse 80i upright microscope. Micrographs were taken with a Nikon DS-Fi1 digital camera.

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

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

Results

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

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

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

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

Liver toxicity of acute treatment with acetaminophen

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

Contribution of CB_1 receptors to anti-hyperalgesia by acetaminophen

In order to test for a possible contribution of the cannabinoid systems to acetaminophen and AM 404-mediated analgesia in inflammatory pain conditions, we tested the effects of acetaminophen and AM 404 in global CB_1 receptor deficient ($CB_1^{-/-}$) mice with an inflamed hindpaw. Wild-type and $CB_1^{-/-}$ mice did not differ in their baseline mechanical sensitivities (PWTs

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

Analgesic effect of acetaminophen in PGE_2*-induced inflammatory pain*

It has previously been suggested that acetaminophen might act through an inhibition of COX-dependent prostaglandin formation in the central nervous system (Flower and Vane, 1972; Hanel and Lands, 1982; Chandrasekharan et al., 2002; Graham and Scott, 2005). To test whether acetaminophen reduces inflammatory hyperalgesia through a mechanism downstream of central prostaglandin production, we induced hyperalgesia through intrathecal PGE_2 injection (Taiwo and Levine, 1986; Uda et al., 1990; Reinold et al., 2005). One hour after PGE_2 injection (0.4 nmol), PWTs decreased from a baseline value of 3.50 ± 0.08 g to 0.90 ± 0.06 g (*n* = 13) (Fig. 4A). Acetaminophen (p.o., 200 mg/kg) but not vehicle (p.o. 0.9% NaCl) administered 1 hour after PGE_2 injection partially reversed PGE_2-induced hyperalgesia. The AUCs ([g·h]) were calculated between the post-drug PWTs and a straight line between the PWT at 1.5 and 4.0

hours after PGE_2 injection. In wild-type mice, the average AUC (anti-hyperalgesia) in acetaminophen-treated mice (AUC: 1.51 ± 0.14 g·h, n = 7) was significantly higher than that of the vehicle treated group (AUC. 0.073 ± 0.073 g·h, n = 6 mice, P < 0.001, unpaired Student's t-test) (Fig. 4B). We also assessed the hyperalgesic effect of intrathecal PGE_2 in $CB_1^{-/-}$ mice and the potential reversal of PGE_2-induced hyperalgesia by acetaminophen in these mice. PGE_2 induced the same level of hyperalgesia, but acetaminophen was again completely devoid of anti-hyperalgesic effects in $CB_1^{-/-}$ mice. Average AUCs in acetaminophen-treated $CB_1^{-/-}$ mice (AUC: 0.20 ± 0.58 g·h, n = 6) were virtually identical to those in vehicle-treated $CB_1^{-/-}$ mice (AUC: 0.064 ± 0.46 g·h, n = 6, P = 0.95, unpaired Student's t-test). Two-way ANOVA yielded a significant genotype x treatment interaction $F(1,25) = 5.46$, P = 0.03. These results suggest that acetaminophen alleviates inflammatory hyperalgesia through a mechanism independent of prostaglandin formation.

Ablation of CB_1 receptors from the periphery and the spinal cord does not block anti-hyperalgesia by systemic acetaminophen

We next aimed at identifying the anatomical origin of acetaminophen-induced anti-hyperalgesia. Our first analyses concentrated on CB_1 receptors in the spinal cord for two reasons. First, intrathecal injection of AM 404 mimicked the anti-hyperalgesia induced by systemic treatment with acetaminophen in several respects and, second, activation of spinal CB_1 receptors inhibits transmission for nociceptive signals between primary nociceptors and second order dorsal horn neurons *in vitro* (Liang et al., 2004; Kato et al., 2012). The latter action might be considered a prime candidate mechanism for acetaminophen-induced anti-hyperalgesia. To distinguish a peripheral/spinal from a supraspinal site of action, we made use of *hoxb8*-$CB_1^{-/-}$ mice, which were generated by crossing hoxb8-cre mice with $CB_1^{fl/fl}$ mice. During development, *hoxb*8-cre is expressed in all DRG neurons and in all neurons and astrocytes of the spinal cord up to level C4. *hoxb*8-cre is however virtually absent from the brain (Witschi et al., 2010). We verified the specific ablation of CB_1 receptors from the spinal cord by comparing CB_1 receptor expression in the spinal dorsal horn and in the periaqueductal grey (PAG), a midbrain area rich in CB_1 receptors (Fig. 5). In wild-type ($CB_1^{fl/fl}$) mice, intense CB_1 receptor staining was observed in the grey matter of the superficial dorsal horn and in the dorsolateral funiculus as well as around the cerebral aqueduct in the PAG (Fig. 5A,D,D′,G). This staining was completely absent in spinal cord and PAG sections obtained from global $CB_1^{-/-}$ mice (Fig. 5B,E,E′,H) indicating the specificity of the CB_1 receptor antibody (see also Nyilas et al., 2009). As expected, *hoxb*8-$CB_1^{-/-}$ mice exhibited a drastic reduction in CB_1 receptor expression in the spinal dorsal horn (Fig. 5C,F,F′), but not in the PAG (Fig. 5I). A side-by-side comparison of global $CB_1^{-/-}$ and conditional *hoxb*8-

$CB_1^{-/-}$ mice showed some remaining CB_1 immunoreactivity in the dorsal horn of the *hoxb8*-$CB_1^{-/-}$ mice, especially in the most superficial layers of the dorsal horn, which might result from terminals of axons descending from supraspinal sites to the dorsal horn.

In behavioral experiments, *hoxb8*-$CB_1^{-/-}$ mice and wild-type (hoxB8-cre negative $CB_1^{fl/fl}$) littermates did not differ in their baseline sensitivity to mechanical stimulation (PWT were 4.21 ± 0.10 g (*n* = 15) and 4.39 ± 0.07 g (*n* = 12) in naïve *hoxb8*-$CB_1^{-/-}$ mice and $CB_1^{fl/fl}$ littermates) and developed virtually identical inflammatory hyperalgesia with PWTs of 0.79 ± 0.07 g and 0.73 ± 0.08 g in *hoxb8*-$CB_1^{-/-}$ mice and $CB_1^{fl/fl}$ littermates. Both genotypes also exhibited virtually identical anti-hyperalgesic responses to systemic acetaminophen treatment. AUC were 2.15 ± 0.08 g·h (*n* = 6) and 1.59 ± 0.27 g·h (*n* = 6) for *hoxB8*-$CB_1^{-/-}$ and cre-negative wild-type ($CB_1^{fl/fl}$) mice, respectively (Fig. 5J). Very similar results were obtained with AM 404. AUCs were 1.41 ± 0.12 g·h (*n* = 9) and 1.38 ± 0.11 g·h (*n* = 6), for *hoxB8*-$CB_1^{-/-}$ and cre-negative littermates (Fig. 5K). Together with the complete lack of anti-hyperalgesia by acetaminophen and AM 404 in $CB_1^{-/-}$ mice, these results suggest that acetaminophen acted through CB_1 expressed at supraspinal sites. Alternatively, acetaminophen might act via CB_1 receptors expressed in the spinal cord on the terminals of neurons descending from supraspinal sites, which are not targeted by the *hoxB8*-cre (compare Fig. 5C,F,F'). To distinguish between these two possibilities we continued with local injections of AM 404 and of the CB_1 receptor antagonist rimonabant.

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

The RVM serves well-established roles in endogenous pain control (Heinricher and Fields, 2013) and as a site of action of centrally acting analgesic drugs including cannabinoid ligands (Meng et al., 1998; Suplita et al., 2005). We therefore tested whether the RVM was also involved in the anti-hyperalegsic actions of acetaminophen. To this end, we analyzed whether local injection into the RVM of the CB_1 receptor antagonist rimonabant would interfere with anti-hyperalgesia by systemic acetaminophen (Fig. 6). Rimonabant (and vehicle) injections were made via chronic cannulas that had been pre-implanted into the RVM one week before the experiment. Proper RVM injections were verified by addition of a small amount of Evans Blue to the injection solution and post-hoc anatomical analysis of mouse brain sections (Fig. 6A,B). Injection of rimonabant (0.67 µg in 300 nl) completely prevented the anti-hyperalgesic action of systemic acetaminophen (200 mg/kg) (Fig. 6C,D). The AUCs were 4.89 ± 1.35 g·h (*n* = 5) versus 0.67 ± 0.54 g·h (*n* = 6), in aCSF and rimonabant pretreated mice, respectively (*P* = 0.013, unpaired Student's t-test). RVM injection of rimonabant *per se* did not affect inflammatory hyperalgesia and RVM injection of vehicle did neither affect the inflammatory hyperalgesia nor change the

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

Distribution of CB_1 receptor mRNA and protein in the RVM.

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

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

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

adeno-associated virus (AAV) carrying a cre recombinase expression cassette. AAV-cre virus injections were performed one week before acetaminophen treatment. Successful cre-mediated ablation of the CB_1 receptor gene was verified with real time RT-PCR. The number of CB_1 receptor transcripts in the RVM was reduced to about 25% (Fig. 8A). However, despite this significant down-regulation of CB_1 receptors, acetaminophen-induced anti-hyperalgesia remained largely unaffected (Fig. 8B,C). These results suggest that the relevant CB_1 receptors reside on axon terminals of neurons projecting to the RVM rather than on intrinsic RVM neurons. Figure 9 illustrates a possible scenario: AM 404 in the RVM would increase the concentration of endocannabnoids (anandamide and 2-AG) and thereby indirectly activate CB_1 receptors on inhibitory neurons that project to the RVM to tonically inhibit antinociceptive fiber tracts descending to the spinal cord. Increased activation of CB_1 receptors on these neurons will reduce GABA release and dis-inhibit endogenous descending pain control units. Since many of the descending fibers release serotonin (Heinricher and Fields, 2013), this scenario is consistent with previous reports proposing not only a central site of action of acetaminophen but also a contribution of spinal serotonin receptors (Pelissier et al., 1995; Bonnefont et al., 2005).

Discussion

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

As shown in a previous study from our group, zymosan A-induced hyperalgesia strongly depends on spinally produced PGE_2 (Reinold et al., 2005). This model is therefore well-suited to investigate mechanisms of drugs with anti-hyperalgesic actions in inflammatory conditions and should permit a straightforward detection of prostaglandin-dependent drug actions. The reversal of inflammatory hyperalgesia by acetaminophen observed in our study would hence be consistent with a block of PGE_2 production by acetaminophen. However, acetaminophen was still active when hyperalgesia was induced by local spinal injection of PGE_2 favoring a mechanism different from inhibition of prostaglandin formation. Several results of the present study support instead the involvement of central CB_1 receptors: the reversal of PGE_2-induced hyperalgesia by acetaminophen was absent in $CB_1^{-/-}$ mice, and both AM 404 and acetaminophen failed to reverse zymosan A-induced hyperalgesia in $CB_1^{-/-}$ mice. Furthermore, the congruent pattern of efficacy of acetaminophen and of AM 404 in different (global and spinal cord-specific) CB_1 receptor-deficient mouse lines supports the contribution of AM 404 to the anti-hyperalgesic actions of acetaminophen. These results also correspond well with previous findings demonstrating that acetaminophen-induced analgesia was lost in $FAAH^{-/-}$ mice, which do not convert acetaminophen into AM 404 (Högestätt et al., 2005; Dalmann et al., 2015). However, neither the present nor previously published results (Ottani et al., 2006; Dani et al., 2007; Mallet et al., 2008) exclude an involvement of COX-1 or COX-2 (Flower and Vane, 1972; Hanel and Lands, 1982; Muth-Selbach et al., 1999; Boutaud et al., 2002; Graham and Scott, 2005). An *ex vivo* study performed in human volunteers demonstrated inhibition of COX-1 and COX-2 following the oral administration of acetaminophen (Hinz et al., 2008), and AM 404 has also been shown to block COX-1 and COX-2 in lipopolysaccharide-stimulated macrophages (Högestätt et al., 2005). In this context, it is important to note that COX-2 contributes to the metabolism of endocannabinoids (Yu et al., 1997; Kozak et al., 2000). The extent to which inhibition of COX-dependent endocannabinoid degradation or blockade of endocannabinoid transporters contribute to acetaminophen-induced analgesia remains to be determined.

Our results can also be reconciled with a report by (Mallet et al., 2010), who have proposed a role of supraspinal TRPV1 receptors as additional targets in acetaminophen and AM 404-induced analgesia. AM 404 is not only an inhibitor of anandamide reuptake but also an agonist at TRPV1 receptors (De Petrocellis et al., 2000). The observation that AM 404-induced analgesia was absent in $TRPV1^{-/-}$ mice and abolished by intracerebroventricular injection of the TRPV1 receptor antagonist capsazepine may suggest functional interactions of CB_1 and TRPV1 receptors in the CNS (Fioravanti et al., 2008). More difficult to reconcile with our findings is the report by (Andersson et al., 2011). These authors ascribe the analgesic action of acetaminophen to the activation of TRPA1 channels on the spinal terminals of nociceptive fibers by the

acetaminophen metabolites NPQI and p-benzoquinone, and a subsequent inhibition of transmitter release via primary afferent depolarization. Since anti-hyperalgesia by acetaminophen was retained in *hoxb8*-$CB_1^{-/-}$ mice, an interaction of TRPA1 channels with CB_1 receptors cannot explain these findings. It is likely that distinct mechanisms underlie the acute analgesic and the anti-hyperalgesic actions of acetaminophen.

Comparing the effects of classical cannabinoids with those of acetaminophen reveals similarities and differences. Classical cannabinoids exert a tetrad of actions in rodents, which includes analgesia, hypothermia, sedation (reduced locomotor activity), and catalepsy (Little et al., 1988). Analgesia, sedation and hypothermia do also occur in mice in response to acetaminophen (Mallet et al., 2010). While our data provide strong support for the involvement of cannabinoid signaling in acetaminophen-induced anti-hyperalgesia, cannabinoid independent actions are likely more relevant for the hypothermic and antipyretic effects of acetaminophen (Gentry et al., 2015). Such CB_1 receptor-independent mechanisms include the inhibition of hypothalamic COX by AM 404 (Högestätt et al., 2005) and the activation of TRPA1 via the acetaminophen metabolite NAPQI (Gentry et al., 2015). The mechanisms of acetaminophen-induced sedation in mice have not been identified so far and catalepsy is not seen in mice. Furthermore, the psychotropic actions seen with classical CB_1 receptor agonists in humans do not occur with acetaminophen. Local differences in the conversion of the acetaminophen metabolite p-aminophenol into pharmacologically active AM 404, caused for example by varying FAAH activity in different CNS regions, or differences in the local activity of endocannabinoid system may explain these discrepancies. Such differences may also account for another discrepancy. While a previous report has suggested that CB_1 receptor agonists exert most of their analgesic action through CB_1 receptors on peripheral nociceptors (Agarwal et al., 2007), our experiments in *hoxB8*-$CB_1^{-/-}$, which lack CB_1 receptors also from these cells, suggest that this is not the case for acetaminophen (see also Dalmann et al., 2015).

In our experiments, we also aimed at a better definition of the site of acetaminophen's action. To this end, we used *hoxb8*-$CB_1^{-/-}$ mice, which lack CB_1 receptors specifically from the spinal cord and peripheral sensory neurons. Because CB_1 receptors are densely expressed on different types of intrinsic spinal dorsal horn neurons and on sensory fiber terminals (Tsou et al., 1998; Farquhar-Smith et al., 2000; Bridges et al., 2003; Hegyi et al., 2009; Nyilas et al., 2009), experiments first focused on a possible spinal site of action. However, the anti-hyperalgesia by acetaminophen were completely preserved in *hoxb8*-$CB_1^{-/-}$ mice.

At least two explanations may account for these findings. The CB_1 receptors responsible for acetaminophen analgesia might reside on the spinal terminals of fibers descending from supraspinal sites which are spared from *hoxb8*-cre mediated gene deletion. This scenario is

consistent with the presence of CB_1 receptors in the termination area of descending fiber tracts in spinal cords of *hoxb8*-$CB_1^{-/-}$ mice, and with the efficacy of AM 404 after intrathecal injection. However, AM 404 might have diffused to supraspinal sites after lumbar intrathecal injection. Such diffusion has been demonstrated earlier for radioactively labeled morphine (Gustafsson et al., 1985). Alternatively, acetaminophen might act via CB_1 receptors at supraspinal sites located e.g. in the brainstem, where the somata of descending antinociceptive fiber tracts are located. Our experiments with local injection of rimonabant and AM 404 into the RVM provide strong support for this scenario (see also Högestätt et al., 2005; Mallet et al., 2008; Mallet et al., 2010; Dalmann et al., 2015). According to these previous studies, acetaminophen acts through a CB_1 receptor-mediated reinforcement of descending serotonergic bulbospinal pathways originating from the RVM (Mallet et al., 2008) with subsequent activation of pain-suppressing serotonin receptors in the spinal cord (Tjolsen et al., 1991; Pelissier et al., 1995; Pini et al., 1996; Bonnefont et al., 2005). Our results are thus in line with the important role of supraspinal CB_1 receptors in stress-induced analgesia (Hohmann et al., 2005; Suplita et al., 2006).

Strong CB_1 receptor immune reactivity but weak *in situ* hybridization signals in the RVM suggest that the relevant CB_1 receptors reside on processes of neurons that project to the RVM from other brain areas. In this scenario, it is likely that the acetaminophen metabolite AM 404 promotes the activation of CB_1 receptors on GABAergic axon terminals that tonically inhibit serotonergic antinociceptive fiber tracts descending from the RVM to the spinal cord. Since the periaqueductal grey (PAG) controls RVM activity via descending axons (Heinricher and Fields, 2013), it is conceivable that the CB_1 receptors relevant for the analgesic action of acetaminophen reside on the terminals of fibers reaching the RVM from the PAG. Acetaminophen would thus indirectly reduce GABA release from these projections and dis-inhibit descending serotonergic fibers to facilitate endogenous pain control.

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

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Figure legends

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

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

Fig. 3 Effect of CB_1 receptor ablation on the antihyperalgesic actions of by acetaminophen and AM 404. **(A)** Acetaminophen (200 mg/kg, p.o.). Time course of changes in PWT. Acetaminophen was given 24 hours after injection of zymosan A to wild-type mice ($n = 6$) and to $CB_1^{-/-}$ mice ($n = 8$). Bar chart: AUCs (g·h, mean ± SEM). *, $P \leq 0.05$, unpaired Student's t-test. **(B)** AM 404 (10 nmol, i.t.) was administered 24 hours after injection of zymosan A in wild-type and $CB_1^{-/-}$ mice ($n = 7$ each). ***$P < 0.001$, unpaired Student's t-test. (**C**) Systemic pretreatment with rimonabant (rim, 5 mg/kg, i.p.) completely blocked anti-hyperalgesia by acetaminophen. Two-way ANOVA $F(1,22) = 9.08$, $P = 0.007$ for pretreatment x treatment interaction, $n = 4 – 8$ per group. **, $P < 0.01$, $n = 6$ and 8 mice for vehicle and rimonabant pretreated mice (unpaired Student's t-test).

Fig. 4 Effect of acetaminophen (200 mg/kg, p.o.) on mechanical hyperalgesia evoked by intrathecal PGE_2 (0.4 nmol) in wild-type and $CB_1^{-/-}$ mice. **(A)** Change in PWTs (mean ± SEM). PGE_2 was injected i.t. at time 0. Acetaminophen or vehicle were given p.o. (1 hour after PGE_2 injection. $n = 7$ and 6 for acetaminophen and vehicle, respectively. **(B)** AUC (mean ± SEM). Two-way ANOVA yielded a significant genotype x treatment interaction $F(1,25) = 5.46$, $P = 0.03$. $n = 6$ - 7 mice per group.

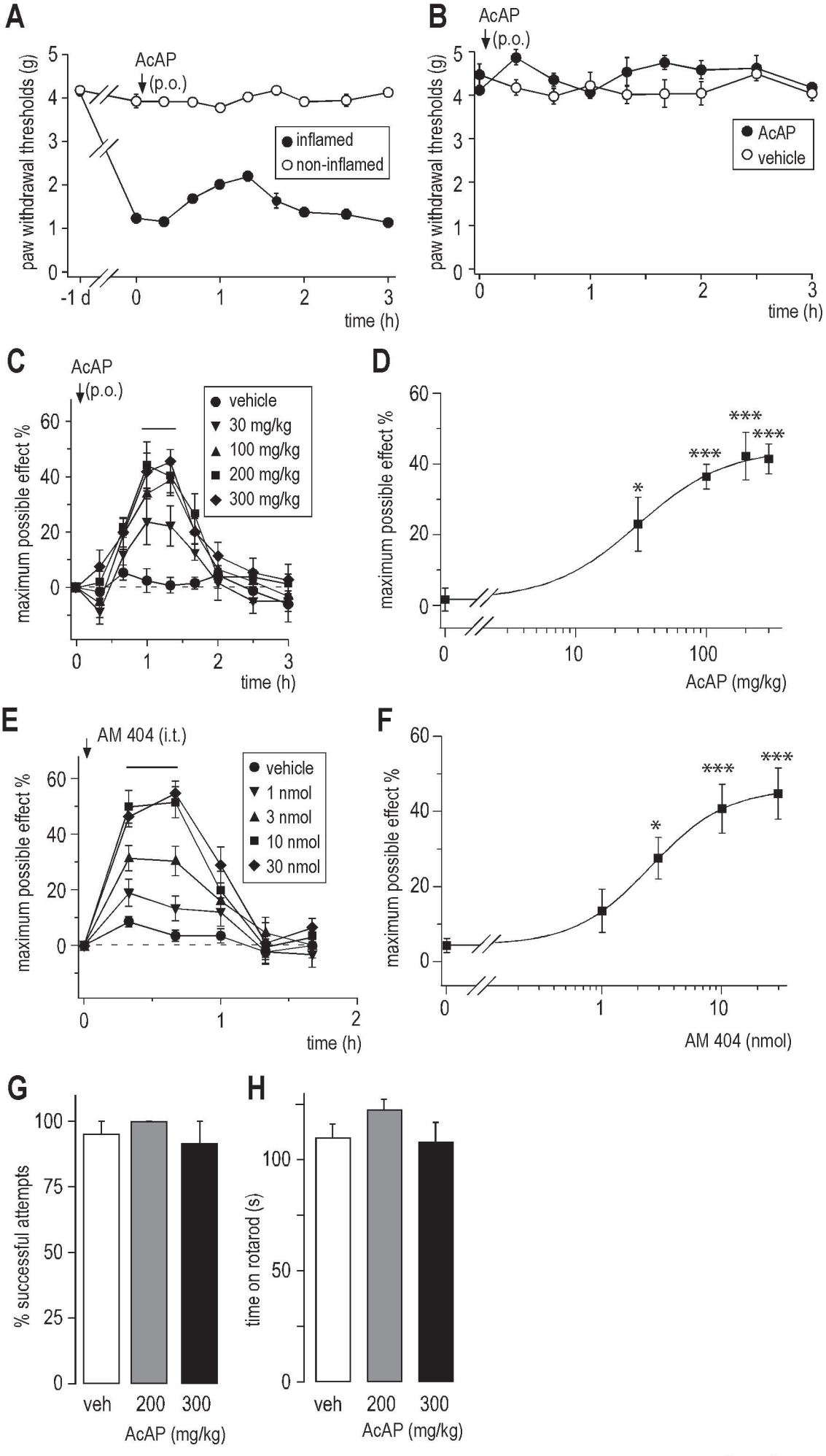
Fig. 5 Morphological and behavioral analysis of *hoxb8*-$CB_1^{-/-}$ mice. **(A-I)** CB_1 receptor expression in the spinal dorsal horn and PAG of wild-type, $CB_1^{-/-}$ and *hoxb*8-$CB_1^{-/-}$ mice. **(A)** High density of CB_1 receptor-immunostaining is found in the superficial layers in the dorsal horn of wild type ($CB_1^{fl/fl}$) mouse spinal cord. **(D,D')** At higher magnification, an abundant punctate staining pattern corresponding mostly to axon terminals is observed. **(B,E,E')** The specificity of this staining pattern is validated by the complete lack of immunostaining on spinal cord sections derived from global $CB_1^{-/-}$ animals. **(C,F,F')** Deletion of CB_1 receptors from DRG and spinal neurons as well as from astrocytes in *hoxb8*-$CB_1^{-/-}$ animals did not fully eliminate CB_1 receptor immunostaining. A remaining weak staining pattern was found in lamina I and II, where most descending monoaminergic fibers terminate. **(G)** Immunostaining for CB_1 receptors in the midbrain periaqueductal grey nucleus (PAG) is concentrated around the dorsal and central part of the PAG. **(H)** This staining pattern is completely eliminated in the global $CB_1^{-/-}$ animals, but remains fully intact in *hoxB8*-$CB_1^{-/-}$ mice. Similar results were obtained in three mice of both genotypes. Scale bars are: (C valid also for A,B) 100 µm; (F applies also for D,E) 20 µm; (F' applies also for D',E') 10 µm; and (I valid also for G,H) 200 µm. **(J,K)** Behavioral analysis. Changes in PWTs induced by the acetaminophen (200 mg/kg, p.o., **J**) in *hoxb8*-$CB_1^{-/-}$ (n = 6) and wild-type ($CB_1^{fl/fl}$) mice (n = 6), and by AM 404 (10 nmol, i.t., **K**) in *hoxb8*-$CB_1^{-/-}$ (n = 6) and wild-type ($CB_1^{fl/fl}$) mice (n = 9). Acetaminophen and AM 404 were administered 24 hours after zymosan A injection. Differences in AUCs were statistically insignificant (unpaired Student's t-test).

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

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

822 **Fig. 7** CB_1 receptor immunoreactivity and in situ hybridization in the RVM.
823 (**A,B**) CB_1 receptor immunoreactivity in coronal sections through the brainstem of wild-type (A)
824 and $CB_1^{-/-}$ mice (B). The lack of brownish color of the DAB precipitate in the $CB_1^{-/-}$ tissue (B)
825 confirms the specificity of CB_1 immunolabeling. Squares indicate the area of the RVM shown at
826 hight magnification in (**C-F**). (**C**) CB_1 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 CB_1 immunostaining can be found in control sections from $CB_1^{-/-}$ 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 dehidration. (**E**) CB_1 in situ hybridization signal in the RVM. Only a few scattered neurons
832 (blue) express CB_1 receptor mRNA at rather low levels. (**F**) No labelled cells are present in
833 control sections prepared from CB_1 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 CB_1 receptor expression in intrinsic RVM neurons fails to prevent
837 acetaminophen-induced anti-hyperalgesia.
838 (**A**) Changes in CB_1 receptor mRNA levels seven days after AAV-cre injection in $CB_1^{fl/fl}$ 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 CB_1 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 CB_1 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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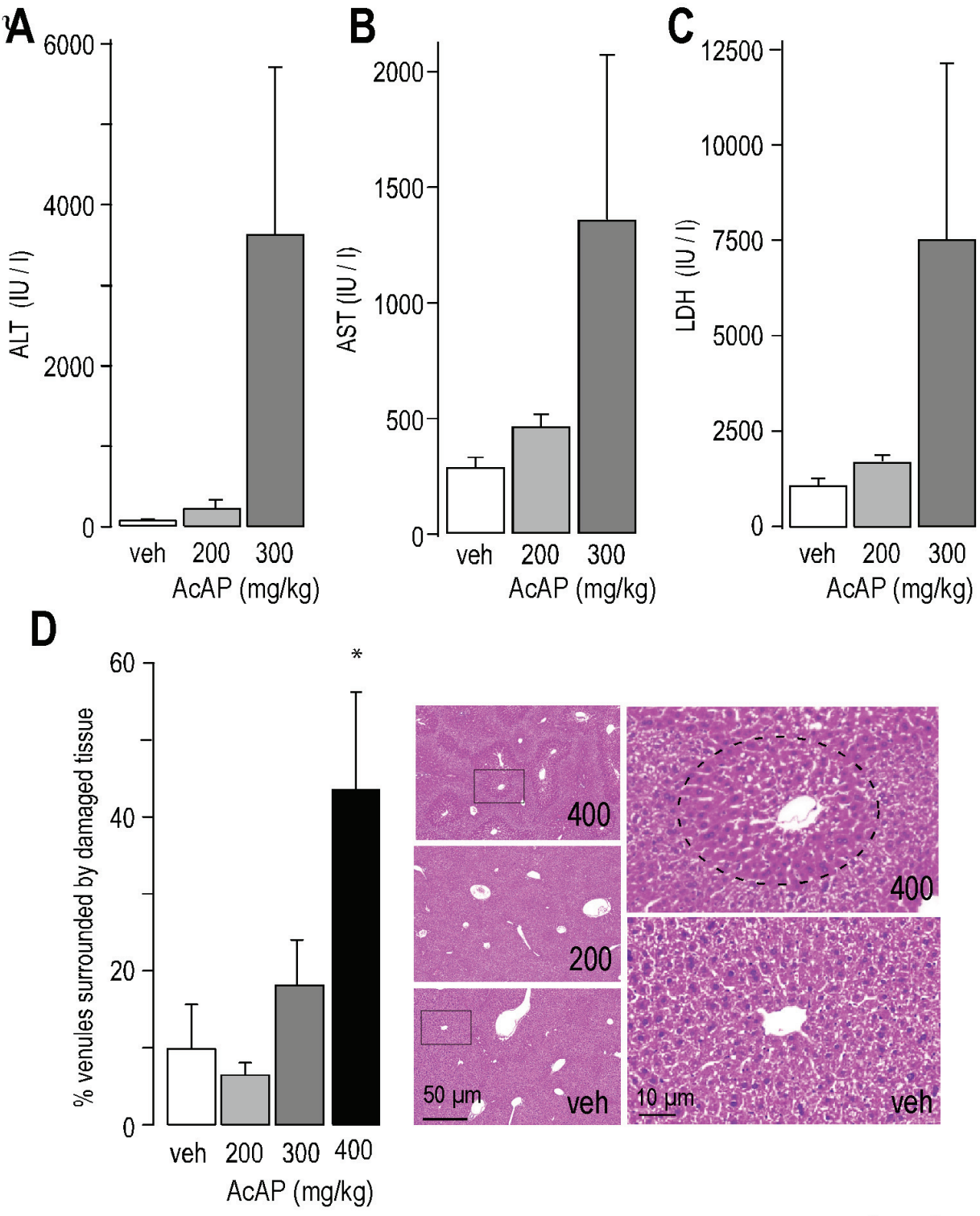

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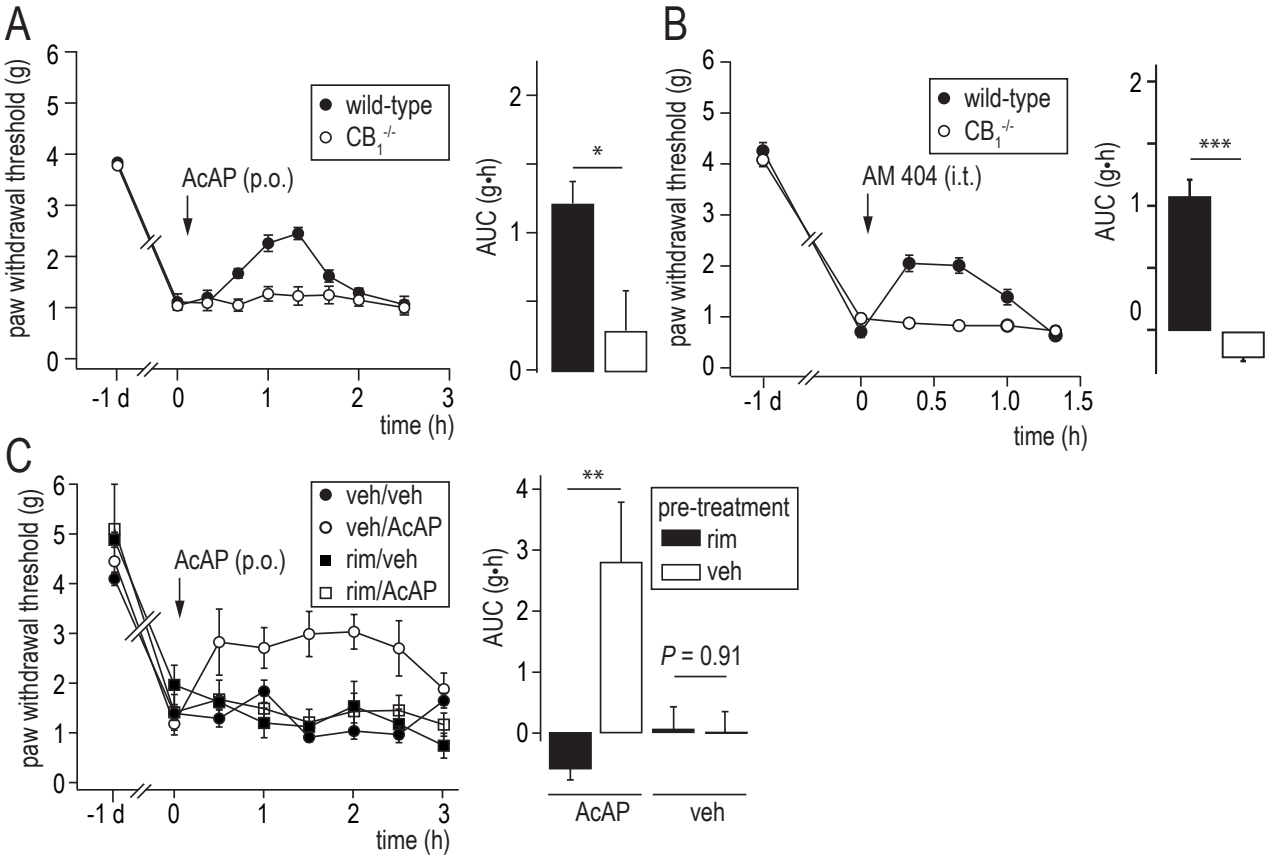

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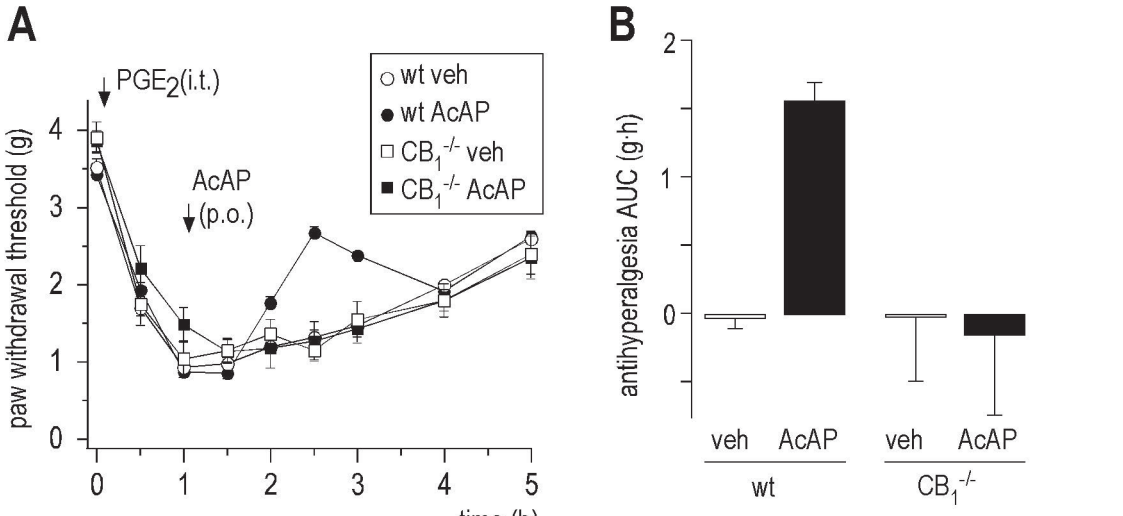

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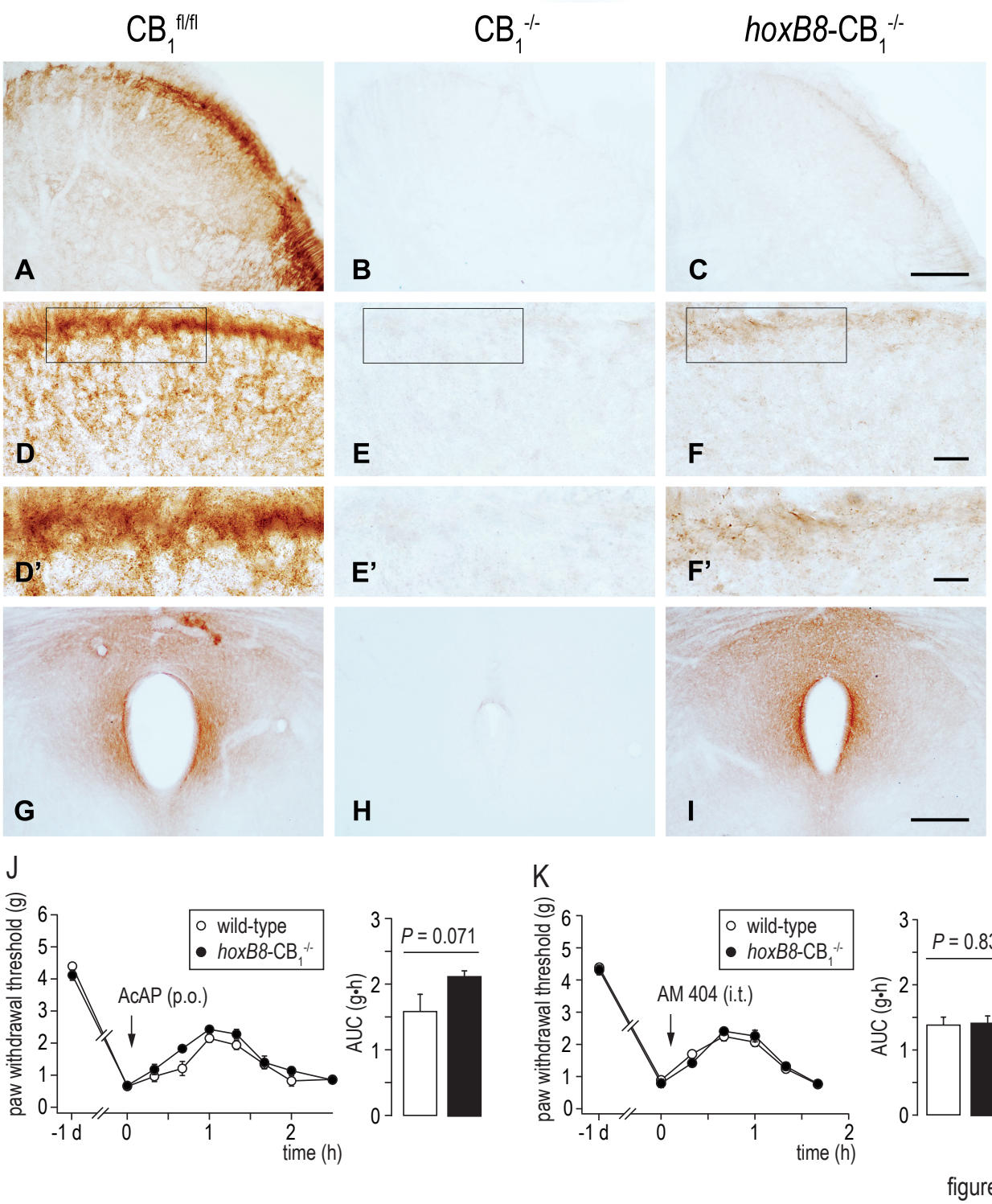

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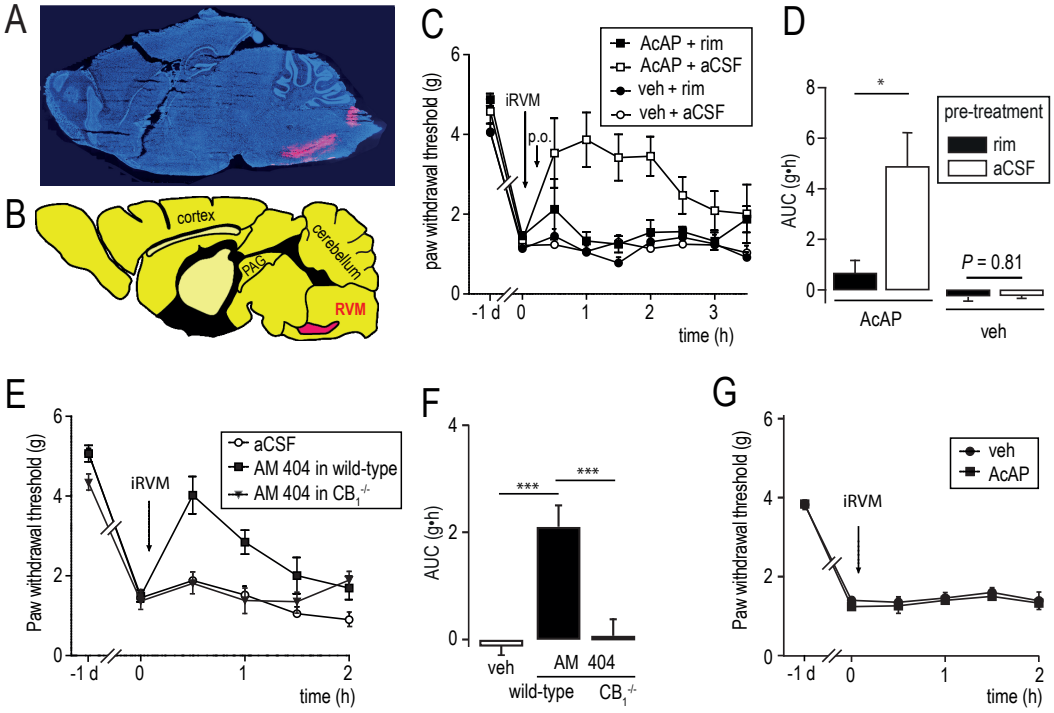

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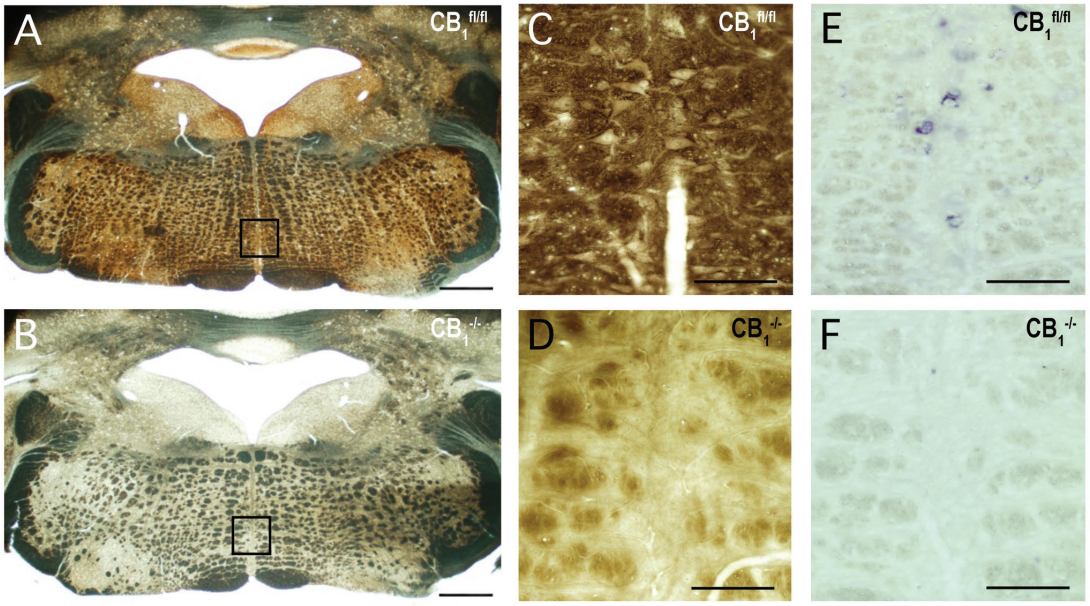

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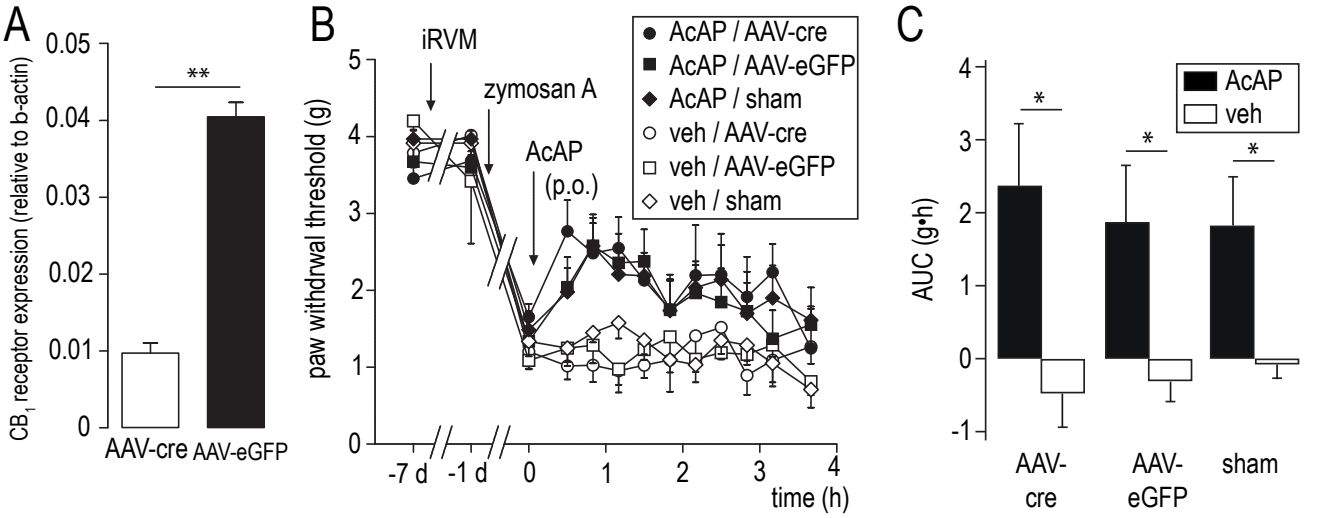

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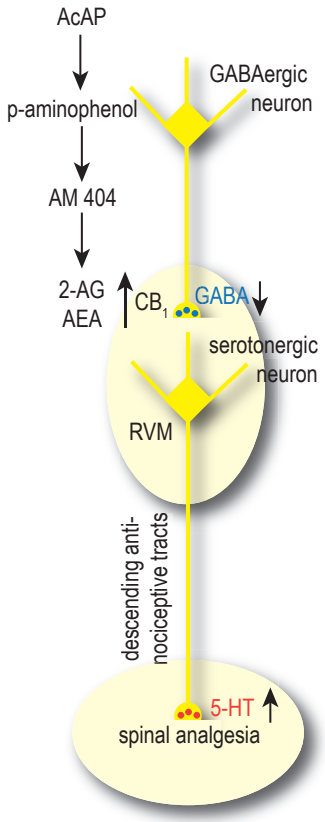

figure 9