



Immunohistochemical distribution of the cannabinoid receptor 1 and fatty acid amide hydrolase in the dog claustrum



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ABSTRACT

Cannabinoid receptor 1 (CB1R) and fatty acid amide hydrolase (FAAH) are part of the endocannabinoid system (ECB) which exerts a neuromodulatory activity on different brain functions and plays a key role in neurogenesis. Although many studies have reported FAAH and CB1R expression in the brain of different animal species, to the best of our knowledge they have never been described in the canine claustrum. Claustrum samples, obtained from necropsy of four neurologically normal dogs, were formalin fixed for paraffin embedding. Sections were either stained for morpho-histological analysis or immunostained for CB1R and FAAH. Analysis of adjacent sections incubated with the two antisera showed a complementary labeling pattern in the claustrum, with CB1R antibody staining fibers while anti-FAAH antibody stained cell bodies and the proximal portion of dendrites; this particular anatomical relationship suggests a retrograde endocannabinoid action via CB1R. CB1R and FAAH complementary immunostaining and their cellular localization reported here provide the first anatomical evidence for existence of the ECB in the dog claustrum.

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1. Introduction

The herbaceous plant *Cannabis sativa* (marijuana) contains an array of pharmacologically active compounds, including the cannabinoids known for their psychoactive effects. Among these phytochemicals is Δ^9 -tetrahydrocannabinol the chief psychoactive component in marijuana. This phytocannabinoid exerts its action by binding to cannabinoid receptor 1 (CB1R), the most abundant G-protein-coupled receptor in brain (Covey et al., 2014). CB1R was first identified by Devane et al. (1988) and later cloned by Matsuda et al. (1990). A second cannabinoid receptor (CB2R), mainly expressed in immune cells, was subsequently identified by Munro et al. (1993). In the central nervous system (CNS) CB1R receptor is activated by the endogenous cannabinoids (endocannabinoids; ECs) anandamide (Devane et al., 1992) and 2-arachidonoyl-glycerol (2-AG) (Mechoulam et al., 1995). Anandamide and 2-AG are degraded by fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MAGL), respectively (Di Marzo et al., 1999; Di Marzo et al., 1998; Goparaju et al., 1999; Goparaju et al., 1998). MAGL is mainly

localized to the presynaptic axon terminals, while FAAH is a cytoplasmic enzyme localized in somata and dendrites of postsynaptic elements (Dinh et al., 2002; Egertova et al., 2003; Gulyas et al., 2004; Tsou et al., 1998b). Both anandamide and 2-AG are synthesized postsynaptically whereas CB1R is located on presynaptic terminals, suggesting a ECs retrograde release mechanism which suppresses presynaptic neurotransmitter release (Kreitzer and Regehr, 2001; Maejima et al., 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Termination of the retrograde signal depends on EC uptake from its site of action and degradation by FAAH and MAGL (Ohno-Shosaku and Kano, 2014).

CB1R, ECs and their degrading/synthetic enzymes make up the ECB which exerts a neuromodulatory activity on different brain functions (Moreira and Lutz, 2008; Solinas et al., 2008) and plays a key role in neurogenesis (Prenderville et al., 2015). Moreover, it is well known that the ECB is implicated in a variety of psychological and psychiatric disorders, including drug addiction (Gardner, 2005; Maldonado et al., 2006). The analgesic properties of cannabinoids have been demonstrated in animal models of pain (Rice, 2001).

The topographical distribution of CB1R in the brain has been extensively investigated, with immunohistochemical findings showing its presence in mouse (Cristino et al., 2006) and dog brain (Campora et al., 2012). In a knock-in mouse line genetic

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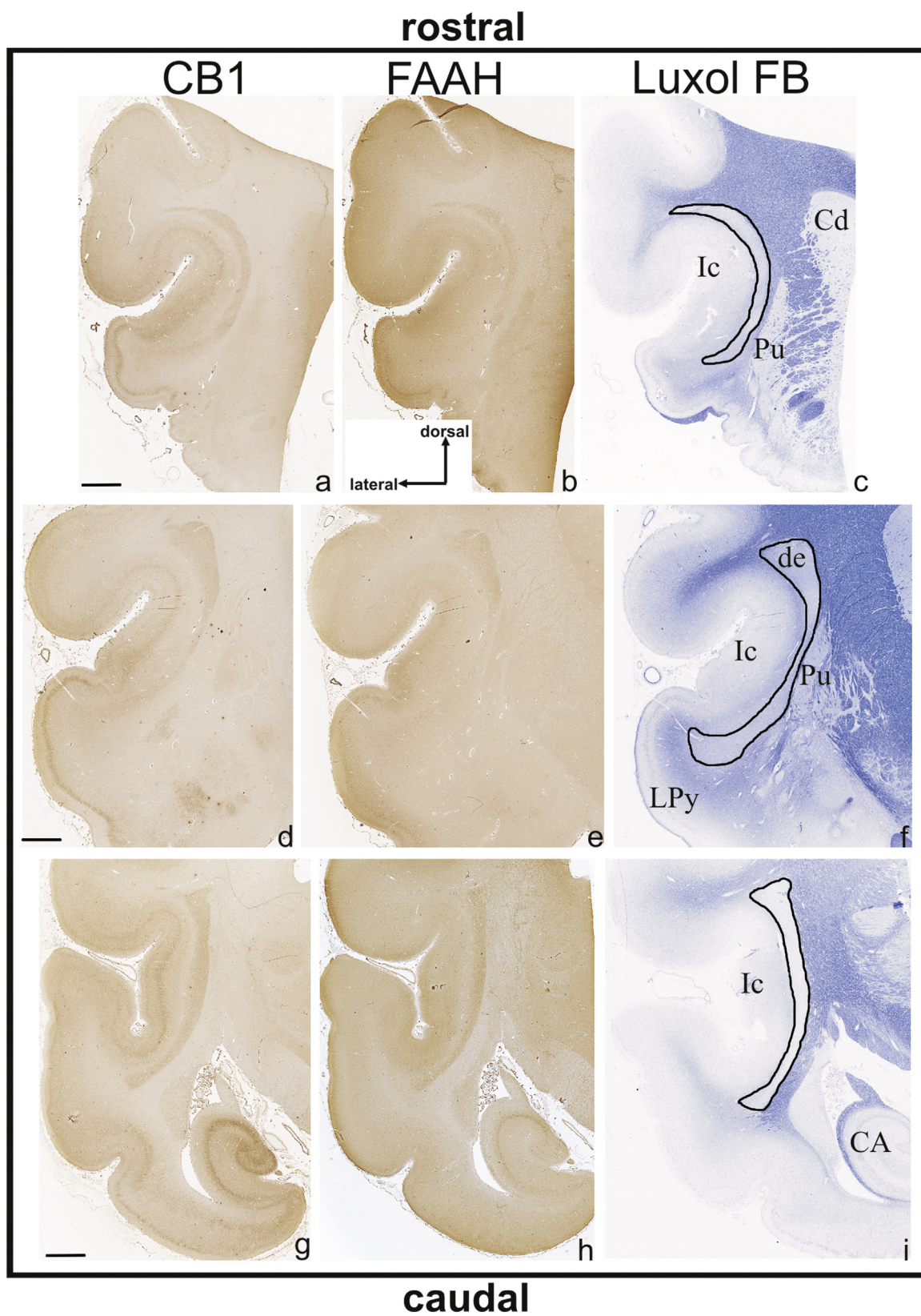


Fig. 1. Photomicrographs of immunohistochemical and histological staining of adjacent transverse brain sections. Low magnification image of the CB1R (a, d, g) and FAAH (b, e, h) immunostaining distribution in the left hemisphere including the claustrum. (c, f, i) Low magnification image of a Luxol Fast Blue stained section, with the claustrum outlined in black. CA, hippocampus; Cd, caudate nucleus; de, dorsal enlargement; Ic, insular cortex; LPy, pyriform lobe; Pu, putamen. Scale bars = 2 mm.

labeling of CB1R was found throughout the brain; in particular, low CB1R expression was found in the nucleus accumbens and also in the claustrum (Winters et al., 2012). CB1R was described also in rat brain (Herkenham et al., 1990; Moldrich and Wenger, 2000; Thibault et al., 2013), in particular, localized in striatum and hippocampus (Rivera et al., 2014; Van Waes et al., 2012). FAAH immunostained somata were observed in the latter structure, as well. Previous studies employing immunocytochemistry and autoradiography showed CB1R presence in the brain of guinea pig, dog, rhesus monkey and human (Eggan and Lewis, 2007; Herkenham et al., 1990).

To the best of our knowledge, CB1R and FAAH have never been reported within the claustrum. The structure, function and origin of the claustrum is still a matter of debate (Crick and Koch, 2005; Deutch and Mathur, 2015; Edelstein and Denaro, 2004; Goll et al., 2015; Mathur, 2014; Pirone et al., 2012). Although neurophysiological data are scarce, several reports demonstrated the distribution of neurons expressing various molecules in the claustrum of different species (Cozzi et al., 2014; Hinova-Palova et al., 2014; Pirone et al., 2014; Rockland, 2014). Recently, we studied the expression of parvalbumin in the dog claustrum, whose anatomical and chemical features have received little attention until now (Pirone et al., 2015). The dog may represent a good model for

investigating the cellular mechanism of pathologies affecting humans, and some neurological disorders of this species phenotypically resemble human diseases (Colle et al., 2000; Rofina et al., 2003; Skoumalova et al., 2003). To this end, in the present report we have investigated the neuroanatomical, and hence functional, relationships between the ECB and the claustrum using an immunohistological analysis of CB1R and FAAH.

2. Material and methods

2.1. Animals and tissue sampling

Clastrum samples were obtained from necropsy of four dogs (three males and one female) of different breeds and age referred to the Department of Veterinary Science of the University of Pisa for post-mortem examination. Clinical history of these animals was reported previously (Pirone et al., 2015). CNS abnormalities were excluded by histopathological examination. The brains, extracted within 6 h of death for routine diagnostic purposes, were cut in transverse sections (0.5 cm thick), fixed by immersion in buffered formalin and processed for paraffin embedding. Since archival samples were used, Institutional Animal Care and Use Committee approval was not required. Use of archival samples is

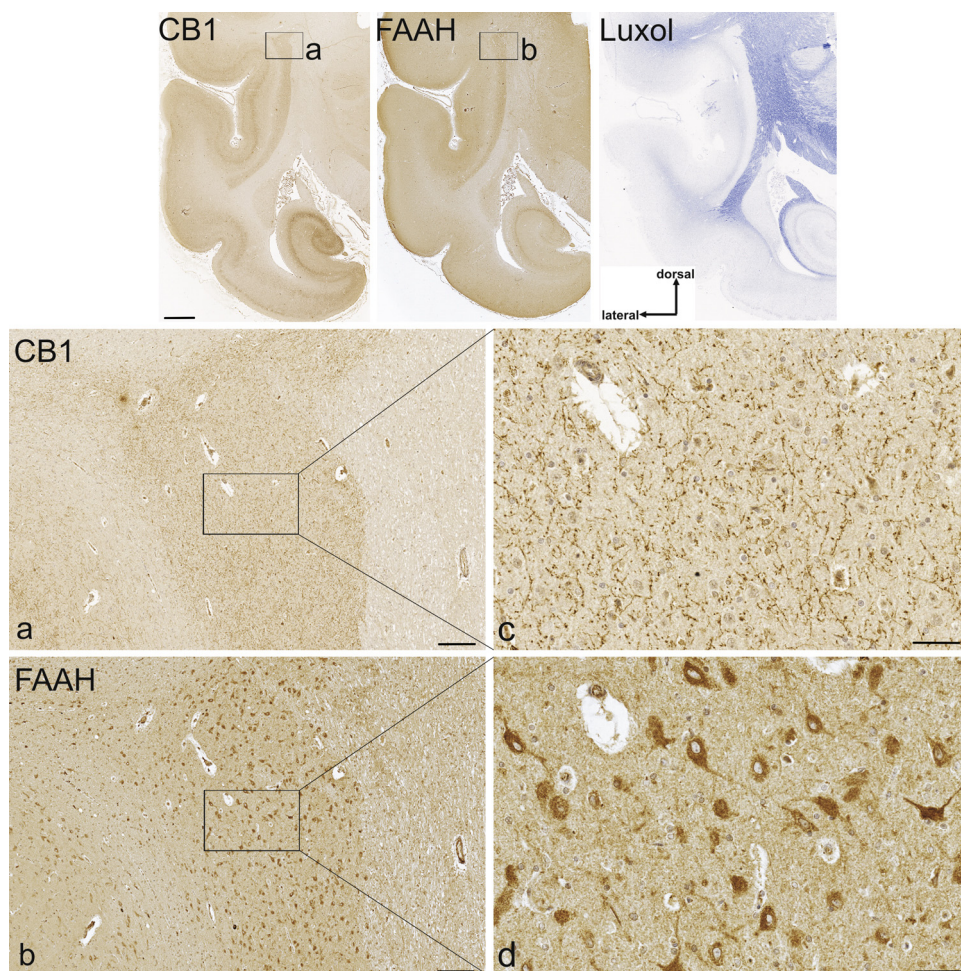


Fig. 2. Low magnification image (first row) of immunohistochemical and histological staining of adjacent transverse brain sections. (a) Magnification of the black square in the CB1R stained sections. (b) Magnification of the black square in the FAAH stained sections. (c) Magnification of (a) showing CB1R-ir fibers; (d) magnification of (b) showing polymorphic somata and proximal dendrites immunoreactive for FAAH. Scale bars: CB1R, FAAH and Luxol low magnification images = 2 mm; a, b = 200 μ m, c, d = 50 μ m.

encouraged by the EU Directive 2010/63/of 22 September 2010 on the protection of animals used for scientific purposes.

2.2. Immunohistochemistry

Immunohistochemistry was performed on serial sections of the left hemisphere using a rabbit polyclonal anti-CB1R antibody (1:50, Abcam, ab23703) and a goat polyclonal anti-FAAH (1:100, abcam, ab110840). Epitope retrieval was carried out at 120 °C in a pressure cooker for 5 min with a Tris/EDTA buffer, pH 9.0. Sections were pretreated with 1% H₂O₂ (in 0.1 M phosphate-buffered saline (PBS), pH 7.4, 10 min) to quench endogenous peroxidase activity, then rinsed with 0.05% Tween-20 detergent (in 0.1 M PBS, 3 × 10 min), and blocked for 1 h with 5% normal goat serum (s-1000, Vector Labs, Burlingame, CA) for CB1R and 2.5% normal horse serum (PK-7200, Vector Labs) for FAAH (in 0.1 M PBS). To enable comparison of FAAH expression with CB1R, adjacent sections were incubated overnight at 4 °C in a solution containing the rabbit anti-CB1R with 2% normal goat serum or the goat anti-FAAH with 2% normal horse serum, 0.05% Triton X-100 (in 0.1 M PBS). Sections were then rinsed in 0.1 M PBS, (3 × 10 min), followed by incubation with a biotinylated anti-rabbit IgG (BA-1000, Vector Labs) for CB1R or biotinylated anti-goat IgG (BA-9500, Vector Labs) for FAAH, each diluted 1:300 in PBS. Sections were again rinsed in 0.1 M PBS, for 3 × 10 min. Staining was visualized by incubating the sections in diaminobenzidine (sk-4105, Vector Labs) solution. The specificity of immunohistochemical staining was tested by substituting either the primary antibody, anti-rabbit/goat IgG, or the ABC complex with PBS or non-immune serum. Under these conditions staining was abolished. Moreover, the anti-CB1R antibody specificity has already been tested in the canine species by pre-adsorption with the corresponding blocking peptide (Campora et al., 2012). To better localize the claustrum boundaries, selected sections were stained with the Luxol Fast Blue method. The claustrum and adjoining structures were examined in accordance with a stereotaxic atlas (Palazzi, 2011). Whole slide images were acquired with D-Sight (A. Menarini Diagnostics srl,

Italy). Microphotographs were taken with a light microscope (Leitz Diaplan, Wetzlar, Germany) connected to a PC via a Nikon digital system (Digital Sight DS-U1, NIS-Elements BR-4.13.00 software).

3. Results

3.1. Immunohistochemistry

Three subsequent rostro-caudal levels were considered for the histological and immunohistochemical analysis of each claustrum (Fig. 1). CB1R and FAAH staining patterns and intensity were quite homogenous within the claustrum and clearly delineated its shape against the white matter. Analysis of adjacent sections incubated with the two antisera showed a complementary labeling pattern in the claustrum, insular cortex, and hippocampus (Fig. 1a,b,d,e,g,h): fibers were labeled with the CB1R antibody, whereas the anti-FAAH antibody stained cell bodies. All the structures identified in the serial stained sections revealed that FAAH-immunoreactive (ir) neuronal somata and dendrites were surrounded by CB1R-ir fibers (Fig. 2a,b) while the putamen failed to reveal immunolabeling with either antisera (Fig. 3).

3.1.1. CB1R

A high density of CB1R-ir fibers, characterized by numerous varicosities, were present in the claustrum. The CB1R-ir processes surrounded immunonegative cell bodies (Fig. 2a; 5). Although CB1R immunostaining was mainly localized in fibers (Fig. 2a), CB1R positivity was also observed in the cytoplasm of some neurons close to the nucleus (Fig. 5).

CB1R immunolabeling appeared to be present throughout the rostro-caudal extent of the claustrum. The intermediate section of each claustrum revealed a higher staining intensity in the dorsal enlargement (Fig. 1d), and immunostaining was also evident in the adjoining structures. In particular, in the insular cortex CB1R immunoreactivity was distributed through the cortex layers, with the fourth layer being the most immunoreactive (Fig. 1a,g; 3a). The extreme capsule showed positive fibers (Fig. 4a,b) while no

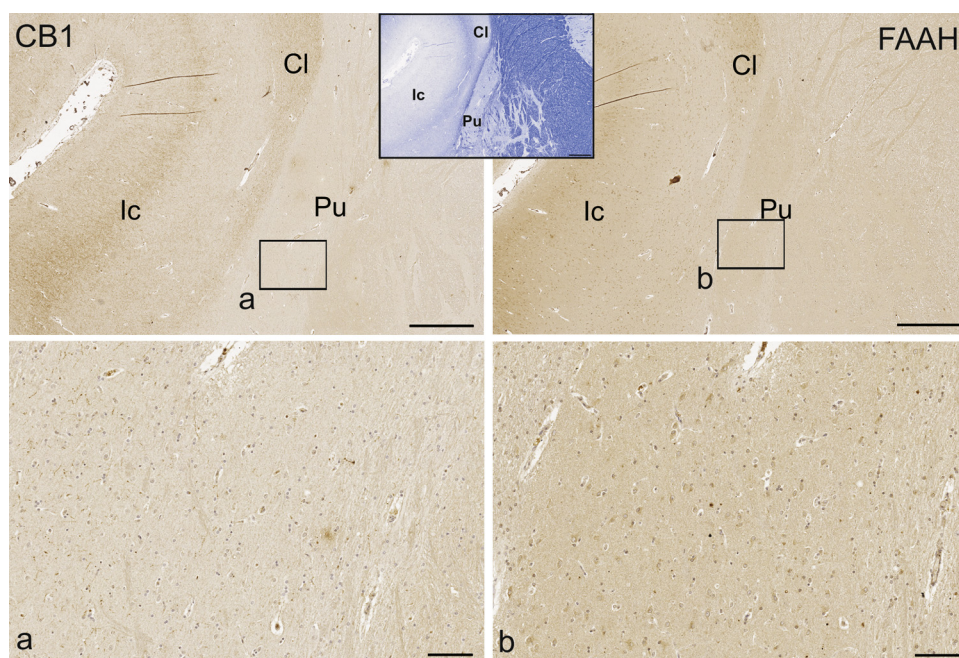


Fig. 3. Photomicrographs of CB1R (first row, left side) and FAAH (first row, right side) immunostained sections. The inset outlined in black is a Luxol FB stained section showing the morphology of immunolabeled structures. (a) Magnification of the black square in the CB1R stained section displaying the absence of CB1R immunoreactivity in the putamen. (b) Magnification of the black square in the FAAH stained section displaying the absence of FAAH immunoreactivity in the putamen. Cl, claustrum; Ic, insular cortex; Pu, putamen. Scale bars: microphotographs in the first row = 1 mm; a, b = 100 μ m.

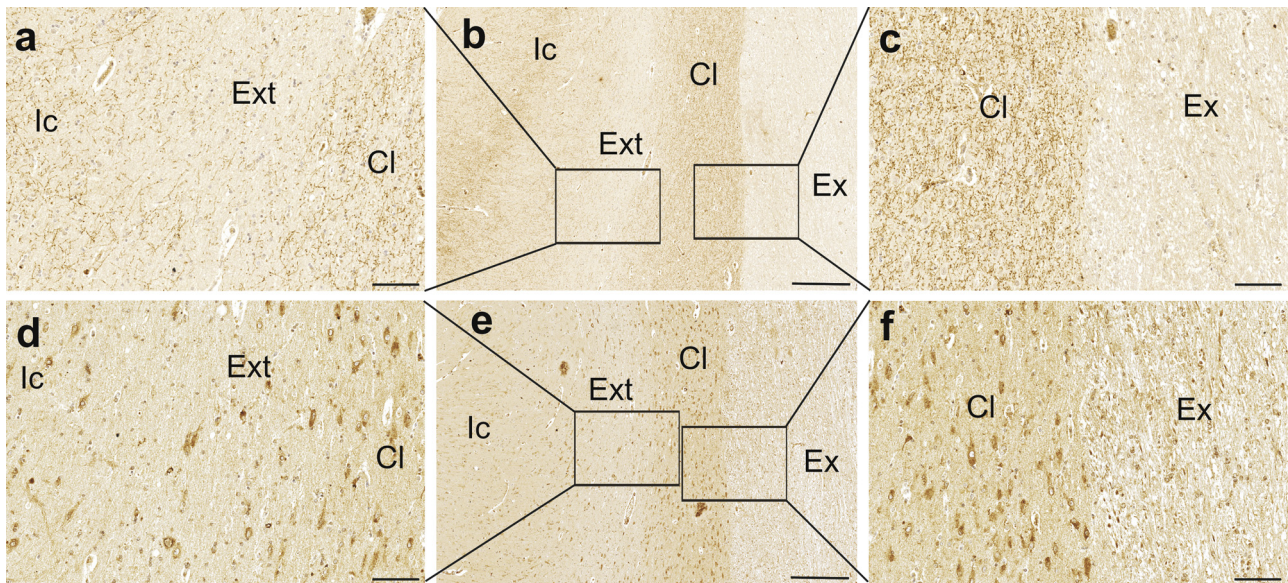


Fig. 4. Photomicrographs showing CB1R (b) and FAAH (e) immunoreactivity in the external and extreme capsules. (a) detail of (b) revealing CB1R-ir fibers in the extreme capsule. (c) detail of (b) displaying the absence of CB1R-ir in the external capsule. (d) detail of (e) revealing the presence of FAAH-ir somata in the extreme capsule. (f) detail of (e) showing the absence of FAAH labeling in the external capsule. Cl, claustrum; Ic, insular cortex; Ext, extreme capsule; Ex, external capsule. Scale bars: b, e = 500 μ m; a, c, d, f = 100 μ m.

immunoreactivity was revealed in either the putamen (Fig. 3a) or the external capsule (Fig. 4b,c).

3.1.2. FAAH

FAAH immunolabeling was mainly detected in the somata and proximal portion of the dendrites (Fig. 2b). Immunostaining revealed neurons throughout the rostro-caudal extent of the claustrum, which were mainly concentrated in the dorsal enlargement in the intermediate region (Fig. 1e). These immunostained cells showed polymorphic somata (Fig. 2b). In the dorsal and ventral parts of the claustrum the main axes of immunopositive neurons were randomly oriented while in the central region, flanked by the capsules, the majority of labeled cells showed elongated cell bodies parallel to the long axis of the claustrum (Fig. 4f). Immunoreactive somata were found in the extreme capsule and in the insular cortex (Fig. 4 d–f), but not in the external capsule or putamen.

4. Discussion

In the present study we report the immunohistochemical analysis of CB1R and FAAH in the claustrum and adjoining structures of the dog brain. FAAH and CB1R expression showed a pattern that was broadly consistent with that reported by previous studies in the brain of different animal species (Egertova et al., 2003; Egertova and Elphick, 2000; Gulyas et al., 2004; Tsou et al., 1998a; Tsou et al., 1998b; Yoshida et al., 2006). Our study utilized an antiserum against a synthetic peptide corresponding to C-terminal amino acids 461–472 of the human CB1R, which demonstrated 100% identity with the predicted CB1R of *Canis lupus familiaris* by BLAST analysis (Boratyn et al., 2013). This CB1R antiserum labeled CB1R in plasma membrane fibers and those internalized in the cell body cytoplasm. Previous studies employing antibodies to the N-terminus of CB1R described immunoreactivity in the neuronal somata of rat brain (Pettit et al., 1998; Tsou et al., 1998a). Conversely, antibodies raised against the C-terminal

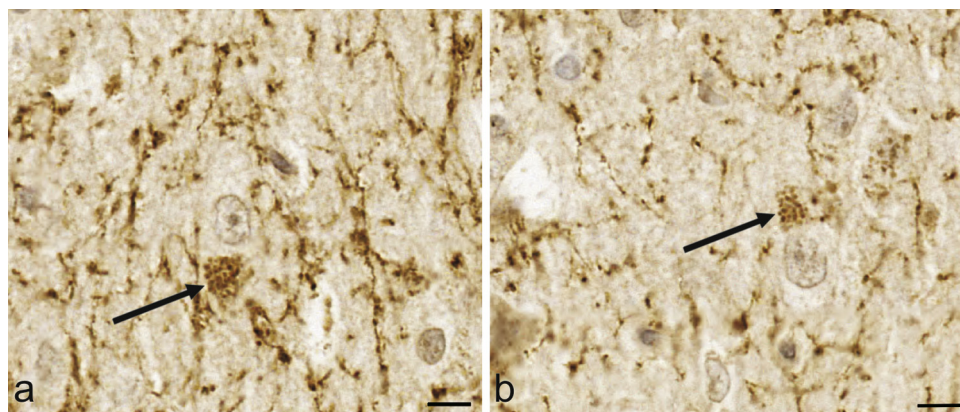


Fig. 5. Photomicrographs (a,b) showing the presence of CB1R immunoreactivity in the cytoplasm of two neurons (black arrows). Scale bars = 10 μ m.

portion of CB1R did not detect immunostaining in cell bodies (Egertova and Elphick, 2000). The present observations concerning CB1R cellular localization are supported by earlier studies in the primate cortex using an antiserum against the C-terminus, where CB1R was expressed at both the plasma membrane level and intracellularly (Eggen and Lewis, 2007).

CB1R is reported to be present in hippocampus, basal ganglia, thalamus, hypothalamus and cerebellum of the mouse (Cristino et al., 2006; Winters et al., 2012). A similar localization has been observed in rat brain (Herkenham et al., 1990; Matyas et al., 2006; Moldrich and Wenger, 2000; Thibault et al., 2013). Ultrastructural studies identified CB1R in the dendritic spines and axons in the prefrontal cortex, cerebellum, hippocampus and basal ganglia of non-human primates (Eggen and Lewis, 2007; Ong and Mackie, 1999). The presence of CB1R was studied in the brain of different species, including the dog, by autoradiographic techniques (Herkenham et al., 1990). Although these authors described CB1R binding sites in human and monkey claustrum they did not report any data for dog claustrum. In contrast, Eggen and Lewis (Eggen and Lewis, 2007) in their immunocytochemical analysis of CB1R distribution in primate neocortex observed a high density of CB1R-ir fibers in the claustrum and adjacent insular cortex. Our results on the distribution and localization of FAAH agree with previous immunohistochemical studies on rat brain (Tsou et al., 1998b). FAAH mRNA was mostly localized to neurons of the neocortex, hippocampal formation, amygdala and cerebellum (Thomas et al., 1997). Moreover, ultrastructural analysis demonstrated the presence of FAAH immunoreactivity in the somata and dendrites of the hippocampus, cerebellum and amygdala (Gulyas et al., 2004).

Precedence exists for the distribution of CB1R and FAAH in the same CNS structures, for example in rat hippocampus (Egertova et al., 1998; Rivera et al., 2014). Co-labeling of CB1R and FAAH has also been described in mouse, notably in the olfactory cortex, amygdala, dentate gyrus, transitional cortex and neocortex (Egertova et al., 2003). These authors reported an anatomical association with FAAH-ir neuronal somata surrounded by CB1R-ir fibers. Here, we observed the same immunostaining pattern in dog claustrum. Moreover, we noted a complementary immunostaining in the insular cortex and extreme capsule, but not in the external capsule or putamen. These findings might further support the theory of the pallial origin of claustrum (Edelstein and Denaro, 2004; Pirone et al., 2012).

Our results confirm the cellular localization of CB1R and FAAH as described in other studies, with FAAH being a cytoplasmic enzyme localized in somata and dendrites of postsynaptic elements, and CB1R located on presynaptic terminals. This particular anatomical relationship underlies a retrograde ECs action via CB1R (Di Marzo et al., 2015; Dinh et al., 2002; Egertova et al., 2003; Gulyas et al., 2004; Tsou et al., 1998b).

The high density of CB1R-ir fibers in the claustrum raises the question of whether they derive from intrinsic or extrinsic sources. The hypothesis of their extrinsic source is supported by the presence of the extensive cortical and subcortical claustral connections (Mathur, 2014); on the other hand, the cytoplasmic CB1R labeling does not exclude an intrinsic source. Former studies suggest a double origin for CB1R-ir fibers in the primate (Eggen and Lewis, 2007) and mouse (Galarreta et al., 2004) neocortex. The idea of a role for CB1R/FAAH in the intrinsic neuronal circuits of canine claustrum is supported by a former study suggesting inhibitory local circuit interneurons as a source of CB1R-ir axons (Eggen and Lewis, 2007). Although only a hypothesis, our findings may indicate that the ECB in claustrum could mediate depolarization-induced suppression of inhibition, as previously described in rodent neocortex and hippocampus (Bodor et al., 2005; Pitler and Alger, 1994).

The presence of the ECB in the claustrum points to involvement of this telencephalic structure in important brain functions, as already shown in primate neocortex (Eggen and Lewis, 2007). The ECB might be viewed as a tool to finely modulate local and extrinsic circuits in this structure, in order to act as “a conductor coordinating a group of players in the orchestra, the various cortical regions” (Crick and Koch, 2005). New hypotheses regarding claustrum function have recently been proposed (Goll et al., 2015; Jankowski and O'Mara, 2015; Patru and Reser, 2015). Goll et al. (2015) suggest that it plays a key role in the segregation of attention, while Patru and Reser (2015) view the claustrum as a potential common center for delusional states. In this context the latter authors highlight the kappa opioid receptor as a modulator of the dopaminergic pathway in the claustrum. Given the apparent synergy ('crosstalk') between the opioid and cannabinoid signaling pathways (Cichewicz, 2004) one may speculate that the ECB of the claustrum has a neuromodulatory role in these proposed functions.

In conclusion, the CB1R and FAAH complementary immunostaining and their cellular localization described here provide the first anatomical evidence for existence of the ECB in dog claustrum. Further investigation of the neuronal cell types involved in this circuitry may contribute to a more complete understanding of the role played by the ECB.

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