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Spatial distribution of cannabinoid receptor 1 and fatty acid amide hydrolase in the cat ovary and oviduct

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ABSTRACT

Involvement of the endocannabinoid system in female reproduction has been extensively described in humans with the cognate receptors and ligands being found in the ovaries and genital tract. In human, an imbalance of the endocannabinoid system is linked with both ectopic pregnancy and infertility. In bovine species anandamide levels regulate aspects of sperm-oviduct interaction. Here we report the immunohistochemical distribution of cannabinoid receptor 1 (CB1R) and fatty acid amide hydrolase (FAAH) in cat ovary and oviduct, using paraffin-embedded tissue samples and commercially available antibodies. We found a differential expression of both CB1R and FAAH during different stages of ovarian function and in the oviduct. CB1R was detected only in tertiary follicle granulosa cells while more immature follicles were negative. FAAH was instead found in ovarian pre-antral follicles, the oocyte cytoplasm, and in granulosa cells of primary, secondary and tertiary follicles. Secondary and tertiary follicles were also FAAH immunoreactive. Luteal cells were immunopositive for both CB1R and FAAH. Because CBR1 in oviduct was found only in ciliated cells, it might represent a specific marker at least in cats. In contrast, FAAH immunoreactivity was observed in both ciliated and non-ciliated cells. The present study may thus serve as the starting point for further investigations on the role of the endocannabinoid system in cat reproduction. Additional work will be needed to assess whether the morphological distribution of CB1R and FAAH changes in different conditions such as pre-pubertal age, follicular phase of the sexual cycle and pregnancy.

1. Introduction

Cannabinoid receptors together with a group of endogenous lipid ligands and the enzymes for their biosynthesis and degradation constitute the endocannabinoid system (ECS) (Iannotti et al., 2016). The ECS mediates the effects of cannabinoids, including Δ9-tetrahydrocannabinol (THC) the chief psychoactive component in marijuana (Skaper and Di Marzo, 2012). At least two cannabinoid receptors are known, cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R). CB1R is a G-protein-coupled receptor abundantly expressed in the brain (Covey et al., 2015) that mediates the effects of the endogenous cannabinoids anandamide (AEA) (Devane et al., 1992) and 2-arachidonoyl-glycerol (2-AG) (Mechoulam et al., 1995). Although CB1R was first described as a central nervous system receptor, CB1R-mediated actions were proposed in the liver, pancreas, gastrointestinal system, skeletal muscle, adipose tissue, skin and embryo development (Campora et al., 2012; Kunos and Tam, 2011; Pirone et al., 2015; Stander et al., 2005). Fatty acid amide hydrolase (FAAH) is the principal catabolic enzyme for AEA and other fatty acid amides, and in brain is found in soma and dendrites of postsynaptic elements (where AEA is also synthetized). CB1R, in contrast, is located on presynaptic terminals (Pirone et al., 2015). This anatomical distribution underlies the so-called retrograde inhibitory mechanism which suppresses presynaptic neurotransmitter release (Kreitzer and Regehr, 2001).

The ECS also plays a complex role in the female reproductive system. Its components are located throughout the reproductive tract, including the rat and human ovary (Bagavandoss and Grimshaw, 2010; El-Talatini et al., 2009), oviduct (Horne et al., 2008), myometrium (Brighton et al., 2011), and endometrium (Taylor et al., 2010). Studies performed in a human granulosa cell line indicated that the intrinsic ovarian ECS plays a role in estradiol synthesis (Ernst et al., 2016). AEA is found in human follicular fluid (Schuel et al., 2002), and alterations in its plasma levels are related to fertility/infertility in healthy women (Maccarrone et al., 2002). Moreover, ECS signaling was shown to be involved in the oocyte maturation (Agirregoitia et al., 2015; Agirregoitia et al., 2016; Lopez-Cardona et al., 2016; Peralta et al.,

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2011) and the fertilization process as well, as the fertilizing ability of sperm and sperm–oocyte interaction depends on AEA binding to either CB1R or the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor. This occurs in a two-step process: first, AEA negatively affects human sperm motility in a CB1R-dependent manner while acting as a capacitating signal at physiological concentrations (Rossato et al., 2005); second, once capacitation is completed, AEA effects are mediated by TRPV1 activation to prevent spontaneous acrosomal reaction that results only from sperm–egg interactions which maximize sperm-fertilizing potential (Francavilla et al., 2009). AEA also plays key roles post-fertilization, being involved in the implantation process, placentation and pregnancy (Battista et al., 2015). Aberrant plasma levels of AEA, whether too high or too low, are deleterious for pregnancy (Wang et al., 2004).

Domestic cats are well-known for their reproductive proclivity, and suppression of fertility in this species is often desirable. Although surgical contraception is highly effective, but its cost and irreversibility may desirable for professional cat breeders (Goericke-Pesch et al., 2014).

As far as we are aware, a role for the ECS in domestic animal reproduction has only been described in bovine species (Gervasi et al., 2013) in terms of the effects that ECS ligands (i.e AEA) might play in sperm-oviduct interaction. Morphological data on the distribution of CB1R and FAAH is limited to human (El-Talatini et al., 2009) and rats (Bagavandoss and Grimshaw, 2010).

Targeting the ECS with cannabinoid receptor agonists is under investigation for a number of therapeutic indications (Abramo et al., 2014; Campora et al., 2012; Pertwee, 2012). The potential use of cannabimimetic compounds in companion animals as analgesic/anti-inflammatory drugs was reviewed recently (Re et al., 2007). In a pilot study in cats, palmidrol [palmitoylethanolamide (PEA)], an AEA congener, was used to treat eosinophilic granuloma with full resolution of clinical signs (Scarampella et al., 2001). Immunophenotypical characterization of the ECS components might be of utility in predicting wanted and unwanted effects when a new cannabimimetic compound is used in a new target species. The present study was designed to begin addressing this issue by assessing the morphological distribution of CB1R and FAAH in cat ovary and oviduct.

2. Materials and methods

2.1. Animals and samples

Five non-pregnant female domestic cats in the luteal phase of the sexual cycle (diestrus), referred for spaying to our local veterinary teaching hospital, were used in the study. A written informed consent was obtained from each cat's owner prior to surgery. After premedication with dexmedetomidine and methadone IM and intravenous induction with propofol, hair was clipped and the surgical site aseptically prepared. Anaesthesia was then maintained with isoflurane in oxygen and fentanyl infusion for the nociception control. Gonads and oviducts were collected and promptly immersed in modified Davidson's solution (Latendresse et al., 2002) for 24 h. Tissues were then processed for routine paraffin embedding and 5 µm thick sections prepared for morphological evaluation (hematoxylin and eosin staining) and immunohistochemistry.

This research was carried out according to the international regulation on the use of animals for scientific purposes (Directive 2010/63/EU).

2.2. Immunohistochemistry

Immunohistochemistry was performed on three different section per animal in a single session in order to expose tissues to analogous experimental conditions. A rabbit polyclonal anti-CB1R antibody (1:50, abcam, ab23703) and a goat polyclonal anti-FAAH antibody (1:100, abcam, ab110840) were used. 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 in 1% H_2O_2 (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 with 5% normal goat serum (NGS) (s-1000, Vector Labs, Burlingame, CA) for CB1R and 2.5% normal horse serum (NHS) (PK-7200, Vector Labs) for FAAH (in 0.1 M PBS, 1 h). To enable comparison of FAAH and CB1R expression, adjacent sections were incubated overnight at 4 °C in a solution containing anti-CB1R antibody with 2% NGS or anti-FAAH antibody with 2% NHS, 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 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. Photomicrographs were obtained using a Nikon Ni-e microscope equipped with the Nis Elements Br Microscope Imaging Software (Nikon Instruments, Calenzano, Italy). Immunostaining was graded by two independent investigators as minimal, moderate or strong.

Specificity of immunohistochemical staining was also confirmed as follows: blocking with the corresponding peptide (abcam, ab50542), substitution of primary antibody, anti-rabbit/goat IgG, or ABC complex with PBS or non-immune serum. Under these conditions staining was abolished. Moreover, specificity of the anti-CB1R antibody used has been demonstrated previously in dogs (Campora et al., 2012); *felis catus* CB1R is 91% identical to canine CB1R at the gene level. Further, the FAAH antibody used was previously tested in dogs in our laboratory (Pirone et al., 2016); in analogy to CB1R, the f*elis catus* FAAH nucleotide sequence is predicted to be 91% identical to the canine one.

To further verify the specificity of the used antibodies, a second set of cat ovaries were collected during spaying and promptly frozen in liquid nitrogen for subsequent western blot analyses. Briefly, 30 µg of proteins were resolved by 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (0.2 µm) using a voltage of 25 V for 7 min (Trans-Blot®TurboTM Transfer System; Bio-Rad). After electrophoresis, the membranes were blocked and then incubated with appropriately diluted primary antibodies. HRP-conjugated goat anti-rabbit (1:10,000, Enzo life science, ADI-SAB-300J) and HRP-conjugated donkey anti-goat SantaCruz (1:10,000, Santa Cruz Biotechnology, Inc, sc 2020) were used as secondary antibodies. The chemiluminescent images were acquired by LAS 4010 (GE Health Care).

3. Results

3.1. CB1R immunoreactivity

Western blot analyses showed the presence of two immunoreactive bands (of 66 and 81 kDa); either primary antibody omission and blocking with the corresponding peptide completely abolished immunostaining (Fig. 1). Cat ovaries were characterized by the presence of primordial, primary, secondary, tertiary and pre-ovulatory follicles as well as active corpora lutea. Primordial follicles did not show CB1R immunoreactivity either in their squamous epithelium or oocyte structures (Fig. 2a). The same was the case for primary and secondary follicles (Fig. 2c and Fig. 3a, respectively) while tertiary follicles showed minimal cytoplasmic immunoreactivity for both granulosa and corona radiata (Fig. 3c). The same minimal staining was observed in the secondary oocyte cytoplasm while both theca interna and externa cells where devoid of staining, as were stromal structures and ovarian surface epithelium (OSE).

Corpora lutea showed moderate immunostaining of the secretory cell cytoplasm (Fig. 4a) while strong immunoreactivity was observed for oviduct ciliated cells (Fig. 5a). Non-ciliated epithelial cells of the oviduct and stroma were consistently devoid of staining.

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