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Running head: Cannabinoid receptors in horse sensory ganglia

Abstract

BACKGROUND: Growing evidence recognises cannabinoid receptors as potential therapeutic targets for pain. Consequently, there is ~~an~~increasing interest in developing cannabinoid receptor agonists for treating pain. As a general rule, to better understand the actions of a drug, it would be of extreme importance to know the cellular distribution of its specific receptors. The localisation of cannabinoid receptors in the dorsal root ganglia of the horse has not yet been investigated.

OBJECTIVES: To localise the cellular distribution of canonical and putative cannabinoid receptors in the equine cervical dorsal root ganglia (DRG).

STUDY DESIGN: Qualitative and quantitative immunohistochemical study.

METHODS: Cervical (C6-C8) DRG were collected from six horses (1.5 years of age) at the slaughterhouse. The tissues were fixed and processed to obtain cryosections which were used to investigate the immunoreactivity of canonical cannabinoid receptors 1 (CB1R) and 2 (CB2R), and for three putative cannabinoid-related receptors: nuclear peroxisome proliferator-activated receptor alpha (PPARα), transient receptor potential ankyrin 1 (TRPA1) and serotonin 5-HT1a receptor (5-HT1aR).

RESULTS: The neurons showed immunoreactivity for CB1R (100%), CB2R (80±13%), PPARα (100%), TRPA1 (74±10%) and 5-HT1aR (84±6%). The neuronal satellite glial cells showed immunoreactivity for CB2R, PPARα, TRPA1 and 5-HT1aR.

MAIN LIMITATIONS: The number of horses included in the study.

CONCLUSIONS: The present study highlighted the expression of cannabinoid receptors in the sensory neurons and glial cells of the DRG. These findings could be of particular relevance for future functional studies assessing the effects of cannabinoids in horses to manage pain.

Introduction

The dorsal root ganglia (DRG) reside within the dorsal root of the spinal nerve and are mainly constituted of a cluster of primary sensory neurons. Previous research has described the DRG as passive neural structures which merely "supported" the physiological communication between the peripheral nervous system and the central nervous system [1]. Nonetheless, novel evidence shows that the DRG neurons play a critical role in carrying sensory messages from various receptors, including those for pain and temperature, and transmitting them to the spinal cord [2].

The cell bodies of the DRG neurons are separated from each other by an envelope of satellite glial cells (SGCs), which play important roles in both healthy and pathological states. Since the SGCs carry receptors for numerous neuroactive agents, they have a plethora of roles including receiving signals from other cells and influencing neighboring cells and other DRG neurons. These mechanisms likely influence signal processing and transmission within the DRG [3] and possibly contribute to the sensitisation of pain transmission nociceptors [4]. Altogether, this evidence suggests that the study of the DRG can have significant clinical applications for pain modulation and novel targeted therapeutic treatment (Hogan, 2010) [5].

A growing body of literature demonstrates that cannabinoid receptors play a critical role in nociception also through peripheral mechanisms (Calignano et al., 1998) [6]. Specifically, upon activation by medical cannabis, beneficial effects have been recorded on pain perception in humans [7-9].

Even though limited empirical research has been carried out concerning the use of medical marijuana for pain treatment in domestic animals [10] and horses [11], the business of cannabis products for use in animals is expanding [12]. Of the cannabis products, cannabidiol (CBD), a non-psychoactive compound found in *cannabis sativa*, seems to be one of the most promising therapeutic substances. Due to its numerous health-related benefits, CBD has found multiple clinical applications in the medical field, including analgesic, anti-inflammatory, anti-spasmodic and anti-anxiety uses [13, 14]. For many years, it was assumed that the beneficial effects of the cannabinoids were mediated exclusively by cannabinoid receptors 1 (CB1R) and 2 (CB2R). However, it is currently known that phytocannabinoids may act on multiple targets outside the

endocannabinoid system, such as other G-protein coupled receptors (GPRs), the transient receptors potential (TRPs) channel, nuclear peroxisome proliferator-activated receptors (PPARs), and serotonin receptors [15]. In particular, CBD, which shows indirect interaction with CB1R and CB2R, seems to be involved in the modulation of receptors, such as the serotoninergic 5-HT1a receptor (5-HT1aR), and the transient receptors potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1), the latter two being excitatory ion channels expressed by the sensory neurons mediating somatic and visceral pain [7].

As a general rule, to better understand the effects exerted by a drug, it would be of extreme importance to know the cellular distribution of its specific receptors. Currently, only a small number of studies have been published regarding the expression of cannabinoid receptors in the DRG of animals [16-19] whereas, to the best of the Authors' knowledge, no analogous studies have yet been carried out involving horses.

Thus, the present study was designed to immunohistochemically localise, ~~in the equine DRG,~~ two canonical cannabinoid receptors (CB1R and CB2R) and three cannabinoid-related reeptors (PPARα, TRPA1, and 5-HT1aR) in the equine DRG.

Material and Methods

Animals

The cervical (C6-C8) DRG were collected from the left and the right halves of the carcasses of six horses (4 males and 2 females) which were slaughtered for human food purposes. The horses (1.5 years of age) were of two breeds, 3 Polish and 3 half-breeds. The complete cell blood count (CBC) and routine serum biochemical analyses were carried out with blood samples taken at the time of exsanguination. The horses were considered healthy on the basis of normal results of the CBC count and routine serum biochemical analyses. In addition, the horses did not show lameness of either the thoracic or the pelvic limbs.

The tissues were fixed and processed to obtain cryosections, as described elsewhere [20].

Since the suppliers of the primary antibodies used in the present study stated that they were rat-specific (CB2R, TRPA1, and 5-HT1aR) or reacted with rat tissues (CB1R, PPARα), the experiments on the equine DRG were carried out by including rat C6-C8 DRG (authorization no. 112/2018-PR of 12 February 2018) as positive controls (Supplementary Item 1~~3~~). The distribution of the study receptors in the subclasses of the rat sensory neurons was outside the scope of the present study and was therefore not evaluated.

Immunofluorescence

The cryosections (14-16 µm thickness), were hydrated in phosphate–buffered saline (PBS) and processed for immunostaining. To block non-specific bindings, the sections were incubated in a solution containing 20% normal goat serum[a], 0.5% Triton X-100[b] and bovine serum albumin (1%) in PBS for 1 h at room temperature (RT) (22-25°C). The cryosections were incubated overnight in a humid chamber at RT with the primary antibodies (Table 1) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After washing in PBS (3 x 10 min), the sections were incubated for 1 h at RT in a humid chamber with the secondary antibody [goat F(ab)2 anti-rabbit FITC; ab98430][c] diluted in PBS. After washing in PBS (3 x 10 min), to identify the DRG neurons and the SGCs and to determine the proportion of neurons immunoreactive for each of the markers studied, the sections were counterstained with Blue fluorescent Nissl stain solution (NeuroTrace®, # N-21479, dilution 1:200)[d]. The cryosections were then washed in PBS (3 x 10 min) and mounted in buffered glycerol at pH 8.6. A minimum of one hundred Nissl-stained neurons were counted for each marker. Data were collected from preparations obtained from four animals (*n=4*). The percentages of immunopositive neurons were expressed as mean ± standard deviation.

Specificity of the primary antibodies

The choice of the primary antibodies utilised in the study was based on the homology of the aminoacidic sequence between the immunogen of the commercially available antisera and the horse proteins, verified by the "alignment" tool available on the Uniprot database (www.uniprot.org) and the BLAST tool of the National Center for Biotechnology information (NCBI) (www.ncbi.nlm.nih.gov). ~~Details are summarized in Supplementary item 1.~~

CB1R – The immunogen used to obtain antibody ab23703 was the synthetic peptide MSVSTDTSAEAL, corresponding to carboxy-terminal amino acids 461-472 of Human cannabinoid receptor I. The homology between the full amino acid sequences of Horse (F6SIU9) and Human (P21554) CB1R was 97.88%, and the correspondence with the specific sequence of the immunogen was 100%.

CB2R - The immunogen used to obtain antibody ab45942 was the synthetic peptide conjugated to keyhole limpet haemocyanin (KLH) derived from within residues 200 - 300 of Rat cannabinoid receptor II. The homology between the full amino acid sequences of Horse (F7CUS7) and Rat (Q9QZN9) CB2R was 80.9%, and the correspondence with the specific sequence of the immunogen was 83.33%.

PPARα - Antibody NB600-636 was prepared from whole rabbit serum produced by repeated immunisations using a synthetic peptide corresponding to amino acids 1 to 18 of Mouse PPARα. The homology between the full aminoacidic sequences of Horse (F7DSM8) and Mouse (P23204) PPARa was 90.81%, and the correspondence with the specific sequence of the immunogen was 100%.

TRPA1 - The immunogen used to obtain antibody ab58844 was peptide EKQHELIKLIIQKME corresponding to amino acids 1060-1075 of Rat TRPA1. The homology between the full amino acid sequences of Horse (F7DXW9) and Rat (Q6RI86) TRPA1 was 82%, and the correspondence with the specific sequence of the immunogen was 100%.

Serotonin 5-HT1aR - The immunogen used to obtain antibody ab85615 was the synthetic peptide corresponding to Rat 5HT1aR amino acids 100-200 conjugated to keyhole limpet hemocyanin. The homology between the full amino acid sequences of Horse (Q0EAB6) and Rat (P19327) 5HT1aR was 89.3%, and the correspondence with the specific sequence of the immunogen was 99%.

In addition, the specificity of the primary antibodies employed was also tested using Western blot (Wb) analysis.

Specificity of the secondary antibody

The secondary antibody specificity was tested by the lack of signal after omission of the primary antibody on DRG tissues

Fluorescence microscopy

The preparations were analysed by the same observer, and the images were recorded and adjusted as described elsewhere [21].

Western blot: specificity of the primary antibodies

Cervical (C-6-C8) DRG and spinal cord samples were collected, frozen in liquid nitrogen, and stored at − 80°C until sample processing. The primary antibodies were tested in our laboratories according to standardized protocols [21]. The Wb analysis of CB1R (1:500) revealed double bands of ~ 100 and 120 kDa (the theoretical molecular weight of CB1R is 53-60 kDa) (Fig. 1 a~~Supplementary item 2a~~), that of CB2R (1:1000) revealed a single band of ~ 90 kDa (the theoretical molecular weight of CB2R is 45 kDa) (Fig. 1 b~~Supplementary item 2b~~), that of PPARα (1:2000) revealed a single band of ~ 50 kDa (the theoretical molecular weight of PPARα is 52 kDa) (Fig. 1 c~~Supplementary item 2c~~), that of TRPA1 (1:500) revealed a major band at

~ 100 kDa (the theoretical molecular weight 100 kDa) (Fig. 1 d~~Supplementary item 2d~~) and that of the 5-HT1aR (1:3000) revealed a major band at ~ 50 kDa (theoretical molecular weight 46 kDa) (Fig. 1 e~~Supplementary item 2e~~). Western blot analysis confirmed the specificity of the anti-PPARα,-TRPA1 and-5-HT1aR primary antibodies; the latter finding is consistent with the 100% homology of the amino acid sequence between the immunogen of the antisera and the horse proteins (see above). The CB1R (100% homology) and CB2R (83% homology) showed bands with apparent double molecular weights; it is known that G-protein receptors, such as CB1R, may show dimerisation, and form functionally active homodimer and heterodimer complexes, and that polyclonal antibodies directed against the carboxy-terminal may only recognise the high-molecular weight form of CB1R [22]. ~~We have also observed~~An apparent dimeric form for the other G-protein receptor CB2R was also observed; however, no studies regarding the dimeric form of this receptor are available in the literature. Nevertheless, ~~it is not excluded~~the possibility that the anti-CB2R antibody may recognize only the band in which the receptor is conjugated to another CB2R (or to another protein), as observed for the anti-CB1R antibody, cannot be excluded.

Results

CB1R

Bright cytoplasmic and nucleolar CB1R immunoreactivity (CB1R-IR) was displayed, with different degrees of intensity, by 100% of the neurons (604/604 cells counted, *n*=4) (Fig. 2~~1~~ a-c); no distinction of CB1R immunolabelling was observed among neurons of different sizes. The nerve processes also showed CB1R-IR, although it was fainter than that observed in the neuronal somata. The SGCs also showed faint CB1R-immunolabeling. This finding is partially consistent with what has been observed in the rat DRG in which neurons and SGCs expressed faint cytoplasmic and nuclear CB1R-IR [19].

CB2R

The CB2R-IR was expressed by 80±13% of the neurons (665/799 cells counted, *n*=4) and by the SGCs. In general, there was an inverse correlation between the brightness of CB2R-IR in the neurons and SGCs; small-to-medium-sized neurons showed brighter granular CB2R cytoplasmic immunolabeling in

comparison with larger ones which were encircled by brightly labeled SGCs (Fig. 2~~1~~ d-i). In the rat DRG, CB2R-IR was expressed by the neuronal nuclei [19].

TRPA1

The TRPA1-IR was expressed by the cytoplasm (moderate staining) and nucleus (bright staining) of both the neurons (74±10%; 497/698 cells counted, *n*=4) and the SGCs, and by the nerve processes (Fig. 3~~2~~ a-c). Of the nerve processes, thin and unmyelinated nerve fibres showed brighter TRPA1 immunolabelling than large myelinated nerve fibres. This finding is partially consistent with what has been observed in the rat DRG in which only the neuronal cytoplasm expressed TRPA1-IR (Supplementary item 1~~3~~ a-c).

PPARα

Faint cytoplasmic PPARα-IR was displayed by all the neurons (100%; 456/456 cells counted, *n*=4) whereas it was brightly displayed by the cytoplasm of the SGCs (Fig. 3~~2~~ d-f~~g-i~~). The PPARα-IR was also expressed by the endothelial cells of the blood vessels (data not shown). This finding is partially consistent with what has been observed in the rat DRG in which neurons and SGCs expressed PPARα-IR [19].

5-HT1aR

Moderate cytoplasmic 5-HT1aR-IR was expressed by 84±6% of the neurons (462/547 cells counted, *n*=4); faint to moderate 5-HT1aR-IR was also expressed by the SGCs (Fig. 3 g-i) and the Schwann cells (Fig. 3 j-l). This finding is partially consistent with what has been observed in the rat DRG in which only the neuronal cytoplasm expressed 5-HT1aR-IR (Supplementary item 1~~3~~ d-f).

The results of the cellular distribution and intensity of the immunolabeling in the equine DRG are summarised in semiquantitative Table 2.

Discussion

This is the first study aimed at investigating the expression and localisation of cannabinoid receptors (CB1R and CB2R) and cannabinoid-related receptors (PPARalpha, TRPA1, 5-HT1aR) in the equine DRG.

Cannabinoid CB1R is widely expressed throughout the nociceptive system and its activation by endogenous or exogenous cannabinoids modulates neurotransmitter release. Previous studies carried out on humans and rodents have shown CB1R-IR expression limited to the sensory neurons [16,19,23] whereas the present study showed that CB1R-IR is localised in both the equine sensory neurons and SGCs in line with data recently reported for dogs [18,19].

In humans, the CB2R-IR has been localised exclusively in nociceptive DRG neurons [23]. In the present study, CB2R-IR was observed ~~either~~ in the equine DRG neurons and SGCs, as previously observed in rats [17]. The CB2R-IR has recently been observed in the neuronal nuclei of rats and dogs as well as in the Schwann cells of dogs [19]. It has been shown that the application of CB2R agonists functionally inhibits nociceptive signalling in human DRG neurons [24]. Based on the Authors' findings, it can be hypothesised that, in horses, the same agonists could be useful for the peripheral modulation and treatment of painful sensation [24].

~~Beside of~~In addition to canonical cannabinoid receptors, multiple targets can be influenced by phytocannabinoids unravelling the complexity of this endocannabinoid system. For instance, PPARs belong to the family of intranuclear receptors which act as transcription factors, modulating different physiological functions. Once activated by their ligand, PPARs induce the expression of hundreds of genes in each cell type [25]. However, their activation has also been shown to result in rapid cellular changes which do not require transcription, including reduction of inflammation [26, 27]. Recent studies have shown that cannabinoids activated PPARs [15, 27, 28], and that this activation is associated with some of the pain-relieving, anti-inflammatory and neuroprotective properties of cannabinoids.

In the present study, PPARα-IR expression was observed in both the sensory neurons and the SGCs as has previously been shown in rats [19] whereas ~~in mice and dogs,~~ PPARα was detected exclusively in the neurons or in the SGCs, respectively in mice and dogs [19, 29]. ~~In the present study,~~ PPARα-IR was also observed in the endothelial cells of the DRG blood vessels. Recent evidence has shown that endocannabinoids exert a pro-homeostatic function on vascular biology by means of complex mechanisms involving canonical as well as putative cannabinoid receptors [30]. Specifically, different cannabinoid receptors

can actively influence vasodilation at different cellular sites, i.e., nerves, endothelial cells, vascular smooth muscle cells or pericytes [31].

The TRPA1 is required for normal mechano- and chemo-sensory functions in specific subsets of vagal, splanchnic and pelvic afferents [32]. The TRPA1 also mediates somatic and visceral pain in response to stimulation of a chemical, mechanical or thermal origin [33, 34], and can be desensitised by different mechanisms [35]. Data from the present study demonstrated that TRPA1 was expressed by the sensory neurons and by the SGCs, with bright immunolabelling in thin unmyelinated nerve fibres. This is partially in line with previous studies carried out on humans and rodents showing that the TRPA1-IR sensory neurons are involved in nociception [36]. As a consequence, the analgesic effects of CBD might be due, in part, to its ability to activate and desensitise the TRPA1 [37].

Another finding which emerged from this study was the expression of 5-HT1aR in sensory neurons, SGCs and Schwann cells. It is well established that 5-HT exerts a pivotal role in sensory information processing [38]. At the level of the spinal cord, 5-HT is primarily released by the descending bulbospinal serotonergic neurons and causes analgesia by inhibiting dorsal horn neuronal responses to noxious stimuli by means of the activation of the 5-HT1aR [39]. In addition, the activation of the 5-HT1aR inhibits glutamate release from the sensory neurons, reducing pain transmission [40]. Functional studies have suggested the presence of the 5-HT1aR in the DRG and its role in nociception [41]. In the present study, the 5-HT1aR-IR was observed in the sensory neurons and the SGCs of horses, and in the neurons of rats. Therefore, it is plausible to consider that the 5-HT1aR might play a role in pain modulation. ~~We~~ The Authors also observed the 5-HT1aR in Schwann cells, a finding which is consistent with the expression of other serotoninergic receptors on Schwann cells in rodents [42].

In summary, this study showed a notable distribution of cannabinoid and cannabinoid-related receptors in the equine DRG.

The reduced number of animals and ganglia considered, as well as the specificity of the anti-CB2R antiserum, represent the limitations of the present study. Additional biomolecular studies as well as the neurochemical characterisation of the immunoreactive DRG neurons are needed to reinforce the data in the present. Nonetheless, these findings represent novel insight regarding the complex processing of nociceptive imput in the equine peripheral nervous system and will hopefully encourage new studies regarding the role of cannabinoid-related receptors in the equine DRG and their interactions with non-psychotic cannabis-derived

molecules. In addition, cannabinoids, specifically CBD, has been proposed as an opioid alternative in human medicine, having a comparable efficacy with a better safety profile [43].

Therefore, considering the present results, it would be appropriate to also test them in horses with preclinical and clinical studies to verify safety, dosages, the kinetics of the substance and whether similar benefical effects also occur in equines with the possible presence or absence of side effects.

Conclusions and relevance

Cannabinoid and cannabinoid-related receptors had a wide distribution in the sensory neurons and SGCs of the equine DRG. -These findings represented an important anatomical basis upon which it would be possible to continue with other preclinical and clinical studies aimed at investigating and possibly supporting the specific therapeutic uses of non-psychotropic cannabinoid agonists against noxius stimulation in horses.

Manufacturers' addresses

[a] Colorado Serum Co., Denver, CO, USA

[b] Sigma Aldrich, Milan, Italy, Europe

[c] abcam, Cambridge, UK

[d] Molecular Probes, Eugene, OR

Figure legends

Fig. 1: Western blot (WB) analysis showing the specificity of the following primary antibodies utilised: a) rabbit anti-cannabinoid receptor 1 (CB1); b) rabbit anti-cannabinoid receptor 2 (CB2); c) rabbit anti-nuclear peroxisome proliferator-activated receptor alpha (PPARα); d) rabbit anti-transient receptor potential ankyrin 1 (TRPA1) and e) rabbit anti-serotonin receptor 5-HT1a (5-HT1a).

The antibody anti-CB1 receptor showed double bands of ~ 100 and 120 kDa (the theoretical molecular weight of the CB1 receptor is 53-60 kDa), more evident in the spinal cord. The antibody anti-CB2 receptor

showed a double band (only in the DRG) of ~ 90 kDa (the theoretical molecular weight of the CB2 receptor is 45 kDa). The antibody anti-PPARα showed a single band of ~ 50 kDa (the theoretical molecular weight of PPARα is 52 kDa). The antibody anti-TRPA1 showed a major band at ~ 100 kDa (theoretical molecular weight 100 kDa). The antibody anti-5-HT1A receptor showed a double band (only in the spinal cord) at ~ 50 kDa (theoretical molecular weight 46 kDa). The numbers on the DRG and the spinal cord lines indicate the molecular weights. The images of the immunoblots were slightly adjusted in brightness and contrast to match their backgrounds.

Fig. 2~~1~~: Photomicrographs of cryosections of an equine cervical (C8) dorsal root ganglion showing cannabinoid receptor 1 (CB1R) (a-c) and cannabinoid receptor 2 (CB2R) (d-i) immunoreactivity (IR). a-c) CB1R-IR was expressed by the sensory neurons whereas the satellite glial cells, the nuclei of which are indicated by arrows, were CB1R-negative. d-i) The arrows indicate the satellite glial cells which were CB2R immunoreactive. Sensory neurons, in particular the smallest ones, showed very faint granular CB2R immunolabelling.

Scale bar = 50µm

Fig. 3~~2~~. Photomicrographs of cryosections of an equine cervical (C8) dorsal root ganglion showing TRPA1- (a-c), ~~TRPV1- (d-f),~~ PPARα- (d-f~~g-i~~) and 5-HT1a receptor- (g-l)~~PPARγ- (j-l)~~ immunoreactivity (IR). a-c) Sensory neurons and satellite glial cells (small arrows) expressed TRPA1-IR. Large arrows indicate groups of amyelinic sensory fibres which showed very bright TRPA1-IR whereas the nerve fibres with a larger diameter (stars) showed weaker immunostaining. ~~d-e) Only the sensory neurons were TRPV1-immunoreactive whereas the satellite glial cells (arrows) were TRPV1-negative.~~ d-f~~g-i~~) Weak PPARα-IR was expressed by the cytoplasm of all the neurons whereas the satellite glial cells (arrows) showed bright PPARα-IR. ~~j-l) Bright PPARγ-IR was expressed by neuronal nuclei (large arrows) whereas the nuclei of the glial cells (small arrows) showed fainter immunolabelling.~~ g-i) Sensory neurons expressed bright 5-HT1a receptor-IR; satellite glial cells (arrows) also showed moderate 5-HT1a receptor-IR. j-l) The Schwann cells (arrows) showed 5-HT1a receptor-IR.

Scale bar = 50µm

~~**Fig. 3.** Photomicrographs of cryosections of an equine cervical (C8) dorsal root ganglion showing GPR3- (a-c), GPR55- (d-f) and 5-HT1a receptor- (d-i) immunoreactivity (IR). a-c) The three arrows indicate the sensory neurons expressing weak GPR3-IR. The star indicates the nucleus of one large sensory neuron expressing moderate GPR3-IR. d-f) The white stars indicate the sensory neurons expressing bright GPR55-IR whereas the open stars indicate the neurons with weaker immunolabeling. The arrows indicate some SGCs showing GPR55-IR. g-i) Sensory neurons expressed bright 5-HT1a receptor-IR; satellite glial cells (arrows) also showed moderate 5-HT1a receptor-IR. j-l) The Schwann cells (arrows) showed 5-HT1a receptor-IR.~~

~~Scale bar = 50 µm~~

References

1 Krames, E.S. (2015) The dorsal root ganglion in chronic pain and as a target for neuromodulation: a review. *Neuromodulation*. **18**, 24-32.

2 Esposito, M.F., Malayil, R., Hanes, M., Deer, T. (2019) Unique Characteristics of the Dorsal Root Ganglion as a Target for Neuromodulation. *Pain Med*. 20:S23-S30.

3 Hanani, M. (2005) Satellite glial cells in sensory ganglia: from form to function. *Brain Res Brain Res Rev.* **48**, 457–476.

4 Ji, R.R., Berta, T., and Nedergaard, M. (2013) Glia and pain: is chronic pain a gliopathy? *Pain*. **154** Suppl 1:S10-28.

5 Hogan, Q.H. (2010) Labat lecture: the primary sensory neuron: where it is, what it does, and why it matters. *Reg Anesth Pain Med*. **35**, 306-311.

6 Calignano, A., La Rana, G., Giuffrida, A., Piomelli, D. (1998) Control of pain initiation by endogenous cannabinoids. *Nature*. **394**, 277–281.

7 Ligresti, A., De Petrocellis, L., and Di Marzo, V. (2016) From phytocannabinoids to cannabinoid receptors and endocannabinoids: pleiotropic physiological and pathological roles through complex pharmacology. *Physiol Rev*. **96**, 1593-659.

8 Pergolizzi, J.V. Jr., Lequang, J.A., Taylor, R. Jr., Raffa, R.B., and Colucci, D. (2018) NEMA Research Group. The role of cannabinoids in pain control: the good, the bad, and the ugly. *Minerva Anestesiol*. **84**, 955-969.

9 Yanes, J.A., McKinnell, Z.E., Reid, M.A., Busler, J.N., Michel, J.S., Pangelinan, M.M., Sutherland, M.T., Younger, J.W., Gonzalez, R., Robinson, J.L. (2019) Effects of cannabinoid administration for pain: A meta-analysis and meta-regression. *Exp Clin Psychopharmacol*. **27**, 370-382.

10 Kogan, L., Schoenfeld-Tacher, R., Hellyer, P., Rishniw, M. (2019) US Veterinarians' Knowledge, Experience, and Perception Regarding the Use of Cannabidiol for Canine Medical Conditions. *Front Vet Sci*. **5**, 338.

11 Ellis, K.L., and Contino, E.K. (2019) Case Report: Treatment using cannabidiol in a horse with mechanical allodynia. *Equine vet Educ*. doi: 10.1111/eve.13168.

12 Williamson, E.M., Liu, X., Izzo, A.A. (2019) Trends in use, pharmacology and clinical applications of emerging herbal nutraceuticals. *Br J Pharmacol*. doi: 10.1111/bph.14943.

13 Mechoulam, R., Peters, M., Murillo-Rodriguez, E., and Hanus, L.O. (2007) Cannabidiol-recent advances. *Chem Biodivers*. **4**, 1678-1692.

14 Pertwee, R.G. (2008) The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *Br J Pharmacol*. **153**, 199–215.

15 Morales, P., Hurst, D.P., and Reggio, P.H. (2017) Molecular targets of the phytocannabinoids: a complex picture. *Prog Chem Org Nat Prod*. **103**, 103-131.

16 Ahluwalia, J., Urban, L., Capogna, M., Bevan, S., Nagy, I. (2000) Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neuroscience*. **100**, 685-688.

17 Svízenská, I.H., Brázda, V., Klusáková, I., and Dubový, P. (2013) Bilateral changes of cannabinoid receptor type 2 protein and mRNA in the dorsal root ganglia of a rat neuropathic pain model. *J Histochem Cytochem*. **61**, 529-547.

18 Freundt-Revilla, J., Kegler, K., Baumgärtner, W., Tipold, A. (2017) Spatial distribution of cannabinoid receptor type 1 (CB1) in normal canine central and peripheral nervous system. *PLoS One*. **12**, e0181064.

19 Chiocchetti, R., Galiazzo, G., Tagliavia, C., Stanzani, A., Giancola, F., Menchetti, M., Militerno, G., Bernardini, C., Forni, M., Mandrioli, L. (2019) Cellular distribution of canonical and putative cannabinoid receptors in canine cervical dorsal root ganglia. *Front Vet Sci.* **6**, 313.

20 Russo, D., Bombardi, C., Castellani, G., and Chiocchetti, R. (2011) Characterization of spinal ganglion neurons in horse (*Equus caballus*). A morphometric, neurochemical and tracing study. *Neuroscience*. **176**, 53-71.

21 Giancola, F., Rambaldi, A.M., Bianco, F., Iusco, S., Romagnoli, N., Tagliavia, C., Bombardi, C., Clavenzani, P., De Giorgio, R., Chiocchetti, R. (2017) Localization of the 5-hydroxytryptamine 4 receptor in equine enteric neurons and extrinsic sensory fibers. *Neurogastroenterol Motil*. **29**. doi: 10.1111/nmo.13045.

22 Wager-Miller, J., Westenbroek, R., Mackie, K. (2002) Dimerization of G protein-coupled receptors: CB1 cannabinoid receptors as an example. *Chem Phys Lipids.* **121**, 83-89.

23 Anand, U., Otto, W.R., Sanchez-Herrera, D., Facer, P., Yiangou, Y., Korchev, Y., Birch, R., Benham, C., Bountra, C., Chessell, I.P., Anand, P. (2008a) Cannabinoid receptor CB2 localisation and agonist-mediated inhibition of capsaicin responses in human sensory neurons. *Pain*. **138**, 667-680.

24 Sánchez-Aparicio, P., Florán, B., Rodríguez Velázquez, D., Ibancovichi, J.A., Varela Guerrero, J.A., Recillas, S. (2020) Cannabinoids CB2 Receptors, One New Promising Drug Target for Chronic and Degenerative Pain Conditions in Equine Veterinary Patients. *J Equine Vet Science*. **85**, 102880.

25 Issemann, I., and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*. **347**, 645-650.

26 Lo Verme, J., Fu, J., Astarita, G., La Rana, G., Russo, R., Calignano, A., and Piomelli, D. (2005) The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the antiinflammatory actions of palmitoylethanolamide. *Mol Pharmacol*. **67**, 15-19.

27 O'Sullivan, S. E. (2007) Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. *Br J Pharmacol*. **152**, 576-582.

28 Burstein, S. (2005) PPAR-gamma: a nuclear receptor with affinity for cannabinoids. *Life Sci*. **77**, 1674-1684.

29 LoVerme, J., Russo, R., La Rana, G., Fu, J., Farthing, J., Mattace-Raso, G., Meli, R., Hohmann, A., Calignano, A., Piomelli, D. (2006) Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor-alpha. *J Pharmacol Exp Ther*. **319**, 1051–1061

30 Ho, W.S.V., Kelly, M.E.M. (2017) Cannabinoids in the cardiovascular system. *Adv Pharmacol*. **80**, 329–366.

384 31 Benyó, Z., Ruisanchez, É., Leszl-Ishiguro, M., Sándor, P., Pacher, P. (2016) Endocannabinoids in
385 cerebrovascular regulation. *Am J Physiol Heart Circ Physiol*. **310**, 785–801.

386 32 Brierley, S.M., Hughes, P.A., Page, A.J., Kwan, K.Y., Martin, C.M., O'Donnell, T.A., Cooper, N.J.,
387 Harrington, A.M., Adam, B., Liebregts, T., Holtmann, G., Corey, D.P., Rychkov, G.Y., and Blackshaw, L.A.
388 (2009) The ion channel TRPA1 is required for normal mechanosensation and is modulated by algesic stimuli.
389 *Gastroenterology*. **137**, 2084-2095.e3.

390 33 McNamara, C.R., Mandel-Brehm, J., Bautista, D.M., Siemens, J., Deranian, K.L., Zhao, M., Hayward, N.J.,
391 Chong, J.A., Julius, D., Moran, M.M., and Fanger, C.M. (2007) TRPA1 mediates formalin-induced pain. *Proc*
392 *Natl Acad Sci U S A*. **104**, 13525-13530.

393 34 Wang, X.L., Cui, L.W., Liu, Z., Gao, Y.M., Wang, S., Li, H., Liu, H.X., and Yu, L. (2019) Effects of TRPA1
394 activation and inhibition on TRPA1 and CGRP expression in dorsal root ganglion neurons. *Neural Regen Res*.
395 **14**, 140-148.

396 35 Akopian, A. N., Ruparel, N. B., Jeske, N.A., and Hargreaves, K. M. (2007) Transient receptor potential
397 TRPA1 channel desensitization in sensory neurons is agonist dependent and regulated by TRPV1-directed
398 internalization. *J. Physiol. London.* **583**, 175-193.

399 36 Anand, U., Otto, W.R., Facer, P., Zebda, N., Selmer, I., Gunthorpe, M.J., Chessell, I.P., Sinisi, M., Birch,
400 R., Anand, P. (2008b) TRPA1 receptor localisation in the human peripheral nervous system and functional
401 studies in cultured human and rat sensory neurons. *Neurosci Lett*. **438**, 221-227.

402 37 De Petrocellis, L., Vellani, V., Schiano-Moriello, A., Marini, P., Magherini, P.C., Orlando, P., and Di Marzo,
403 V. (2008) Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin
404 type-1 and melastatin type-8. *J Pharmacol Exp Ther*. **325**, 1007-1015.

405 38 Richardson, B.P. (1990) Serotonin and nociception. *Ann. N.Y. Acad. Sci*. **600**, 511–519.

406 39 Liu, Z., Zhuang, D., Lunderberg, T. and Yu, L. (2002) Involvement of 5-hydroxytryptamine(1A) receptors in
407 the descending anti- nociceptive pathway from periaqueductal gray to the spinal dorsal horn in intact rats, rats
408 with nerve injury and rats with inflammation. *Neuroscience*. **112,** 399–407.

409 40 Haleem, D.J. (2018) Serotonin-1A receptor dependent modulation of pain and reward for improving therapy
410 of chronic pain. *Pharmacol Res*.**134**, 212-219.

411 41 Todorovic, S., and Anderson, E.G. (1992) Serotonin preferentially hyperpolarizes capsaicin-sensitive C
412 type sensory neurons by activating 5-HT1A receptors. *Brain Res*. **585**, 212–218.

413 42 Yoder, E.J., Tamir, H., Ellisman, M.H. (1997) Serotonin receptors expressed by myelinating Schwann cells
414 in rat sciatic nerve. *Brain Res*. **753**, 299-308.

415 43 Capano, A., Weaver, R., Burkman, E. (2019) Evaluation of the effects of CBD hemp extract on opioid use
416 and quality of life indicators in chronic pain patients: a prospective cohort study. *Postgrad Med*. 1-6. doi:
417 10.1080/00325481.2019.1685298.

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419 **Supplementary Information Items~~s~~**

420 ~~Supplementary item 1 - Homology of the aminoacidic sequence between the immunogen of the commercially~~
421 ~~antisera employed in the study and the horse proteins.~~

422 ~~Supplementary item 2 - Specificity of the primary antibodies tested with Western blot analysis.~~

423 Supplementary item 1~~3~~ – Immunohistochemistry on rat dorsal root ganglia (positive control).

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