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Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors

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Abstract

Although endocannabinoids constitute one of the first lines of defense against pain, the anatomical locus and the precise receptor mechanisms underlying cannabinergic modulation of pain are uncertain. Clinical exploitation of the system is severely hindered by the cognitive deficits, memory impairment, motor disturbances and psychotropic effects resulting from the central actions of cannabinoids. We deleted the type 1 cannabinoid receptor (CB_1) specifically in nociceptive neurons localized in the peripheral nervous system of mice, preserving its expression in the CNS, and analyzed these genetically modified mice in preclinical models of inflammatory and neuropathic pain. The nociceptor-specific loss of CB_1 substantially reduced the analgesia produced by local and systemic, but not intrathecal, delivery of cannabinoids. We conclude that the contribution of CB_1-type receptors expressed on the peripheral terminals of nociceptors to cannabinoid-induced analgesia is paramount,

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which should enable the development of peripherally acting CB_1 analgesic agonists without any central side effects.

Chronic pain is a major health problem. Although opioids are widely used in the clinical management of chronic pain syndromes, their long-term usage is accompanied by side effects that seriously diminish the quality of life in a large portion of patients suffering from chronic pain, leading to poor compliance and rejection of therapy. In recent years, cannabinoids have emerged as attractive alternatives or supplements to therapy with opioids for chronic pain states[1,2]. However, in humans the activation of cannabinoid receptors is associated with psychotropic side effects, temporary memory impairment and dependence, which arise via the effects of cannabinoids on forebrain circuits[2,3]. For clinical exploitation of the analgesic properties of opioids and cannabinoids, a major challenge is to devise strategies that reduce or abolish their adverse effects on cognitive, affective and motor functions without attenuating their analgesic effects.

In animal studies, the anti-nociceptive efficacy of cannabinoids has been unequivocally demonstrated in several models of inflammatory and neuropathic pain (reviewed in ref. [1]). However, there are marked inconsistencies between different reports with respect to the locus of these pain-protective effects. Indeed, receptors for cannabinoids are distributed across many key loci in pain-modulating pathways, including the peripheral and central terminals of primary afferents, second-order spinal dorsal-horn neurons, pain-regulatory circuits in the brainstem, and brain regions involved in sensory discrimination, affective states and the emotional responses to nociceptive stimuli[1–3]. Although numerous studies have demonstrated that activation of cannabinoid receptors individually at several of these diverse loci can reduce nociceptive transmission, the relative contributions of each of these sites to the global analgesic effects of systemic cannabinoids remains ambiguous[1].

The biological effects of cannabinoids are mediated via binding to type 1 and type 2 G protein–coupled cannabinoid receptors (CB_1 and CB_2, respectively)[3,4], which activate inhibitory $G_{i/o}$ proteins. In addition, several endocannabinoids have been shown to modulate the activity of ion channels, including diverse transient receptor potential (TRP) channels[5] and potassium channels[6], which are implicated in the modulation of pain processing. Therefore, not only the site, but also the mechanism, of cannabinergic modulation of pain and analgesia are uncertain.

Studies of global-knockout mice have confirmed that CB_1 and CB_2 are involved in cannabinoid-induced analgesia[7–9], but have not revealed their site of action. Conditional gene targeting of cannabinoid receptors at distinct loci in the pain pathway presents the means for identifying this site. Because a delineation of the relative contribution of the peripheral and the central components of cannabinoid-induced analgesia could help in the development of therapeutic strategies free of central side effects, we specifically targeted peripheral nociceptor neurons. Using the Cre/*loxP* system for conditional gene deletion[10], we generated transgenic mice lacking CB_1 in nociceptors, preserving expression in the spinal neurons, brain and all other organs. The phenotype of these cell type–specific knockout mice with respect to pain closely resembled that of the conventional global-knockout mice (that is, CB_1 receptor deficiency in all somatic cells) in nature as well as magnitude. By using a combination of electrophysiological, behavioral and pharmacological methods, we have shown that specific loss of CB_1 in nociceptors leads to a major reduction in the analgesia produced by endocannabinoids as well as systemically administered cannabinoids, indicating that these CB_1 receptors, and not those within the CNS, constitute the prime target for producing cannabinoid analgesia.

RESULTS

Conditional and specific deletion of CB_1 in nociceptors

We generated mice that lacked CB_1 specifically in primary nociceptors (homozygous mice referred to henceforth as SNS-CB_1^-) via Cre/*loxP*-mediated recombination by mating homozygous mice carrying the *loxP*-flanked (floxed) *Cnr1* allele (CB_1^{fl})[11] with a mouse line expressing Cre recombinase under the control of the $Na_v1.8$ promoter (SNS-Cre)[12] (Fig. 1a,b). The SNS-Cre mice enable gene recombination selectively in nociceptive ($Na_v1.8$-expressing) sensory neurons, commencing at birth, without affecting gene expression in the spinal cord, brain or any other organs in the body[12]. *In situ* mRNA hybridization using CB_1-specific riboprobes[11,13] showed Cre/*loxP*-mediated CB_1 deletion in the dorsal root ganglia (DRG) (Fig. 1a). Quantitative size-frequency analysis revealed a significant loss of CB_1 in DRG neurons with a diameter <30 μm (Fig. 1b; $P < 0.01$), but not in neurons with a cell diameter ≥30 μm, exactly as expected from the profile of SNS-Cre mice[12]. A C-terminal antibody to CB_1 (anti-CB_1), which yields specific staining in wild-type DRGs but not in those from global homozygous CB_1^- mice[13] (Fig. 1c), was used to further probe the specificity of the CB_1 deletion in DRG neuron subtypes in the SNS-CB_1^- mice. Confocal analysis of dual immunofluorescence experiments revealed CB_1 immunoreactivity in more than 40% of isolectin-B_4 (IB_4)-labeled nonpeptidergic nociceptors, substance P–expressing peptidergic nociceptors and $Na_v1.8$-expressing nociceptors in wild-type and CB_1^{fl} mice (typical examples in Fig. 1d and quantitative summary in Fig. 1e). In contrast, SNS-CB_1^- mice demonstrated a near-complete loss of specific staining in these nociceptor populations (Fig. 1d,e). Moreover, nearly all TRPV1-expressing neurons had anti-CB_1 immunoreactivity in wild-type and CB_1^{fl} mice, but only a minor population continued to express CB1 in SNS-CB_1^- mice (Fig. 1d,e). In contrast, nearly all large-diameter, neurofilament 200–immunoreactive neurons retained expression of CB_1 in the SNS-CB_1^- mice (Fig. 1d,e). Taken together, these results show that CB_1 is normally expressed in a significant proportion of nociceptors and is selectively lost from C- and A-δ neurons, but not from large-diameter DRG neurons, in SNS-CB_1^- mice. Consistent with a loss of CB_1 in DRG neurons, binding of ^{3}H-CP-55940 (ref. 14), a cannabinoid agonist, was significantly decreased in SNS-CB_1^- mice as compared with CB_1^{fl} littermates in the DRG (8.14 ± 0.64 versus 11.81 ± 0.76 fmol of bound ligand per mg of tissue, respectively), as well as in zones of central terminals of nociceptive afferents in the superficial spinal dorsal horn (94.77 ± 2.59 versus 121.1 ± 4.91 fmol of bound ligand per mg of tissue, respectively; $^*P < 0.001$, ANOVA, *post hoc* Fisher's test).

In contrast to the DRG, the brain and spinal cord showed normal expression of CB_1 mRNA (Fig. 2a,b) and CB_1 protein (Fig. 2c,d) in SNS-CB_1^- mice, whereas globally CB_1^- mice[13] had a complete loss of CB_1 mRNA and anti-CB1 immunoreactivity (Fig. 2a–d). Similarly, binding of ^{3}H-CP-55940 remained unaffected in several brain regions in SNS-CB_1^- and $CB1^{fl}$ mice (Fig. 2e). SNS-CB_1^- mice appeared normal and were fertile, and the development of the spinal cord and brain was normal (data not shown). No abnormalities were observed in the spinal termination of peptidergic or nonpeptidergic nociceptors, as shown by immunostaining for substance P and binding of IB_4 (Fig. 2f).

Tonic inhibition of pain via peripheral CB1

Compared with CB_1^{fl} littermates, SNS-CB_1^- mice had significantly reduced reaction latencies to noxious heat and reduced response thresholds to mechanical stimuli applied via a dynamic aesthesiometer, showing that physiological, basal pain sensitivity is exaggerated in SNS-CB_1^- mice (Fig. 3a; $P = 0.001$ and 0.003, respectively). Similarly, acute responses elicited by intraplantar injections of the irritants capsaicin and formalin were significantly greater in SNS-CB_1^- mice than in CB_1^{fl} mice (Fig. 3b; $P = 0.002$ and 0.049 for capsaicin and formalin, respectively), which is indicative of enhanced chemogenic pain. In contrast, motor

performance on a Rotarod was unaffected in SNS-CB_1^- mice (Fig. 3c; $P = 0.203$). After intraplantar formalin injection, SNS-CB_1^- mice showed a significantly higher number of neurons expressing markers of activity[15], such as Fos and phosphorylated ERK1/2 (pERK), in the DRG and spinal cord than did CB_1^{fl} mice (Fig. 3d,e; $P < 0.02$ in all cases). Dual immunofluorescence revealed that 81 ± 4% of Cre-expressing DRG neurons in formalin-treated SNS-CB_1^- mice expressed Fos, whereas only 42 ± 2% expressed Fos in SNS-Cre mice (controls). This shows that the enhanced induction of Fos in the SNS-CB_1^- mice in response to formalin takes place in those nociceptive neurons in which CB_1 expression was genetically deleted.

In contrast to SNS-CB_1^- mice, SNS-Cre mice showed no alteration in acute responses to noxious heat and pressure[12] or to noxious chemical stimuli, such as capsaicin and formalin (Supplementary Fig. 1; $P > 0.05$), nor did they differ from wild-type littermates with respect to development of chronic inflammatory pain or neuropathic pain (Supplementary Fig. 1; $P > 0.05$), showing that the alterations in nociception observed in SNS-CB_1^- do not arise from expression of Cre recombinase in the sensory neurons.

To address whether CB1-mediated inhibitory tone on nociceptors is maintained by endocannabinoids released constitutively in peripheral tissue, we analyzed endocannabinoid abundance in the paw skin of wild-type mice. The amount of anandamide (AEA) was low, albeit detectable, whereas 2-arachidonoyl glycerol (2-AG), 1-arachidonoyl glycerol (1-AG) and the precursor molecule arachidonic acid were found at moderate to high levels in the paw tissue of naive mice (Fig. 4a). The abundances of AEA, 1-AG, 2-AG and arachidonic acid significantly increased in the paw skin of mice 24 h after the induction of localized peripheral inflammation by intraplantar injection of complete Freund's adjuvant[16] (CFA; $P < 0.05$ in all cases; Fig. 4a), but not in the spinal cord segments receiving sensory inputs from the hindlimb (L4–L6) in the same animals ($P \geq 0.05$ in all cases; Fig. 4b). These results suggest that peripherally synthesized endocannabinoids regulate basal pain and may have an enhanced action on inflammatory pain sensitivity via CB_1 that is expressed on cutaneous nociceptors. Basal peripheral and spinal endocannabinoid levels did not differ between SNS-CB_1^- mice and $CB1^{fl}$ mice, suggesting that alterations in endocannabinoid availability do not account for these phenotypic differences (Fig. 4c,d).

Exaggerated inflammatory hyperalgesia in SNS-CB_1^- mice

We assessed the development of somatic inflammatory pain and hyperalgesia in SNS-CB_1^- mice at 6–7, 17, 27 and 52 h after CFA- induced unilateral hindpaw inflammation. SNS-CB_1^- mice had an enhanced basal response to von Frey hairs as compared with their respective wild-type littermates (Fig. 5a; $P = 0.002$). Upon CFA injection, the magnitudes of both allodynia (defined as responses to 0.16–0.4*g* of force) and mechanical hyperalgesia (defined as responses to 0.6–4*g*) were significantly higher in SNS-CB_1^- mice than in their wild-type littermates (Fig. 5a,b; $P < 0.005$). Similar to the SNS-CB_1^- mice, globally CB_1^- mice showed exaggerated basal pain and developed significantly more hyperalgesia and allodynia after intraplantar CFA than did their corresponding control littermates (Fig. 5a; $P < 0.002$), but to a similar extent as did SNS-CB_1^- mice. The relative drop in response thresholds (defined as the minimum force required to elicit 40% response frequency) over the basal (pre-CFA) state or over control littermates was comparable between SNS-CB_1^- mice and CB_1^- mice (Fig. 5b; $P > 0.05$). These results imply that an additional loss of CB_1 in spinal cord, brain or non-neuronal tissues (as it is the case in globally CB_1^- mice) does not produce a greater effect on pain behavior than is produced by a loss of CB_1 that is restricted to peripheral nociceptor neurons.

Using the caerulein model of acute pancreatitis in CB_1^{fl} and SNS-CB_1^- mice[17], we observed that SNS-CB_1^- mice developed hypersensitivity to abdominal mechanical stimuli after

pancreatic inflammation to a significantly higher extent than did CB_1^{fl} mice (Fig. 5c; $P < 0.01$). This suggests that CB_1 expressed in peripheral nociceptive neurons also exerts an inhibitory tone on visceral inflammatory pain.

Hypersensitivity of nociceptors in SNS-CB_1^- mice

The exacerbation of somatic and visceral inflammatory pain that we observed in the SNS-CB_1^- mice could result from the deletion of CB_1 from the peripheral terminals of the nociceptor neurons and/or from their central terminals in the spinal cord. To clarify the specific contribution of CB_1 on peripheral terminals, we carried out electrophysiological recordings on peripheral mechanosensitive C-fiber nociceptors that were identified on the basis of stimulation and conduction properties in a hindpaw skin-nerve preparation[18] isolated from SNS-CB_1^- or wild-type mice at 24 h after hindpaw injection of CFA. The median mechanical threshold of unmyelinated C-fibers was lower in SNS-CB_1^- mice (16 mN; range 1–362 mN; n =29) than in the CB_1^{fl} mice (22.6 mN; n = 31; Fig. 5d). Furthermore, in SNS-CB_1^- mice, a significantly higher proportion of mechanosensitive C-fibers had very low activation thresholds, of 1–2 mN, compared with the CB_1^{fl} group (24% versus 3%; $P < 0.05$, χ^2 analysis; Fig. 5d), suggesting that CB_1 localized on the peripheral terminals of nociceptors limits the excitability of mechanosensitive C-fibers in inflammatory states.

Requirement of peripheral CB_1 for cannabinoid analgesia

In addition to clarifying the peripheral component of endocannabinoid-mediated analgesia, SNS-CB_1^- mice represent a useful tool for delineating what proportion of the analgesia produced by exogenous cannabinoids is mediated by CB_1 expressed on nociceptors. In CB_1^{fl} mice, intraperitoneal administration of 1 mg per kg of body weight WIN 55212-2 (WIN, ref. [19]), a synthetic agonist of CB_1 and CB_2, 24 h after CFA-induced hindpaw inflammation attenuated mechanical hyperalgesia by >50%, as determined by a dynamic aesthesiometer (Fig. 6a; $P = 0.005$). In contrast, SNS-CB_1^- mice showed only a 17% attenuation of mechanical hyperalgesia with 1 mg per kg systemic WIN (not significant; Fig. 6a). Application of WIN at 3 mg per kg produced results very similar to those produced by 1 mg per kg WIN (Fig. 6a). The sedative effects elicited by doses of 4 mg per kg or higher precluded an analysis of analgesia. Similarly, on application of von Frey hairs to the same cohort of animals, mechanical hyperalgesia and allodynia 17 h after hindpaw CFA injection were nearly fully reversed after intraperitoneal injection of WIN (1 mg per kg) in CB_1^{fl} mice (mean force required to elicit a response in 50% of cases was 2g in the control group, 1g in the CFA-treated group and 1.9g after acute WIN treatment in the CFA group; Fig. 6b). In contrast, in SNS-CB_1^- mice, systemic WIN reduced CFA-induced hyperalgesia and allodynia only slightly (mean force required to elicit a response in 50% of cases was 1.7g in the control group, 0.2g in the CFA-treated group and 0.6g after acute WIN treatment in the CFA group; Fig. 6b). Thus, the analgesia induced by systemically administered WIN was strongly reduced in SNS-CB_1^- mice as compared with CB_1^{fl} mice. In globally CB_1^- mice, systemically administered WIN did not evoke statistically significant analgesia, as determined using either a dynamic aesthesiometer (Fig. 6c; 1 or 3 mg per kg WIN) or von Frey hairs (Fig. 6d; 1 mg per kg WIN). This suggests that the residual WIN-induced analgesia seen in SNS-CB_1^- mice is mediated via CB_1 receptors that are expressed somewhere other than in the DRG: for example, in central neurons. In contrast to the reduction in WIN-induced analgesia in SNS-CB_1^- mice, we observed that catalepsy[20], an effect of cannabinoids attributed to central receptors, occurred at comparable magnitudes in SNS-CB_1^- and CB_1^{fl} mice after systemic administration of WIN (Fig. 6e; $P = 0.005$ and 0.002, respectively). We therefore conclude that a large component of the inflammatory pain relief produced by systemic administration of a CB_1 agonist is mediated by activation of CB_1 receptors expressed on primary afferent nociceptors.

Delineation of peripheral and central contributions

CB_1 receptors localized on the central terminals of nociceptors in the spinal dorsal horn and on peripheral terminals in the paw could contribute to the analgesic effect of systemic CB_1 agonists. In an effort to delineate the respective contributions of the central and peripheral terminals of nociceptors, we delivered WIN intrathecally (10 μg) to the lumbar spinal cord 17 h after CFA-induced hindpaw inflammation. Following this mode of delivery, WIN can act on the central terminals of nociceptors and on spinal dorsal-horn neurons to modulate pain sensitivity. Intrathecally applied WIN significantly attenuated CFA-induced mechanical hypersensitivity in CB_1^{fl} mice (Fig. 7a,b). The antinociceptive effect of the intrathecally applied WIN was, moreover, entirely preserved in SNS-CB_1^- mice when examined using a dynamic aesthesiometer (Fig. 7a) or von Frey hairs (Fig. 7b). These results indicate that the loss of CB_1 on the central terminals of nociceptors does not reduce the analgesic effects of WIN applied locally to the spinal cord, which must therefore be acting on spinal dorsal-horn neurons.

Given our observations that systemically administered WIN requires CB_1 expressed by primary nociceptive afferents, but intrathecally applied WIN does not, we surmised that CB_1 receptors expressed on peripheral, rather than spinal, terminals of nociceptor neurons are likely to be critical for the action of systemically applied WIN. Consistent with this, peripherally administered cannabinoids produce analgesia[19,21]. However, owing to the highly lipophilic nature of cannabinoids, which results in rapid systemic uptake and efficient transfer across the blood-brain barrier, as well as the issue of enhanced capillary permeability in inflamed tissue, some studies have raised concerns that central loci contribute to the analgesia observed after peripheral injection of cannabinoids[22–24]. If CB_1 expressed on nociceptors were a prime mediator of the analgesia produced by peripherally administered cannabinoids, SNS-CB_1^- mice would be expected to be largely resistant to peripherally applied cannabinoids. Indeed, intraplantar injection of 10, 20 or 30 μg WIN into the hindpaw 17 h after CFA-induced paw inflammation strongly decreased mechanical hyperalgesia in CB_1^{fl} mice ($P < 0.01$), but not in SNS-CB_1^- mice ($P > 0.5$; Fig. 7c). In this regard, the behavior of the SNS-CB_1^- mice was essentially identical to that of globally CB_1^-mice (Supplementary Fig. 2). Furthermore, in the von Frey test, intraplantar injection of WIN to CB_1^{fl} mice not only fully reversed CFA-induced hyperalgesia and allodynia, but also produced hypoalgesia (mean force required to elicit a response in 50% of cases was 1.4*g* in the control group, 0.7*g* in the CFA-treated group and 2*g* after intraplantar WIN treatment in the CFA group; Fig. 7d). Compared with the above, intraplantar injection of WIN in SNS-CB_1^- mice decreased CFA-induced hypersensitivity only slightly (mean force required to elicit a response in 50% of cases was 1*g* in the control group, 0.29*g* in the CFA-treated group and 0.5*g* after intraplantar WIN treatment in the CFA group; Fig. 7d). We conclude that CB_1 receptors expressed on the peripheral terminals of primary nociceptive neurons are an important mediator of the antinociceptive effects of exogenous cannabinoids in inflammatory pain states.

Neuropathic pain and peripheral CB_1

We then asked whether a similar scenario exists with respect to neuropathic pain, as therapy with cannabinoids holds substantial promise[23,25]. To assess whether peripheral endocannabinoid synthesis is regulated by nerve lesions, we used the spared nerve injury (SNI) model of neuropathic pain[26]. At 7 d after injury to the tibial and common peroneal branches of the sciatic nerve, there were no marked changes in endocannabinoid levels in skin samples derived from the tibial, saphenous or sural nerve innervation territories after SNI (Fig. 8a). In contrast, the sciatic nerve proximal to the lesion site after SNI showed a 3–4-fold increase in levels of 1-AG ($P = 0.04$), 2-AG ($P = 0.001$) and arachidonic acid ($P = 0.029$), whereas an increase in the concentration of AEA did not reach statistical significance ($P = 0.062$) (Fig.

8a), suggesting that local synthesis of endocannabinoids in proximal nerve stumps or leukocytes invading the lesion may regulate nociceptive drive following nerve lesions.

We therefore compared the responses of SNS-CB_1^- mice with those of CB_1^{fl} mice to nociceptive stimuli after SNI. Both SNS-CB_1^- and CB_1^{fl} mice showed reduced latencies to mechanical stimuli applied with a dynamic aesthesiometer in comparison with sham-treated littermates of the same genotype (Fig. 8b). Quantification of the response magnitude as the area under the response-versus-time curve (AUC) revealed an exaggerated mechanical hypersensitivity in SNS-CB_1^- mice as compared with CB_1^{fl} mice after SNI (Fig. 8c, $P = 0.014$). Similarly, SNI-treated SNS-CB_1^- mice demonstrated an exaggerated sensitivity to cold (5 °C) as compared with sham-treated SNS-CB_1^- mice or SNI- treated CB_1^{fl} mice (Fig. 8d,e; $P = 0.012$ and 0.05, respectively). When we tested mechanical and cold sensitivity via manual application of von Frey hairs and acetone, respectively, we did not observe significant differences between SNS-CB_1^- mice and CB_1^{fl} mice, which might result from technical aspects of these methods, especially in light of a ceiling effect after SNI (Supplementary Fig. 3).

To clarify whether CB_1 expression in peripheral sensory neurons contributes to cannabinoid-induced analgesia in neuropathic pain states, we compared the magnitude of analgesia produced by systemic delivery of WIN (1, 3 or 10 mg per kg body weight) in SNS-CB_1^- and CB_1^{fl} mice 7 d after SNI. In CB_1^{fl} mice, WIN significantly increased the response latency to thermal stimuli at a dose of 3 mg per kg (Fig. 8f) and raised the response threshold to von Frey hairs starting at a dose of 1 mg per kg (Fig. 8g). These antinociceptive effects of WIN were significantly weaker in the SNS-CB_1^- mice than in the CB_1^{fl} mice at 1 and 3 mg per kg WIN with respect to thermal nociception (Fig. 8f; $P < 0.001$ and $P = 0.018$, respectively) and mechanically evoked pain (Fig. 8g; $P = 0.002$ and 0.02, respectively). However, differences between SNS-CB_1^- mice and CB_1^{fl} mice were greater with respect to cannabinoid effects on mechanical sensitivity than to those on thermal responses. Only at a dose of 10 mg per kg, which caused motor rigidity and sedation in all mice, did SNS-CB_1^- mice and CB_1^{fl} mice show comparable responses. From these data, we infer that CB_1 expressed by nociceptor neurons mediates a large proportion of the cannabinoid-induced antinociception produced in neuropathic pain.

DISCUSSION

Expression analyses have reported highly variable distributions of CB_1 in nociceptive and non-nociceptive neurons of the DRG[27–30], likely due to differences in the sensitivity and specificity of techniques, differential detection of splice variants[31] and species differences. Using a riboprobe[11] and an antibody[32] that detect all forms of CB_1 and completely fail to elicit signals in globally CB_1^- mice, a thorough quantitative analysis revealed that CB_1 mRNA and protein are abundantly expressed in a major population of nociceptive neurons in adult mouse DRG. Moreover, we observed that CB_1 is lost specifically from nociceptive neurons, but preserved in large-diameter DRG neurons and in the CNS, in SNS-CB_1^- mice. Using a combination of pharmacology, electrophysiology and genetic manipulations, we demonstrate here a critical role for CB_1 expressed by nociceptors in a tonic inhibition of pain by endocannabinoids, as well as in exogenous cannabinoid–induced analgesia for chronic inflammatory or neuropathic states.

This study addresses a number of important questions about cannabinoid analgesia. First, our study helps to clarify the anatomical locus of cannabinoid-induced analgesia. Pharmacological and electrophysiological studies have shown that cannabinergic modulation of neuronal circuits in the cortex[33], amygdala[34], rostroventral medulla[35], periaqueductal gray[36] and the spinal cord[37] can inhibit nociceptive processing. Which of these sites mediates cannabinoid analgesia, however, has been an issue of some debate. Our data indicate that CB_1 expressed

by nociceptors accounts for the largest proportion of the antinociception produced by endocannabinoids, as well as by systemically or topically applied cannabinoids. Furthermore, electrophysiological recordings from isolated nociceptors innervating the skin, and pharmacological experiments comparing intrathecal (spinal) delivery with intraplantar (peripheral) administration, suggest that the peripheral, rather than the central, terminals of nociceptors are the important site of cannabinergic modulation.

We have ruled out several potentially confounding factors, such as developmental defects or unspecific deletion of CB_1, that could have complicated the interpretation of this study. Thus, although it has been known for several years that cannabinoids can activate peripheral receptors on nociceptors[38], our findings show that peripheral CB_1-mediated inhibitory mechanisms on these neurons are paramount in the production of cannabinoid analgesia. Because centrally, unlike systemically, applied cannabinoids elicit analgesia in SNS-CB_1^- mice, it is conceivable that the peripheral effects on CB_1 exceed any central effects in response to systemic treatment because the initiation, rather than the processing, of pain is inhibited. Furthermore, analogous to the described synergy between various sites of opioid actions[39], a synergy between spinal and peripheral sites of cannabinoid action has been reported[19], which may be disrupted by a loss of peripheral CB_1, leading to a large deficit in systemic cannabinoid-induced analgesia.

Second, this study highlights the potential significance of peripheral CB_1–mediated cannabinoid analgesia. Although analgesia resulting from an action on nociceptor peripheral terminals is well established for opioids, including in clinical settings[40], studies on the peripheral administration of cannabinoids in diverse states of chronic pain yielded equivocal effects[23,24], with reports of substantial analgesia from some studies[41–43], but not from others[22]. Owing to the highly lipophilic nature of cannabinoids and the high doses of pharmacological agents required in some studies to elicit peripheral analgesia[44], systemic effects can occur with peripheral administration[23]. Furthermore, some reports have questioned the involvement of CB_1 in the analgesia evoked by peripherally administered cannabinoids[42–44]. We found that comparatively low doses of a peripherally applied synthetic cannabinoid reduced inflammatory and neuropathic pain, and that this was nearly completely lost on nociceptor-specific deletion of CB_1. It will be interesting in future studies to determine whether a nociceptor-specific rescue of CB_1 expression in globally CB_1^- mice can fully or partially reinstate cannabinoid analgesia on systemic or peripheral application.

Finally, the results derived from these experiments reveal important insights into how the peripheral endocannabinoid system works in controlling pain. Some studies have reported hyperalgesia in response to systemically administered antagonists at cannabinoid receptors, whereas several others have reported evidence against a role for the endocannabinoid system in the tonic inhibition of pain[1]. Global, classical CB_1 knockout mice from two different genetic backgrounds have yielded conflicting results in this regard[7,8]. Therefore, the role of the endocannabinoid system in the tonic regulation of physiological pain has remained unclear. Our conditional gene targeting strategy has revealed that CB_1 expressed by primary nociceptors mediates an inhibitory tone on nociceptive activity in naive states. Nevertheless, a note of caution is warranted in directly comparing the phenotype of SNS-CB_1^- mice with those of previously reported mutants because of potential differences in genetic background. Consistent with the increased pain sensitivity in SNS-CB_1^- mice, endocannabinoids were detectable in peripheral tissues of naive mice and their abundance increased severalfold locally in the skin after inflammation or in nerve stumps after nerve injury. In contrast, persistent activation of nociceptors did not lead to elevated abundance of endocannabinoids in the vicinity of their central terminals in the spinal cord. We conclude, therefore, that the peripheral endocannabinoid system is an important component of endogenous pain control mechanisms.

The pain phenotypes and the near-complete and complete loss of systemic cannabinoid-induced analgesia in SNS-CB_1^- and CB_1^- mice, respectively, suggest that CB_1 receptors are a major target for pain control via endocannabinoids and exogenous cannabinoids *in vivo*. CB_2 cannabinoid receptors expressed on immune cells and in the nervous system have also been implicated in cannabinoid analgesia[1,9]. Our study was not designed to elucidate the relative contributions of CB_1 and CB_2, and it is possible that CB_1, CB_2, as yet unidentified cannabinoid receptors[45] and potential synergistic effects between them contribute to cannabinoid analgesia.

In summary, our results show that by targeting CB_1 expressed on the peripheral axons of primary sensory neurons, substantial analgesia can be achieved in somatic and visceral pain, as well as in inflammatory and neuropathic pain. Taken together with previous reports[9,19–22,30,42–44], this study presents a strong basis for the design of novel synthetic cannabinoids that do not cross the blood-brain barrier as a new class of peripherally acting analgesics without the psychotropic liability of centrally acting CB_1 agonists.

METHODS

Genetically modified mice

Mice homozygous for the floxed allele of the mouse *Cnr1* gene, which encodes the cannabinoid receptor 1 (CB_1^{fl} mice), have been described previously[11]. CB_1^{fl} mice were crossed with SNS-Cre mice[12] to obtain homozygous CB_1^{fl};SNS-Cre^+ and CB_1^{fl} mice (control littermates). Genotyping was done on mouse genomic tail DNA using sense primer 5′-GCTGTCTCTGGTCCTCTTCTTAAA-3′ and antisense primer 5′-GGTGTC ACCTCTGAAAACAGA-3′ to detect the *Cnr1* floxed allele, and sense primer 5′-GAAAGCAGCCATGTCCAATTTACTGACCGTAC-3′ and antisense primer 5′-GCGCGCCTGAAGATATAGAAGA-3′ to detect SNS-Cre transgene expression. Both SNS-Cre and $CB1^{fl}$ mice were backcrossed individually into the C57BL/6 background for more than eight generations before being crossed with each other. Mice lacking CB_1 globally (CB_1^-mice)[13] and their wild-type littermates had the genetic background C57Bl/6-N. SNS-Cre mice and their corresponding wild-type littermates had the background C57Bl/6-J. SNS-CB_1^- mice and their CB_1^{fl} littermates had the background C57BL/6-J mixed with C57Bl6-N. Littermates were used in all experiments to control for background effects. All animal use procedures were in accordance with ethical guidelines imposed by the local governing body (Regierungspräsidium Karlsruhe, Germany). All behavioral measurements were done in awake, unrestrained, age-matched male mice that were more than 3 months old by individuals who were blinded to the genotype of the mice being analyzed (see Supplementary Methods online for details).

Endocannabinoid measurements

For measuring endocannabinoid levels, mice were decapitated and their paws, spinal cords, nerves or skin were rapidly removed and frozen in liquid nitrogen (Supplementary Methods). Endocannabinoid levels were determined by liquid chromatography/mass spectrometry as described previously[46].

Afferent recordings in skin-nerve preparation

A total of 32 mice (17 CB_1^{fl} and 15 SNS-CB_1^-) were used in the electrophysiological investigations. An *in vitro* skin-nerve preparation[18] was used to study the properties of the afferent fibers that innervate the skin in the inflamed area 24 h after CFA inoculation (20 μl; Supplementary Methods).

Data analysis and statistics

All data are presented as mean ± s.e.m. Analysis of variance (ANOVA) for random measures was carried out, followed by *post hoc* Fisher's test or Dunnett's test to determine statistically significant differences for all data with the exception of nerve recordings (below). $P < 0.05$ was considered significant. To compare activation thresholds of populations of C-fibers across mice, we used χ^2 analysis.

Additional details on methods are provided in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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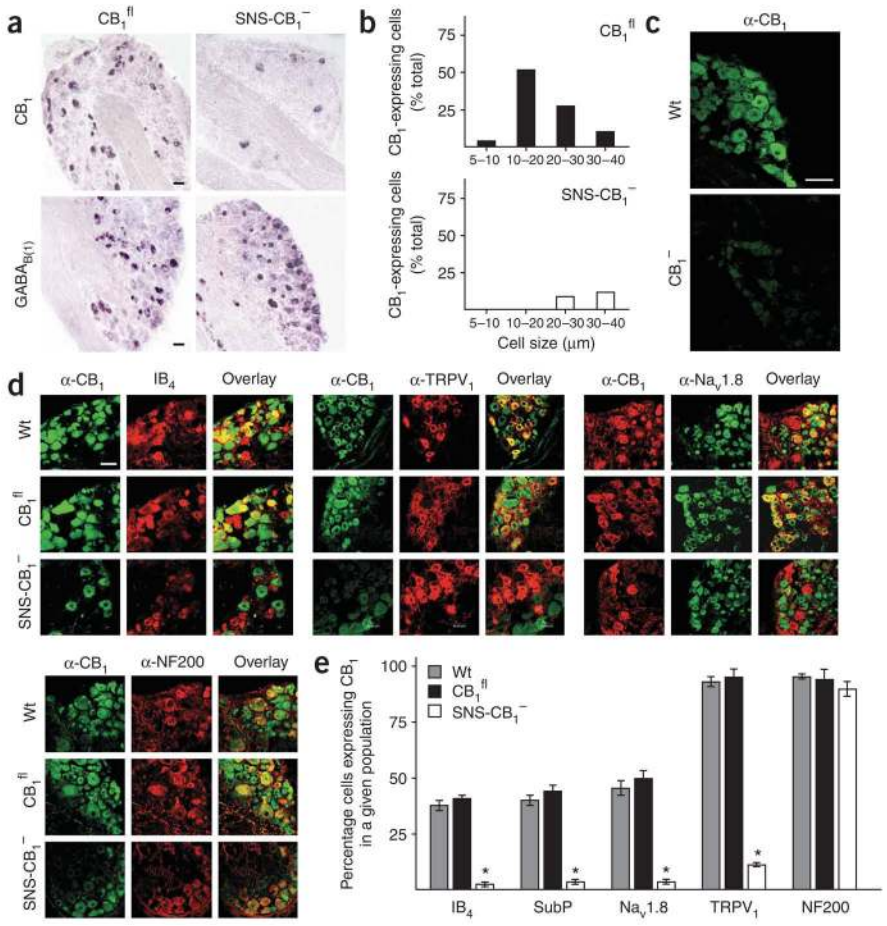

Figure 1.

Demonstration of conditional deletion of CB_1 specifically in nociceptive neurons of the DRG in sensory neuron–specific CB_1 knockout mice (SNS-CB_1^-). (**a**) mRNA *in situ* hybridization for expression of CB_1 or $GABA_{B(1)}$ (control) on DRG sections from SNS-CB_1^- mice and control littermates (CB_1^{fl}). (**b**) Quantitative size analysis of DRG neurons expressing CB_1 mRNA showed that small-diameter neurons lost and large-diameter neurons maintained CB_1 expression in SNS-CB_1^- mice. (**c**) A goat anti-CB_1 used throughout this study yielded specific labeling of DRG neurons that was entirely lost in the DRG of globally CB_1^- mice. (**d**) Typical examples of anti-CB_1 immunoreactivity in subpopulations of DRG neurons labeled using binding to IB_4 or using antibodies to TRPV1, $Na_v1.8$ and neurofilament 200 (NF200) in wild-type, CB_1^{fl} and SNS-CB_1^- mice. In SNS-CB_1^- mice, CB_1 immunoreactivity was nearly abrogated from nociceptors (IB_4-, substance P– or $Na_v1.8$-positive neurons) and reduced in a large fraction of TRPV1-positive C- and A-δ neurons, but entirely preserved in NF200-positive large-diameter neurons. (**e**) Quantitative summary of DRG cell populations expressing CB_1 protein in wild-type (Wt), CB_1^{fl} mice and SNS-CB_1^- mice from experiments represented in **d** (mean ± s.e.m.; $n = 10–15$ DRG sections each). *$P < 0.001$, ANOVA, *post hoc* Fisher's test. Scale bars, 40 μm in (**a**,**c**,**d**).

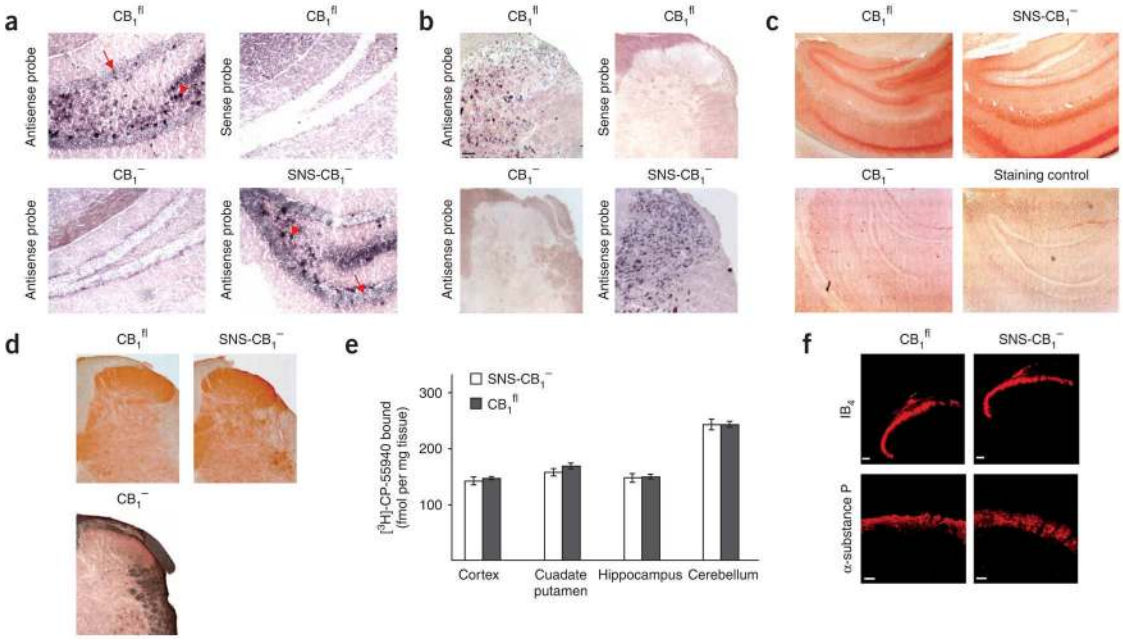

Figure 2.

Expression of CB_1 mRNA and CB_1 protein is similar in the brain and spinal cord of CB_1^{fl} mice and SNS-CB_1^{-} mice. (**a**,**b**) Antisense mRNA riboprobes revealed comparable expression of CB_1 in hippocampal interneurons (arrows, **a**) and spinal neurons (**b**) of SNS-CB_1^{-} mice and their $CB_1^{fl/fl}$ littermates, but a loss of signal in global CB_1^{-} mice or on usage of the sense probes. (**c**,**d**) Immunostaining with a goat anti-CB_1 revealed comparable expression of CB_1 in the brain (hippocampus shown in **c**) and in the spinal cord (**d**) of SNS-CB_1^{-} mice and their CB_1^{fl} littermates, but a loss of signal in global CB_1^{-} mice or in staining controls. (**e**) Autoradiography with a synthetic cannabinoid ^{3}H-CP-55940 revealed similar levels of binding (mean ± s.e.m.) in various brain regions of SNS- CB_1^{-} as compared to CB_1^{fl} mice. (**f**) The pattern of termination of primary nociceptive afferents in the spinal dorsal horn was similar in CB_1^{fl} and SNS-CB_1^{-} mice, as shown via binding to TRITC-labeled isolectin-B4 (IB_4) and immunoreactivity for substance P. Scale bars, 150 (**a**–**d**) and 100 μm (**f**).

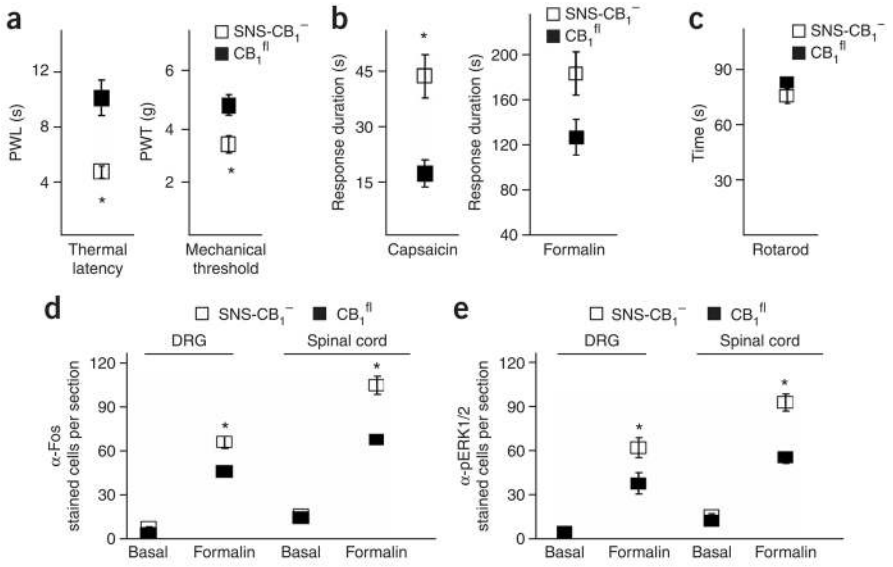

Figure 3.

Nociceptive responses, locomotive performance and nociceptive activity–induced expression of proteins in SNS-CB_1^- mice and their CB_1^{fl} littermates. (**a**) SNS-CB_1^- mice ($n = 12$) showed significant reductions in paw withdrawal latency (PWL; $P = 0.001$) in response to radiant heat and in paw withdrawal threshold (PWT; $P = 0.003$) in response to punctuate pressure in comparison with CB_1^{fl} mice ($n = 12$). (**b**) SNS-CB_1^- mice ($n = 8$) showed a significant reduction in the duration of acute nocifensive responses to intraplantar paw injection of capsaicin ($P = 0.002$) or formalin (phase I; $P = 0.049$), as compared with CB_1^{fl} mice ($n = 8$). (**c**) Latency to fall from a rotating rod was similar in SNS-CB_1^- mice ($n = 6$) and CB_1^{fl} mice ($n = 6$; $P = 0.203$). (**d**,**e**) Quantitative analysis of neurons immunoreactive for either Fos or phosphorylated ERK1/2 per section of DRG or spinal dorsal horn in the basal state (naive) or 1 h after intraplantar hindpaw injection of formalin in SNS-CB_1^- mice ($n = 6$) and CB_1^{fl} mice ($n = 6$). *$P < 0.05$, ANOVA, *post hoc* Fisher's test. All data points represent mean ± s.e.m.

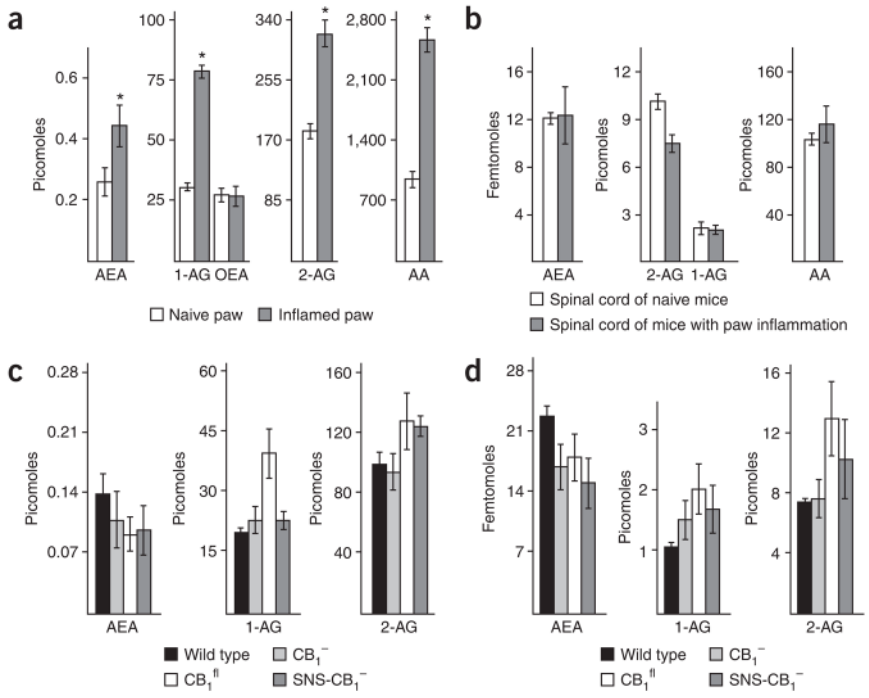

Figure 4.

Analysis of endocannabinoid levels in the paws and spinal segments (L4–L6) of SNS- CB_1^- mice, CB_1^{fl} mice, global CB_1^- mice and their wild-type controls ($n = 6$ each) in the basal state (naive) or in wild-type mice after injection of CFA into the hindpaw. (**a**) Levels of AEA, 1-AG, 2-AG and arachidonic acid (AA) rose in the paw skin after inflammation, as compared with naive state ($P < 0.05$; $n = 8$ paws in each group), whereas oleoylethanolamide (OEA) levels remained unchanged ($P = 0.9$). (**b**) Levels of endocannabinoids did not change significantly in the L4–L6 spinal cord after paw inflammation over the naive state ($P > 0.05$; $n = 6$ mice in each group). (**c**,**d**) Levels of endocannabinoids in the paw or in the spinal cord were not significantly different across SNS-CB_1^-, CB_1^{fl}, global CB_1^- and wild-type mice. *$P < 0.05$, ANOVA, *post hoc* Fisher's test. All data points represent mean ± s.e.m.

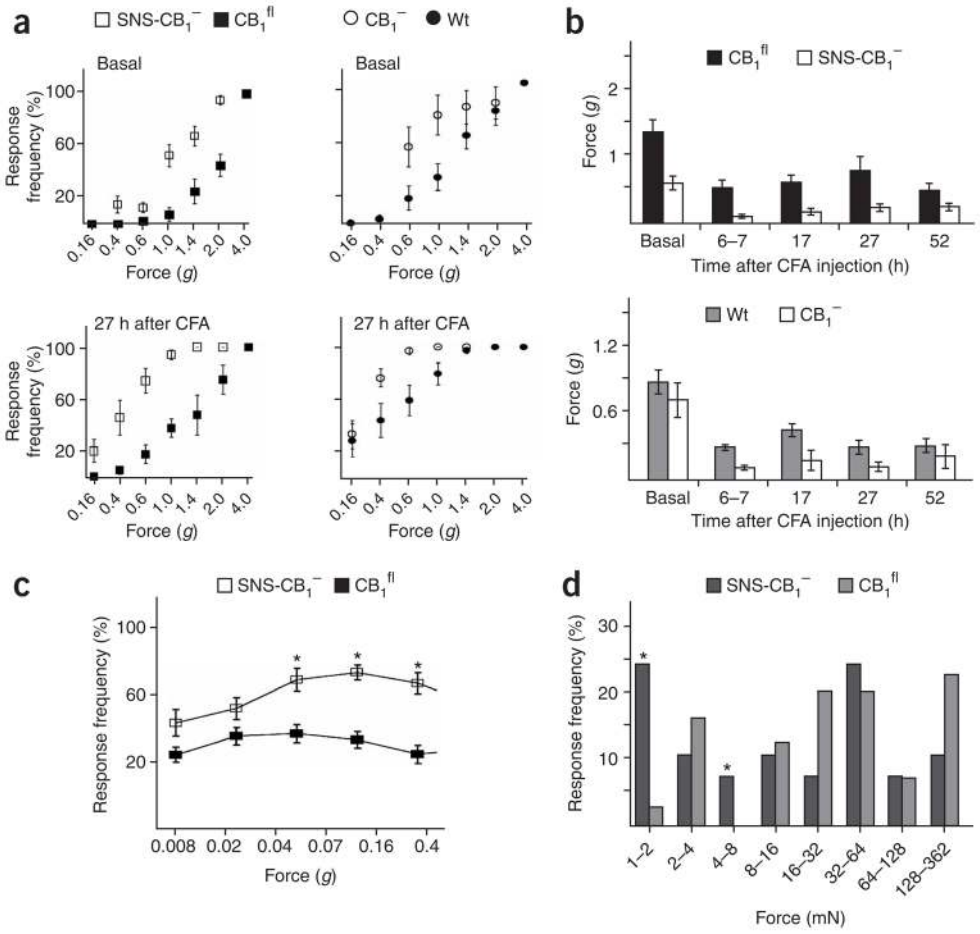

Figure 5.

Behavioral and electrophysiological analysis of SNS-CB_1^- mice in models of inflammatory pain. (**a**) Comparison of response frequency to von Frey hairs in SNS-CB_1^- mice ($n = 12$), CB_1^{fl} mice ($n = 12$), global CB_1 knockout mice (CB_1^-; $n = 6$) and their wild-type littermates (Wt; $n = 6$) before and 27 h after intraplantar injection of CFA. Note that SNS-CB_1^- mice and CB_1^- mice demonstrated comparable deviations from their respective control littermates. (**b**) Summary of response thresholds (defined as a force eliciting a response frequency of at least 40%) before and at 6–7 h, 14 h, 27 h or 52 h after intraplantar injection of CFA to SNS-CB_1^-, CB_1^{fl}, global CB_1^- and Wt mice. (**c**) Response frequency to abdominal application of von Frey filaments after induction of acute pancreatitis was significantly greater in SNS-CB_1^- mice ($n = 7$) than in CB_1^- mice ($n = 8$) ($P < 0.01$, ANOVA, *post hoc* Fisher's test). (**d**) Electrophysiological recordings from C-mechanoreceptors in the skin-nerve preparation derived from the paw showed that the frequency of responsive C-fibers was significantly greater at 1–2 mN force in SNS-CB_1^-mice ($n = 29$ fibers) than in CB_1^{fl} mice ($n = 31$ fibers) ($P < 0.05$, chi square analysis). *y* axes in **a**–**c** indicate force exerted by individual von Frey filaments. All data points represent mean ± s.e.m.

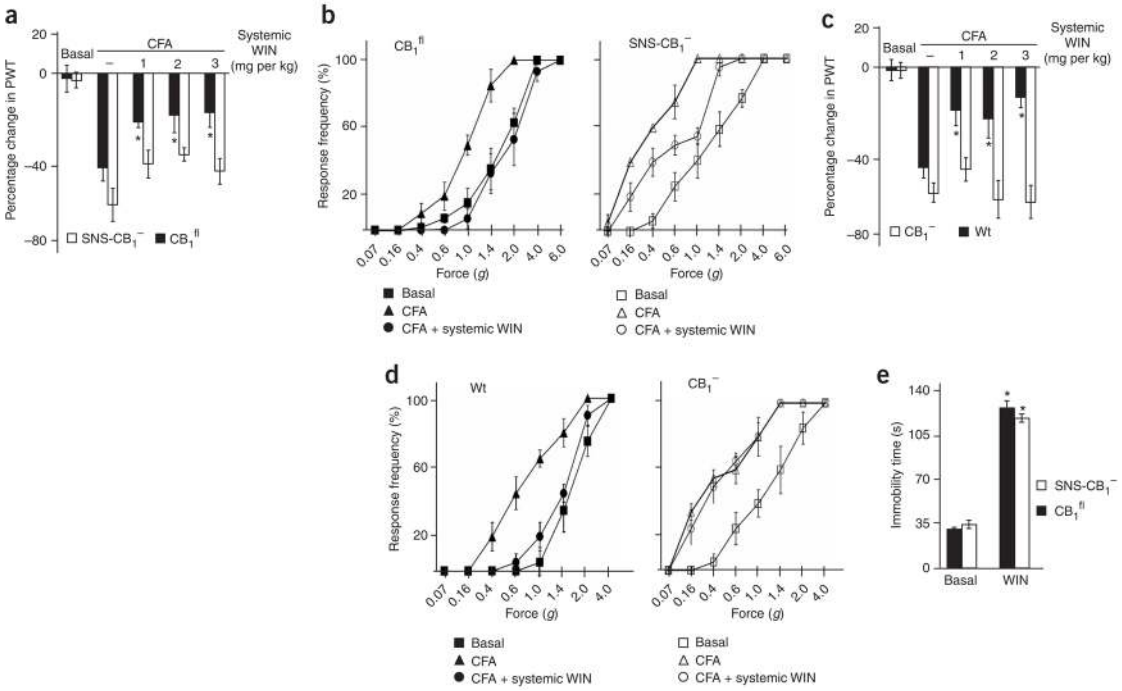

Figure 6.

Effects of a systemically applied CB_1/CB_2-agonist, WIN, on inflammation-induced mechanical hypersensitivity and immobilization behavior. (**a**–**d**) Paw inflammation was induced by unilateral intraplantar injection of CFA and mechanical hypersensitivity was derived as the percentage change in paw withdrawal threshold (PWT) over uninjected paw using an automated dynamic aesthesiometer (**a**,**c**) or by recording stimulus force-response frequency curves upon manual application of von Frey filaments (**b**,**d**) on the same cohort of animals. Systemically applied WIN (1 or 3 mg per kg) reduced CFA-induced mechanical hypersensitivity to a greater extent in CB_1^{fl} mice ($n = 5$ or 6 mice for each dose) than it did in SNS-CB_1^- mice ($n = 5$ or 6 for each dose) (**a**,**b**). Systemically applied WIN (1 or 3 mg per kg) reduced CFA-induced mechanical hypersensitivity in wild-type mice (Wt; $n = 5$ or 6 for each dose), but not in classical CB_1 knockout mice (CB_1^-; $n = 5$ or 6 for each dose) (**c**,**d**). $^*P < 0.05$ as compared with CFA-induced mechanical hyperalgesia in (**a**,**c**), ANOVA, *post hoc* Fisher's test. (**e**) Intraperitoneal injection of WIN induced immobilization responses in the ring catalepsy test in both CB^{fl} mice and SNS-CB_1^- mice. $^*P < 0.05$ over basal state, ANOVA, *post hoc* Fisher's test. All data points represent mean ± s.e.m.

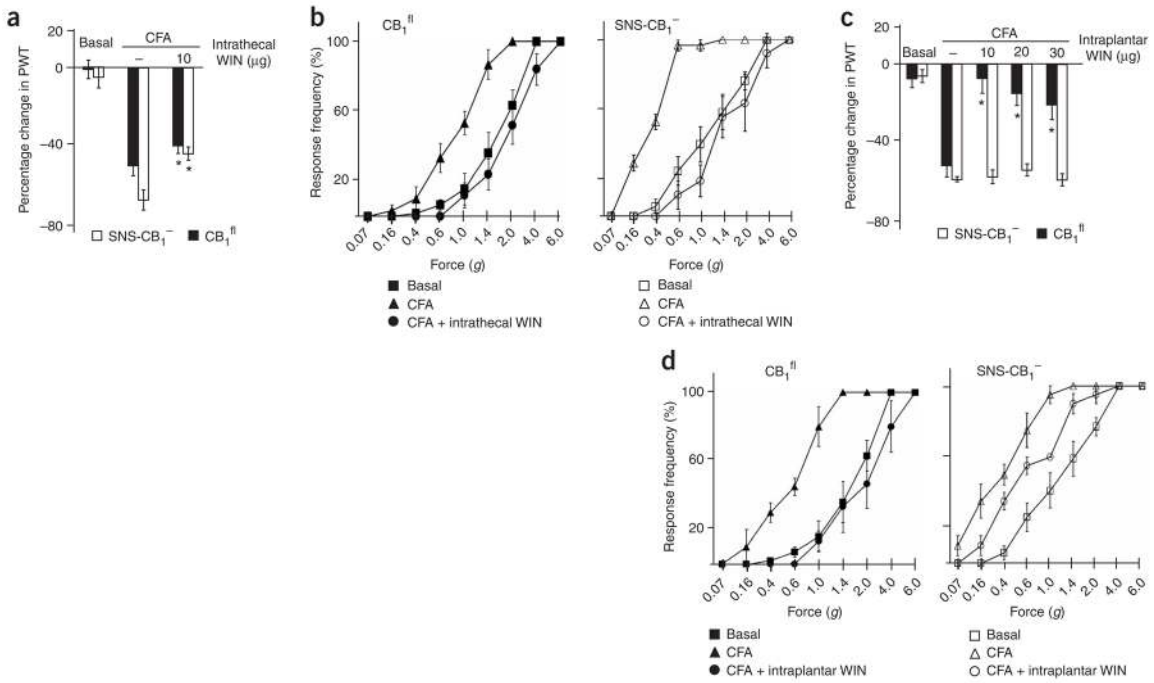

Figure 7.

Effects of WIN. (**a**–**d**) WIN was applied via intrathecal (**a**,**b**) or intraplantar (**c**,**d**) routes of administration on inflammation-induced mechanical hypersensitivity. Intrathecally applied WIN reduced CFA-induced mechanical hypersensitivity in both CB_1^{fl} mice ($n = 6$) and in SNS-CB_1^- mice ($n = 6$). Intraplantar application of WIN (10–30 μg) significantly reduced CFA-induced mechanical hypersensitivity in CB_1^{fl} mice ($n = 5$ or 6 for each dose), but not in SNS-CB_1^- mice ($n = 5$ or 6 for each dose). $^*P < 0.05$ as compared with CFA-induced mechanical hyperalgesia in **a** and **c**, ANOVA, Fisher's test. All data points represent mean ± s.e.m.

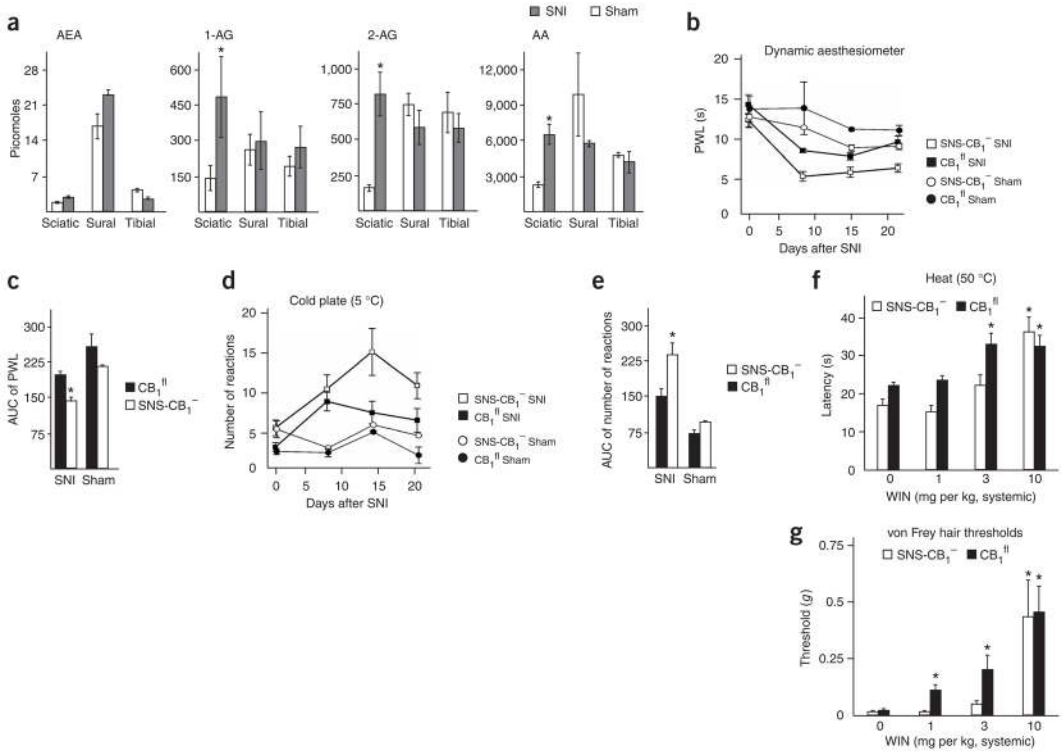

Figure 8.

Endocannabinoid levels, pain behavior and analgesic effects of WIN in SNS-CB_1^-mice and CB_1^{fl} mice in the SNI model for neuropathic pain. (**a**) Levels of endocannabinoids in innervation territories of the 'sural' and 'saphenous/tibial' branches of the sciatic nerve or in the sciatic nerve just proximal to the site of ligation. *P < 0.02, ANOVA, Fisher's test; n = 3 or 4 samples in each group. (**b**,**c**) Latency of PWL in response to mechanical stimuli (represented as integrated area under the curve, AUC, in **c**) in SNS-CB_1^- mice and CB_1^{fl} mice (n = 7 each for SNI and 3 each for sham). SNS-CB_1^- mice showed an exaggerated drop in PWL as compared with controls after SNI (*P < 0.05, ANOVA, Fisher's test). (**d**,**e**) Number of reactions to a cold stimulus (5 °C) (represented as integrated AUC in panel **e**) in SNS-CB_1^- mice and CB_1^{fl} mice (n = 6 each). *P < 0.05, ANOVA, Fisher's test. (**f**,**g**) Effects of intraperitoneal injections of WIN (1, 3 or 10 mg per kg) on latency of paw withdrawal to heat at 50 °C (**f**) or plantar response threshold to von Frey hairs (**g**) in SNS-CB_1^- mice (n = 7) and CB_1^{fl} mice (n = 9). *P < 0.05 as compared with values before WIN application (0) in the respective group, ANOVA, Fisher's test. All data points represent mean ± s.e.m.