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# Differential distribution of functional cannabinoid $CB_1$ receptors in the mouse gastroenteric tract

Maria Antonietta Casu[a], Anna Porcella[b], Stefania Ruiu[a], Pierluigi Saba[c], Giorgio Marchese[a], Mauro Antonio Maria Carai[a], Roberta Reali[a], Gian Luigi Gessa[a,c], Luca Pani[a,b,*]

[a] *Neuroscienze Scarl, via Palabanda, 9 09124-I, Cagliari, Italy*
[b] *Institute of Neurogenetic and Neuropharmacology, C.N.R, via Palabanda, 9 09124-I, Cagliari, Italy*
[c] *Department of Neuroscience, University of Cagliari, Cagliari, Italy*

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**Abstract**

Recently, the gastrointestinal pharmacology of cannabinoid $CB_1$ receptors has been extensively explored. We employed western blotting and immunohistochemistry techniques to study the distribution of the cannabinoid $CB_1$ receptor protein in the mouse gastroenteric tract. The cannabinoid $CB_1$ receptor peptide was detected by western blotting only in its glycosylated form (63 kDa) with a significant differential distribution. The highest levels of expression were detected in the stomach and in the colon, while the pyloric valve was devoid of any cannabinoid $CB_1$ receptor protein. The immunohistochemical study showed intense cannabinoid $CB_1$ receptor immunoreactivity in ganglia subadjacent to the gastric epithelium and in the smooth muscle layers of both the small and large intestine. Only the small intestine showed (-)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-(3-hydroxylpropyl) cyclohexan-1-ol ([$^3$H]CP 55,940) specific binding (27%). These receptors mediated pharmacologically significant effects since the cannabinoid $CB_1$ receptor agonist *R*(-)-7-hydroxy-delta-6-tetra-hydrocannabinol-dimethylheptyl (HU 210) dose dependently inhibited gastrointestinal transit up to 70%, while the cannabinoid $CB_1$ receptor antagonist *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (SR 141716A) increased gastrointestinal transit. Moreover, the dose of 0.3 μg/kg of HU 210, devoid per se of any activity on mouse intestinal propulsion, blocked the increased gastroenteric transit induced by the cannabinoid $CB_1$ antagonist SR 141716A.


*Keywords:* Marijuana; Gene expression; Drug abuse

## 1. Introduction

*Cannabis sativa* preparations were used for centuries in the medicinal treatment of gastrointestinal disorders (Merck & Co., 1899; Grinspoon and Bakalar, 1997). Delta (9)-tetrahydrocannabinol, 1 of the more than 60 cannabinoids contained in *Cannabis*, has been shown to inhibit defaecation (Dewey et al., 1972) and decrease spontaneous (Rosell et al., 1976; Shook and Burks, 1989) as well as electrically evoked (Pertwee et al., 1996) gastrointestinal motility in rodents.

Two cannabinoid receptor types $CB_1$ and $CB_2$ have been cloned (Matsuda et al., 1990; Munro et al., 1993) and characterized both biochemically and pharmacologically (Pertwee, 1997). Their distribution has been mapped using different techniques (Herkenham et al., 1991; Lynn and Herckenham, 1994; Galiègue et al., 1995). From these studies, it becomes clear that the widespread distribution of cannabinoid receptors from the brain throughout the periphery mirrors the variety of effects produced by Delta (9)-tetrahydrocannabinol or by synthetic or endogenous cannabinoids such as anandamide (Devane et al., 1992).

A large body of evidence has demonstrated that (a) cannabinoids inhibit electrically evoked contraction of isolated small intestine; (b) enteric cannabinoid $CB_1$ receptors mediate inhibition of evoked acetylcholine release; and (c) cannabinoids delay gastric emptying, inhibit gastric acid secretion and intestinal motility in the whole animal (reviewed in Pertwee, 2001).

* Corresponding author. Neuroscienze Scarl, via Palabanda, 9 09124-I, Cagliari, Italy. Tel: +39-070-254-8079, fax: +39-070-254275.
*E-mail address:* l.pani@ca.cnr.it (L. Pani).

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Very little is known, however, about the precise localization of cannabinoid $CB_1$ receptor distribution in the mammalian gastrointestinal system. The presence of cannabinoid $CB_1$ receptor mRNA in the enteric ganglia of rat embryos (Buckley et al., 1998) and of cannabinoid $CB_1$ receptor immunoreactivity in ganglionated neurons and fibers of porcine ileum (Kulkarni-Narla and Brown, 2000) has been reported. The purpose of the present study was to characterize the anatomical distribution of cannabinoid $CB_1$ receptor protein in the gastroenteric tract of the mouse. In addition, we wanted to extend previous observations from this laboratory (Colombo et al., 1998; Carai et al., 2000) on the inhibiting effect of cannabinoid $CB_1$ receptor agonists on mouse gastrointestinal motility by studying the effect of the potent cannabinoid agonist HU 210.

## 2. Materials and methods

### 2.1. Animals

Male CD-1 mice (Charles River, Calco, LC, Italy) weighing from 20 to 35 g were used. After delivery to our animal facility, the mice were left undisturbed for 7 days to adapt to the new housing conditions. The mice were housed 20/cage in standard plastic cages ($55 \times 33 \times 19$ (*h*) cm) with wood chip bedding under a 12 h artificial light–dark cycle (lights on at 8:00 a.m.) at a constant temperature of $22 \pm 2$ °C and relative humidity of 60%. Tap water and standard laboratory rodent chow (MIL Morini, San Polo D'Enza, RE, Italy) were provided ad libitum. All experimental protocols were approved by the Ethical Committee at the University of Cagliari and performed in strict accordance with the EC regulation for care and use of experimental animals (EEC N°86/609).

### 2.2. One-dimensional western blotting

Mice were killed by cervical dislocation and stomach, small intestine, caecum, colon and liver were rapidly removed and placed on an ice-cold plate. In brief, tissue samples were homogenized at 4 °C with a homogenizer system (GlasCol, Terre Haute, IN, USA), 200 μl of 20 mM HEPES buffer (pH 7.9) containing in mM: NaCl, 125; $MgCl_2$, 5; glycerol, 12%; ethylenediaminetetracetic acid (EDTA), 0.2; Nonidet P-40, 0.1%; dithithreitol, 5; phenylmethylsulphonyl fluoride, 0.5; leupeptin, 0.5 μg/ml; and pepstatin A, 0.7 μg/ml. The extracts were then centrifuged at $15\,000 \times g$ (at 4 °C) for 20 min and the resulting supernatant was collected as total cell extracts. An aliquot was analysed for protein concentration with a Protein assay kit II (Bio-Rad Laboratories, Hercules, CA, USA), and the rest was frozen at −80 °C until assayed.

Sodium dodecyl sulfate (SDS)-stop solution (final concentration, 2% SDS, 10% glycerol and 5% β-mercaptoethanol) was added to aliquots of total gastroenteric preparation extracts containing 30 μg of total protein. Samples were then treated as previously described (Porcella et al., 2000). The blot was blocked with 4% nonfat dry milk and incubated with diluted (8 μg/ml) $CB_1$ rabbit polyclonal antibody from Biosource International (Camarillo, CA, USA) followed by peroxidase-labelled anti-rabbit antibody (1:1500; Amersham Life Science, Milan, Italy). Immunoreactivity was visualized as enhanced chemiluminescence. A specificity control was run by pre-absorbing (1 h) and co-incubating the antiserum (8 μg/ml) with the immunizing protein (50 μg/ml).

### 2.3. Analysis of immunoblots

Gels were visualized on a transilluminator and the images were processed with the aid of western blotting gel analysis software (Media Cybernetics, Silver Spring, MD, USA). Each sample was measured on the basis of total absorbance in arbitrary units of optical density (OD).

### 2.4. Immunohistochemistry

Mice were perfused transcardially with 4% paraformaldehyde in 0.1% phosphate buffer (PB) at pH 7.4. The gastroenteric tract and cerebellum were subsequently post-fixed in the same fixative for 2 h and then cryoprotected overnight with a solution of 30% sucrose in 0.1 M PB at 4 °C. Transverse sections (10 μm) of different gastroenteric tracts and cerebellum were cut on a cryostat (Leica CM 3050, Leica Microsystems, Nussloch, Germany), mounted on gelatin-coated glass slides and cytochemical staining was performed. Briefly, after rinsing in phosphate buffered saline with 0.2% Triton X-100 (PBS + T), the sections were incubated with 0.3% $H_2O_2$ to eliminate endogenous peroxidase activity and after extensive washing in a blocking solution containing 1% bovine serum albumin and 20% normal goat serum in PBS + T. Sections were then incubated overnight at 4 °C with a cannabinoid $CB_1$ receptor polyclonal antiserum (40 μg/ml) from Biosource International. After rinsing, the sections were incubated with goat anti-rabbit biotinylated IgG (1:200; Vector, Burlingame, CA, USA) for 1 h followed by an avidin–biotin complex (1:500; Vectastain ABC kit, Vector) for an additional hour. The sections were then exposed to 3,3' diaminobenzidine (Sigma, St. Louis, MO) containing cobalt chloride and nickel ammonium sulfate for 15 min. Immunostaining was developed by adding 5 μl of $H_2O_2$ (0.1% in PBS) to each 500 μl of 3,3′ diaminobenzidine. Finally, after washing in PBS + T, all sections were dehydrated in ascending concentrations of ethanol, cleared with xylene and cover slipped with Entellan before observation under light microscopy (Olympus BX60, Olympus Optical, Hamburg, Germany). A specificity control was run by pre-absorbing (1 h) and co-incubating the antiserum (40 μg/ml) with the immunizing protein used in 200-fold M excess as suggested by Biosource International (76 μg/ml).

### 2.5. [$^3$H]CP 55,940 binding

Mice were killed by cervical dislocation and brain (minus cerebellum), stomach, small intestine, caecum and colon were rapidly removed and placed on an ice-cold plate. After thawing, the tissues were homogenised in 20 vol. (w/v) of ice-cold TME buffer (50 mM of Tris–HCl, 1 mM of EDTA and 3.0 mM of $MgCl_2$, pH 7.4) with an Ultra-Turrex homogenizer. The homogenates were centrifuged at $1086 \times g$ for 10 min at 4 °C, and the resulting supernatants were centrifuged at $45{,}000 \times g$ for 30 min at 4 °C.

[$^3$H]CP 55,940 binding was performed by a modification of the method previously described (Rinaldi-Carmona et al., 1994). Briefly, the membranes (30–100 μg of protein) were incubated with 0.5 nM of [$^3$H]CP 55,940 for 1 h at 30 °C in a final volume of 0.5 ml of TME buffer containing 5 mg/ml of fatty acid-free bovine serum albumin. Specific binding of [$^3$H]CP 55,940 was defined as the difference between the binding that occurred in presence and absence of 10 μM of CP 55,940. All binding studies were performed in disposable glass tubes pre-treated with Sigmacote (Sigma, Poole, UK) in order to reduce non-specific binding. The reaction was terminated by rapid filtration through Whatman GF/C filters pre-soaked in 0.5% polyethyleneimine using a Brandell 96-sample harvester (Gaithersburg, MD, USA). Filters were washed five times with 4 ml of ice-cold Tris–HCl buffer (pH 7.4) containing 1 mg/ml of fatty acid-free bovine serum albumin. The filter bound radioactivity was measured in a liquid scintillation counter (Tricarb 2900, Packard, Meridien, USA) with 4 ml of scintillation fluid (Ultima Gold MV, Packard). Protein determination was performed with the Bradford (1976) protein assay using bovine serum albumin as a standard according to the protocol of the supplier (Bio-Rad, Milan, Italy). All experiments were performed in triplicate and results were confirmed in at least four independent experiments. In saturation binding assays, the concentrations of the radiolabelled ligands used ranged between 0.05 and 3 nM.

The dissociation constant of [$^3$H]CP 55,940 ($K_d$) and the concentration of specific binding sites ($B_{max}$) were calculated using the GraphPad Prism program (GraphPad Software, San Diego, CA, USA).

### 2.6. Gastrointestinal transit

Gastrointestinal transit in mice was measured according to a procedure validated by Nagakura et al. (1996). HU 210 (0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 μg/kg) was administered i.p. 20 min before the i.g. administration of the marker (0.3 ml/mouse carmine) to groups of $n = 9–10$ mice. Twenty minutes later, the mice were killed by cervical dislocation, the stomach and small intestine were removed and the omentum separated, avoiding stretching. The distance travelled by the head of the marker was measured and expressed as percent of the total length of the small intestine (from the pyloric sphincter to the ileocecal junction). In the antagonism test, the cannabinoid antagonist SR 141716A (0.3 mg/kg) was administered i.p. 10 min prior to the injection of HU 210 (0.3 μg/kg). The marker was administered i.g. 20 min afterwards. Twenty minutes later, the mice were killed by cervical dislocation, and the stomach and small intestine were removed as described above.

### 2.7. Chemicals and drugs

All drugs were suspended in saline with 0.1% Tween 80 and injected in a 12.5 ml/kg volume. Carmine (Sigma) was suspended at the concentration of 6% (w/v) in distilled water containing 0.5% methylcellulose. HU 210 (*R*(-)-7-hydroxy-delta-6-tetra-hydrocannabinol-dimethylheptyl) and CP 55,940 ((-)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-(3-hydroxylpropyl) cyclohexan-1-ol)) were purchased from Tocris Cookson (Bristol, UK), SR 141716A ((*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-

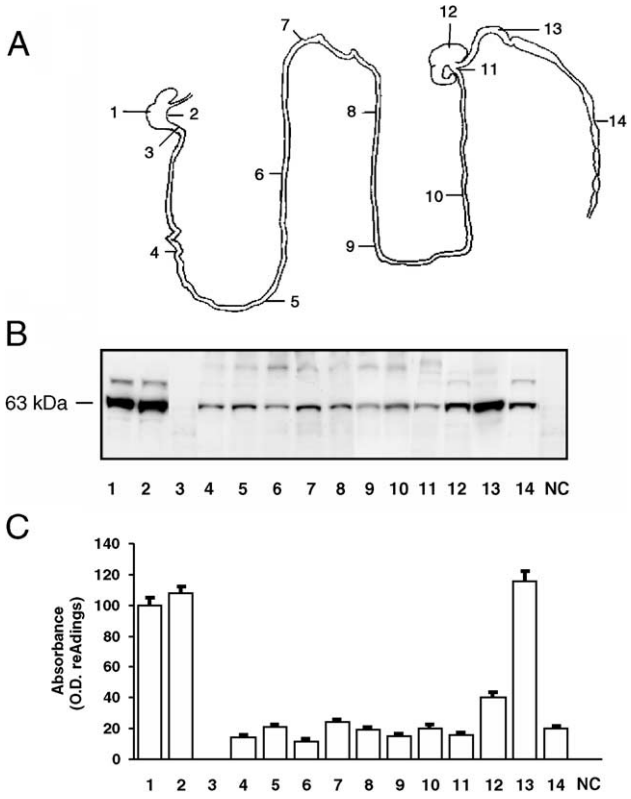


Fig. 1. Diagrammatic scheme (Panel A), western blotting of whole cell extracts (Panel B) and relative differences of $CB_1$ peptide (Panel C) of the mouse gastroenteric tract: 1 = stomach greater curvature; 2 = stomach lesser curvature; 3 = pyloric valve; from 4 to 11 = small intestine (each number represents a section 6 cm in length); 12 = caecum; 13 = colon; 14 = rectum; NC = negative control (liver). Differences in $CB_1$ peptide were expressed in arbitrary units of OD; each OD value represents the mean ± S.E.M. for four mice.

pyrazole-3-carboxamide)) was a generous gift from Sanofi ~ Synthélabo Recherche (Montpellier, France). [$^3$H]CP 55,940 (specific activity of 180 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). All other chemicals were from commercial sources and were of analytical grade.

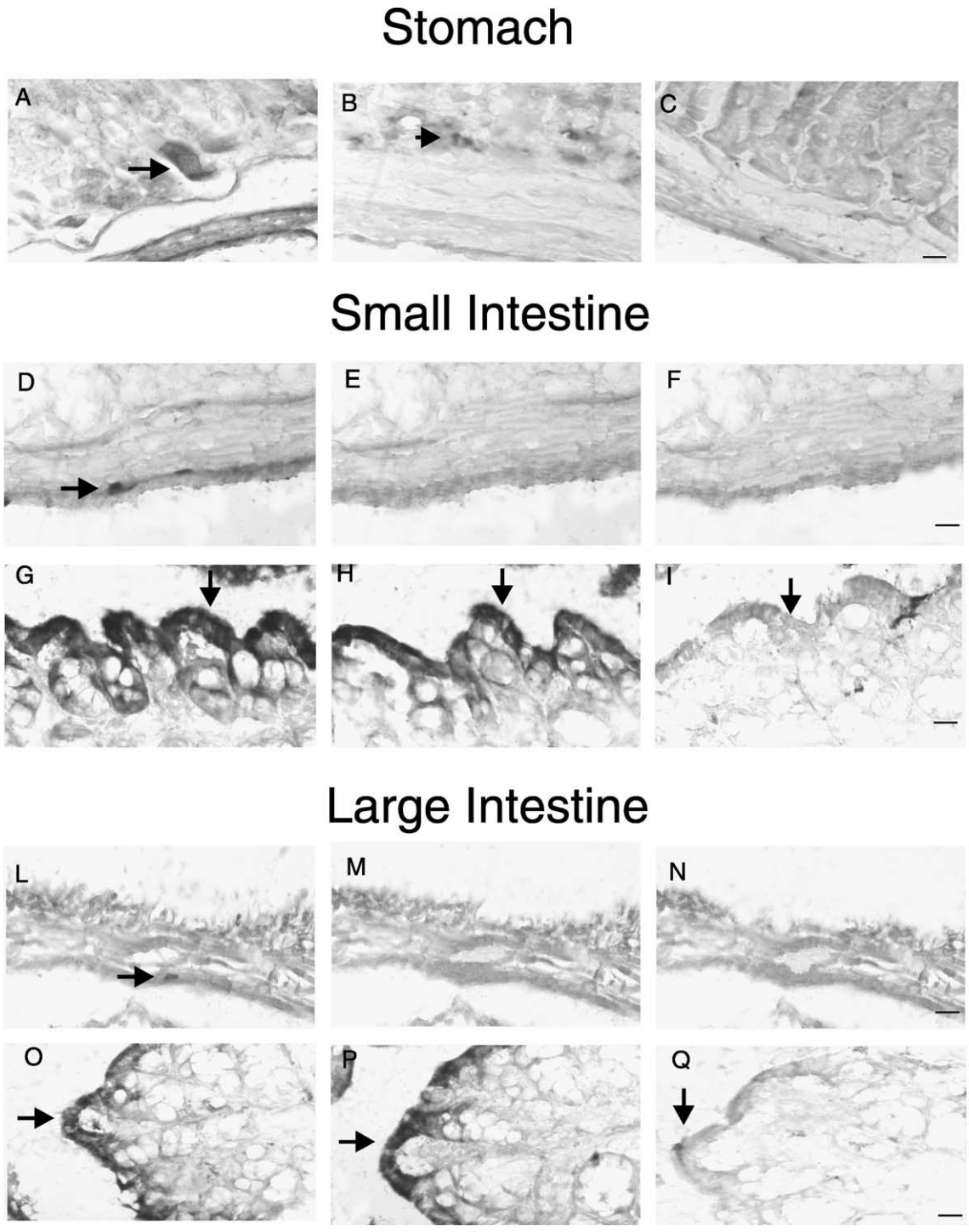


Fig. 2. A dense $CB_1$ receptor immunoreactivity was found in the ganglia subadjacent to the gastric epithelium (A, long arrow). Non-specific labelling persisted in preabsorption control in the gastric epithelium (B, short arrow) and no positive staining was present in the omission control (C). In the small intestine, $CB_1$ receptor immunoreactivity was present in the smooth muscle layers (D, arrow). No positive staining was present in preabsorption and omission control in the smooth muscle layers (E–F). Non-specific labelling was present in the epithelial cells of the villi of the small intestine (G, arrow) persisting in preabsorption and omission controls (H–I, arrow). Intense $CB_1$ receptor immunoreactivity was also found in the large intestine in the smooth muscle layers (L, arrow). No positive staining was shown in preabsorption and omission control in the smooth muscle layers (M–N). Non-specific labelling was present in the epithelial cells of the villi of the large intestine (O, arrow) persisting in preabsorption and omission controls (P–Q, arrow). Scale bar = 20 μm.

### 2.8. Data analyses

In each experiment, statistical evaluation of the gastrointestinal transit, expressed as percentage of the distance travelled by the head of the marker over the total length of the small intestine, was performed with a one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for post hoc comparisons. The inhibitory effects of cannabinoids on gastrointestinal transit are also expressed as percentages of inhibition of transit in cannabinoid-treated animals (test gastrointestinal transit) compared with the mean transit obtained in a group of vehicle-treated mice: percent inhibition=[(vehicle gastrointestinal transit − test gastrointestinal transit)/(vehicle gastrointestinal transit)]×100.

## 3. Results

### 3.1. Western blotting

The cannabinoid $CB_1$ receptor protein was differentially expressed in the sections (Fig. 1 Panel A) of mouse gastroenteric tract analysed. Panel B of Fig. 1 shows a representative western blotting with affinity-purified polyclonal antibody raised against the N-terminus of the cannabinoid $CB_1$ receptor, detecting only one major band of approximately 63 kDa which corresponds to the expected molecular weight of the glycosylated form of the cannabinoid $CB_1$ receptor (Song and Howlett, 1995; Porcella et al., 2000). A specificity control experiment (data not shown), done by incubating the cannabinoid $CB_1$ receptor antibody with the immunizing protein as previously described (Porcella et al., 2000), showed no immunoreactivity. The 63-kDa band gave an OD reading (Fig. 3C) of 100 ± 4.8 and 108 ± 4.0 in the greater and lesser curvature of the stomach, respectively. In the small intestine, the immunoreactivity OD reading ranged between 12 ± 1.7 and 24 ± 1.9, while in caecum, proximal colon and rectum, it was 40 ± 3.2, 116 ± 6.5 and 20 ± 1.8, respectively. Each OD value represents the mean ± S.E.M. from four mice.

### 3.2. Immunocytochemistry

Intense cannabinoid $CB_1$ receptor immunoreactivity was observed in all tracts of the mouse gut. In the stomach, a dense $CB_1$ receptor immunoreactivity was found in the ganglia subadjacent to the gastric epithelium (Fig. 2A). Non-specific labelling persisted in the pre-absorption control in the gastric epithelium (Fig. 2B) and no positive staining was present in the omission control (Fig. 2C). In the small intestine, $CB_1$ receptor immunoreactivity was present in the smooth muscle layers (Fig. 2D). No positive staining was present in pre-absorption and omission controls in the smooth muscle layers (Fig. 2E–F). Nevertheless, non-

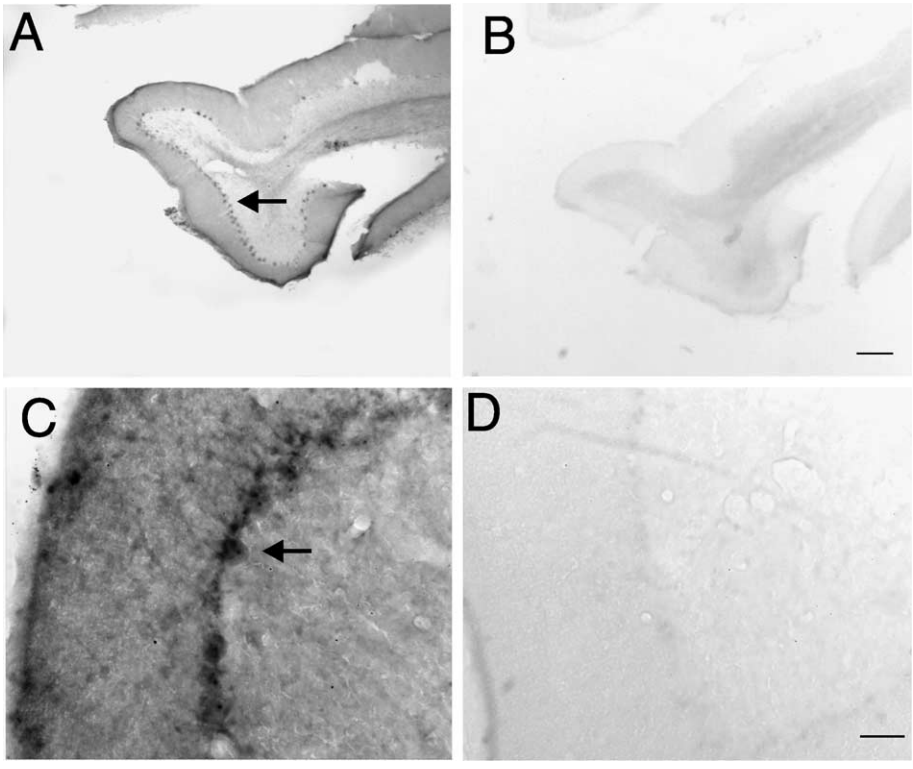


Fig. 3. Uniform intense $CB_1$ staining was exhibited in the cerebellum molecular layer (A, arrow) while moderate positivity was present in the granular layer. At high magnification, the intense $CB_1$ receptor immunoreactivity surrounds the Purkinje cell bodies (C, arrow). Moreover, the negative control experiment with the immunizing protein showed the absence of positive staining in the molecular layer of the cerebellum (B–D). Scale bar = 100 μm (A–B), 20 μm (C–D).

Table 1
Distribution of [$^3$H]CP 55,940 binding in mouse gastroenteric trait and forebrain

| Tissue | Binding (pmol/mg protein) | | | |
|---|---|---|---|---|
| | Total | Non-specific | Specific | Specific binding (%) |
| Stomach | 0.14 ± 0.005 | 0.124 ± 0.005 | 0.016 | 11 |
| Small intestine | 0.22 ± 0.010 | 0.160 ± 0.005 | 0.060 | 27 |
| Caecum + colon | 0.25 ± 0.020 | 0.225 ± 0.030 | 0.025 | 10 |
| Forebrain | 0.60 ± 0.050 | 0.095 ± 0.010 | 0.505 | 84 |

Values are means ± S.E.M. of at least four different experiments, each performed in triplicate.

specific labelling was present in the epithelial cells of the villi of the small intestine (Fig. 2G) persisting in pre-absorption and omission controls (Fig. 2H–I). A $CB_1$ receptor immunoreactivity was also found in the large intestine in the smooth muscle layers (Fig. 2L). No positive staining was shown in pre-absorption and omission controls in the smooth muscle layers (Fig. 2M–N). Non-specific labelling was present in the epithelial cells of the villi of the large intestine (Fig. 2O) persisting in preabsorption and omission controls (Fig. 2P–Q). Standard hematoxylin and eosin staining was also performed in order to check the histological pattern of the intestinal tract that we used for this study (data not shown). In addition, uniform intense $CB_1$ staining was seen in the cerebellum molecular layer (Fig. 3A) that we used as a positive control since the cerebellum has a high $CB_1$ receptor density while moderate positivity was present in the granular layer. At high magnification, the intense $CB_1$ receptor immunoreactivity surrounds the Purkinje cell bodies (Fig. 3C). Moreover, the negative control experiment with the immunizing protein showed the expected absence of positive staining in the molecular layer of the cerebellum (Fig. 3B–D).

### 3.3. [$^3$H]CP 55,940 binding

The total [$^3$H]CP 55,940 specific binding and the specific binding percent in the different tissues examined are presented in Table 1. A lower level of [$^3$H]CP 55,940 specific binding (27%, $n=4$) was found in the small intestine crude homogenates when compared to the forebrain (84%, $n=4$), while no other tissue examined had specific binding greater than 11%. As shown in Fig. 4, binding of [$^3$H]CP 55,940 to the small intestine was dose-dependent and saturable, yielding a $K_d = 0.41 \pm 0.084$ nM and $B_{max} = 42 \pm 6$ fmol/mg protein, while in the forebrain, the kinetic parameters were $K_d = 0.32 \pm 0.052$ nM and $B_{max} = 805 \pm 57$ fmol/mg protein (graph not shown). No significant differences were detected in the $K_d$ values between the small intestine and forebrain ($P = 0.3770$, Student's $t$-test). $V_{max}$ values were much higher in the forebrain than in small intestine ($P < 0.001$, Student's $t$-test). We were unable to increase the specific binding percent by using sucrose gradient fractionation (data not shown) as reported for the guinea pig small intestine (Ross et al., 1998).

### 3.4. Gastrointestinal transit

Administration of HU 210 (0.1–1000 μg/kg i.p.) resulted in a dose-dependent reduction, up to approximately 70%, at doses equal to or greater than 100 μg/kg in comparison to vehicle-treated mice of the propulsive activity in the small intestine [one-way ANOVA $F(9,89) = 19.75$, $P < 0.01$] (Fig. 5A). In addition, SR 141716A (0.3 mg/kg)

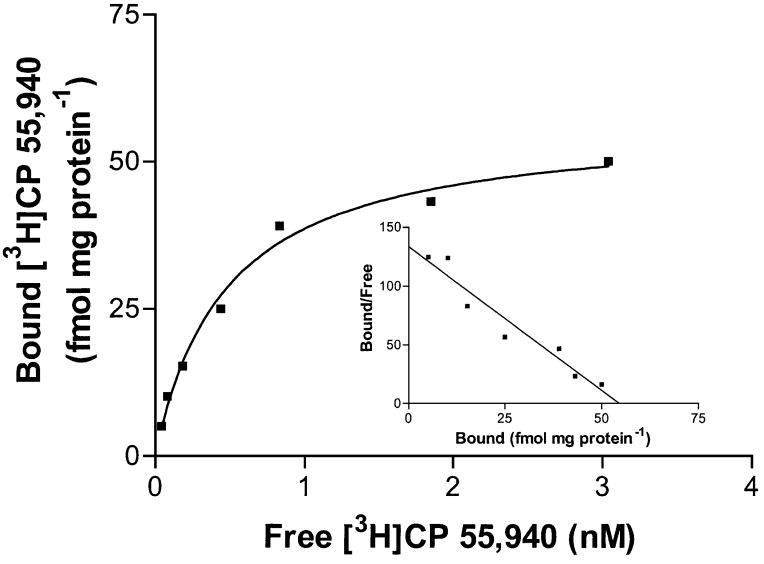


Fig. 4. Saturation of [$^3$H]CP 55,940 specific binding to mouse small intestine. Different concentrations of [$^3$H]CP 55,940 were incubated with membrane suspension (30–100-μg protein) for 60 min at 30 °C. Each point represents the mean of triplicate determinations obtained in a single representative experiment. Inset represents a Scatchard transformation of the same data.

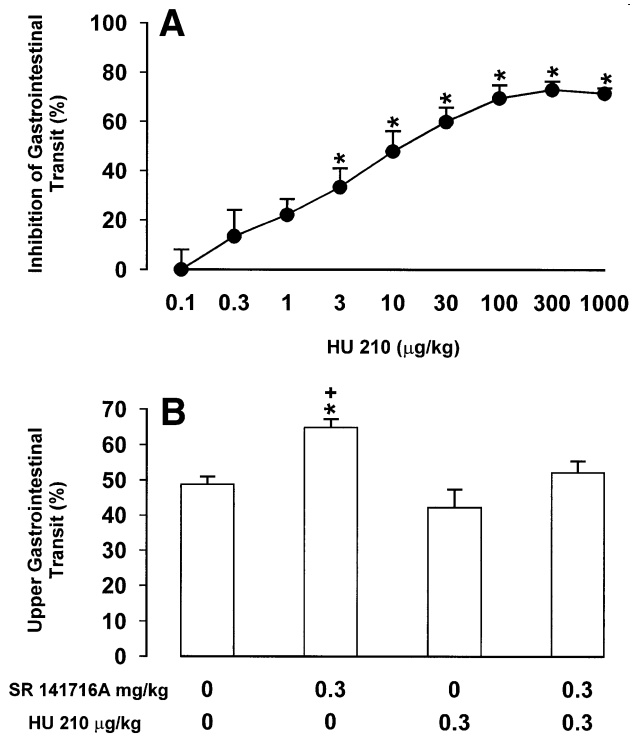


Fig. 5. Panel A: effect of the cannabinoid $CB_1$ receptor agonist HU 210 (0.1–1000 μg/kg, i.p.) on gastrointestinal transit in CD-1 mice. HU 210 was administered 20 min prior to the i.g. administration of the marker. Gastrointestinal transit was evaluated 20 min after the administration of the marker. Each point is the mean ± S.E.M. from 9 to 10 mice. $^{*}P<0.01$ with respect to vehicle-treated mice (Newman–Keuls test). Panel B: effect of HU 210 (0–0.3 μg/kg, i.p.) alone or in combination with SR 141716A (0.3 mg/kg, i.p.). SR 141716A was given 10 min before the administration of HU 210, the latter 20 min before the marker. Gastrointestinal transit was evaluated 20 min after the administration of the marker. Each bar represents the mean ± S.E.M. from 10 to 15 mice. $^{*}P<0.01$ with respect to 0 mg/kg HU 210+0 mg/kg SR 141716A-treated mice (Newman–Keuls test). $^{+}P<0.05$ with respect 0.3 μg/kg HU 210+0.3 mg/kg SR 141716A-treated mice (Newman–Keuls test).

produced an increase in gastrointestinal transit [one-way ANOVA $F(3,43)=8.91$, $P<0.01$] (Fig. 5B) to an extent comparable to that previously observed in other studies from this laboratory using the same procedure (Colombo et al., 1998; Carai et al., 2000). HU 210, administered at the dose of 0.3 μg/kg, which per se did not affect propulsion in the small intestine, reversed the propulsive effect of SR 141716A (Fig. 5B).

## 4. Discussion

In this study, we demonstrated the presence of functional cannabinoid $CB_1$ receptors in the mouse gastroenteric tract.

In the mouse gut, the cannabinoid $CB_1$ receptor protein was detected by western blotting with a differential expression supporting the recognised link between cannabinoids and the gastroenteric system (reviewed in Pertwee, 2001). In accord with the present western blotting experiments, polymerase chain reaction, performed on cDNA prepared from human stomach and colon (Shire et al., 1995), guinea pig small intestine (Griffin et al., 1997) and from the myenteric and submucosal plexus of the rat embryo digestive tract (Buckley et al., 1998), demonstrated the presence of cannabinoid $CB_1$ receptor mRNA. The absence of $CB_1$ protein signal in the pyloric valve, a dense muscular ring, is consistent with the reported lack of a Delta (9)-tetrahydrocannabinol effect on antro-duodenal intraluminal pressure (Shook and Burks, 1989). This would suggest that the stomach epithelial wall rather than the muscle layer might indirectly reduce the frequency of stomach contractions produced by cannabinoids (Shook and Burks, 1989; Izzo et al., 1999).

When we studied the cannabinoid $CB_1$ receptor cellular distribution in the mouse gut by immunohistochemistry, using the same antibody which identified the cannabinoid $CB_1$ receptor protein in the western blotting experiments, we found intense positive $CB_1$ receptor immunoreactivity in all regions of the mouse gut.

A dense $CB_1$ receptor immunoreactivity was found in the ganglia subadjacent to the gastric epithelium. Moreover, to confirm neuronal morphology, several studies showed $CB_1$ receptor immunoreactivity on acetylcholine-containing neurones that innervate smooth muscle, mucosa and submucosal blood vessels of the rat stomach (Izzo et al., 2000a; Adami et al., 2002). It is consistent with this that cannabinoid receptor agonists have been reported to delay gastric emptying (Izzo et al., 1999) and to inhibit gastric acid secretion stimulated either by pentagastrin (Adami et al., 2000) or by stress (Germanò et al., 2001) in rats.

We also found an intense $CB_1$ receptor immunoreactivity in the smooth muscle layers of mouse small and large intestine. $CB_1$ receptor immunoreactivity has been observed in the myenteric and submucosal ganglionated plexus of porcine ileum (Kulkarni-Narla and Brown, 2000) and of mouse colon (Pinto et al., 2002) where it was coexpressed with a cholinergic marker.

These findings, together with the results of the pharmacological study, further support the role of cannabinoid receptors in the inhibition of intestinal motility in the mouse upper gastrointestinal tract (Chesher et al., 1973; Shook and Burks, 1989; Calignano et al., 1997; Colombo et al., 1998; Carai et al., 2000; Izzo et al., 2000b) and electrically evoked peristaltic activity of the mouse isolated distal colon (Mancinelli et al., 2001). Moreover, the presence of $CB_1$ receptor immunoreactivity in the mouse intestinal mucosa supports the results of studies in the rat where cannabinoid $CB_1$ receptors were found to be involved in the regulation of small intestinal water and electrolyte transport (Tyler et al., 2000).

To further characterize the expression of the cannabinoid $CB_1$ receptor in the mouse gastrointestinal tract, we performed [$^3$H]CP 55,940 binding on crude membrane homogenates. [$^3$H]CP 55,940 specific binding was present in the

various mouse gut tracts, even if, only in the small intestine, the specific binding percent was sufficient to allow a kinetic parameter study. It should be noticed that the percentage of specific binding (27%) found in the crude homogenate fraction of mouse small intestine was similar to what has been observed in guinea pig small intestine (21.6%) (Ross et al., 1998). However, Ross et al. (1998), who used membranes separated by sucrose gradient fractionation from the myenteric plexus-longitudinal muscle preparation of the guinea pig small intestine, found an increase of [$^3$H]CP 55,940 specific binding percentage (65.2%). One notable difference between the present binding experiments and the results reported by Ross et al. (1998) is that we were unable to enrich the specificity of binding by using sucrose gradients. This is most likely due to the technical difficulties of obtaining sufficient amounts of myenteric plexus-longitudinal muscle preparation from the mouse gastroenteric tract. Although, we found a low specific-binding percent in the small intestine, our results indicated that the [$^3$H]CP 55,940 affinity was similar to that observed in the forebrain, a region known to highly express cannabinoid $CB_1$ receptors. The binding site density was, however, much lower in the small intestine than in the mouse forebrain.

The low receptor density observed in the mouse gut and the related technical problem might explain the different receptor distribution found when using western blotting and [$^3$H]CP 55,940 binding in the different gastrointestinal tracts.

The results of the study investigating the effect of HU 210 on mouse gastrointestinal transit are in agreement with previous reports showing that the endogenous cannabinoid, anandamide (Calignano et al., 1997) and the cannabinoid receptor agonists WIN 55,212-2 and CP 55,940 (Colombo et al., 1998; Izzo et al., 2001; Carai et al., 2000) inhibit gastrointestinal transit in a dose-dependent manner. HU 210 proved to be more potent to inhibit mouse gastrointestinal transit than other receptor agonists such as WIN 55,212-2 (Colombo et al., 1998; Carai et al., 2000) and CP 55,940 (Izzo et al., 2001). The fact that SR 141716A per se increased the gastrointestinal transit suggests the existence of an inhibitory, cannabinoid receptor-mediated endogenous tone in the mouse gastroenteric tract. Further, a dose of HU 210 devoid per se of any activity on mouse intestinal propulsion blocked the increased gastroenteric transit induced by the cannabinoid $CB_1$ receptor antagonist SR 141716A.

The cellular and pharmacological evidence shown here supports the notion that cannabinoids may have a direct influence on the whole mouse gastroenteric tract. In addition, the identification of the gastroenteric regions where cannabinoids receptors are expressed will permit further investigation of their differential physiology and their possible role in pathological states. We confirm that cannabinoid agonists or antagonists may have therapeutic applications as potential modulators of gastrointestinal physiopathology.

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## References

Adami, M., Bertini, S., Frati, P., Soldani, G., Corazzi, G., 2000. Cannabinoid $CB_1$ receptors are involved in the regulation of gastric acid secretion. Pharm. Pharmacol. Commun. 6, 273–275.

Adami, M., Frati, P., Bertini, S., Kulkarni-Narla, A., Brown, D.R., de Caro, G., Corazzi, G., Soldani, G., 2002. Gastric antisecretory role and immunohistochemical localization of cannabinoid receptors in the rat stomach. Br. J. Pharmacol. 135, 1598–1606.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buckley, N.E., Hansson, S., Harta, G., Mezey, E., 1998. Expression of the $CB_1$ and $CB_2$ receptor messenger RNAs during embryonic development in the rat. Neuroscience 82, 1131–1149.

Calignano, A., La Rana, G., Makriyannis, A., Lin, S.Y., Beltramo, M., Piomelli, D., 1997. Inhibition of intestinal motility by anandamide, an endogenous cannabinoid. Eur. J. Pharmacol. 340 (2–3), R7–R8.

Carai, M.A.M., Agabio, R., Lobina, C., Pani, M., Reali, R., Vacca, G., Colombo, G., Gessa, G.L., 2000. Relationship between cannabinoid $CB_1$ and dopamine $D_2$ receptors in intestinal motility in mice. Pharm. Pharmacol. Commun. 6, 287–291.

Chesher, G.B., Dahl, C.J., Everingham, M., Jackson, D.M., Marchant-Williams, H., Starmer, G.A., 1973. The effect of cannabinoids on intestinal motility and their antinociceptive effect in mice. Br. J. Pharmacol. 49 (4), 588–594.

Colombo, G., Agabio, R., Lobina, C., Reali, R., Gessa, G.L., 1998. Cannabinoid modulation of intestinal propulsion in mice. Eur. J. Pharmacol. 344 (1), 67–69.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949.

Dewey, W.L., Harris, L.S., Kennedy, J.S., 1972. Some pharmacological and toxicological effects of 1-*trans*-8 and 1-*trans*-9-tetrahydrocannabinol in laboratory rodents. Arch. Int. Pharmacodyn. Ther. 196 (1), 133–145.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P.M., Bouaboula, M., Shire, D., Le Fur, G., Casellas, P., 1995. Expression of central and peripheral cannabinoid receptor in human immune tissues and leukocyte subpopulations. Eur. J. Biochem. 232, 54–61.

Germanò, M.P., D'Angelo, V., Mondello, M.R., Pergolizzi, S., Capasso, F., Capasso, R., Izzo, A.A., Mascolo, N., De Pasquale, R., 2001. Cannabinoid $CB_1$-mediated inhibition of stress-induced gastric ulcers in rats. Naunyn-Schmiedebergs Arch. Pharmacol. 363 (2), 241–244.

Griffin, G., Fernando, S.R., Ross, R.A., McKay, N.G., Ashford, M.L., Shire, D., Huffman, J.W., Yu, S., Lainton, J.A., Pertwee, R.G., 1997. Evidence for the presence of $CB_2$-like cannabinoid receptors on peripheral nerve terminals. Eur. J. Pharmacol. 339 (1), 53–61.

Grinspoon, L., Bakalar, J.B., 1997. The Forbidden Medicine. Yale Univ. Press, New Haven, CT.

Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., De Costa, B.R., Rice, K.C., 1991. Characterization and localization of cannabinoid receptors in the rat brain: a quantitative in vitro autoradiographic study. J. Neurosci. 11, 563–583.

Izzo, A.A., Mascolo, N., Capasso, R., Germano, M.P., De Pasquale, R., Capasso, F., 1999. Inhibitory effect of cannabinoid agonists on gastric emptying in the rat. Naunyn-Schmiedebergs Arch. Pharmacol. 360 (2), 221–223.

Izzo, A.A., Mascolo, N., Capasso, F., 2000a. Marijuana in the new millennium: perspectives for cannabinoid research. Trends Pharmacol. Sci. 21 (8), 281–282.

Izzo, A.A., Pinto, L., Borrelli, F., Capasso, R., Mascolo, N., Capasso, F., 2000b. Central and peripheral cannabinoid modulation of gastrointestinal transit in physiological states or during the diarrhoea induced by croton oil. Br. J. Pharmacol. 129 (8), 1627–1632.

Izzo, A.A., Fezza, F., Capasso, R., Bisogno, T., Pinto, L., Iuvone, T., Esposito, G., Mascolo, N., Di Marzio, V., Capasso, F., 2001. Cannabinoid $CB_1$-receptor mediated regulation of gastrointestinal motility in a model of intestinal inflammation. Br. J. Pharmacol. 134, 563–570.

Kulkarni-Narla, A., Brown, D.R., 2000. Localization of $CB_1$-cannabinoid receptor immunoreactivity in the porcine enteric nervous system. Cell Tissue Res. 302 (1), 73–80.

Lynn, A.B., Herckenham, L., 1994. Localization of cannabinoid receptors and no saturable high-density cannabinoid binding sites in peripheral tissues of the rat: implications for receptor-mediated immune modulation by cannabinoids. J. Pharmacol. Exp. Ther. 268, 1612–1623.

Mancinelli, R., Fabrizi, A., Del Monaco, S., Azzena, G.B., Vargiu, R., Colombo, G.C., Gessa, G.L., 2001. Inhibition of peristaltic activity by cannabinoids in the isolated distal colon of mouse. Life Sci. 69 (1), 101–111.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Merck & Co., 1899. Merck's Manual of the Materia. Medica Merck, New York.

Munro, S., Thomas, K.L., Abu-Shire, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61–65.

Nagakura, Y., Naitoh, Y., Kamato, T., Yamano, M., Miyata, K., 1996. Compounds possessing 5-HT3 receptor antagonistic activity inhibit intestinal propulsion in mice. Eur. J. Pharmacol. 311 (1), 67–72.

Pertwee, R.G., 1997. Pharmacology of cannabinoid $CB_1$ and $CB_2$ receptors. Pharmacol. Ther. 74 (2), 129–180.

Pertwee, R.G., 2001. Cannabinoids and the gastrointestinal tract. Gut 48 (6), 859–867.

Pertwee, R.G., Fernando, S.R., Nash, J.E., Coutts, A.A., 1996. Further evidence for the presence of cannabinoid $CB_1$ receptors in guinea-pig small intestine. Br. J. Pharmacol. 118 (8), 2199–2205.

Pinto, L., Izzo, A.A., Cascio, M.G., Bisogno, T., Hospodar-Scott, K., Brown, D.R., Mascolo, N., Di Marzo, V., Capasso, F., 2002. Endocannabinoids as physiological regulators of colonic propulsion in mice. Gastroenterology 123, 227–234.

Porcella, A., Maxia, C., Gessa, G.L., Pani, L., 2000. The human eye expresses high levels of $CB_1$ cannabinoid receptor mRNA and protein. Eur. J. Neurosci. 12, 1123–1127.

Rinaldi-Carmona, M., Barth, F., Heaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Neliat, G., Caput, D., Ferrara, P., Soubriè, P., Breliere, J.C., Le Fur, G., 1994. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. 350 (2–3), 240–244.

Rosell, S., Agurell, S., Martin, B., 1976. Effect of cannabinoids on isolated smooth muscle preparations. In: Nahas, G.G. (Ed.), Marihuana: Chemistry, Biochemistry and Cellular Effects. Springer, New York, pp. 397–406.

Ross, R.A., Brockie, H.C., Fernando, S.R., Saha, B., Razdan, R.K., Pertwee, R.G., 1998. Comparison of cannabinoid binding sites in guinea-pig forebrain and small intestine. Br. J. Pharmacol. 125 (6), 1345–1351.

Shire, D., Carillon, C., Kaghad, M., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Caput, D., Ferrara, P., 1995. An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. J. Biol. Chem. 270 (8), 3726–3731.

Shook, J.E., Burks, T.F., 1989. Psychoactive cannabinoids reduce gastrointestinal propulsion and motility in rodents. J. Pharmacol. Exp. Ther. 249 (2), 444–449.

Song, C., Howlett, A., 1995. Rat brain cannabinoid receptors are *N*-linked glycosilated proteins. Life Sci. 56, 1983–1989.

Tyler, K., Hillard, C.J., Greenwood-Van Meerveld, B., 2000. Inhibition of small intestinal secretion by cannabinoids is $CB_1$ receptor-mediated in rats. Eur. J. Pharmacol. 409 (2), 207–211.