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Evaluation of the effect of age on cannabinoid receptor functionality and expression in guinea-pig ileum longitudinal muscle–myenteric plexus preparations

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Abstract

Cannabinoid drugs exert a wide range of biological effects and are currently under study for their multiple potential therapeutic uses. Cannabinoids reduce gastrointestinal (GI) motility and this is mediated by the CB1 cannabinoid receptor (CB1R) present in the myenteric neurones. GI motility can also be affected by a variety of pathophysiological situations, including ageing. The purpose of this work was to study the influence of age on the functionality and expression of CB1R in the myenteric plexus. Ileal longitudinal muscle–myenteric plexus (LMMP) preparations from young, adult and old guinea-pigs were used in two sets of experiments: in vitro assessment of the inhibitory cannabinoid effect upon electrically stimulated contractions and immunohistochemical quantification of myenteric neurones expressing CB1R. LMMP preparations responded to the synthetic cannabinoid WIN 55,212-2, and the endogenous cannabinoid ligand anandamide in an age-independent manner. The total number of CB1R-immunoreactive (IR) myenteric neurones, which included at least part of the motor neurones to the longitudinal smooth muscle, decreased in proportion to the general neuronal population; however, the proportion of CB1R-IR neurones was preserved in old animals. These data may justify the preservation of the effectiveness of the cannabinoids in the isolated guinea-pig ileum. This age-related independency of CB1R expression and effect on GI motility could be of interest if cannabinoids are to be used therapeutically.

Keywords: Cannabinoid; Myenteric plexus; Ageing; Guinea-pig; Isolated ileum

Marijuana (*Cannabis sativa*) has been used for recreational and medicinal purposes for centuries. The therapeutic effects of marijuana derivatives and synthetic analogues, known as cannabinoid drugs, are surprisingly broad. This variety of effects is related to the widespread distribution of cannabinoid receptors in the brain and throughout the periphery [13,24].

In the gastrointestinal (GI) tract, it has been shown that cannabinoids exert many biological functions, including gastroprotection, reduction of intestinal secretion and reduction of gastric and intestinal motility. Most cannabinoid effects on gastrointestinal motility depend on the activation of CB1 receptors (CB1R; Table 1). CB1R mRNA has been detected in the guinea-pig ileum and rat colon [2,11], and numerous myenteric neurones, responsible for GI motility, show immunoreactivity for the receptor itself [4,20].

Although the CB1R has been found in the rat GI tract as early as in prenatal development [2], the influence of ageing on expression and functionality of CB1R in the enteric nervous system has not been addressed so far. However, it also deserves attention because cannabinoids are being studied and likely to be used in the near future for the treatment of a variety of health challenges frequently encountered in the elderly. Thus, some cannabinoid agonists are being used for the treatment of emesis induced by cancer chemotherapy [25]. Furthermore, the CB1R antagonist, rimonabant, is expected to be imminently approved for the treatment of obesity [7]. It is not known, though, to what extent responses to cannabinoid drugs could be altered in aged patients. Furthermore, in the GI tract, ageing has been associated with functional impairments

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Table 1
Summary of experimental data reporting cannabinoid effects on gastrointestinal motility

Cannabinoid effect	Species
CB1 cannabinoid receptor	
Prejunctional inhibition of electrically-evoked twitches of small intestine longitudinal smooth muscle	Guinea-pig [30], Human [6]
Inhibition of intestinal peristalsis	Guinea-pig [12]
Depression of fast cholinergic neurotransmission in S-myenteric neurones	Guinea-pig [23]
Reduction in gastric emptying	Mouse, rat [36], Rat [21]
Reduction in gastrointestinal transit	Mouse [17], Rat [21]
Reduction in faecal pellet output	Rat [17,18]
Involvement of enteric endocannabinoid system in the induction of experimental paralytic ileus by peritoneal irritation	Mouse [26]
CB2 cannabinoid receptor	
Reduction of intestinal motility induced by endotoxic inflammation	Rat [28]

such as decreased oesophageal and colonic motility, delayed gastric emptying and increased intestinal transit times [9,37]. These alterations have generally been attributed to a loss of myenteric neurones, which could be particularly greater in the case of those mediating excitatory responses [1,10,33]. It is likely that a higher endocannabinoid tone could also be involved in these age-related alterations in GI motility.

Therefore, we aimed to study the influence of age on the expression and functionality of CB1R in the myenteric plexus, which is responsible for GI motility. As a first approach to reach this objective, we used guinea-pig ileum longitudinal muscle myenteric plexus (LMMP) preparations. Myenteric neurones in this preparation have been well characterised functionally in terms of their immunohistochemical contents [3].

Dunkin–Hartley female guinea-pigs of three different ages were used: 1–3 months (YOUNG, weight 330–450 g), 8–10 months (ADULT, weight 800–950 g) and 22–26 months (OLD, weight 900–1200 g). All experimental protocols were approved by the Ethical Committee at the Universidad Rey Juan Carlos and performed in strict accordance with the EC regulation for care and use of experimental animals (EEC N° 86/609). In order to minimize pain and discomfort, animals were killed by cervical dislocation and segments of distal ileum, at least 10 cm oral to the ileocaecal junction, were obtained, opened along the mesenteric border, rinsed and pinned flat on a Sylgard-coated Petri-dish filled with modified Krebs solution (mM: NaCl; 118, KCl; 4.75, NaH_2PO4; 1.0, $NaHCO_3$; 25, $MgSO_4$; 1.2, $CaCl_2$; 2.5, glucose; 11, pH 7.4). Whole-mount LMMP preparations were obtained by removal of mucosa, submucosa and circular muscle layers. LMMP preparations were used in two different sets of experiments.

For the functional studies, LMMP preparations were suspended, under a tension of 1 g, in an organ bath containing Krebs solution (36 °C) and aerated with carbogen (95% O_2–5% CO_2). Contractile activity of the preparations was recorded by means of an isometric transducer connected to a PowerLab/4e system. Electrical field stimulation (pulses of 2 ms duration, 0.3 Hz and supramaximal voltage) was used to induce atropine-sensitive twitches of the preparations. Those preparations whose contractions did not reach 0.5 g were discarded. No atropine-sensitive differences were apparent in the number of usable preparations. The influence of age on the responses to two well-known cannabinoid agonists was tested in electrically stimulated preparations. Either WIN 55,212-2 (WIN, non-selective CB1-CB2 synthetic cannabinoid drug) or anandamide (endogenous ligand of CB1R) were added in increasing cumulative concentrations (10^{-8} to 2.4×10^{-6} M) at intervals of 15 min; the effect of the cannabinoids was evaluated as the percentage of inhibition of the initial twitch amplitude. The CB1R-selective antagonist SR141716A (10^{-6} M) was added at the end of the experiment to revert the effect of the cannabinoids. All drugs were purchased from Tocris and dissolved in ethanol following the method previously described by Pertwee et al. [32]. The effect of the vehicle was tested in preparations from young animals. At the higher concentration used, it produced a slight inhibition (less than 10%) of the electrically stimulated contractions.

Methods for the immunohistochemical study of whole-mount LMMP preparations have been described in detail in a previous report [1]. Briefly, each LMMP preparation was stretched to its maximal extension, fixed in Zamboni's fixative and cleared with dimethylsulfoxide. After several washes with phospate buffer saline (PBS), tissues were incubated overnight at room temperature with a mixture of mouse anti-Hu C/D (1:500, Molecular Probes) and rabbit anti-CB1 (1:100, generous gift from Dr Mackie, University of Washington [4]). After washing with PBS (3×10 min), tissues were exposed for 90 min at room temperature to a mixture of avidin-AlexaFluor 488 (1:1000, Molecular Probes) and donkey anti-rabbit-CY5 (1:100, Jackson). All mixtures of antibodies were diluted with hypertonic PBS (1.7% NaCl) [3]. Some preparations were further labelled for calretinin (CR), which is present in the motor neurones that innervate the longitudinal muscle in guinea-pig ileum [3]. In this case, goat anti-CR (1:1000, Chemicon) and donkey anti-goat (1:100, Jackson) antibodies were added to the mixture of primary and secondary antibodies, respectively. The preparations were first observed under a fluorescent microscope (Nikon Eclipse TE2000-U, with appropriate filters). Using a confocal microscope (LSM 510 Zeiss, lasers: 488 and 453 nm), micrographs were taken and mounted on mosaics for the quantitative analysis [1]. All counts of myenteric neurones were made by an experimenter blind to the age of the guinea-pig from which the tissue originated. For each preparation labelled for Hu and CB1 no less than 400 cells immunoreactive to Hu were counted and the proportion of CB1R-immunoreactive (IR) neurones was obtained. In preparations immunolabelled for CR, the proportion of CR-IR neurones also positive for CB1R was determined. Controls for double-labelling were performed by pairing the wrong primary and