THE NEURONAL DISTRIBUTION OF CANNABINOID RECEPTOR TYPE 1 IN THE TRIGEMINAL GANGLION OF THE RAT

T. J. PRICE, G. HELESIC, D. PARGHI, K. M. HARGREAVES AND C. M. FLORES

Abstract—Cannabinoid compounds have been shown to produce antinociception and antihyperalgesia by acting upon cannabinoid receptors located in both the CNS and the periphery. A potential mechanism by which cannabinoids could inhibit nociception in the periphery is the activation of cannabinoid receptors located on one or more classes of primary nociceptive neurons. To address this hypothesis, we evaluated the neuronal distribution of cannabinoid receptor type 1 (CB1) in the trigeminal ganglion (TG) of the adult rat through combined in situ hybridization (ISH) and immunohistochemistry (IHC). CB1 receptor mRNA was localized mainly to medium and large diameter neurons of the maxillary and mandibular branches of the TG. Consistent with this distribution, in a de facto nociceptive sensory neuron population that exhibited vanilloid receptor mRNA in TG neurons, these findings suggest that the peripheral sites in the pain pathway. For example, peripherally administered anandamide, an endocannabinoid neurotransmitter, was shown to be antihyperalgesic when administered directly into the hind paw of carrageenan-inflamed rats, and this effect was reversed by the selective CB1 antagonist SR141716A (Richardson et al., 1998). In addition to having direct effects on nociception, cannabinoids may have anti-inflammatory properties, as peripherally administered anandamide decreased carrageenan-evoked plasma extravasation and decreased capsaicin-evoked calcitonin gene-related peptide (CGRP) release from rat hind-paw skin (Richardson et al., 1998). Similarly, Ko and Woods (1999) demonstrated that Δ9-THC, injected at the site of capsaicin-induced hyperalgesia, reduced nociceptive responses in rhesus monkeys and that this effect was CB1 receptor-mediated, insofar as it was blocked by SR141716A.

Key words: calcitonin gene-related peptide, vanilloid receptor type 1, substance P, isoleucin B4, in situ hybridization.

The primary psychoactive component of Cannabis sativa, Δ9-tetrahydrocannabinol, has long been recognized for its medicinal properties. Over the last two decades, a large body of work has elucidated the receptor-mediated actions of natural and synthetic cannabinoid compounds (for review, see Khanolkar et al., 2000). Two G protein-coupled cannabinoid receptors have been cloned, the cannabinoid receptor type 1 (CB1; Matsuda et al., 1990), found primarily in neurons, and the cannabinoid receptor type 2 (CB2; Munro et al., 1993), found predominantly in immune cells. CB1 receptors couple to inhibitory G proteins and have been shown to produce multiple cellular effects, including decreases in cyclic AMP accumulation (Felder et al., 1993), as well as the modulation of neurotransmitter release through the inhibition of calcium currents (Twitchell et al., 1997; Shen and Thayer, 1998) and the activation of G protein-coupled inwardly-rectifying potassium channels (Mackie et al., 1995). On a whole organism level, endogenous cannabinoids as well as exogenously administered cannabinoids have been shown to, among other things, alleviate pain and inflammation.

While there is ample evidence for antinociceptive effects of cannabinoids in the CNS (Lichtman and Martin, 1991; Martin et al., 1993, 1995; Smith et al., 1994; Hohmann et al., 1995; Lichtman et al., 1996; Meng et al., 1998), there is a growing body of work indicating that cannabinoid antinociception also may be mediated at peripheral sites in the pain pathway. For example, peripherally administered anandamide, an endocannabinoid neurotransmitter, was shown to be antihyperalgesic when administered directly into the hind paw of carrageenan-inflamed rats, and this effect was reversed by the selective CB1 antagonist SR141716A (Richardson et al., 1998).

In conclusion, our results indicate that the cannabinoid receptor type 1, CB1, is expressed predominantly in neurons, and the cannabinoid receptor type 2, CB2, is expressed sparsely (<5%). Furthermore, very few neurons (approximately 5%) in the peptidergic (defined as calcitonin gene-related peptide- or substance P-immunoreactive) or the isolectin B4-binding sensory neuron populations contained CB1 mRNA. In contrast, and consistent with the neuron-size distribution for CB1, near 75% of CB1-positive neurons exhibited N52-immunoreactivity, a marker of myelinated axons. These results indicate that the distribution of cannabinoid receptor type 1 in the TG is consistent with the neuron-size distribution for CB1, near 75% of CB1-positive neurons, and with the absence of an above-background signal for CB2 mRNA in TG neurons, these findings suggest that the peripheral sites in the pain pathway. For example, peripherally administered anandamide, an endocannabinoid neurotransmitter, was shown to be antihyperalgesic when administered directly into the hind paw of carrageenan-inflamed rats, and this effect was reversed by the selective CB1 antagonist SR141716A (Richardson et al., 1998). In addition to having direct effects on nociception, cannabinoids may have anti-inflammatory properties, as peripherally administered anandamide decreased carrageenan-evoked plasma extravasation and decreased capsaicin-evoked calcitonin gene-related peptide (CGRP) release from rat hind-paw skin (Richardson et al., 1998). Similarly, Ko and Woods (1999) demonstrated that Δ9-THC, injected at the site of capsaicin-induced hyperalgesia, reduced nociceptive responses in rhesus monkeys and that this effect was CB1 receptor-mediated, insofar as it was blocked by SR141716A.

Nociceptors (i.e. neurons activated by painful stimuli) can be grossly assigned to two classes based on certain neurochemical markers: 1) a peptidergic class, which contains either or both of the proinflammatory neuropeptides CGRP or substance P (SP), and 2) an unmyelinated class, which contains glycoproteins that bind isoleucin B4 (IB4; Averill et al., 1995). A proportion of both classes of nociceptors, the majority of which are polymodal (Wood and...
Perl, 1999), is activated by chemical stimuli including capsaicin, the pungent ingredient of hot peppers. Recently, a capsaicin-activated ion channel receptor, called vanilloid receptor type 1 (VR1), was isolated (Caterina et al., 1997), and its cell-type distribution was defined (Tominaga et al., 1998). VR1 is also activated by noxious heat or protons (Tominaga et al., 1998). Accordingly, the expression of VR1 by a neuron defines it as a nociceptor. It has been shown that activation of VR1 induces the release of neurotransmitters, including CGRP and SP, which cause vasodilation and increased vaso-permeability, respectively (Brain et al., 1985; Gamse and Saria, 1985; Yonehara et al., 1998). VR1 is also activated by noxious heat or protons (Tominaga et al., 1998). VR1 by a neuron defines it as a nociceptor. It has been shown that activation of VR1 induces the release of neurotransmitters, including CGRP and SP, which cause vasodilation and increased vaso-permeability, respectively (Brain et al., 1985; Gamse and Saria, 1985; Yonehara et al., 1998). VR1 is also activated by noxious heat or protons (Tominaga et al., 1998).

While it is likely that the peripheral antinociceptive effects of cannabinoids arise in part via activation of one or more cannabinoid-responsive receptors located on primary nociceptive neurons, the identity of these neurons, with respect to their neurochemical and functional phenotypes, is unknown. Therefore, an elucidation of the precise localization of cannabinoid receptors in nociceptive sensory neuron populations should provide a better understanding of cannabinoid analgesic mechanisms. In this regard, Hohmann and Herkenham (Hohmann and Herkenham, 1999b) have demonstrated that CB1 mRNA is present in dorsal root ganglion (DRG) neurons and that this transcript colocalizes with α-CGRP and preprotachykinin A mRNA, although to only a modest degree (approximately 10%). Furthermore, CB1 receptors produced in the DRG undergo axonal transport to the periphery (Hohmann and Herkenham, 1999a) suggesting a functional role for CB1 receptors on peripheral nerve terminals. Neonatal capsaicin treatment, which abolishes capsaicin-sensitive sensory neurons (Jancso et al., 1978), reduced cannabinoid binding sites in the spinal dorsal horn by 16% (Hohmann and Herkenham, 1998), implying that cannabinoid binding sites are localized, at least partially, to capsaicin-sensitive nociceptors. In contrast, in cultured DRG neurons, CB1-immunoreactivity overlaps almost completely with VR1-immunoreactivity (Ahuwalia et al., 2000), suggesting, at least in primary culture that CB1 and VR1 are found in the same neuronal populations. To date, CB1 expression in trigeminal ganglion (TG), however, has not been evaluated. Because of the physiological differences between the spinal and trigeminal systems (Tal and Devor, 1992; Bongenhielm et al., 1998), an evaluation of the neurochemical phenotype of CB1-expressing neurons in TG is an important research objective. Furthermore, an assessment of CB1 mRNA expression as it relates to the VR1-positive and IB4-binding nociceptive neuronal populations has not been executed through direct double-labeling studies in sensory ganglia. Thus, an analysis of CB1 mRNA expression in these nociceptive populations should shed light on potential mechanisms through which cannabinoids inhibit nociception in the periphery. Herein, we have characterized the neuronal distribution of CB1 in TG using combined in situ hybridization (ISH) and CGRP, SP, VR1 or N52 immunohistochemistry (IHC) or IB4–binding histochemistry (IBH) double-labeling.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing approximately 175–225 g were used in this study. All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health. Every attempt was made to minimize the number of animals used and to reduce their suffering in the present study. Rats were killed by decapitation, and their TGs were promptly removed (approximately 120 s) and fresh frozen at −80 °C in OCT compound (Sakura, Torrance, CA, USA). Tissue sections were cut (20 μm) on a Leica CM1800 cryostat (Bannockburn, IL, USA), thaw-mounted (10 min at room temperature) onto Superfrost Plus glass slides (VWR, West Chester, PA, USA) and stored at −80 °C until use. For each experimental condition, one TG section was randomly chosen from each of three animals, and all ISH and IHC or IBH experiments were performed concurrently.

Single-labeling ISH

All chemicals were from Sigma (St. Louis, MO, USA) unless otherwise stated. A 5’ CB1 fragment (11–554) and a 5’ CB2 fragment (106–373) were separately subcloned into Topo-TA PCR2.1 (Invitrogen, Carlsbad, CA, USA) from the full length rat CB1 cDNA (Accession number NM_012784), generously provided by Dr. Lisa Matsuda and full length rat cannabinoid receptor type 2 (CB2) cDNA (Accession number NM_020543), generously provided by Dr. Mary Abdo, respectively. [35S]-labeled riboprobes were synthesized from the linearized CB1 and CB2 fragment-containing constructs using The Riboprobe Combination System SP6/T7 (Promega, Madison, WI, USA) incorporating 250 μCi each of [35S]-CTP and [35S]-UTP (specific activity ~800 Ci/mmol; Amersham, Piscataway, NJ, USA). Riboprobes were purified by G-50 column chromatography (Ambion, Austin, TX, USA) and stored in hybridization buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, 50 μg/ml yeast tRNA (Roche, Indianapolis, IN, USA) and 10 mM dithiothreitol at a concentration of 1×106 cpm/ml.

Tissue sections were prepared for hybridization by fixing in ice cold 3.7% formaldehyde for 1 h and permeabilizing with 0.5% Triton X-100 in 0.1 M Tris/0.05 M EDTA for 30 min. Sections were then acylated with acetic anhydride for 10 min, dehydrated, depigidated with chloroform, rehydrated and allowed to air dry. Dry sections were then hybridized in a humidified chamber with CB1 or CB2 riboprobes (40 μl 1×107 cpm/ml per TG section) in hybridization buffer for 16 h at 55 °C. Following hybridization, sections were washed with 4× SSC four times and treated with RNase 1 (20 μg/ml; Roche) for 30 min at 37 °C. Washes were performed in decreasing concentrations of SSC, culminating in a final high stringency wash in 0.1× SSC at 55 °C for 30 min, then dehydrated and allowed to air dry. Finally, slides were emulsion dipped in NTB-3 (Kodak, Rochester, NY, USA) and developed 17 days later (for development of optimal signal).
RESULTS

CB1 mRNA is localized to a sub-population of neurons in rat TG while CB2 mRNA is absent

Hybridization of tissue sections with CB1 anti-sense riboprobes yielded specific labeling that was restricted to TG neurons (Fig. 1, upper panel), while sense riboprobes exhibited no signal above background (data not shown). Interestingly, these CB1-positive neurons were found almost exclusively in those areas of the ganglia in which reside the somata that give rise to the mandibular and maxillary divisions of the trigeminal nerve but not the ophthalmic division (Fig. 1, upper panel). In contrast, immunoreactivity for peripherin, a marker of small diameter sensory neurons, was observed throughout the ganglion (Fig. 1, lower panel). CB1 mRNA was expressed mostly by neurons with diameters ranging from 40 to 80 μm (98.8%), with 61.3% of neurons having diameters greater than 60 μm (Fig. 2). CB1 mRNA-positive neurons comprised 29.6% ± 5.6 of the total TG neuronal population (3034 positive neurons of 9913 total neurons counted over eight entire TG sections from eight different animals). On the other hand, CB2 mRNA was not found in rat TG while the control tissue, spleen, displayed high levels of CB2 mRNA confirming the activity of the riboprobe by autoradiography (Fig. 3).

CB1 mRNA rarely colocalizes with the sensory neuron markers CGRP, SP, VR1 or IB4

Few CB1-positive neurons (4.9% ± 2.2) contained VR1 immunoreactivity, and 3.5% ± 1.4 of VR1-positive neurons were CB1-positive. Furthermore, just 1.9% ± 0.9 of CB1-positive neurons exhibited IB4 binding sites, while 2.0% ± 1.5 of IB4-binding neurons expressed CB1 mRNA. Similarly, colocalization of CB1 mRNA with the neuropeptides CGRP or SP was relatively rare. Of CB1-positive neurons, only 7.5% ± 2.0 contained CGRP immunoreactivity, and only 5.4% ± 0.8 contained SP immunoreactivity. Likewise, CB1 expression in these peptidergic neuronal populations was small with 7.5% ± 1.2 of CGRP-positive neurons containing CB1 mRNA and 4.4% ± 0.4 of SP-positive neurons containing CB1 mRNA. Representative samples of photomicrographs showing combined ISH/IHC or IBH are shown in Fig. 4 and the extent of this colocalization is quantitatively depicted in Fig. 5.

CB1 mRNA colocalizes extensively with N52-immunoreactivity, a marker of myelinated axons

The monoclonal antibody N52 selectively labels large diameter, myelinated neurons in native sensory ganglia. The majority of CB1 mRNA-positive neurons were N52-immunoreactive (75.1% ± 6.9) while 39.9% ± 7.3 of N52-immunoreactive neurons also contained CB1 mRNA (Fig. 6). This colocalization is depicted through a representative photomicrograph in Fig. 6.
DISCUSSION

In the present studies, the neuronal distribution and neurochemical colocalization of CB1 mRNA in TG was evaluated. The purpose of this investigation was to elucidate the neurochemical phenotype of certain cannabinoid-responsive trigeminal sensory neurons and, therefore, help to inform efforts to deduce the mechanisms that subserve peripheral cannabinoid analgesia. To this end, we found that approximately 30% of TG neurons contain CB1 mRNA and that the majority of these give rise to fibers in the maxillary and mandibular divisions of the trigeminal nerve, while we found no evidence for the existence of CB2 transcripts in TG. Interestingly, the size distribution for CB1 mRNA-positive neurons indicates that these cells are likely members of the rapidly conducting Aβ-fiber type involved with light touch and vibration sensation (Harper and Lawson, 1985; Lawson et al., 1993). Nevertheless, because the focus of peripheral actions of cannabinoids has been largely concentrated on nociception, we set out to explore the colocalization of CB1 mRNA with a number of markers that help define various nociceptor populations.

To directly assess the distribution of CB1 mRNA in a de facto nociceptive neuronal population, colocalization of CB1 mRNA with VR1 immunoreactivity was explored. VR1-positive sensory neurons encode sensory information
pertaining to noxious heat and pH and are thought to be, by definition, nociceptors. Only 5% of CB1 positive cells contained VR1 immunoreactivity, while even fewer VR1 immunopositive cells contained CB1 mRNA, indicating that only 1.5% of all TG neurons contain both CB1 mRNA and VR1 immunoreactivity. These results in native sensory ganglion tissue disagree with those from a study in cultured DRG neurons, wherein nearly all neurons containing CB1 immunoreactivity also contained VR1 immunoreactivity (Ahluwalia et al., 2000). This discrepancy may be due to a lack of antibody specificity and/or changes in neuronal phenotype that occur with neuronal culturing. In any case, such disparities point out important caveats to the comparison and interpretation of in vivo cellular localization data from primary cultures and native tissues.

In addition, IB4 binding sites, which are markers for a subset of unmyelinated nociceptors, rarely colocalized with CB1 mRNA. These data indicate that CB1 receptors are likely to be found mostly outside both the VR1 and IB4-binding nociceptor populations. Neuronal expression of the neuropeptides CGRP and SP roughly defines the peptidergic class of primary sensory neurons, many of which are thought to be nociceptive (Averill et al., 1995). Here again, very little colocalization was found between CB1 mRNA and either CGRP or SP immunoreactivity, in agreement with similar studies in DRG (Hohmann and Herkenham, 1999b).

Because we saw very little overlap between the neuronal markers examined here and CB1 mRNA, and due to the neuronal size of the CB1-positive population, we examined CB1 colocalization with the marker of myelinated sensory neurons, N52. More than 75% of CB1-positive neurons contained N52-immunoreactivity, indicating that CB1 is found nearly exclusively in myelinated, large diameter neurons. Furthermore, CB1 mRNA is in nearly half of the N52-positive population indicating that cannabinoid compounds acting through CB1 receptors may be major modulators of neurotransmission in this subclass of sen-
sory neurons. These findings further support the notion that CB1 receptors are found predominantly in Aβ-fibers.

A growing body of evidence suggests that cannabinoids inhibit peripheral nociceptor activation. Thus, the endocannabinoid anandamide inhibits capsaicin-evoked CGRP release from rat hind paw skin in vitro (Richardson et al., 1998), and the nonselective cannabinoid receptor agonist WIN 55,212-2, injected directly into the hind paw, inhibits mechanical allodynia and thermal hyperalgesia induced by capsaicin injection in vivo (Johanek et al., 2001). Similarly, utilizing the partial sciatic ligation model of neuropathic pain, Fox et al. (2001) showed that intraplantar injection of WIN 55,212-2 decreased mechanical allodynia. Collectively, these data indicate that cannabinoids activate peripheral mechanisms that reduce nociception and, taken together with the present results, that such mechanisms likely do not involve the direct inhibition of nociceptors via CB1 activation.

In spinal dorsal horn slice preparations, either anandamide or WIN 55,212-2 attenuated mEPSCs evoked by capsaicin perfusion, indicating that cannabinoid agonists also are capable of inhibiting the central terminals of capsaicin-sensitive primary afferents (Morisset and Urban, 2001). However, in the medullary dorsal horn, WIN 55,212-2 inhibited only GABAergic and glycinegic but not glutamatergic neurotransmission, suggesting that CB1 receptors are present on interneurons but not on primary sensory inputs to this region (Jennings et al., 2001). This is particularly significant, because it indicates a potential difference between the spinal and trigeminal systems with respect to cannabinoid-mediated inhibition of nociceptive neurotransmission, which is supported by the finding here that CB1 mRNA is not expressed by putative nociceptive primary afferents of the TG.

Clearly cannabinoid agonists have effects on primary sensory neurons in the nociceptive pathway. The specific receptors through which these actions are mediated, as well as the mechanism of action through which this inhibition occurs, however, remain a point of scrutiny. Because colocalization of CB1 with the putative nociceptor markers explored here is sparse, it seems most probable that cannabinoid agonists do not act directly on nociceptors to inhibit their activation, at least not via CB1 receptors. However, a number of other possibilities exist. The evidence presented here indicates that CB1 receptors are likely found predominantly on Aβ-fibers. It has been shown that Aβ fiber stimulation in inflamed rat skin induces CGRP-mediated vasodilatation caused by antidromic firing of C-fibers stimulated by the Aβ-fiber afferent barrage at the level of the spinal dorsal horn (Garcia-Nicas et al., 2001). Furthermore, non-noxious Aβ-fiber stimulation prolongs mechanical hyperalgesia induced by topical capsaicin application (from 2 to 24 h) to rat hind paw skin (Kim et al., 2001). Hence, the CB1-mediated antihyperalgesic actions of cannabinoid agonists might be mediated by inhibition of Aβ-fiber firing rates, which, in turn, decrease nociceptor activation through as yet undescribed processing events at the level of the dorsal horn.

However, inhibition of Aβ-fibers does not explain all of the evidence for cannabinoid inhibition of nociceptor activation. A number of recent reports have described cannabinoid effects in the CB1 knockout mouse. For example, Hajos et al. (2001) demonstrated that CB1−/− mice display WIN 55,212-2-mediated inhibition of evoked EPSCs from hippocampal glutamatergic terminals, and this effect was blocked by SR141716A. This evidence is further supported by the demonstration of a SR141716A-sensitive cannabinoid receptor in CB1−/− mouse brain membranes using GTPγS binding (Breivogel et al., 2001). While peripheral effects of cannabinoid agonists on nociceptive responses have not been explored in sensory neurons of CB1−/− mice, the data shown here, when considered in the context of cannabinoid effects on nociceptors that are sensitive to SR141716A, raise the possibility

---

**Fig. 5.** Summary data for colocalization of CB1 mRNA with CGRP-, SP- or VR1-immunoreactive or IB4-binding neurons. TG sections were double-labeled as described in Experimental Procedures and colocalization was assessed. Colocalization is expressed as mean percentage ± S.E.M. (n = 3 sections) of all neurons that were positive for one analyte and that also contained signal for another analyte.
of a novel cannabinoid-responsive receptor with similar pharmacology to CB1 that is expressed by nociceptive primary sensory neurons. Interestingly, while some studies found that the peripheral antihyperalgesic effects of cannabinoids were blocked by the CB1 selective antagonist SR141716A (Richardson et al., 1998), another found only partial (60%) inhibition (Johanek et al., 2001), consistent with a non-CB1 receptor mechanism for peripheral cannabinoid effects on nociception.

In conclusion, we have demonstrated that CB1 mRNA is expressed in a subset of primary sensory afferents in TG. Somewhat surprisingly, based on cell size distributions and colocalization analysis, CB1 receptors in TG likely are restricted almost entirely to neurons that give rise to Aβ fibers that are not involved in nociception in the normal animal and that are generally not in the ophthalmic division of the trigeminal nerve. Interestingly, a subset of large diameter, CGRP-negative neurons that exhibit calbindin D-28K immunoreactivity and that innervate myelinated fibers of the tooth pulp (Ichikawa et al., 1996) and the palatal mucosa (Ichikawa and Sugimoto, 1997) are also found almost exclusively in the maxillary and mandibular divisions of the rat TG (Ichikawa et al., 1996). While colocalization between CB1 mRNA and calbindin D-28K immunoreactivity was not explored here, the similarities between their patterns of localization and neuronal size distributions merit attention. Further studies are required to define the precise neurochemical and functional phenotype of CB1 containing neurons in TG as well as the mechanisms by which CB1 activation in these neurons may underlie peripherally-mediated antinociception. Such an understanding will be critical to the research and development of a peripherally active analgesic drug class.

Acknowledgements—The authors wish to thank Dr. Lisa Matsuda and Dr. Mary Abood for providing the CB1 and CB2 cDNA constructs, respectively, used as templates for the CB1 and CB2 subclones used for riboprobe synthesis. The authors also thank Dr. Evangeline Loh for assistance in the creation of the CB1 subclone used for riboprobe synthesis.

REFERENCES


(Accepted 6 April 2003)