

## The presence of the $\mu$ -opioid receptor in the isthmus of mare oviduct

S. Desantis<sup>1</sup>, M. Albrizio<sup>2</sup>, G. Ventriglia<sup>1</sup>, M. Deflorio<sup>1</sup>, A.C. Guaricci<sup>2</sup>, R. Minoia<sup>2</sup> and G. De Metro<sup>1</sup>

<sup>1</sup>Department of Animal Health and Well-being and

<sup>2</sup>Department of Animal Production, Faculty of Veterinary Medicine, University of Bari, Italy

**Summary.** The presence of the  $\mu$ -opioid receptor and the type of glycosylation in the third extra-cellular loop of this receptor was investigated in the isthmus of mare oviduct during oestrus by means of immunoblotting and immunohistochemistry combined with enzymatic (N-glycosidase F and O-glycosidase) and chemical ( $\beta$ -elimination) treatments. Immunoblotting analysis showed that the  $\mu$ -opioid receptor consists of two peptides with molecular weights of around 65 and 50 kDa. After N-deglycosylation with N-glycosidase F an additional immunoreactive peptide was observed at around 30 kDa. The cleavage of O-glycans by O-glycosidase failed in immunoblotting as well as in immunohistochemistry investigations, revealing that the third extra-cellular loop of the  $\mu$ -opioid receptor expressed in mare isthmus oviduct contains some modifications of the Gal $\beta$ (1-3)GalNAc core binding to serine or threonine. Immunohistochemistry revealed the  $\mu$ -opioid receptor in the mucosal epithelium, some stromal cells, muscle cells and blood vessels. In ciliated cells the  $\mu$ -opioid receptor showed N-linked glycans, since the immunoreactivity was abolished after N-glycosidase F treatment, whereas it was preserved in the apical region after  $\beta$ -elimination. Most non-ciliated cells expressed the  $\mu$ -opioid receptor with both N- and O-linked oligosaccharides, as revealed by the abolition of immunostaining after N-glycosidase F and  $\beta$ -elimination. Stromal cells, endothelial and muscle cells of blood vessels expressed the  $\mu$ -opioid receptor containing both N- and O-linked oligosaccharides. Myosalpinx myocytes expressed the  $\mu$ -opioid receptor with O-linked oligosaccharides. The immunopositive myocytes formed a circular coat in the intrinsic musculature, whereas they were arranged in some isolated, oblique bundles in the extrinsic musculature. In conclusion, the  $\mu$ -opioid receptor could have a role in

the production and the movement of isthmus lumen content that contributes to ensuring the effective condition of the sperm in the mare oviduct.

**Key words:**  $\mu$ -opioid receptor, Glycosylation, Oviduct, Mare

### Introduction

The isthmus of the mammalian oviduct is considered to be a spermatozoal reservoir for several species, such as cattle (Suarez et al., 1990; Hunter et al., 1991), hamster (Smith and Yanagimachi 1991), mouse (Suarez, 1987), pig (Hunter 1981; Suarez et al., 1991), rabbit (Overstreet and Cooper, 1978), sheep (Hunter and Nichol, 1983), and horse (Thomas et al., 1994; Dobrinski et al., 1996). This oviduct region accounts for a unique biochemical milieu able to i) prevent polyspermic fertilization, ii) maintain the fertility of sperm, and iii) regulate capacitation and motility hyperactivation in order to ensure the sperm are in effective condition when ovulation occurs (Suarez, 2002). Thus, the role of the isthmus appears to be of the utmost importance in the horse where fertilization may occur up to 6 days after mating (Day, 1942; Burkhardt, 1949).

The epithelium of the isthmus is of simple columnar type and consists of two types of cells: ciliated and non-ciliated (secretory) cells. Both kinds of cell have been found to be involved in sperm trapping in cattle (Hunter et al., 1991; Gualtieri and Talevi, 2000) and pig (Flechon and Hunter, 1981; Suarez et al., 1991). However, non-ciliated cells are mainly involved in the synthesis and release of secretory glycoproteins that are dissolved in the oviductal fluid, together with a selective transudate of serum (Leese, 1988).

Investigations on processes occurring at the oviduct epithelium level have provided evidence of the implication of the luminal surface (Abe, 1996; Buhi, 2002) but the exact nature of the primary receptors and channels activated has not been determined in the

isthmus.

Recent studies have demonstrated that endogenous opioid peptides can influence  $Ca^{2+}$  turnover, and, by acting on voltage-gated  $Ca^{2+}$  channels, create  $Ca^{2+}$  oscillations within the cell and thus modify downstream  $Ca^{2+}$ -dependent cellular activity (Bourinet et al., 1996). Opioid receptors are target molecules for analgesia, reward and many of the physiological functions of opiates. Opioid peptides act through three main subtypes of opioid receptors, termed  $\mu$  (Chen et al., 1993a; Wang et al., 1993),  $\gamma$  (Evans et al., 1992; Kieffer et al., 1992) and  $\kappa$  (Chen et al., 1993b; Meng et al., 1993; Yasuda et al., 1993). These subtypes are defined on the basis of their pharmacological and ligand binding properties. In particular, the  $\mu$ -opioid receptor is the primary site of action of several endogenous opioid peptides, including  $\beta$ -endorphin (Zadina et al., 1997) and of most of the therapeutic effects and side effects of  $\mu$ -opioid receptorphine. This receptor has been reported to modulate somatic  $Ca^{2+}$  channel currents in mouse periaqueductal gray neurons (Connor et al., 1999).

The presence of  $Ca^{2+}$  in oviduct epithelial cells has been demonstrated (Cox and Leese, 1997).  $Ca^{2+}$  plays an important role in oviduct functions, since it permits binding of oviductal secretory proteins to spermatozoa (Lapointe and Sirard, 1996) and it is involved in maintaining ciliary beat (Barrera et al., 2004; Andrade et al., 2005).

Although the  $\mu$ -opioid receptor has been immunolocalized mainly in the nervous system, the expression of this opioid receptor has been shown by means of immunohistochemical studies in other tissues, such as the pancreas and liver (Khawaja et al., 1990), gastrointestinal tract (Lang et al., 1996; Bagnol et al., 1997; Fickel et al., 1997), follicular cells and pre-implantation mouse embryos (Kalyuzhny et al., 1997), keratinocytes (Biardi et al., 1998; Bigliardi-Qi et al., 1999, 2004), kidney (Di Sole et al., 2001), cumulus-oocyte complex (Dell'Aquila et al., 2002), inner ear (Popper et al., 2004), pituitary (Carretero et al., 2004), pineal gland (Phansuwan-Pujito et al., 2006) and sperm cells (Albrizio et al., 2006). As regards the horse, the  $\mu$ -opioid receptor has been immuno-localized in synovial membranes (Sheehy et al., 2001) and ejaculated spermatozoa (Albrizio et al., 2005).

The  $\mu$ -opioid receptor is a glycosylated protein (Gioannini et al., 1982; Chen et al., 1995; Singh et al., 1997; Chaturvedi et al., 2001). The oligosaccharide chains of glycoproteins are classified into two families: N- and O-linked oligosaccharides (Spicer and Schulte, 1992). The  $\mu$ -opioid receptor has been demonstrated to be an N-glycosylated protein in Chinese hamster ovary (Chen et al., 1995) and human embryonic kidney (HEK)-293 (Chaturvedi et al., 2000), whereas in equine spermatozoa the possibility of O-glycosylation cannot be discarded (Albrizio et al., 2005). These findings are not surprising since the glycosylation of a glycoprotein can differ between cell types and species (Spiro, 2002).

The aim of the present study was to describe the

localization of the  $\mu$ -opioid receptor in the mare oviductal isthmus and to characterize the type of glycosylation of its third extra-cellular loop of the  $\mu$ -opioid receptor in the mare isthmus, as well as on the glycosylation pattern of its third extra-cellular loop. This region appears to be a key element for the binding of  $\mu$ -opioid receptor ligands (Guarna et al., 2003) by means of immunoblotting and immunohistochemistry combined with enzymatic and chemical treatments.

## Materials and methods

Oviducts from five oestrus mares (with a follicle >35 mm) were obtained from a local slaughterhouse. Immediately after collection, on the basis of gross appearance, the isthmus was separated from the ampulla and processed for Western blot analysis and immunohistochemistry.

### Western blot analysis

Crude plasma membranes from equine isthmus were prepared by a modification of the procedure reported by Albrizio and coworkers (Albrizio et al., 2005). Briefly, 10 mg of equine isthmus was frozen in liquid nitrogen, then fine pulverized in a mortar and suspended in ice-cold homogenizing buffer (0.25M sucrose, 10mM Tris-HCl, pH 7.5) containing a cocktail of protease inhibitors (Sigma, Milano, Italy). The suspension was homogenized in a motor-driven homogenizer and centrifuged at 10000xg for 30 min at 4°C. The resulting supernatant was recovered and the protein concentration was spectrophotometrically assessed using the BCA assay (Pierce, Rockford, IL, USA).

Western blot analysis was performed using a polyclonal anti- $\mu$ -opioid receptor antibody (Chemicon, Temecula, CA, USA) against the third extra-cellular loop of the receptor that selectively binds  $\mu$ -agonists (Xue et al., 1995). Thirty micrograms of isolated proteins were denatured for 4 min at 90°C, loaded and run on a 12% (w/v) pre-cast polyacrylamide gel (BioRad, Milano, Italy). Separated proteins were transferred by means of a Trans-Blot semidry apparatus (BioRad, Milano, Italy) onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). After transfer, the membrane was blocked with Blotto (20mM Tris-HCl pH 7.5, 0.15M NaCl, 1% (v/v) Triton X-100) containing 5% (w/v) non-fat dry milk (blocking buffer) for 1h and then incubated with the rabbit polyclonal anti- $\mu$ -opioid receptor antibody diluted 1:7500 in blocking buffer. After washing, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma, Milano, Italy) diluted 1:10000, and then tested for horseradish peroxidase activity using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

For N-glycosidase F (PNGase F) and O-glycosidase (Roche, Milano, Italy) digestion, 90 mg of the enriched membranes fraction was treated for 5 min at 90°C with

## $\mu$ -opioid receptor in the isthmus oviduct

0.1% SDS, 1%  $\beta$ -mercaptoethanol, 0.5mM PBS. Thirty micrograms of the denatured proteins were incubated overnight at 37°C in the presence of incubation buffer (0.5% IGEPAL, 1xPMSF, 40mM PBS) plus 4U PNGase F, or 2U O-glycosidase, or without any enzyme (control). The enzymatic reaction was stopped by adding 4x running buffer and the samples were loaded on the gel, run and immunoblotted as previously described.

Negative controls were carried out by incubating blots containing equine isthmus plasma membranes with 1) a solution where the primary antibody was absorbed with a molar excess of the immunizing peptide, 2) a rabbit pre-immune serum.

### Light microscopy

The tissue fragments were fixed overnight in 4% (w/v) phosphate-buffered paraformaldehyde at 4°C. After fixation, the tissues were washed and dehydrated in ethanol series, cleared in xylene, and embedded in paraffin wax. 4- $\mu$ m thick sections were cut and, after de-waxing with xylene and hydration in an ethanol series of descending concentration, they were stained with Mayer's haematoxylin and eosin (to study the general morphology) or processed for immunohistochemistry.

### Immunohistochemistry

De-waxed and re-hydrated tissue sections were incubated for 30 min in a solution of 0.3%  $H_2O_2$  in methanol to inhibit endogenous peroxidase activity. Then, they were rinsed with PBS-1% BSA, and non-specific binding sites for immunoglobulins were blocked by 5% NGS in PBS-BSA for 30 min. Sections were incubated for 17 hours at 4°C in a moist chamber with a 1:500 dilution of primary rabbit polyclonal antibody against the third extra-cellular loop of the mouse  $\mu$ -opioid receptor (anti- $\mu$ -opioid receptor; Chemicon, Temecula, CA, USA). The sections were incubated for 30 min with diluted biotinylated goat anti-rabbit IgG.

After washing for 15 min in PBS-BSA, immunohistochemical visualisation was obtained using the Vecta-lab "Elite" (ABC) kit (Vector, Burlingame, CA). Peroxidase activity was visualized by incubating with both 0.01%  $H_2O_2$  and 0.05% diaminobenzidine-tetrahydrochloride (DAB) (Sigma, Milano, Italy) in 0.05M Tris buffer, pH 7.2, for 5 min to reveal the brown immuno-reactive cells. Finally, the sections were counterstained with Haematoxylin, dehydrated and mounted.

To confirm the specificity of the immunoreaction, the following control procedures were performed: 1) replacement of primary antibody with NGS, 2) omission of the primary antibody incubation step.

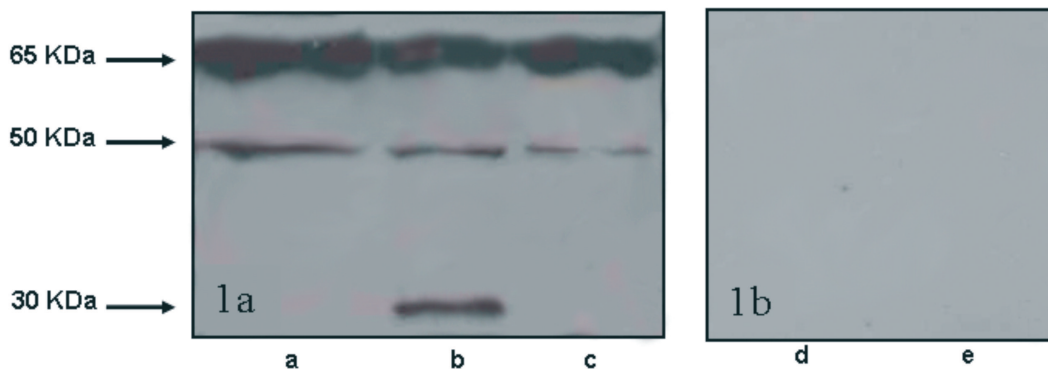
### Deglycosylation

Hydrolysis of N-linked oligosaccharides was carried out by enzymatic treatment with 10U/ml of N-Glycosidase F (Roche, Milano, Italy) diluted in 20mM sodium phosphate buffer (PBS), pH 7.2, containing 10mM EDTA and 0.5% Triton X-100, overnight at 37°C (Desantis et al., 2006). Cleavage of O-glycans was performed by enzymatic and chemical treatments. Enzymatic hydrolysis was carried out by incubating sections with 5U/ml of O-glycosidase (Roche, Milano, Italy) diluted in PBS, pH 7.4, overnight at 37°C. Chemical cleavage consisted of the  $\beta$ -elimination reaction by means of incubation of sections with 0.5 N NaOH in 70% (v/v) ethanol at 4°C for 5 days (Ono et al., 1983). After deglycosylation, the sections were immunostained under the above experimental conditions.

## Results

### Western blot analysis

Western blot analysis showed the presence of the  $\mu$ -opioid receptor in the crude plasma membrane fraction



**Fig. 1.** Immunoblotting analysis of the  $\mu$ -opioid receptor (MOR) in plasma membrane from the isthmus of mare oviduct. Blot with equine plasma membranes from isthmus incubated with antiserum to a peptide related to the third extra-cellular loop of the mouse MOR. **a.** lane a, two bands of 50 and 65 KDa were observed in the isthmus of mare oviduct incubated in deglycosylation reaction buffer; lane b, an additional 30 KDa band was observed in

isthmus after N-deglycosylation treatment; lane c, no differences were observed in the isthmus immunoblotting profile after O-glycosidase treatment. **b.** Negative controls immuno-blotting of equine isthmus plasma membranes incubated with pre-absorbed antibody (lane d) or with a rabbit pre-immune serum (lane e).



obtained from the isthmus of equine oviduct. The protein appeared as two clear bands with molecular weights of around 65 and 50 kDa (Fig. 1a, lane a).

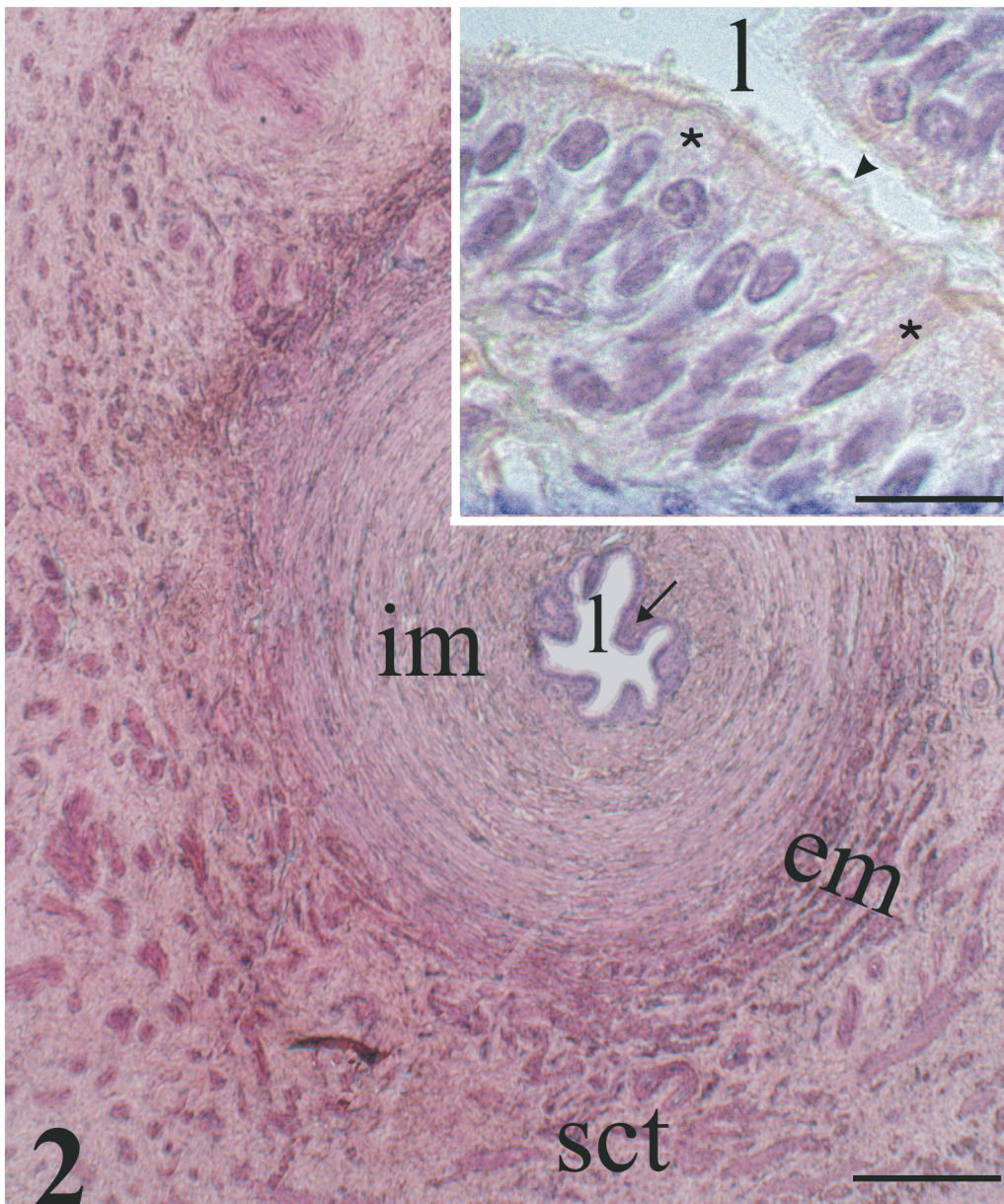
After N-glycosylation, an additional immunoreactive peptide at around 30 kDa was observed (Fig. 1a, lane b), whereas O-deglycosilation did not show any additional band (Fig. 1a, lane c). No immunoreactivity was observed in control blots incubated with the antiserum depleted of anti- $\mu$ -opioid receptor antibodies by pre-adsorption with a molar excess of the immunizing peptide (Fig. 1b, lane d) or in blots incubated with rabbit pre-immune serum (Fig. 1B, lane e)

#### *Light microscopy*

The isthmus of the mare oviduct is characterized both by well-developed muscle layers and non-branched mucosal folds (Fig. 2). The epithelium lining the mucosa consisted of ciliated cells and non-ciliated (secretory) cells (Fig. 2).

#### *Immunohistochemistry*

The results of immunostaining experiments in the mare isthmus oviduct are summarized in Table 1.



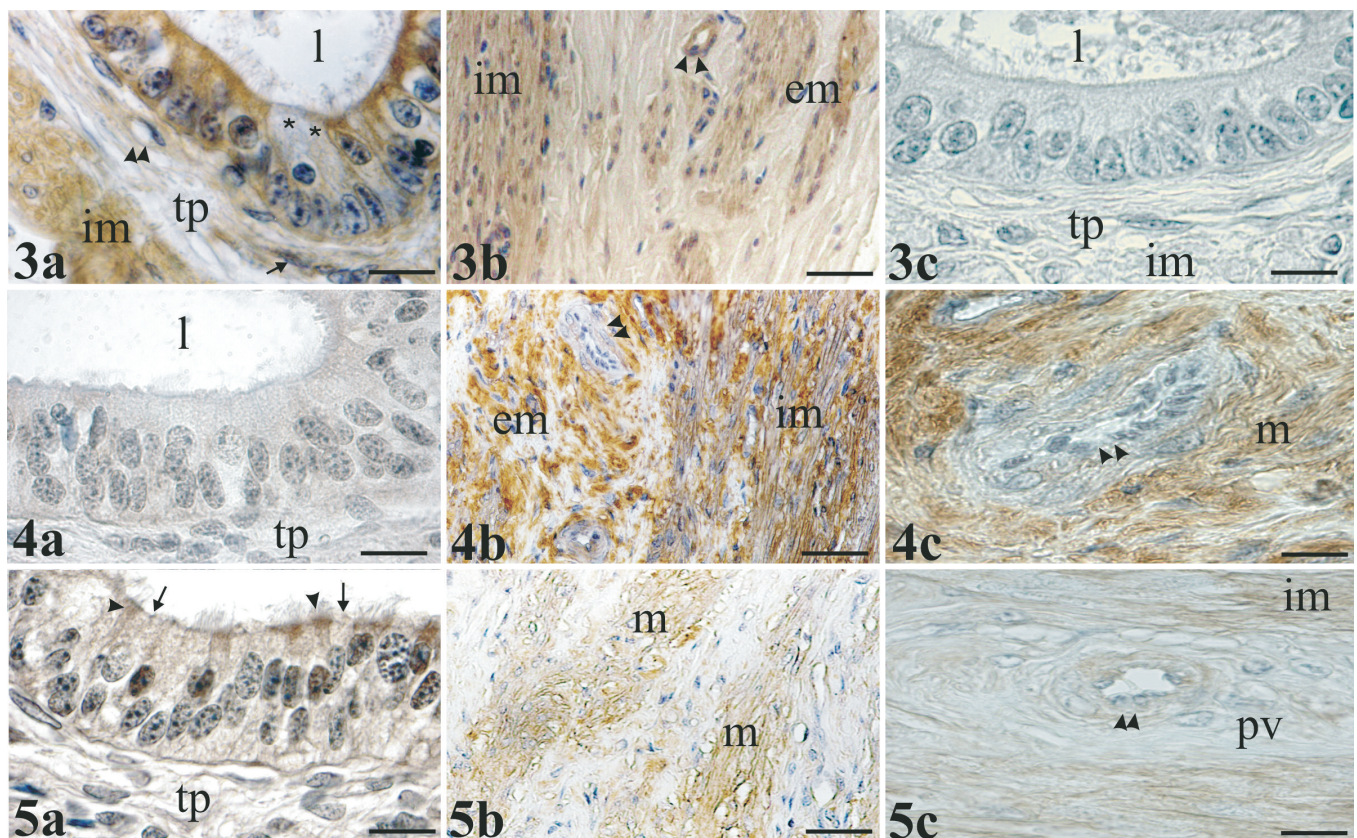
**Fig. 2.** Cross-section of the horse oviductal isthmus. The isthmus shows a well-developed muscle layer and non-branched mucosal folds lined by a columnar epithelium containing ciliated cells and non-ciliated cells. em, extrinsic musculature; im, intrinsic musculature; l, lumen; sct, sub-peritoneal connective tissue; arrow, mucosal fold; arrowhead, ciliated cells; asterisk, non-ciliated cells. Mayer's haematoxylin-eosin staining. Scale bar: 350  $\mu$ m. Scale bar in inset: 18  $\mu$ m.



*$\mu$ -opioid receptor in the isthmus oviduct*

The  $\mu$ -opioid receptor antiserum immunoreacted with the mucosa, the musculature and blood vessels. In the epithelium lining the mucosa, ciliated cells and non-ciliated cells were immunostained with  $\mu$ -opioid receptor antiserum (Fig. 3a). Some non-ciliated cells did not show immunostaining (Fig. 3a). In the tunica propria the endothelial cells and the vascular muscle cells, as well as some stromal cells, showed immunoreactive sites (Fig. 3a). Immunopositive smooth muscle cells were found all along the whole thickness of the isthmus. The  $\mu$ -opioid receptor immunopositive myocytes were more

numerous in the intrinsic musculature than the extrinsic musculature (Fig. 3b). The immunopositive myocytes formed a compact circular coat in the intrinsic musculature, whereas they were arranged in oblique, sometimes isolated, muscle bundles mixed with unstained myocytes in the extrinsic musculature (Fig. 3b). Blood vessels with immunopositive endothelial cells and smooth muscle cells were scattered in the muscular wall of the isthmus. No stained sites were observed when the primary antibody incubation was omitted in the immunostaining procedure (Fig. 3c).



**Fig. 3.** Light micrographs of horse oviductal isthmus immunostained with anti- $\mu$ -opioid receptor. **a.** Anti- $\mu$ -opioid receptor immunoreactivity was found in ciliated cells and non-ciliated cells (some of the latter were negative) of the epithelium lining, in endothelial cells of mucosal blood vessels, and in intrinsic musculature. **b.**  $\mu$ -opioid receptor was expressed in the intrinsic and extrinsic muscle cells, as well as in the endothelial cells of capillaries scattered in the muscular wall. **c.** No staining sites were observed when the primary antibody incubation was omitted in the immunostaining procedure. em, extrinsic musculature; im, intrinsic musculature; l, lumen; tp, tunica propria; arrow, positive stromal cell; double arrowhead, blood vessel; asterisk, negative non-ciliated cells. Scale bars: a, 16  $\mu$ m; b, 81  $\mu$ m; c, 16  $\mu$ m.

**Fig. 4.** Light micrographs of horse oviductal isthmus immunostained with N-glycosidase F/anti- $\mu$ -opioid receptor procedure. **a.** No anti- $\mu$ -opioid receptor immunoreactivity was found in ciliated cells or non-ciliated cells of lining epithelium. **b.** Anti- $\mu$ -opioid receptor immunoreactivity occurs in the muscle cells, whereas immunostaining was lacking in blood vessels. **c.** High-magnification micrograph of a blood vessel dispersed in the muscular wall of the isthmus showing no anti- $\mu$ -opioid receptor reactivity in endothelial cells or smooth muscle cells. em, extrinsic musculature; im, intrinsic musculature; l, lumen; m, muscle cells; tp, tunica propria; double arrowhead, blood vessel. Scale bars: a, 18  $\mu$ m; b, 81  $\mu$ m; c, 21  $\mu$ m.

**Fig. 5.** Light micrographs of horse oviductal isthmus immunostained with  $\beta$ -elimination/anti- $\mu$ -opioid receptor procedure. **a.** The mucosal epithelium only showed anti- $\mu$ -opioid receptor immunoreactivity in the apical region of ciliated cells. **b.** Weak immunostaining with anti- $\mu$ -opioid receptor serum was observed in a lower number of muscle cells of the muscular wall (compare with 3b and 4b). **c.** High-magnification micrograph of a blood vessel scattered in the muscular wall showing no anti- $\mu$ -opioid receptor reactivity in endothelial cells or smooth muscle cells. im, muscle cells; pv, peri-vascular connective tissue; tp, tunica propria; arrow, negative non-ciliated cells; arrowhead, ciliated cells; double arrowhead, blood vessel. Scale bars: a, 18  $\mu$ m; b, 58  $\mu$ m; c, 16  $\mu$ m.

**Table 1.** Results of immunostaining experiments.

	Ciliated cells	Non-ciliated cells	Stromal cells	Muscle cells	Blood vessels
anti $\mu$ -opioid receptor	+	+/*	+*/-	+	+
N-glycosidase F	-	-	-	+	-
O-glycosidase	+	+/*	-	+	+
$\beta$ -elimination	+a	-	-	-/ $\pm$	-

a, apical zone; -, negative reaction;  $\pm$ , little and weak immunoreactive cells; +, moderate/strong immunoreactivity; \* few cells.

Removal of N-linked oligosaccharides by N-glycosidase F pre-treatment abolished the immunostaining of the ciliated cells, non-ciliated cells and stromal cells in the mucosa (Fig. 4a), as well as in the endothelial cells and smooth muscle cells of the blood vessels (Fig. 4b).

Cleavage of O-glycans by O-glycosidase did not modify the  $\mu$ -opioid receptor antiserum binding pattern, whereas  $\beta$ -elimination procedure abolished the immunostaining of the non-ciliated cells, stromal cells (Fig. 5a), the most muscle cells (Fig. 5b) and the blood vessels (Fig. 5c). The immunoreactive muscle cells showed weak staining. After  $\beta$ -elimination the ciliated cells of the mucosal epithelium showed immunoreactivity in the apical region (Fig. 5a).

## Discussion

The present study provides evidence that the  $\mu$ -opioid receptor is expressed in the isthmus of the horse oviduct. In addition, deglycosylation methods show that a different type of glycosylation occurs in the third extra-cellular loop of the  $\mu$ -opioid receptor.

The mucosal epithelium expressed the  $\mu$ -opioid receptor in ciliated cells and non-ciliated cells. The presence of this receptor in the ciliated cells could be related to ciliary motility. Previous studies have shown that opioids regulate cilia beat frequency in humans (Rutland et al., 1982; Roth et al., 1991) as well as in invertebrate respiratory tissues (Mantione et al., 2006). Calcium ions are involved in maintaining ciliary beat frequency in oviductal ciliated cells (Barrera et al., 2004; Andrade et al., 2005), thus providing the progression of capacitated spermatozoa. The deglycosylation treatments used in the immunohistochemical study indicate that ciliated cells express the  $\mu$ -opioid receptor with N-linked oligosaccharides in the third extra-cellular loop. This was shown by the abolition of staining in N-glycosidase F pre-treated sections and the preservation of immunoreactivity in the apical region of ciliated cells after  $\beta$ -elimination. Since this ciliated cells zone contains the ciliary beat machinery, the presence of the  $\mu$ -opioid receptor could be related to ciliary motility. The presence of N-oligosaccharides in the  $\mu$ -opioid receptor has also been reported in human embryonic kidney (HEK)-293 cells (Chaturvedi et al., 2000).

The immunoreactivity of the  $\mu$ -opioid receptor in

non-ciliated cells proves that this receptor is expressed in many, but not all, non-ciliated cells, because some non-ciliated cells were unstained. The immunostaining abolition of the non-ciliated cells after the N-glycosidase-F and  $\beta$ -elimination procedure suggests that non-ciliated cells express both N- and O-linked oligosaccharides in the third extra-cellular loop of the  $\mu$ -opioid receptor. The expression of this receptor in the isthmus is consistent with the presence of  $\text{Ca}^{2+}$  in the oviduct (Cox and Leese, 1997). The concentration of  $\text{Ca}^{2+}$  in the oviductal media modulates sperm capacitation, zona pellucida binding, and penetration and sperm-egg fusion in an Australian marsupial, the brushtail possum (*Trichosurus vulpecula*) (Sidhu et al., 2003). The lack of the  $\mu$ -opioid receptor in some non-ciliated cells indicates the presence of non-ciliated cell sub-types. Histochemistry studies have shown different non-ciliated cell sub-types in the mare oviduct (Desantis et al., 2005). Different non-ciliated cell sub-types might indicate that non-ciliated cells carry out different roles along the mucosal epithelium of the mare isthmus oviduct. In non-ciliated epithelial cells the  $\mu$ -opioid receptor may mediate exocrine secretion (Lang et al., 1996), as well as paracrine or autocrine signalling (Popper et al., 2004).

Some stromal cells showed immunoreactivity with the anti- $\mu$ -opioid receptor serum in the tunica propria of mare isthmus oviduct. The immunostaining was abolished after the N-glycosidase-F and  $\beta$ -elimination procedures. This indicates that  $\mu$ -opioid receptor contains both N- and O-linked oligosaccharides in the isthmus stromal cells of mare oviduct. Recent non immunohistochemical studies revealed the presence of this receptor in stromal cells of pregnant mouse uterus (Zhu and Pintar, 1998) and of deep infiltrating endometriosis in humans (Matsuzaki et al., 2007). Although expression of the  $\mu$ -opioid receptor is regulated by GnRH and progesterone, its functional role in stromal cells has not been clarified (Matsuzaki et al., 2007).

The immunostaining of the  $\mu$ -opioid receptor was demonstrated in the isthmus muscle cells. This indicates that  $\mu$ -opioid receptor could have some influence on the contraction of mare isthmus musculature. Calcium is an important second messenger involved in exciting-contraction coupling and plays a key role in regulation of smooth muscle tone (Jiang and Stephens, 1994).



*μ-opioid receptor in the isthmus oviduct*

Calcium channels have been isolated in myocytes from the cricket lateral oviduct (Mutoh and Yoshino, 2004). Non immunohistochemical studies have shown the μ-opioid receptor on the smooth muscle cells of guinea pig, human (Bitar and Makhlof, 1985) and cat intestine (Venkova et al., 1992), mouse vas deferens (Ramme and Illes, 1986), canine gallbladder (Severi et al., 1988) and guinea pig stomach (Grider and Makhlof, 1991). In our study, isthmus showed a greater presence of myocytes with the μ-opioid receptor in the intrinsic musculature than in the extrinsic musculature. The immunopositive myocytes formed a compact, circular coat in the intrinsic musculature, whereas they were arranged in some isolated, oblique bundles scattered among unreactive muscle cells in the extrinsic musculature. A scanning electron microscopy study revealed that in mare isthmus the myosalpinx has a plexiform structure. The intrinsic musculature consists of a circular inner coat, mixed with oblique bundles, and the extrinsic musculature contains isolated, oblique muscle bundles scattered among longitudinally arranged fibre bundles (Germanà et al., 2002). The different arrangement of μ-opioid receptor expressing myocytes could generate a stirring movement of lumen content which is peculiar to plexiform musculature (Hodgson et al., 1977). The stirring movement can promote the contact between lumen content molecules. This process can lead to an optimization of the micro-environment which ensures the effective condition of the sperm when ovulation occurs, as well as early embryo development during its flow to the uterus (Motta et al. 1995). A different presence of the μ-opioid receptor in muscle cells has been found in guinea pig, human (Bitar and Makhlof, 1985) and cat intestine (Venkova et al., 1992), where opiate receptors are present on circular muscle cells and absent on longitudinal muscle cells. Contrary to the plexiform architecture of the mare myosalpinx, the muscular architecture of the intestine generates peristaltic movements.

β-elimination abolished immunoreactivity in most muscle cells, whereas the other deglycosylation procedures did not change immunostaining. These results suggest that the third extra-cellular loop of the μ-opioid receptor in muscle cells of the mare isthmus oviduct contains O-linked oligosaccharides. The possibility of O-linked glycosylation has been suggested but not demonstrated in the μ-opioid receptor of equine spermatozoa (Albrizio et al., 2005). O-glycosidase releases the disaccharide Galβ(1-3)GalNAc core from O-glycans binding to serine or threonine. Substitution of the disaccharide by sialic acid, N-acetylgalactosamine or fucose prevents this hydrolysis (Umemoto et al., 1977). Thus, the failure in the cleavage of O-glycans by this enzyme observed in both the western blot and immunohistochemistry procedures suggests that the third extra-cellular loop of the μ-opioid receptor expressed in the mare isthmus contains some modification of the Galβ(1-3)GalNAc unit.

Blood vessels showed the presence of the μ-opioid

receptor in endothelial cells and smooth muscle cells. The immunostaining was abolished after N-glycosidase F and β-elimination incubation. This indicates that the μ-opioid receptor expressed in the endothelial cells and smooth muscle cells of blood vessels of the mare isthmus contains both N- and O-linked oligosaccharides in the third extracellular loop. Pharmacological and immunological investigations have revealed the expression of the μ-opioid receptor in human neural microvascular (Vidal et al., 1998; Cadet et al., 2000), saphenous vein, atria and internal thoracic artery endothelium (Cadet et al., 2001). The presence of the μ-opioid receptor in vascular smooth muscle cells has been revealed in the mesenteric arterial smooth muscle cells of rats (Kai et al., 2002). Some studies show that the expression of the μ-opioid receptor can be up-regulated by pro-inflammatory cytokine, such as interleukin-1 (IL-1), and suggest that opioid-dependent pathways in endothelial tissue may be modulated in the disease state (Maier et al., 1990). Immunoreactive IL-1 has been found in the epithelium and stroma of the bovine isthmus oviduct (Paula-Lopes et al., 1999). The μ-opioid receptor regulates the concentration of Ca<sup>2+</sup>, an important second messenger involved in excitation-contraction coupling, playing a key role in the regulation of vascular smooth muscle tone (Jiang and Stephens, 1994) during pathological events (Kai et al., 2002, 2004).

In conclusion, this study describes the expression sites of the μ-opioid receptor in the mare isthmus oviduct and that this receptor is a protein glycosylated in the third extra-cellular loop, which appears to be a key element for the binding of μ-opioid receptor ligands (Guarna et al., 2003). Since this is not a functional study, and having analysed oestrus specimens, we can infer that in the isthmus oviduct this receptor could have a role in ensuring the best sperm condition when ovulation occurs, as well as in facilitating gamete transport.

---

*Acknowledgements.* The authors wish to thank Mrs. Annunziata Marinelli for her technical assistance.

This work is dedicated to the memory of Prof. Paolo Minoia whose recent death deprives our team of the pioneer in the investigations on the relationship between the opioid receptors and animal reproduction.

---

## References

- Abe H. (1996). The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells. *Histol. Histopathol.* 11, 743-768.
- Albrizio M., Guaricci A.C., Maritato F., Sciorsci R.L., Mari G., Calamita G., Lacalandra G.M., Aiudi G.G., Minoia R., Dell'Aquila M.E. and Minoia P. (2005). Expression and sub-cellular localization of the mu-opioid receptor in equine spermatozoa: evidence for its functional role. *Reproduction* 129, 39-49.
- Albrizio M., Guaricci A.C., Calamita G., Zarrilli A. and Minoia P. (2006). Expression and immunolocalization of the mu-opioid receptor in human sperm cells. *Fertil. Steril.* 86, 1776-1779.

- Andrade Y.N., Fernandes J., Vazquez E., Fernandez-Fernandez J.M., Arniges M., Sanchez T.M., Villalon M. and Valverde M.A. (2005). TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *J. Cell Biol.* 168, 869-874.
- Bagnol D., Mansour A., Akil H. and Watson S.J. (1997). Cellular localization and distribution of the cloned mu and kappa opioid receptors in rat gastrointestinal tract. *Neuroscience* 81, 579-591.
- Barrera N.P., Morales B. and Villalon M. (2004). Plasma and intracellular membrane inositol 1,4,5-trisphosphate receptors mediate the Ca(2+) increase associated with the ATP-induced increase in ciliary beat frequency. *Am. J. Physiol. Cell. Physiol.* 287, 114-124.
- Bigliardi P.L., Bigliardi-Qi M., Buechner S. and Ruffli T. (1998). Expression of mu-opiate receptor in human epidermis and keratinocytes. *J. Invest. Dermatol.* 111, 297-301.
- Bigliardi-Qi M., Bigliardi P.L., Buchner S. and Ruffli T. (1999). Characterization of mu-opiate receptor in human epidermis and keratinocytes. *Ann. NY Acad. Sci.* 885, 368-371.
- Bigliardi-Qi M., Sumanovski L.T., Buchner S., Ruffli T. and Bigliardi P.L. (2004). Mu-opiate receptor and beta-endorphin expression in nerve endings and keratinocytes in human skin. *Dermatology* 209, 183-189.
- Bitar K.N. and Makhlof G.M. (1985). Selective presence of opiate receptors on intestinal circular muscle cells. *Life Sci.* 37, 1545-1550.
- Bourinet E., Soong T.W., Stea A. and Snutch T.P. (1996). Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proc. Natl. Acad. Sci. USA* 93, 1486-1491.
- Buhi W.C. (2002). Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* 123, 355-362.
- Burkhardt J. (1949). Sperm survival in the genital tract of the mare. *J. Agric. Sci.* 39, 201.
- Cadet P., Bilfinger T.V., Fimiani C., Peter D. and Stefano G.B. (2000). Human vascular and cardiac endothelia express mu opiate receptor transcripts. *Endothelium* 7, 185-91.
- Cadet P., Weeks B.S., Bilfinger T.V., Mantione K.J., Casares F. and Stefano G.B. (2001). HIV gp120 and morphine alter mu opiate receptor expression in human vascular endothelium. *Int. J. Mol. Med.* 8, 165-169.
- Carretero J., Bodego P., Rodriguez R.E., Rubio M., Blanco E. and Burks D.J. (2004). Expression of the mu-opioid receptor in the anterior pituitary gland is influenced by age and sex. *Neuropeptides* 38, 63-68.
- Chen Y., Mestek A., Liu J., Hurley J.A. and Yu L. (1993a). Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol. Pharmacol.* 44, 8-12.
- Chen Y., Mestek A., Liu J. and Yu L. (1993b). Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. *Biochem. J.* 295, 625-628.
- Chen C., Chun Xue J., Zhu J., Chen Y.W., Kunapuli S., Kim J., Riel de, Yu L., Yuan L. and Chen L. (1995). Characterization of irreversible binding of beta-funaltrexamine to the cloned rat mu opioid receptor. *J. Biol. Chem.* 28, 17866-17870.
- Connor M., Borgaland S.L. and Christie M.J. (1999). Continued morphine modulation of calcium channel currents in acutely isolated locus coeruleus neurons from morphine-dependent rats. *Br. J. Pharmacol.* 128, 1561-1569.
- Cox C.I. and Leese H.J. (1997). Retention of functional characteristics by bovine oviduct and uterine epithelia in vitro. *Anim. Reprod. Sci.* 46, 169-178.
- Day F.T. (1942). Survival spermatozoa in the genital tract of the mare. *J. Agric. Sci.* 32, 108-111.
- Dell'Aquila M.E., Casavola V., Reshkin S.J., Albrizio M., Guerra L., Maritato F. and Minoia P. (2002). Effects of beta-endorphin and naloxone on in vitro maturation of bovine oocytes. *Mol. Reprod. Dev.* 63, 210-222.
- Desantis S., Ventriglia G., Zubani D., Corriero A., Deflorio M., Acone F., Palmieri G. and De Metrio G. (2005). Differential lectin binding patterns in the oviductal ampulla of the horse during oestrus. *Eur. J. Histochem.* 49, 139-149.
- Desantis S., Ventriglia G., Zubani D., Deflorio M., Megalofonou P., Acone F., Zarrilli A., Palmieri G. and De Metrio G. (2006). Histochemical analysis of glycoconjugates in the domestic cat testis. *Histol. Histopathol.* 21, 11-22.
- Di Sole F., Guerra M., Bagorda A., Reshkin S.J., Albrizio M., Minoia P. and Casavola V. (2001). Naloxone inhibits A6 cell Na(+)/H(+) exchange by activating protein kinase C via the mobilization of intracellular calcium. *Exp. Nephrol.* 9, 341-348.
- Dobrinski I., Ignatz G.G., Thomas P.G. and Ball B.A. (1996). Role of carbohydrates in the attachment of equine spermatozoa to uterine tubal (oviductal) epithelial cells in vitro. *Am. J. Vet Res.* 57, 1635-1639.
- Evans C.J., Keith D.E. Jr, Morrison H., Magendzo K. and Edwards R.H. (1992). Cloning of a delta opioid receptor by functional expression. *Science* 258, 1952-1955.
- Fickel J., Bagnol D., Watson S.J. and Akil H. (1997). Opioid receptor in the rat gastrointestinal tract: a quantitative study with comparison to the brain. *Brain Res. Mol. Brain Res.* 46, 1-8.
- Flechon J.E. and Hunter R.H. (1981). Distribution of spermatozoa in the utero-tubal junction and isthmus of pigs, and their relationship with the luminal epithelium after mating: a scanning electron microscope study. *Tissue Cell* 13, 127-139.
- Germanà A., Cassata R., Cristarella S., Scirpo A. and Muglia U. (2002). Three-dimensional architecture of the myosalpinx in the mare as revealed by scanning electron microscopy. *Anat. Rec.* 267, 235-241.
- Gioannini T., Foucaud B., Hiller J.M., Hatten M.E. and Simon E.J. (1982). Lectin binding of solubilized opiate receptors: evidence for their glycoprotein nature. *Biochem. Biophys. Res. Commun.* 105, 1128-1134.
- Grider J.R. and Makhlof G.M. (1991). Identification of opioid receptors on gastric muscle cells by selective receptor protection. *Am. J. Physiol.* 260, 103-107.
- Gualtieri R. and Talevi R. (2000). In vitro-cultured bovine oviductal cells bind acrosome-intact sperm and retain this ability upon sperm release. *Biol. Reprod.* 62, 1754-1762.
- Guarna M., Bartolini A., Ghelardini C., Galeotti N., Bracci L., Stefano G.B. and Bianchi E. (2003). Anti-mu opioid antiserum against the third external loop of the cloned mu-opioid receptor acts as a mu receptor neutral antagonist. *Mol. Brain Res.* 119, 100-110.
- Hodgson B.J., Talo A. and Pauerstein C.J. (1977). Oviductal ovum surrogate movement interrelation with muscular activity. *Biol. Reprod.* 16, 394-396.
- Hunter R.H. (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J. Reprod. Fertil.* 63, 109-117.
- Hunter R.H. and Nichol R. (1983). Transport of spermatozoa in the sheep oviduct: preovulatory sequestering of cells in the caudal isthmus. *J. Exp. Zool.* 228, 121-128.
- Hunter R.H.F., Flechon B. and Flechon J.E. (1991). Distribution,



*μ-opioid receptor in the isthmus oviduct*

- morphology and epithelial interactions of bovine spermatozoa in the oviduct before and after ovulation: a scanning electron microscopy study. *Tissue Cell* 23, 641-656.
- Jiang H. and Stephens N.L. (1994). Calcium and smooth muscle contraction. *Mol. Cell. Biochem.* 135, 1-9.
- Kai L., Wang Z.F., Hu D.Y., Shi Y.L. and Liu L.M. (2002). Modulation of  $Ca^{2+}$  channels by opioid receptor antagonists in mesenteric arterial smooth muscle cells of rats in hemorrhagic shock. *J. Cardiovasc. Pharmacol.* 40, 618-24.
- Kai L., Wang Z.F., Shi Y.L., Liu L.M. and Hu D.Y. (2004). Opioid receptor antagonists increase  $Ca^{2+}$  in rat arterial smooth muscle cells in hemorrhagic shock. *Acta Pharmacol. Sin.* 25, 395-400.
- Kalyuzhny A.E., Hensleigh H.C., Arvidsson U. and Elde R. (1997). Immunocytochemical localization of mu-opioid receptors in follicular cells and preimplantation mouse embryos. *Anat. Embryol.* 195, 451-455.
- Khawaja X.Z., Green I.C., Thorpe J.R. and Titheradge M.A. (1990). The occurrence and receptor specificity of endogenous opioid peptides within the pancreas and liver of the rat. Comparison with brain. *Biochem. J.* 267, 233-240.
- Kieffer B.L., Befort K., Gavériaux-Ruff C. and Hirth C.G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc. Natl. Acad. Sci. USA* 89, 12048-12052.
- Lang M.E., Davison J.S., Bates S.L. and Meddings J.B. (1996). Opioid receptors on guinea-pig intestinal crypt epithelial cells. *J. Physiol.* 497, 161-174.
- Lapointe S. and Sirard M.A. (1996). Importance of calcium for the binding of oviductal fluid proteins to the membranes of bovine spermatozoa. *Mol. Reprod. Dev.* 44, 234-240.
- Leese H.J. (1988). The formation and function of oviduct fluid. *J. Reprod. Fertil.* 82, 843-56.
- Maier J.A., Hla T. and Maciag T. (1990). Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.* 265, 10805-10808.
- Mantione K.J., Kim C. and Stefano G.B. (2006). Morphine regulates gill ciliary activity via coupling to nitric oxide release in a bivalve mollusk: opiate receptor expression in gill tissues. *Med. Sci. Monit.* 12, 195-200.
- Matsuzaki S., Canis M., Pouly J.L., Botchorishvili R., Déchelotte P.J. and Mage G. (2007). Both GnRH agonist and continuous oral progestin treatments reduce the expression of the tyrosine kinase receptor B and mu-opioid receptor in deep infiltrating. *Hum. Reprod.* 22, 124-128.
- Meng F., Xie G.X., Thompson R.C., Mansour A., Goldstein A., Watson S.J. and Akil H. (1993). Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc. Natl. Acad. Sci. USA* 90, 9954-9958.
- Motta P.M., Nottola S.A., Pereda J., Coatto H.B. and Familiari G. (1995). Ultrastructure of human cumulus oophorus: a transmission electron microscopic study on oviductal oocytes and fertilized eggs. *Hum. Reprod.* 10, 2361-2367.
- Mutoh H. and Yoshino M. (2004). L-type  $Ca^{2+}$  channel and  $Ca^{2+}$ -permeable non-selective cation channel as a  $Ca^{2+}$  conducting pathway in myocytes isolated from the cricket lateral oviduct. *J. Comp. Physiol.* 174, 21-28.
- Ono K., Katsuyama T. and Hotchi M. (1983). Histochemical application of mild alkaline hydrolysis for selective elimination of O-glycosidically linked glycoproteins. *Stain Technol.* 58, 309-312.
- Overstreet J.W. and Cooper G.W. (1978). Sperm transport in the reproductive tract of the female rabbit: II. The sustained phase of transport. *Biol. Reprod.* 19, 115-122.
- Paula-Lopes F.F., de Moraes A.A. and Hansen P.J. (1999). The presence of interleukin-1beta in the bovine reproductive tract. *J. Interferon Cytokine Res.* 19, 279-285.
- Phansuwan-Pujito P., Ebadi M. and Govitrapong P. (2006). Immunocytochemical characterization of delta-opioid and mu-opioid receptor protein in the bovine pineal gland. *Cells Tissues Organs* 182, 48-56.
- Popper P., Cristobal R. and Wackym P.A. (2004). Expression and distribution of mu opioid receptors in the inner ear of the rat. *Neuroscience* 129, 225-233.
- Ramme D. and Illes P. (1986). Differential effect of stimulation strength in mouse vas deferens on inhibition of neuroeffector transmission by receptor type selective opioids. *Naun. Schmied. Arch. Pharmacol.* 332, 57-61.
- Roth Y., Aharonson E.F., Teichtahl H., Baum G.L., Priel Z. and Modan M. (1991). Human in vitro nasal and tracheal ciliary beat frequencies: comparison of sampling sites, combined effect of medication, and demographic relationships. *Ann. Otol. Rhinol. Laryngol.* 100, 378-384.
- Rutland J., Griffin W.M. and Cole P.J. (1982). Human ciliary beat frequency in epithelium from intrathoracic and extrathoracic airways. *Am. Rev. Respir. Dis.* 125, 100-105.
- Severi C., Grider J.R. and Makhlof G.M. (1988). Characterization of opioid receptors on isolated canine gallbladder smooth muscle cells. *Life Sci.* 42, 2373-2380.
- Sheehy J.G., Hellyer P.W., Sammonds G.E., Mama K.R., Powers B.E., Hendrickson D.A. and Magnusson K.R. (2001). Evaluation of opioid receptors in synovial membranes of horses. *Am. J. Vet. Res.* 62, 1408-1412.
- Sidhu K.S., Mate K.E., Molinia F.C., Berg D.K. and Rodger J.C. (2003). Ionic calcium levels in oviduct explant-conditioned media from an Australian marsupial, the brushtail possum (*Trichosurus vulpecula*) and its relevance to in vitro fertilization. *Zygote* 11, 285-291.
- Singh V.K., Bajpai K., Biswas S., Haq W., Khan M.Y. and Mathur K.B. (1997). Molecular biology of opioid receptors: recent advances. *Neuroimmunomodulation* 4, 285-297.
- Smith T.T. and Yanagimachi R. (1991). Attachment and release of spermatozoa from the caudal isthmus of the hamster oviduct. *J. Reprod. Fertil.* 91, 567-573.
- Spicer S.S. and Schulte B.A. (1992). Diversity of cell glycoconjugates shown histochemically: a perspective. *J. Histochem. Cytochem.* 40, 1-38.
- Spiro R.G. (2002). Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12, 43-56.
- Suarez S.S. (1987). Sperm transport and motility in the mouse oviduct: observations *in situ*. *Biol. Reprod.* 36, 203-210.
- Suarez S.S., Drost M., Redfern K. and Gottlieb W. (1990). Sperm motility in the oviduct. In: *Fertilization in mammals*. Bavister B.D., Cummins J. and Roldan E.R.S. (eds). Norwell. Serono Symposia. pp 111-114.
- Suarez S.S. (2002). Formation of a reservoir of sperm in the oviduct. *Reprod. Dom. Anim.* 37, 140-143.
- Suarez S.S., Redfern K., Raynor P., Martin F. and Phillips D.M. (1991). Attachment of boar sperm to mucosal explants of oviduct in vitro: possible role in formation of a sperm reservoir. *Biol. Reprod.* 44,

*μ-opioid receptor in the isthmus oviduct*

- 998-1004.
- Thomas P.G., Ball B.A. and Brinsko S.P. (1994). Interaction of equine spermatozoa with oviduct epithelial cell explants is affected by estrous cycle and anatomic origin of explant. *Biol. Reprod.* 51, 222-228.
- Umemoto J., Bhavanandan V.P. and Davidson E.A. (1997). Purification and properties of an endo-alpha-N-acetyl-D-galactosaminidase from *Diplococcus pneumoniae*. *J. Biol. Chem.* 272, 8609-8614.
- Venkova K., Davidoff M. and Radomirov R. (1992). Effects of Met-enkephalin on the mechanical activity and distribution of Met-enkephalin-like immunoreactivity in the cat large intestine. *Peptides* 13, 193-201.
- Vidal E.L., Patel N.A., Wu G., Fiala M. and Chang S.L. (1998). Interleukin-1 induces the expression of mu opioid receptors in endothelial cells. *Immunopharmacology* 38, 261-266.
- Yasuda K., Raynor K., Kong H., Breder C.D., Takeda J., Reisine T. and Bell G.I. (1993). Cloning and functional comparison of kappa and delta opioid receptors from mouse brain. *Proc. Natl. Acad. Sci. USA* 90, 6736-6740.
- Wang J.F., Ren M.F., Xue J.C. and Han J.S. (1993). Cyclic AMP mediates mu and delta, but not kappa opioid analgesia in the spinal cord of the rat. *Life Sci.* 52, 1955-60.
- Xue J.C., Chen C., Zhu J., Kunapuli S.P., De Riel J.K., Yu L. and Liu-Chen L.Y. (1995). The third extracellular loop of the mu opioid receptor is important for agonist selectivity. *J. Biol. Chem.* 270, 12977-12979.
- Zadina J.E., Hackler L., Ge L.J. and Kastin A.J. (1997). A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 386, 499-502.
- Zhu Y. and Pintar J.E. (1998). Expression of opioid receptors and ligands in pregnant mouse uterus and placenta. *Biol. Reprod.* 59, 925-932.

Accepted November 16, 2007