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Characterisation of cannabinoid 1 receptor expression in the perikarya, and peripheral and spinal processes of primary sensory neurons

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Abstract

The cannabinoid 1 (CB1) receptor is expressed by a sub-population of primary sensory neurons. However, data on the neurochemical identity of the CB1 receptor-expressing cells, and CB1 receptor expression by the peripheral and central terminals of these neurons are inconsistent and limited. We characterised CB1 receptor expression in dorsal root ganglia (DRG) and spinal cord at the lumbar 4–5 level, as well as in the urinary bladder and glabrous skin of the hindpaw. About 1/3 of DRG neurons exhibited immunopositivity for the CB1 receptor, the majority of which showed positivity for the nociceptive markers calcitonin gene-related peptide (CGRP) or/and *Griffonia* (bandeiraea) *simplicifolia* IB4 isolectin-binding. Virtually all CB1 receptor-immunostained fibres showed immunopositivity for CGRP in the skin, while almost none did in the urinary bladder. No CB1 receptor-immunopositive nerve fibres were IB4 positive in either

peripheral tissue. Spinal laminae I and II-outer showed the highest density of CB1 receptor-immunopositive punctae, the majority of which showed positivity for CGRP or/and IB4 binding. These data indicate that a major sub-population of nociceptive primary sensory neurons expresses CB1 receptors that are transported to both peripheral and central terminals of these cells. Therefore, the present data suggest that manipulation of endogenous CB1 receptor agonist levels in these areas may significantly reduce nociceptive input into the spinal cord.

Keywords

Anti-nociception; Somatosensory; Viscerosensory; Peptidergic; Non-peptidergic

Introduction

The cannabinoid 1 (CB1) receptor is a member of the G protein-coupled cannabinoid receptor family (Howlett et al. 2002). Cannabinoid receptors respond to specific terpenoids found in the plant, *Cannabis sativa*, as well as to functionally related endogenous compounds, called endocannabinoids (Di Marzo et al. 1998; Lauckner et al. 2008; Matsuda et al. 1990; Munro et al. 1993; Pertwee 2005). The CB1 receptor is expressed predominantly by neurons (Matsuda et al. 1990; Howlett 1998; Di Marzo et al. 1998; Mackie 2005; Pertwee 2005). At the cellular level, CB1 receptor activation results in reduced adenylate cyclase activity, and the respective attenuation and enhancement of the activity of several voltage-gated Ca^{2+} and K^{+} channels (Matsuda et al. 1990; Mackie and Hille 1992; Deadwyler et al. 1995; Mackie et al. 1995; Twitchell et al. 1997; Di Marzo et al. 1998; Pertwee 2005). This reduces neuronal excitability and neurotransmitter release (Ahluwalia et al. 2003a; Ellington et al. 2002; Di Marzo et al. 1998; Pertwee 2005; Mahmud et al. 2009; Morisset and Urban 2001; Richardson et al. 1998b; Sagar et al. 2005; Santha et al. 2010a; Soneji et al. 2010; Fischbach et al. 2007).

Previous studies indicated that a major sub-population of dorsal root ganglion neurons, which express various nociceptive markers, such as calcitonin gene-related peptide (CGRP) and binding site for the isolectin B4 (IB4) from *Griffonia (bandeiraea) simplicifolia*, express CB1 receptors (Agarwal et al. 2007; Hohmann and Herkenham 1999; Khasabova et al. 2002; Bridges et al. 2003; Price 1985; Silverman and Kruger 1988; Ahluwalia et al. 2000, 2002; Amaya et al. 2006; Binzen et al. 2006; Mittrirattanakul et al. 2006). In agreement with this expression pattern, application of CB1 receptor agonists to primary sensory neurons reduces depolarisation or TRPV1 activation-evoked release of glutamate and nociception-related neuropeptides, such as substance P (SP) and CGRP, and the activity of TRPV1 (Morisset et al. 2001; Morisset and Urban 2001; Richardson et al. 1998a; Ellington et al. 2002; Mahmud et al. 2009; Santha et al. 2010b; Ahluwalia et al. 2003a; Soneji et al. 2010; Sagar et al. 2005; Fischbach et al. 2007). Behaviourally, CB1 receptor agonists applied to primary sensory neurons produce an anti-nociceptive effect (Sagar et al. 2005; Amaya et al. 2006; Calignano et al. 1998; Richardson et al. 1998a; Jaggar et al. 1998; Khasabova et al. 2008; Agarwal et al. 2007). Accordingly, deletion of the CB1 receptor, specifically from NaV1.8-expressing primary sensory neurons, significantly reduces CB1 receptor agonist-induced anti-nociception (Agarwal et al. 2007). Taken together, these data indicate that peripherally acting CB1 receptor agonists might represent a novel class of analgesics. However, in spite of a great deal of effort studying the possible site of action of peripherally applied CB1 receptor agonists, the available data regarding the proportion and type of primary sensory neurons expressing this receptor are inconsistent (Agarwal et al. 2007; Ahluwalia et al. 2000, 2002; Amaya et al. 2006; Bridges et al. 2003; Binzen et al. 2006; Hohmann and Herkenham 1999; Khasabova et al. 2002; Lever et al. 2009; Mittrirattanakul et al. 2006; Price 1985). Further, data on the characteristics of CB1 receptor expression by the

peripheral and central terminals of primary sensory neurons are limited and also inconsistent (Salio et al. 2002; Pernia-Andrade et al. 2009; Khasabova et al. 2004; Farquhar-Smith et al. 2000; Sanudo-Pena et al. 1999; Ong and Mackie 1999; Stander et al. 2005; Nyilas et al. 2009; Hegyi et al. 2009; Walczak et al. 2009; Salio et al. 2001). However, these data are essential because different types of primary sensory neurons vary in their functions and responses to pathological events (Silverman and Kruger 1988; Plenderleith and Snow 1993; Bennett et al. 1996; Dirajlal et al. 2003; Breese et al. 2005; Choi et al. 2007; Price 1985). Therefore, in the present study, we aimed to assess and characterise CB1 receptor expression in the perikarya and peripheral and central processes of primary nociceptive sensory neurons, which were identified by markers of the two main sub-populations of nociceptive primary sensory neurons, CGRP immunopositivity and IB4 binding (Price 1985; Silverman and Kruger 1988).

Materials and methods

Animals and tissue preparations

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, the revised National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, the European Communities Council Directive (86/609/EEC) and the guidelines of the Committee for Research and Ethical Issues of IASP, published in Pain, 16 (1983) 109–110. Furthermore, all experiments were approved by the Ethics Committee of the University of Debrecen, Debrecen, Hungary, and the Animal Subjects Review Board of the University of Porto, Porto, Portugal. All efforts were made to minimise the number of animals used in the present study. Altogether, ten rats were killed (Wistar-Kyoto, 250–300 g; 6 rats were used for immunohistochemistry and 2 and 2 animals for DNA and protein preparation for RT-PCR and Western blot, respectively). In addition, three wild-type (C57BL/6 J) and three CB1^{-/-} mice (gift from the Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary, transfer approved by Dr. Andreas Zimmer) were used in the experiments.

For reverse transcriptase polymerase chain reaction (RT-PCR), samples of the hippocampus, L4–5 dorsal root ganglia (DRG), dorsal and ventral part of the L4–5 spinal cord, glabrous hindpaw skin and urinary bladder were collected from killed rats (intraperitoneal pentobarbital injection; 100 mg/kg). Tissue samples were immersed in RNA-later (Ambion, Inc., TX, USA) immediately after the dissection and stored at –20 °C until use.

For Western blotting, L4–5 spinal cord, L4–5 DRG, glabrous hindpaw skin, urinary bladder and hippocampus samples were removed from the killed rats. The samples were pulverised in liquid nitrogen and solubilised in ice-cold 20 mM TRIS, 1 mM EDTA (pH 7.5) containing 1 % NP-40, 0.5 % deoxycholic acid and 0.1 % SDS, supplemented with protease inhibitors (0.1 mg/ml benzamide, 1 mM phenyl-methyl-sulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml pepstatin A and 5 µg/ml aprotinin). After 2 h of gentle rocking at 4 °C, cellular debris were removed by centrifugation (16,000 rpm, 10 min). The supernatant was stored at –20 °C until Western blotting.

For immunohistochemistry, six rats and three wild-type and three CB1 receptor^{-/-} mice were deeply anesthetized with pentobarbital (100 mg/kg) and transcardially perfused with Tyrode's solution followed by 4 % paraformaldehyde, in 0.1 M phosphate buffer (0.1 M PB, pH = 7.4). The lumbar 4–5 DRG, the L4–5 spinal cord segment, the hippocampus, the urinary bladder and glabrous hindpaw skin were removed and kept in the same fixative for 4 h at 4 °C. Tissue blocks were first immersed in 20 % and then 40 % sucrose dissolved in 0.1 M PB until they sank. Fifty µm sections from DRG, spinal cord and hippocampus and 40-

µm sections from the urinary bladder were cut on a cryostat and stored in 30 % sucrose at 4 °C until immunostaining.

RT-PCR

Total RNA was isolated using the RNeasy micro kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. For first-strand cDNA synthesis, the Omni-script RT kit (Qiagen GmbH, Germany) was used with 1 µg of total RNA and oligo d(T) primer. CB1-specific primers were designed using the rat CB1 gene (accession no. NM_102784 of the NCBI database). To distinguish amplification of genomic DNA, the primer pairs were placed at the 5' UTR of exon 1 and exon 2, respectively. The primer sequences were: 5' CAAGCAAGGAGCACC CAT (sense primer) and 5' TGAAGGAGGCTGTAACCC (anti-sense primer). The predicted product size was 691 bp. Amplification of β-actin was used as a control for the reaction. The amplicon for β-actin had a 317 bp predicted length, and the primer sequences were as follows: actin sense 5'-TGCCTGACATTAAAGAGAAG-3' and actin antisense 5'-CTGCATCCTGTCAGCAATG-3'. The reaction was performed with Tth polymerase (Promega Corp., WI, USA) and included a 4-min incubation at 95 °C, followed by 30 cycles of 40 s at 94 °C, 1 min at 58 °C, 1.5 min at 70 °C and a final extension step of 5 min at 70 °C. The amplified PCR fragments were separated on 1.5 % agarose gel (Sigma-Aldrich Co., MO, USA) in 1×TBE buffer and stained with acridine-orange dye and documented by a MiniBis Pro geldoc System (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Western blotting

Protein samples were dissolved in reducing sample buffer (50 µg protein/lane) and run on 10 % SDS–polyacrylamide gels. The separated protein bands were electrophoretically transferred onto PVDF membrane (Millipore, Bedford, USA). The membranes were blocked with 10 % normal rabbit serum (Vector, Burlingame, USA) in TTBS solution (20 mM TRIS, 500 mM NaCl, pH 7.5, and 0.05 % Tween-20) and then incubated with an anti-CB1 receptor antibody (raised in guinea pig) for 2 h at room temperature (Berghuis et al. 2007). After extensive washes with TTBS, the membranes were incubated with anti-guinea pig IgGs (DAKOCytomation, Glostrup, Denmark). The labelled protein bands were visualised with 3, 3'-diaminobenzidine (Sigma).

Immunohistochemistry

Free floating sections of DRG, spinal cord, hippocampus and urinary bladder were washed in 0.1 M PB and then transferred into 50 % ethanol for 30 min. Following washes in 0.1 M PB, sections were incubated in 10 % normal donkey serum (Vector Laboratories, Burlingame, CA, USA) for 50 min. Sections were then incubated in primary antisera overnight at room temperature, except sections from the urinary bladder, which were incubated for 48 h at 4 °C.

The following antisera were used either alone or in combination: anti-CB1 receptor antibodies raised in guinea pig or goat. These antibodies were produced in Professor Mackie's laboratory and tested thoroughly (Martini et al. 2010; Rozenfeld and Devi 2008; Berghuis et al. 2007). The immunogen was a GST fusion protein containing the last 72 residues of the rat CB1 receptor (NP_036916; SKD LRHAFRSMFSPCEGTAQPLDNSMGDSCLHKHANN TASMHRAAESCIKSTVKIAKVTMSVSTDTSAEAL). This sequence is 100 and 97 % identical with the last 72 amino acids of the mouse (NP_031752) and human CB1 receptor (NP_001153731.1), respectively. Both anti-CB1 receptor antibodies were used in 1:500 dilution for sections cut from DRG, skin and spinal cord. The goat CB1 receptor antibody was used at a 1:8000 dilution for sections cut from the urinary bladder. The anti-CGRP

antibody that was raised in rabbit was purchased from Peninsula Laboratories (CA, USA—Cat. No: T-4031), immunising peptide sequence SCNTATCVTHRLAGLLSRGGVVDNFVPT NVGSEAF, and used in 1:1000 dilution. In addition to the antibodies, a 1:2000 dilution of biotinylated IB4 (Invitrogen, CA, USA – Cat. No.:I21414) was used to identify the IB4-binding sub-population of primary sensory neurons and their processes. The immunoreactions were visualized using species-specific secondary antibodies conjugated to fluorescent dyes at room temperature for 2 h: donkey anti-rabbit Alexa 555 (1:1000, Invitrogen, CA, USA), donkey anti-guinea pig Alexa 647 (1:1000, Invitrogen, CA, USA) donkey anti-rabbit Alexa 488 (1:1000, Invitrogen, CA, USA) and donkey anti-goat Alexa 568 (1:1000, Invitrogen, CA, USA). IB4-binding was visualized by streptavidin-conjugated Alexa 488 (1:2000, Invitrogen, CA, USA). Sections were then collected onto slides, mounted with Vectashield (Vector, Vectashield, Burlingame, USA) and coverslipped.

Samples from the glabrous hindpaw skin were embedded into paraffin and 10- μ m sections were cut with a microtome. Sections were mounted on glass slides. The paraffin was then removed with xylene and the sections were re-hydrated. Immunostaining was then processed as described above. The skin slides were mounted using Vectashield containing 1.5 μ g/ml DAPI (Vector, Vecta-shield, Burlingame, USA) to show the nuclei and help the orientation in these slides.

Controls for the CB1 receptor antibody included staining of hippocampal or DRG sections from wild-type and CB1 knockout mice. In addition, the two CB1 receptor antibodies were combined for staining of hippocampal sections. For all antibodies, control staining was also done by replacing the primary antibody with 1 % normal donkey serum. Exhausted CB1 receptor antibodies were also used in control staining. No immunostaining could be observed when the primary antibodies were replaced by normal serum or when exhausted antibodies were used (not shown).

Confocal microscopy and image analysis

Fluorescent images were taken either with an Olympus FV1000S S or a Biorad 1024 confocal microscope using appropriate laser lines. Multiple images of 2- μ m optical thickness were taken in a sequential mode using 20 $\times$ or 40 $\times$ oil immersion objectives. Fluorescent signals were detected in three separate channels by the use of dichroic mirrors and properly set spectral detectors. The average pixel time during data acquisition was 4 μ s/pixel to provide high signal/noise ratio.

Analysis of immunostaining in DRGs was done on perikarya of primary sensory neurons showing visible nuclei. Measurements were made using the optical section in which the neuron had the largest diameter. The region of interest was manually selected by excluding the nucleus of the cell. Region of interests were analysed using the ImageJ software package (Abramoff et al. 2004). In addition to the average fluorescent intensity, the maximum diameter of the individual cell was also measured.

The pooled intensity histogram of all cells was used to distinguish negative and positive cells based on the separation of their respective Gaussian distributions. The subtracted background intensity was measured in a large area where no tissue was present. The relative number of immunopositive cells was then established. Co-localisation of the immunoreactions was established using immunopositivity and negativity of cells determined on each channel by the intensity threshold.

Statistics

The relative number and longest diameter of labelled and non-labelled cells were established in each animal, and data were then averaged. Data were compared by ANOVA. Statistical significance of the differences was established by Fisher's least significant test. Differences were regarded as significant at $p < .05$. Data are expressed as mean $\pm$ standard error of the mean.

Results

RT-PCR

In order to find tissues in which CB1 receptor mRNA is present and transcription likely occurs, first we studied CB1 receptor mRNA expression. RT-PCR revealed detectable levels of CB1 mRNA in the hippocampus, DRG, ventral and dorsal spinal cord, urinary bladder and skin (Fig. 1 upper gel). In all tissues, the size of the PCR product was indistinguishable from the expected product size of 691. These findings confirmed previous data that in addition to primary sensory neurons, CB1 receptor mRNA was expressed in the hippocampus and dorsal and ventral spinal cord and skin (Mailleux and Vanderhaeghen 1992; Hohmann and Herkenham 1999; Bridges et al. 2003). Furthermore, these findings also suggested that the CB1 receptor mRNA was expressed in the urinary bladder (Tyagi et al. 2009; Walczak et al. 2009).

Immunoblotting

To confirm that the CB1 receptor mRNA is translated into protein in the tissues in which we revealed its expression, we used anti-CB1 receptor antibody in Western blotting. The results showed the presence of two major immunoreactive proteins in all tissues: one, approximately 50–55 kDa, and a second, approximately 65 kDa (Fig. 1 lower gel) protein. Interestingly, in samples from the hippocampus, the spinal cord and the urinary bladder, a third, more or less distinctive band, near 50 kDa, was also revealed by Western blotting. The 50–55 kDa and 65 kDa proteins correspond to the predicted size of the non-glycosylated and glycosylated forms of the CB1 receptor, respectively (Song and Howlett 1995). These data confirmed that the CB1 receptor mRNA is translated into protein in all the tissues we examined.

Immunohistochemistry

Controls—It has been well established that the hippocampus contains a dense network of CB1 receptor-expressing fibres (Westlake et al. 1994; Egertova and Elphick 2000; Hoffman et al. 2003; Katona et al. 2006). Therefore, for the control of the specificity and selectivity of the anti-CB1 receptor antibodies, first, we stained hippocampal slices of rat and wild-type mouse with both guinea pig and goat CB1 receptor antibodies. As expected, the antibodies revealed the characteristic CB1 receptor expression pattern in both the rat and wild-type mouse hippocampus (Fig. 2a, b). However, no CB1 receptor immunopositivity was seen with either of the antibodies in hippocampal sections taken from the brain of CB1 receptor knockout mice (Fig. 2c).

Next, we used the CB1 receptor antibody raised in guinea pig for staining DRG sections of wild-type and CB1 receptor knockout mice. In wild-type mouse DRG, the antibody revealed several intensely stained perikarya of primary sensory neurons (Fig. 2d). However, no CB1 immunopositivity was seen in sections prepared from CB1 receptor knockout mouse DRGs (Fig. 2e). These findings indicated that the CB1 receptor antibody raised in guinea pig specifically and selectively recognised the CB1 receptor in the mouse nervous system.

Furthermore, these data also suggested that the CB1 receptor antibody we used specifically and selectively recognised the CB1 receptor in rat tissues.

In the final control experiment, we studied whether the two anti-CB1 receptor antibodies gave the same signal. We immunostained rat hippocampal sections with the two anti-CB1 receptor antibodies. Although the intensity of co-staining with the two antibodies showed some differences from pixel to pixel, it was evident that the two antibodies recognised the same target, the CB1 receptor (Fig. 3). Also, there are some differences between the staining pattern obtained with these two antibodies and those reported previously (Mackie 2005; Monory et al. 2006). Antibodies raised against different epitopes of the CB1 receptor and the use of different fixation are known to produce differences in CB1 receptor staining pattern in tissues, including the hippocampus. Nevertheless, the pattern of staining shown in Fig. 3 is consistent with the most widely reported patterns of hippocampal CB1 immunoreactivity (Puighermanal et al. 2009).

Dorsal root ganglia

Next, we used the anti-CB1 receptor antibody raised in guinea pig to assess the expression of this receptor in rat DRG. The antibody produced a clear and easily recognisable cytoplasmic and membrane labelling in all sections (Fig. 4a, Fig. 5b, d). The staining appeared patchy, both in the cytoplasm and on the membrane. Visual inspection suggested that about 1/3 of the perikarya of primary sensory neurons expressed detectable CB1 receptor. Visual inspection also indicated that the great majority of the CB1 receptor-immunopositive neurons were small and medium-sized cells.

The image analysis confirmed that 1/3 of the total number of neurons were CB1 receptor immunopositive ($33.22 \pm 1.8 \%$, 124 of 377 collected from 3 animals). The average diameter of the cells that were regarded as CB1 receptor positive was $31.47 \pm 0.64 \mu\text{m}$ ($n = 124$). The average diameters of the cells judged as CB1 receptor immunonegative were $36.03 \pm 1.91 \mu\text{m}$, ($n = 253$). The difference between the size of the immunopositive and immunonegative cells was significant ($p < 0.05$).

The size distribution of the CB1 receptor-immunopositive and -immunonegative neurons (Fig. 4b) showed that more than half of the small neurons and about half the medium-sized neurons expressed CB1 receptors. Very few CB1 receptor-immunopositive cells were seen among the large cells.

Next, the co-staining pattern produced by the anti-CB1 receptor antibody, the anti-CGRP antibody and biotinylated IB4 was characterised. The CGRP antibody produced clearly visible immunolabelling in primary sensory neurons (Fig. 5a, d). The labelling appeared in large vesicles in the cytoplasm. Visual inspection of the sections suggested that about 1/3 of the neurons were immunopositive for CGRP and that the majority of the positive cells were small/medium diameter neurons. Quantitative image analysis showed that $32.17 \pm 0.75 \%$ (121 out of 377 neurons collected from 3 animals) of the total neuronal population was immunolabelled with the CGRP antibody (Fig. 5e). Results of the image analysis furthermore revealed that the average largest diameter of the CGRP-immunopositive and -immunonegative cells was significantly different ($p < 0.05$), $29.5 \pm 0.75 \mu\text{m}$ ($n = 121$) and $36.29 \pm 1.31 \mu\text{m}$ ($n = 256$), respectively. Comparison of CB1 receptor and CGRP immunopositivity in the sample showed that while $61.14 \pm 5.2 \%$ ($n = 3$) of the CGRP-expressing cells were immunopositive for CB1, $62.99 \pm 5.3 \%$ ($n = 3$) of CB1 receptor-expressing cells were immunopositive for CGRP.

Biotinylated IB4 also produced clear labelling in a sub-population of dorsal root ganglion neurons (Fig. 5c, d). By visual inspection, the proportion of neurons that bound IB4 was

about 1/3 of the total neuronal population. The IB4-binding neurons were small cells. Image analysis showed that the average relative number of the IB4-positive neurons was 33.78 ± 2.55 % (125 out of 377 neurons collected from 3 animals). The average diameter of the IB4-binding cells (31.18 ± 1.4 μm , $n = 127$) was significantly ($p < 0.05$) smaller than that of the IB4-negative cells (36.41 ± 1.14 μm ; $n = 250$). Comparison of IB4 binding and CB1 receptor immunopositivity showed that while 34.48 ± 9.4 % ($n = 3$) of the IB4-binding cells expressed the CB1 receptor, 34 ± 8.8 % ($n = 3$) of the CB1-immunolabelled cells bound IB4.

When all three reactions were analysed together, we found that 7 ± 1.2 % of the total cells population showed positivity for both CGRP and IB4 ($n = 3$). This proportion of neurons corresponded to 20.6 ± 2.89 % ($n = 3$) and 21.91 ± 4.05 % of the IB4-binding and the CGRP-immunopositive cells, respectively. Of the CGRP and IB4 double-positive cells, about 3/4 (5.2 ± 0.7 % of the total neuronal population; $n = 3$) were also positive for CB1 receptor staining. Of the CGRP and IB4 double-negative cells, about 1/6 (6.32 ± 2.7 % of the total neuronal population; $n = 3$) were immunopositive to the CB1 receptor.

Taken together, the data on dorsal root ganglia showed that the CB1 receptor was expressed in a well-defined sub-population of primary sensory neurons and that the CB1 receptor-expressing cells belonged to the CGRP-expressing or IB4-binding nociceptive neurons. Furthermore, these data also showed that CB1 receptor expression has a preference for the peptidergic sub-population of neurons.

Peripheral tissues

CB1 receptor immunostaining labelled two types of structures in the skin. First, in agreement with previous findings, keratinocytes in the basal layer of the epidermis showed CB1 receptor immunostaining (Fig. 6a) (Biro et al. 2009; Maccarrone et al. 2003). Second, in the dermis and subcutaneous connective tissue, CB1 receptor expression was also present in nerve fibres (Fig. 6b) (Stander et al. 2005). All the CB1 receptor-immunopositive fibres appeared to be thin fibres. Moreover, virtually all the CB1 receptor-immunopositive fibres were CGRP immunopositive (Fig. 6c – e). No IB4-positive nerve fibres were found in the skin samples we examined (not shown). In addition to the nerve fibres, occasional round and oval-shaped CB1 receptor immunopositive cells were encountered in the dermis and subcutaneous connective tissue (not shown). Based on previous data (Biro et al. 2009; Stander et al. 2005), these cells were regarded as mast cells and histiocytes. Identification and differentiation of these cells were not conducted, as these were beyond the scope of this work.

In the urinary bladder, CB1 receptor immunoreactivity was found in the mucosa, in varicose nerve fibres below the transitional epithelium (Fig. 7). Some CB1 receptor-immunoreactive fibres were also found in the muscular layer and around blood vessels (not shown). More CB1 receptor-immunoreactive fibres were observed in the trigone and bladder neck than in the body and dome. Unexpectedly, double labelling with CGRP showed only a partial co-localisation between CGRP and the CB1 receptor (Fig. 7a – c). Similarly to our previous findings (Tyagi et al. 2009; Walczak et al. 2009), no IB4 + fibres were seen in the samples we examined (not shown).

Spinal cord

The CB1 receptor antibody produced distinctive punctate labelling in the dorsal horn of the spinal cord (Fig. 8a, d, e). The density of the punctae was the highest in Rexed's lamina I and outer lamina II (Fig. 8a, d). The punctae in many cases seemed to be arranged around oval-shaped, non-stained areas (Fig. 8e, f), giving the impression that the CB1 receptor was

expressed either at the membrane of, or on fibres impinging upon, dorsal horn neurons. In the deeper layers of the dorsal horn, both the intensity and density of the punctae were weaker than in the superficial laminae (Fig. 8a, d).

In the superficial dorsal horn, the great majority of the CB1 receptor-immunopositive punctae were also positive to CGRP (Fig. 8d, f). Occasionally, CB1 receptor-immunolabelled punctae also showed IB4 binding (Fig. 8d, f). In addition, punctae showing only CB1 receptor immunopositivity were also often encountered (Fig. 8d, f). However, the number of these CB1 receptor single-labelled punctae appeared less than the punctae showing double labelling. Nevertheless, in summary, these findings indicated that the CB1 receptor was amply transported to the central terminals of nociceptive primary sensory neurons.

Discussion

Here, we assessed and characterised CB1 receptor expression in primary sensory neuronal perikarya, central processes and those peripheral processes innervating somatic and visceral tissues. First, we studied the presence of CB1 receptor mRNA and protein in DRG and a selection of tissues containing the processes of these neurons. Our RT-PCR and Western blotting experiments revealed the presence of both CB1 receptor mRNA and protein in DRG, skin, urinary bladder and spinal cord. These findings are in agreement with previous reports that while a sub-population of DRG neurons express the CB1 receptor, which might be transported from the perikarya to both terminals of primary sensory neurons, various cells in the skin, urinary bladder and spinal cord also express the CB1 receptor (Hohmann and Herkenham 1999; Farquhar-Smith et al. 2000; Ahluwalia et al. 2000, 2002; Khasabova et al. 2002; Bridges et al. 2003; Casanova et al. 2003; Binzen et al. 2006; Mitirattanakul et al. 2006; Agarwal et al. 2007; Merriam et al. 2008; Hegyi et al. 2009; Lever et al. 2009; Amaya et al. 2006; Ong and Mackie 1999; Sanudo-Pena et al. 1999; Salio et al. 2002; Stander et al. 2005; Nyilas et al. 2009; Pernia-Andrade et al. 2009; Walczak et al. 2009; Biro et al. 2009; Maccarrone et al. 2003).

For studying the characteristics of primary sensory neurons expressing the CB1 receptor in the present study, we used two anti-CB1 receptor antibodies raised against the same epitope of the molecule. While both antibodies produced similar staining patterns in rat and wild-type mouse hippocampus and DRG, neither of them produced staining in either the hippocampus or DRG of CB1 receptor KO mice. These findings indicated that both antibodies identified the same antigen, the CB1 receptor. Hence, both of them produced specific and selective staining. However, it is well established that G protein-coupled receptors, including the CB1 receptor, are subject of homo- and heteromultimerisation and other protein-protein interactions, which may result in structural changes or epitope screening, decreasing epitope accessibility (Mackie 2005; Fuxe et al. 2009). Therefore, we cannot exclude the possibility that CB1 receptors in various molecular complexes remained unidentified by the antibodies we used in the present study. Hence, our data may include false negative findings. Nevertheless, in the context of selectivity and specificity of the immunoreactions, we should also note that the proportion and distribution of the CGRP-immunopositive and the IB4-positive neurons found in DRG in the present study are in good agreement with previously published data (Price 1985; Silverman and Kruger 1988).

Analysis of the CB1 receptor immunostaining in DRG revealed that about a third of the perikarya of primary sensory neurons showed CB1 receptor immunopositivity. The CB1 receptor-immunopositive neurons were small or of medium size. Moreover, CB1 receptor immunostaining occurred almost exclusively in CGRP- or IB4-positive neurons. Analysis of the co-expression of the markers also revealed that about 2/3 of the CGRP-immunopositive

and 1/3 of the cells with IB4-binding sites exhibited CB1 receptor immunopositivity. Though some CGRP-containing cells are not nociceptive in function (Lawson et al. 2002), these findings indicate that a major proportion of the CB1 receptor-expressing neurons identified by the anti-CB1 receptor antibodies we used in the current experiment belong to the nociceptive cells. Further, these data also suggest that about a half of all nociceptive cells express the CB1 receptor at detectable levels.

Data from previous in situ hybridisation and immuno-labelling studies showed that, in a physiological setting, CB1 receptor-expressing primary sensory neurons (1) comprise about 1/4–1/3 of the total sub-population of the cells, and the majority of them belong to the non-nociceptive sub-population of neurons (Hohmann and Herkenham 1999; Khasabova et al. 2002; Bridges et al. 2003; Price et al. 2003); (2) comprise about 30–50 % of the total neuronal population and the majority of them are nociceptive in function (Ahluwalia et al. 2000; Binzen et al. 2006; Agarwal et al. 2007; Zhang et al. 2007); or (3) comprise about 90 % of the total population of neurons and the majority of them belong to the nociceptive sub-populations of neurons (Mitrirattanakul et al. 2006). In addition to an inevitable variation in the threshold that was used to separate immunopositive and immunonegative cells in the various studies, these remarkable differences in the proportion and type of primary sensory neurons showing CB1 receptor expression could also be due to the differences in anti-CB1 receptor antibodies raised against different epi-topes of the receptor. Due to the involvement of the CB1 receptor in molecular complexes (Mackie 2005; Fuxe et al. 2009), it is reasonable to assume that some of the CB1 receptor antibodies employed in previous studies, similarly to the antibodies we used in the present study, might produce false negative data. Based on these considerations and previous findings (Hohmann and Herkenham 1999; Khasabova et al. 2002; Price et al. 2003; Agarwal et al. 2007; Ahluwalia et al. 2000, 2002; Binzen et al. 2006; Mitrirattanakul et al. 2006; Zhang et al. 2007), it is also reasonable to assume that the great majority of nociceptive as well as a proportion of non-nociceptive cells may express the CB1 receptor in various combinations of monomers, homo- and heteromultimers.

Analysis of the co-expression patterns in DRG revealed that twice as many CGRP-immunopositive neurons showed immunopositivity for the CB1 receptor than IB4-binding neurons. This proportion of co-expression is similar to that found by quantifying the expression of CB1 receptor-immunopositive punctae with CGRP immunoreactivity of IB4-binding in rat superficial spinal dorsal horn (Hegyi et al. 2009). The different proportion of CB1 receptor expression in the two main sub-populations of primary sensory neurons might have functional and therapeutic importance, because IB4-binding non-peptidergic and CGRP-containing peptidergic neurons show differences in their target tissues as well as in their responses to various activators and pathological processes. For example, while the non-peptidergic cells innervate mainly the skin and predominantly are involved in transmitting information generated by noxious mechanical stimuli, peptidergic neurons innervate both somatic and visceral tissues and are involved mainly in transmitting signals evoked by heat (Bennett et al. 1996; Perry and Lawson 1993; Plenderleith and Snow 1993; Cavanaugh et al. 2009). Furthermore, in naive conditions, electrical stimulation and some noxious stimulus-evoked responses are greater in peptidergic than in non-peptidergic neurons (Dirajlal et al. 2003; Breese et al. 2005; Choi et al. 2007), and inflammatory mediators and processes evoke greater responses in non-peptidergic than in peptidergic neurons (Vellani et al. 2004; Breese et al. 2005). These data suggest that CB1 receptor agonists might have limited anti-nociceptive effect in mechanical allodynia associated with inflammatory processes. However, the expression pattern of CB1 receptor in primary sensory neurons might change following tissue injury. This possibility should clearly be addressed in future studies.

Previous findings indicated that the CB1 receptor was transported from the perikarya of primary sensory neurons to the peripheral processes (Hohmann and Herkenham 1999). In agreement with these data, we found that CB1 receptor and CGRP-immunopositive nerve fibres occurred in both skin and urinary bladder. In the urinary bladder, the great majority of the CB1 receptor-immunostained fibres were single labelled. The lack of IB4-positive fibres and hence the lack of CB1 receptor-IB4-binding co-expression are not unexpected in the rat urinary bladder, because we found a similar lack of IB4-positive nerve fibres in this area in our previous work (Avelino et al. 2002). However, the lack of co-expression between the CB1 receptor and CGRP in the bladder is surprising. First, peptidergic primary sensory neurons play an important role in regulating the urinary bladder activity in acute cystitis, and CB1 receptor agonists reduce the frequency of contractions in the inflamed urinary bladder (Dinis et al. 2004; Maggi et al. 1987; Walczak et al. 2009). Second, CB1 receptor activation reduces CGRP release in the bladder (Hayn et al. 2008a). Third, the CB1 receptor shows a high degree of co-expression with CGRP in DRG (present study). Nevertheless, in spite of the apparent lack of CB1 receptor-IB4-binding site co-expression, the nerve fibres labelled only with the anti-CB1 receptor antibody in this work could belong to non-peptidergic primary sensory neurons, which due to some unknown reasons might not bind IB4 in the urinary bladder. This assumption is supported by the recent finding that in the urinary bladder, the CB1 receptor is expressed by nerve fibres expressing the P2 × 3 receptor, which is a marker of the great majority of IB4-binding primary sensory neurons (Chen et al. 1995). Alternatively, the single CB1 receptor-immunolabelled fibres could be autonomic fibres. Studying this possibility was beyond the scope of the present work and clearly requires further study.

In contrast to the urinary bladder, in the skin virtually all CB1-positive nerve fibres were also CGRP immunopositive. Co-expression of the CB1 receptor with CGRP in the skin is in agreement with previous findings. However, while Stander et al. (2005) found CB1 receptor-expressing myelinated fibres, our samples apparently lacked such fibres in the skin. Interestingly, the CB1 receptor expressed by myelinated afferent fibres in the skin might not be in a responding configuration in naive conditions, as Potenzieri and colleagues (Potenzieri et al. 2008) reported recently that CB1 receptor agonists reduce Aδ nociceptor activity only in inflammatory conditions.

In addition to nerve fibres, various other structures showed immunopositivity for the CB1 receptor in peripheral tissues. Some of these structures, based on their histological appearance, were identified as mast cells and histiocytes (Biro et al. 2009; Stander et al. 2005). The presence of CB1 receptor-expressing non-neuronal cells in peripheral tissues is in agreement with the results of the RT-PCR experiment of the present work, demonstrating that both urinary bladder and skin express CB1 receptor mRNA. Furthermore, CB1 receptor expression by keratinocytes, mast cells and histiocytes is also in agreement with previous reports (Casanova et al. 2003; Gratzke et al. 2009; Hayn et al. 2008b; Merriam et al. 2008; Tyagi et al. 2009; Walczak et al. 2009).

In contrast to previous reports showing significant CB1 receptor immunopositivity in laminae I, and II of the superficial dorsal horn, as well as in deeper laminae and the lateral spinal nucleus (Farquhar-Smith et al. 2000; Hegyi et al. 2009), here, we found significant CB1 receptor immunostaining only in laminae I and II. Further, while previous data showed a limited co-expression of the CB1 receptor with CGRP and IB4 in the superficial dorsal horn (Farquhar-Smith et al. 2000; Khasabova et al. 2004), our present data showed that the great majority of the CB1 receptor-immunopositive punctae were also immunopositive for CGRP or (and occasionally) positive for IB4 binding. Data from previous studies also suggested that a high proportion of spinal cord CB1 receptors are expressed by spinal cord neurons and glia (Hohmann et al. 1999; Farquhar-Smith et al.

2000; Hegyi et al. 2009; Pernia-Andrade et al. 2009). While our data do show punctae solely immunopositive for CB1 receptor in the spinal cord, particularly in the deep dorsal horn, identification of the cellular origin of these punctae was beyond the scope of the current study. Nevertheless, the differences in CB1 receptor expression reported previously and in the present study may be due to the use of diverse antibodies recognising different epitopes of the CB1 receptor, whose accessibility may vary among different cells or tissues. While keeping these considerations in mind, our findings in the spinal cord do suggest that the CB1 receptor is transported to the central terminals of the majority if not all the CB1 receptor-expressing primary sensory neurons. Further, our present findings also suggest that the majority of the CB1 receptor immunopositive in double-labelled structures belong to peptidergic rather than non-peptidergic terminals. The ratio of CB1 receptor-immunopositive punctae showing CGRP immunostaining or IB4 binding is in agreement with the double labelling pattern we revealed in DRG and the recent findings by Hegyi et al. (2009).

This conclusion is in accordance with recent ultra-structural findings (Salio et al. 2001; Hegyi et al. 2009) as well as previous functional data, which indicated that CB1 receptor activation reduced transmitter release from primary sensory neuron terminals in the superficial dorsal horn (Jennings et al. 2001; Morisset et al. 2001; Morisset and Urban 2001; Nyilas et al. 2009; Pernia-Andrade et al. 2009).

The presence of CB1 receptors on peripheral and spinal terminals of nociceptive primary sensory neurons is in agreement with previous findings that CB1 receptor agonists, applied at the periphery or in the spinal cord, reduce the activity of nociceptive primary sensory neurons and, subsequently, pain (Hohmann 2002; Hohmann et al. 1998; Jaggar et al. 1998; Martin et al. 1993, 1995; Pertwee 2001, 2005; Richardson et al. 1998b; Agarwal et al. 2007; Morisset and Urban 2001; Morisset et al. 2001; Pernia-Andrade et al. 2009). The presence of CB1 receptors on nociceptive primary sensory terminals further suggests that an endogenous activator(s) for the CB1 receptor must be produced both at the periphery and the spinal cord. Indeed, both anandamide and 2-AG are produced both in the spinal cord and various peripheral tissues (Dinis et al. 2004; Petrosino et al. 2007; Suplita et al. 2006; Calignano et al. 1998; Di Marzo 2008; Di Marzo et al. 1994, 1996; Deutsch et al. 1997). In agreement with endocannabinoid production in the spinal cord and peripheral tissues, increasing anandamide or 2-AG levels by blocking their hydrolysis, respectively, produces analgesic effects, which is mediated, at least partly, by the CB1 receptor at both sites (Suplita et al. 2006; Jhaveri et al. 2006; Guindon et al. 2010; Spradley et al. 2010; Clapper et al. 2010). Anandamide and 2-AG may, however, act through different mechanisms, at least in the spinal cord. Anandamide may activate CB1 receptors on primary sensory neurons through both autocrine and paracrine mechanisms, both at the periphery and the spinal cord, because in addition to various cells at the periphery and spinal cord, a major sub-population of nociceptive primary sensory neurons also synthesise anandamide (Ahluwalia et al. 2003b; Calignano et al. 1998; Guasti et al. 2009; Carrier et al. 2004). 2-AG is likely to act in similar autocrine and paracrine mechanisms, both at the periphery and in the spinal cord (Guindon et al. 2010; Spradley et al. 2010; Guasti et al. 2009; Mitirattanakul et al. 2006). However, the expression of the primary 2-AG biosynthetic enzyme, diacylglycerol lipase- α (Stella et al. 1997) in structures, which are postsynaptic to CB1 receptor-expressing spinal terminals of primary sensory neurons (Nyilas et al. 2009), suggests that the 2-AG in the spinal cord, similarly to that in that brain, mediates activity-dependent retrograde inhibition (Pernia-Andrade et al. 2009; Kreitzer and Regehr 2002; Wilson and Nicoll 2001; Di Marzo et al. 1999; Dinh et al. 2002; Ligresti et al. 2005; Sugiura et al. 2006; Szabo et al. 2006; Tanimura et al. (2010); Gao et al. 2010). Taken together, our present data with those showing the presence of endocannabinoids, particularly at the periphery, indicates that targeted inhibition of the hydrolysing, or activation of the synthesising, enzymes for anandamide and/or 2-AG

is a feasible approach to increase CB1 receptor activity in nociceptive primary sensory neurons to control pain.

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Fig. 1.

Expression of CB1 receptor mRNA and protein in the various tissues. RT-PCR (*upper gel image*) and Western blot (*lower gel image*) show the presence of CB1 receptor mRNA and protein in the dorsal half of the spinal cord (*lane 1*), ventral half of the spinal cord (*lane 2*), hippocampus (*hippoc*; *lane 3*), dorsal root ganglion (*lane 4*), urinary bladder (*lane 5*) and skin (*lane 6*). Note that all the tissues expressed an RT-PCR product of ~690 bp, which is the predicted size of a product derived from the CB1 receptor mRNA. Note also that proteins of ~55 kDa and ~65 kDa gave reaction with an anti-CB1 receptor antibody in all tissues. In addition, a distinct band at ~50 kDa was also visible in the hippocampus and urinary bladder. The ~55 kDa and ~65 kDa proteins correspond with the predicted size of the non-glycosylated and glycosylated forms of the CB1 receptor, respectively

Fig. 2.

Immunostaining demonstrating the selectivity and specificity of the anti-CB1 receptor antibody raised in guinea pig. **a** CB1 receptor immunostaining in the rat hippocampus. Note the large number of CB1 receptor-immunopositive nerve fibres. **b** CB1 receptor immunostaining in wild-type mouse hippocampus. Note the presence of CB1 receptor-immunopositive fibres. **c** CB1 receptor immunostaining in the hippocampus of a CB1 receptor^{-/-} mouse. Note that no immunopositive fibres could be seen. **d** CB1 receptor immunostaining in a section prepared from a dorsal root ganglion that was collected from a wild-type mouse. Note that several perikarya exhibit positive immunostaining. **e** CB1

receptor immunostaining in a section prepared from a dorsal root ganglion that was collected from a CB1 receptor^{-/-} mouse. Note that the anti-CB1 receptor antibody in these sections produced no staining. *Scale bar* 50 μ m

Fig. 3.

Immunostaining demonstrating that the anti-CB1 receptor antibodies raised in guinea pig and goat, respectively, recognise the same antigen. Slices cut from rat hippocampus were incubated in a mixture of goat and rabbit anti-CB1 receptor antibodies. Note that the staining produced by the two antibodies shows almost complete overlap. The image shows a 2- μ m thick optical section. Immunopositivity by the guinea pig antibody is shown in *red* (**a**), whereas immunopositivity by the goat antibody is shown in *green* (**b**). Note the overlap of the two reactions (**c**). *Scale bar* 50 μ m

Fig. 4.

CB1 receptor immunostaining in rat dorsal root ganglia. **a** A typical confocal microscopy image (optical thickness is 4 μm). Note that the immunopositive neurons are small/medium diameter cells. *Scale bar* 50 μm . **b** Size distribution of CB1 receptor-immunopositive (*grey bars*) and -immunonegative (*white bars*) dorsal root ganglion neurons. Note that the CB1 receptor-immunopositive cells belong to the small- and medium-sized populations of neurons

Fig. 5.

Co-expression of the CB1 receptor with markers of the two major sub-populations of nociceptive primary sensory neurons in rat dorsal root ganglia. Typical image of a 4-μm thick confocal optical section shows the staining pattern with anti-CGRP antibody (**a, d**), anti-CB1 receptor antibody (**b, d**) and biotinylated IB4 (**c, d**). Note that CB1 receptor immunopositivity (*green*) shows a high degree of co-expression with CGRP immunostaining (*red*) or/and IB4-binding (*blue*). Scale bar 30 μm. **e** Summary of image analysis quantifying the co-expression pattern of CB1 receptor immunostaining, CGRP immunopositivity and IB4 binding

Fig. 6.

CB1 receptor expression in the skin. Confocal photomicrographs of a typical skin section show the expression of CB1 receptor (*red*) in the keratinocytes of epidermis (**a**) and thin nerve fibres in the dermis (**b, d, e**). CGRP (*green*; **b, c, e**) immunostained nerve fibres in the dermis that are CB1 (*red*; **b, d, e**) positive. *Blue* colour indicates DAPI binding to show cell nuclei. *Dashed line* indicates the border between the epidermis and dermis in panels (**a**) and (**b**). *Scale bar* 50 μ m

Fig. 7.

CB1 receptor immunostaining in the urinary bladder. Confo-cal photomicrograph of the urinary bladder showing CB1 receptor (*red*; **a**, **c**) and CGRP immunostaining (*green*; **b**, **c**). **a** Thin varicose CB1 receptor-expressing nerve fibres can be clearly seen in the sub-urothelial connective tissue. **b** CGRP-immunopositive fibres can also be seen in the lamina muscularis layer and submucosa. **c** *Merged images* show partial co-localisation of the CB1 receptor and CGRP. *Arrows* show a few double-labelled fibres, whereas *arrowheads* indicate fibres containing CB1 without CGRP. *Dashed line* indicates the border between the urothelium and the submucosa. *Scale bar* 50 μm

Fig. 8.

CB1 receptor immunostaining in the spinal dorsal horn. Confocal photomicrographs of the superficial spinal dorsal horn. Distribution of CB1 receptor immunopositivity (*red*; **a, d, e, f**), the anti-CGRP immune reaction (*green*; **b, d, f**) and IB4 binding (*blue*; **c, d, f**). Note the overlap and co-expression of CB1, CGRP receptor immunopositivity and IB4 binding (**d, f**). Note also the areas (*arrows* in **(e)** and **(f)**) circumscribed by CB1 receptor-immunopositive punctae. These areas are likely to be dorsal horn neurons which either receive input from CB1 receptor-expressing fibres or/and them selves express the CB1 receptor. Optical section thickness is 2 μ m. *Scale bar* 50 μ m