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The mu (μ) and delta (δ) opioid receptors modulate boar sperm motility

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Abstract

Endogenous and exogenous opioids modulate reproductive functions in target cells via opioid receptors (μ , δ , and κ). Sperm motility is a metric of gamete functionality, and serves as a suitable parameter for in vitro drug-induced toxicity assays. This study identifies the presence and location of opioid receptors in pig spermatozoa as well as their functional response after in vitro challenge with known agonists (morphine [μ]; [D-Pen 2,5]-enkephanile [δ]; and U 50488 [κ]) and antagonists (naloxone [μ]; naltrindole [δ]; and *nor*-binaltrorphimine [κ]). Only the μ - and δ -opioid receptors were present in the sperm plasma membrane, overlying the acrosome, neck, and principal piece. Challenge experiments with agonists and antagonists identified both μ - and δ -opioid receptors as regulators of sperm kinematics, wherein μ maintains or increases sperm movement whereas δ decreases sperm motility over time.

Introduction

Exogenous opioids (opiate-like) are widely used in human and veterinary medicine to treat moderate to severe pain, but can become addictive (World Drug Report 2015).

Endogenous opioid peptides (endorphins, enkephalins, dynorphins, etc) can inhibit the transmission of pain as well as participate in the regulation of reproductive function through the central nervous system (Kiani et al., 2009), inhibiting the release of gonadotropin-releasing hormone and thus blocking the release of luteinizing hormone and/or follicle-stimulating hormone from the hypophysis (Fabbri et al., 1989).

Opioids of exogenous or endogenous nature bind to members of the transmembrane G protein coupled receptors superfamily: the μ -, δ -, and κ -opioid receptors. These receptors inhibit adenylyl cyclase, which suppresses the opening of voltage-gated calcium channels while facilitating opening of potassium channels, leading to cell hyperpolarization and cell-specific responses (Ong and Cahill, 2014). A variety of signal transduction pathways are induced after receptor activation, including activation of the mitogen-activated protein (MAP) kinases and phospholipase C-controlled cascades (Ong and Cahill, 2014). In human spermatozoa, for example, opioid receptor activation facilitates the acrosome reaction via protein kinase C signaling (Urizar-Arenaza et al., 2015).

Exogenous opioids – such as morphine, heroine, or methadone – can negatively affect human reproductive function. In females, these opioids apparently impair estrogen receptor activity (Korneyi et al., 1999; Shuey et al., 2007; Lee and Ho, 2013). In males, exogenous opioids decrease serum levels of luteinizing hormone and testosterone, leading to dysfunctional copulatory behavior (Pfaus and Gorzalka, 1987; Tokunaga et al., 1977), decreased libido, and erectile dysfunction (Succu et al., 2006; Hallinan et al., 2008). They also reduce the size of accessory glands and sperm production (James et al., 1980; Da Silva et al., 2006), as manifested by lower ejaculate volume, fewer spermatozoa, and decreased sperm

motility (Ragni et al., 1985), deviant sperm morphology (el-Gothamy and el Samahy, 1992), and decreased integrity of the genomic sperm DNA (Safarinejad et al., 2012).

Endogenous β -endorphins are released from the anterior pituitary in relation to pain or stress, and are secreted by genital epithelial cells: those produced by granulosa cells (Kaminski et al., 2000) are found in follicular fluid (Petraglia et al., 1986); those produced by human or bovine oviductal or endometrial cells end up in tubal and uterine fluids (Petraglia et al., 1986); and those produced by the accessory sexual glands reside in the seminal plasma (Fujisawa et al., 1996; Subirán et al., 2008). Cells in contact with these fluids, such as oocytes (Agirregoitia et al., 2012a) or somatic epithelial or muscle cells of the genital organs (Petraglia et al., 1986; Desantis et al., 2008), contain opioid receptors – particularly of the μ - and δ -type, which exhibit the highest affinity for β -endorphin (Minoia and Sciorci, 2001) – suggesting that these endogenous endorphins are paracrine regulators of reproductive function (Fabbri et al., 1989). Indeed, β -endorphins and enkephalins present in human seminal plasma can associate with ejaculated spermatozoa (Subirán et al., 2008; Subirán et al., 2012). This interaction has been indirectly ascribed a role in sperm motility, based on the observation that opioid analgesics commonly used at the hospitals reduce sperm motility (Xu et al., 2012). A minimum concentration of opioid peptides in seminal plasma was experimentally determined to be necessary to maintain human sperm motility *in vitro* (Fujisawa et al., 1996).

The receptors targeted by both exogenous and endogenous opioids reside on a plethora of somatic cells in the pituitary, pineal gland, gastrointestinal tract, liver, pancreas, kidney, skin (reviewed by Desantis et al., 2008), cumulus-oophorus (Dell'Aquila et al., 2002), and oviductal isthmus (Desantis et al., 2008). Opioid receptors are present on spermatozoa, although the current –albeit not comprehensive–experimental evidence suggests their presence is not ubiquitous; indeed, positive evidence is limited to human (μ - [Agirregoitia et

al., 2006; Albrizio et al., 2006], δ - and κ - [Agirregoitia et al., 2006]), equine (μ - [Albrizio et al., 2005] and δ - [Albrizio et al., 2010]), sea bream (μ -, Albrizio et al., 2013), and ovine (Vicente-Carrillo et al., 2015a). These opioid receptors are reported to mediate sperm motility in humans (Agirregoitia et al., 2006; 2012b) and equine (Albrizio et al., 2005; Albrizio et al., 2010), although results are contradictory regarding the effects of opioid antagonists. For instance, antagonists of the δ -opioid receptor increase motility of stallion spermatozoa (Albrizio et al., 2010) but decrease human sperm motility (Agirregoitia et al., 2006; 2012b). Determining if these differences are due to species differences requires further research.

Boar spermatozoa are valuable for pharmacological and toxicological *in vitro* testing (Vicente-Carrillo et al., 2015b). This study was undertaken to address (i) the presence and distribution of μ -, δ -, and κ -opioid receptors in these gametes and (ii) their relation to *in vitro* sperm kinematics, sperm viability, mitochondrial integrity, and early membrane destabilization using specific agonists (morphine [μ]; [D-Pen 2,5]-enkephanil (DPDPE) [δ]; and U 50488 [κ]) and antagonists (naloxone [μ]; naltrindole [δ]; and *nor*-binaltorphimine [κ]).

Results

Boar spermatozoa express only μ - and δ -opioid receptors

Western blots established the presence of the μ - and δ -opioid receptors in ejaculated pig spermatozoa, revealing a protein of 45 kDa for the μ -opioid receptor and 70 kDa for the δ -opioid receptor; the κ -opioid receptor was not detected (Fig. 1). Immunocytochemistry further showed that both μ - and δ -opioid receptors reside over the plasma membrane

covering the acrosome, the neck, and the principal piece of pig spermatozoa (μ -opioid receptor, Fig 2E; δ -opioid receptor, 2F); again, κ -opioid receptor was not detectable (Fig 2G).

Human spermatozoa, used as a positive control, contained each of these opioid receptors, as seen by Western blot (Fig. 1) and immunocytochemistry (Fig. 2A-2C).

Immunocytochemical controls where the primary antibody was omitted appeared negative (human sperm, Fig 2D; pig sperm, Fig 2H), supporting the specificity of the staining.

The μ -opioid receptor modulates boar sperm motility

Under baseline conditions, total and progressive sperm motility, but not sperm velocity, were affected by time with a significant ($P<0.05$) reduction after 30 min (Table 1). Exposure to 1 or 10 μ M morphine abolished this time-dependent effect, significantly increasing total and progressive sperm motility for as long as 60 min (Table 1). The μ -antagonist naloxone, at 1 and 100 μ M, significantly reduced sperm motility over time (Table 1).

No apparent time-effect changes were observed in sperm viability, mitochondria integrity, or stability of the plasma membrane among control samples. Incubation with 0.1 and 1 μ M of morphine, however, led to a significant ($P<0.05$) reduction of sperm viability and mitochondrial integrity over time, while 10 μ M of morphine only reduced mitochondrial integrity (Table 1). Incubation with naloxone, on the other hand, did not affect any of the flow-cytometry variables monitored compared to controls or elapsed time (Table 1).

The δ -opioid receptor does not mediate sperm motility

Exposure to 0.1 μM of DPDPE lead to a significant reduction in sperm motility over time ($P<0.05$) compared to the control conditions; conversely, no other concentrations of DPDPE or any concentrations of naltrindole affected sperm motility (Table 2). Sperm viability and mitochondrial integrity of controls were the only measures significantly reduced over time ($p<0.05$ after 30 min, Table 2). The highest concentration of naltrindole tested (100 μM) caused a significant decrease ($P<0.05$) in sperm viability after 60 min (Table 2).

κ -opioid receptor agonists and antagonists appear to be toxic to pig sperm

Total and progressive sperm motility, sperm velocity, sperm viability, and mitochondrial integrity were time-affected in the control, with a significant ($P<0.05$) reduction after 30 min (Table 3). Exposure to 0.1 and 1 μM U 50488 or 10 and 100 μM *nor*-binaltrorphimine did not cause any significant change in time-effect compared to the control; further, 10 μM U 50488 and 1 μM *nor*-binaltrorphimine maintained sperm kinematics over time (Table 3). Both 0.1 and 10 μM U 50488 significantly decreased sperm velocity compared to the control at 15 min incubation (Table 3). The highest concentration of *nor*-binaltrorphimine tested (100 μM) caused a significant ($P<0.05$) decrease in sperm velocity compared to the control at 10, 15, and 30 min time points (Table 3). By 60 min incubation, 100 μM *nor*-binaltrorphimine caused a significant increase in early membrane destabilization and a reduction in sperm viability compared to the control (Table 3).

Discussion

The μ - and δ -opioid receptors, but not the κ -opioid receptor, were hereby identified in ejaculated boar spermatozoa by WB and ICC. Both μ - and δ -receptors shared the same membrane location over the plasma membrane (acrosome, neck and principal piece domains) confirming that sperm-expressed opioid receptors appear to be unmodified among the species that utilize them. The μ -opioid receptor migrates as 65-70 and 50 kDa bands in human spermatozoa (Agirregoitia et al., 2006, Albrizio et al., 2006); 50 and 65 kDa in equine spermatozoa (Albrizio et al., 2005); and 45 kDa in boar spermatozoa. The δ -opioid receptor is 50 kDa in human spermatozoa (Agirregoitia et al., 2006); migrates as 50 and 65 kDa in equine spermatozoa (Albrizio et al., 2010); and is 70 kDa in boar spermatozoa. The κ -opioid receptor was detected as four different bands of 100, 65, 56, and 36.5 kDa in human spermatozoa (Agirregoitia et al., 2006); the commercially available antibodies raised against the κ -opioid receptor failed to detect this subtype in boar spermatozoa, suggesting that it is not abundant or present in these gametes.

The distribution of sperm μ - and δ -opioid membrane receptors appears to be conserved among animals that express it. The human μ -opioid receptor resides in the plasma membrane of the sperm head, at the equatorial and post-acrosomal region, in part of the midpiece, and in the tail (Agirregoitia et al., 2006) or over the acrosomal region of the sperm head and on the neck (Albrizio et al., 2006). In equine spermatozoa, the μ -opioid receptor is similarly found over the membrane of the acrosome and the tail (Albrizio et al., 2005). In sea bream spermatozoa, the μ -opioid receptor was detected over the head (Albrizio et al., 2013). In boar spermatozoa, both μ - and δ -receptors can be found at the plasma membrane over the acrosome, neck, and principal piece. The δ -opioid receptor is more restricted than the μ -opioid receptor in stallion sperm – primarily over the tail (Albrizio et al., 2010) – and in human spermatozoa – limited to the acrosome region, the mid-piece, and uniformly distributed along the tail (Agirregoitia et al., 2006). Finally, the κ -opioid receptor can be

found in the sperm head, the mid-piece, and the tail of human spermatozoa (Agirregoitia et al., 2006).

Considering the relatively conserved localization of the μ - and δ -opioid sperm receptors, we addressed their function in boar spermatozoa by testing the effects of specific agonists (morphine [μ]; DPDPE [δ]; and U 50488 [κ]) and antagonists (naloxone [μ]; naltrindole [δ]; and *nor*-binaltrorphimine [κ]) used in human or veterinary medicine. We included analysis of κ -opioid receptor pharmacological modulators because sub-detectable levels of the receptor may still be active and contribute to sperm physiology. The range of drug concentrations evaluated were informed by previously work performed on human spermatozoa (Agirregoitia et al., 2006), whereas the challenge intervals were shorter in order to minimize toxic time-dependent effects that commonly occur when working with spermatozoa (unpublished).

Morphine-challenge in boar spermatozoa followed a classic biphasic, dose-dependent effect of opioids (Feigenbaum and Howard, 1997), with an immediate decrease in sperm motility and a posterior inversion of effects by 60 min of incubation ($P<0.05$). These results agree with previous observations in equine spermatozoa (Albrizio et al., 2005), but are clearly different from findings in human, in which morphine seems to inhibit sperm motility after 3 h (Agirregoitia et al., 2012b). These differences indicate that the function of μ -opioid receptor is not completely shared among species and the roles it plays are sensitive to exposure time.

DPDPE, the δ -opioid receptor agonist, induced a decrease in boar sperm motility over time at 0.1 μ M, yet no effect was observed from the other DPDPE concentrations or using the δ -antagonist naltrindole. This clearly differs from findings in the stallion, in which naltrindole increased sperm motility at low concentrations but decreased it at high concentrations (Albrizio et al., 2010). On the other hand, naltrindole decreases human sperm motility

(Agirregoitia et al., 2012b). Such extreme differences with the δ -opioid receptor among species warrant further studies using β -endorphins and met-enkephalins.

Exposure of boar spermatozoa to the κ -opioid agonist U 50488 and its antagonist *norbinaltrorphimine* did not elicit changes in any of the viable sperm kinematics studied; however, both were toxic at higher concentrations. These negative findings generally support the absence of the receptor in these spermatozoa – particularly since the toxicity observed *in vitro* might be associated with off-target effects, such as affecting other opioid receptors than the κ -opioid receptor. These data are also consistent with those from human spermatozoa challenged with κ -opioid agonist and antagonist, which had no effect. In this regard, the κ -opioid receptor does not appear to contribute to the regulation of sperm motility in men; alternatively, observing its activity requires longer exposure conditions or drug levels (Agirregoitia et al. 2006; Agirregoitia et al., 2012b). Interestingly, the κ -opioid receptor is found on granulosa cells of growing porcine ovarian follicles (Słomczyńska et al., 1997), suggesting that its function may be gender specific. Understanding these negative sperm data in light of possible gender differences is warranted.

The initial contribution of endogenous opioids to fertility were thought to be minor based on reports that administration of exogenous opiates (Pfaus and Gorzalka, 1987; Succu et al., 2006) does not result in infertility (Miralles-García et al., 1986) or reduction in sperm motility (Davidson et al., 1989) – although this absence of an effect associated with the need of long-exposure of the opiates (Hallinan et al., 2008; Vuong et al., 2010). This perspective was questioned by reports that opioid analgesics impair human sperm motility (Xu et al., 2012), as well as the observed changes to β -endorphin levels in the peripheral blood of stressed sows (Einarsson et al., 2008) or experimentally induced sexual frustration in boars (Bishop et al., 1999).

Natural variation in opiate levels during the estrous cycle implies that these molecules do

participate in female reproductive function. Indeed, concentrations of endorphins and enkephalins in follicular fluid peak pre-ovulation (Kaminski et al., 2000) while β -endorphins vary in coordination with uterine and oviductal contractions induced by oestradiol, which also peak during the peri-ovulatory period (Okresa et al., 2003). Granulosa cells change steroidogenesis pathway in pigs in response to β -endorphins (Kaminski et al., 2004), possibly acting in a feedback loop. The μ -opioid receptor is also present in the oviduct segment where capacitation takes place (Desantis et al., 2008), as well as in the myometrium and the myosalpinx, which respond to β -endorphins via μ -receptors (Desantis et al., 2008), resulting in contractions that are pivotal for sperm transport (Rodriguez-Martinez et al., 2005).

The relationship between opioid receptor activity and the female reproductive organs – in combination with the observations that sperm exposure to μ -opioid receptor agonists enhances total and progressive sperm motility in boar, using the potent μ -agonist morphine (Sato and Minami, 1995), and in humans, when met-enkephalin, a weaker μ -agonist (Leslie 1987), was used (Subirán et al., 2012) – supports a model in which opioid receptors participate in sperm transport, particularly during *in vivo* sperm storage, capacitation, and interaction with the cumulus-oophorus complex: Spermatozoa colonize sperm reservoirs in the lower oviduct, progressing towards the upper segments to encounter the newly ovulated oocytes in coordination with ovulation (Rodriguez-Martinez, 2007). Changes in motility occur when spermatozoa separate from the tubal epithelium, and stronger beating is required for penetration of the cumulus-oophorus and particularly, of the zona pellucida (Gadella and Luna, 2014). Opioid receptors are present in ciliated tubal cells (Desantis et al., 2008), and may specifically regulate ciliary beating, as reported for other organs (Roth et al., 1991); whether or not the sperm tail, with its flagellar core, may act similarly remains to be tested.

The sources of the cellular activity that fosters sperm progression towards an oocyte include calcium and ATP. The oviduct fluid contains Ca^{2+} (Cox and Leese, 1997), which

maintains the ciliary beating that establishes the directed fluid flow. Ca^{2+} influx also occurs during boar sperm capacitation via specific calcium channels, including CatSper channels (unpublished), as well as through opioid ligand-receptor interaction – albeit the latter effects decrease sperm motility (Minoia and Sciorci, 2001). ATP production may also be affected by opiates, as observed in somatic cells (Sciorci et al., 2000). Exposure of boar spermatozoa to the μ -agonist morphine reduces mitochondrial function, despite the maintained or elevated sperm motility. At face value, this would be a paradox; yet boar spermatozoa consume an abundance of glucose through glycolysis and respiratory oxidation (Rodriguez-Gil and Bonet, 2016), so decreased mitochondrial integrity might not lead to an immediate energy deficit.

Finally, boar spermatozoa can be used to detect drug-induced toxicity using kinematics as an indicator in a simple motility assay (Vicente-Carrillo et al., 2015b) as well as for mapping changes in CatSper receptors under capacitation conditions (unpublished). The current findings that μ - and δ -opioid receptors on ejaculated boar spermatozoa regulate motility further highlight the utility of such assays when exploring opiates and their interaction with membrane receptors as well as for other types of receptors.

Material and Methods

Experimental design

Ejaculated, commercially extended boar spermatozoa were examined for the presence and distribution of μ -, δ -, and κ -opioid receptors using Western blot and immunocytochemistry, respectively. Their possible involvement in sperm physiology was tested by challenging semen samples with three different concentrations of the μ -agonist morphine [hydrochloride] (APL Pharma Specials, Sweden) or the μ -antagonist naloxone [hydrochloride dehydrate] (Sigma-Aldrich, Sweden); the δ -agonist DPDPE [D-Pen 2,5]-enkephanile [hydrate] (Sigma-

Aldrich) or the δ -antagonist naltrindole [hydrochloride] (Sigma-Aldrich); or the κ -agonist U 50488 (Sigma-Aldrich) or the κ -antagonist *nor*-binaltrorphimine (Sigma-Aldrich). Changes in sperm kinematics (total and progressive motility and sperm velocity), membrane and mitochondrial integrity, and signs of early membrane destabilization were monitored 5, 10, 15, 30, and 60 min after exposure to each compound. Each experiment was performed at 38°C, and repeated three times with independent samples.

All experiments were performed in accordance with relevant regulations (European Community Directive 2010/63/EU) and in compliance with Swedish current legislation (SJVFS 2012:26) at the Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. The experimental protocol was previously reviewed and approved by the Local Ethical Committee for Experimentation with Animals (Dnr 74-12), and the Local Ethical Committee for Human Studies (Ethical Permission Number, EPN-Dnr 2015/387-31), Linköping, Sweden.

Animals and sperm collection

Mature Swedish Hampshire breeding boars, selected according to normal semen quality and proven fertility, were housed individually on straw beds at Quality Genetics (now Svenska Köttföretagen, SvKF, Hållsta, Sweden). The animals were fed with commercial rations (Läntmännen, Stockholm, Sweden) according to national standards (Simonsson, 1994), and provided with water *ad libitum*.

The sperm-rich fraction of the ejaculate was manually collected twice weekly using the gloved-hand method. Sperm-rich fractions from three boars were pooled, extended in either Beltsville-Thawing Solution (IMV-Technologies, L'Aigle, France) or Durasperm (Jørgen

Kruuse A/S, Langeskov, Denmark) to a concentration of 48 million spermatozoa per mL, cooled to 16-20°C, and sent overnight to the laboratory as 24 h-old commercial artificial insemination-semen doses. Only ejaculates with at least 70% motile and 75% morphologically normal spermatozoa present immediately after collection were used.

Human spermatozoa were used as positive control for detection of the individual opioid receptors. Ejaculates were obtained from anonymous donors after informed, written consent at the Reproductive Medicine Center (RMC), Region Östergötland, Linköping, Sweden (Ethical Permission Number Dnr 2015/387-31).

Western Blot

Sperm proteins were extracted by incubating the spermatozoa in RIPA buffer (Sigma-Aldrich) at 4°C for 40 minutes. The extracted samples were centrifuged 13,000g for 10 min, and the supernatant was collected. Protein quantification was performed using the DC Protein assay kit (Bio Rad, Hercules, CA, USA), according to manufacturer's instructions. Protein suspensions (2.5 µg protein/µL) were denatured by heating at 70°C for 10 min. Ten microliter aliquots of each protein suspension were loaded into a NuPAGE 4-12 % Bis-Tris SDS-PAGE gel (Life Technologies, Carlsbad, CA, USA). Electrophoresis was performed at 180 V for 90 min, followed by transfer to the proteins to a polyvinylidene difluoride (PVDF) membrane [Invitrolon PVDF filter paper sandwich] (Life Technologies) at 125 mA for 90 min.

The membrane was blocked at room temperature for 60 min with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA, USA) containing 0.1 % Tween-20 (Sigma-Aldrich) (PBST). After three washes of 5 min in PBST, the membranes were incubated at 4°C overnight with 1:1000 dilutions of the primary

polyclonal antibodies: rabbit anti- μ -opioid receptor (ab10275), rabbit anti- δ -opioid receptor (ab10272), or rabbit anti- κ -opioid receptor (ab113533) (Abcam, Cambridge, UK). Each membrane was then washed 3 times in PBST, and incubated for 60 min with a 1:7500 dilution of the horseradish peroxidase-conjugated goat anti-rabbit (DC03L) (Calbiochem, Merck Millipore, Darmstadt, Germany), followed by extensive washing in PBST. All membranes were incubated with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific), according to manufacturer instructions, and scanned using the C-Digit (LI-COR Biosciences, Lincoln, NE, USA). Images of the blots were obtained using the Image Studio Digits 4.0.21 software (LI-COR Biosciences).

Immunocytochemistry

Spermatozoa were fixed in 4% paraformaldehyde at room temperature for 20 min. Cell suspensions were then centrifuged at 1,200 x g for 6 min, and the pellet was resuspended in PBS, pH 7.3 to prepare smears on poly-L-lysine slides (LSM, Thermo Scientific, Germany). The smears were allowed to dry, washed three times for 5 min each with PBS, and blocked with 5% BSA in PBS at 4°C for 120 min. After three washes in PBS for 5 min each, the slides were incubated at 4°C overnight with primary antibodies diluted in 1% BSA-PBS: μ - (1:100 dilution of ab10275), δ - (1:100 dilution of ab10272), or κ - (1:100 to 1:25 dilutions of ab113533) opioid receptors (Abcam). The smears were then washed three times in PBS for 5 min each before incubation in the dark at room temperature for 75 min with a 1:1000 dilution of Alexa Fluor 568-conjugated goat anti-rabbit (Invitrogen,) in 1% BSA-PBS. The smears were washed extensively, and then mounted with Prolong Gold anti-fade reagent (Invitrogen). Negative controls were performed by omitting primary antibody. All stained sperm smears were examined on a LSM 700 Zeiss confocal microscope (Carl Zeiss, Sweden) at 400x magnification, and the images were recorded using ZEN Navigator software (Carl

Zeiss). At least 200 cells were counted per replicate. Immunolabelling was consistent in >90% of the spermatozoa.

Preparation and dilutions of the agonists and antagonists of the opioid receptors

Two millimolar (2 mM) stock solutions were prepared by diluting all the above listed agonists and antagonists of the opioid receptors in Beltsville-Thawing Solution (Pursel and Johnson, 1975, IMV-Technologies, L'Aigle, France). Stock solutions were frozen at -20°C, and thawed immediately before analysis.

In vitro challenge of boar spermatozoa with agonists and antagonists of the opioid receptors

Stock solutions were thawed and diluted in Beltsville-Thawing Solution to 20 µM for morphine, DPDPE, and U 50488, and to 200 µM for naloxone, naltrindole, and *nor*-binaltrorphimine. Three 1:10 serial dilutions of each compound were made in Beltsville-Thawing Solution. Subsequently, 200 µL of sperm sample was added to 200 µL of each dilution or Beltsville-Thawing Solution only (control), and then carefully mixed. The final concentrations of morphine, DPDPE, and U 50488 were 10, 1, and 0.1 µM; of naloxone, naltrindole, and *nor*-binaltrorphimine were 100, 10, and 1 µM.

The compound-sperm mixtures were placed on a shaking plate inside an incubator at 38°C, and aliquots taken at 5, 10, 15, 30, and 60 min. Two aliquots were taken per time point: 25 µL for flow cytometry analyses of membrane and mitochondrial integrity and for signs of early membrane destabilization and a 10 µL for sperm kinematics analysis.

Flow cytometry analysis for early membrane destabilization, sperm viability, and mitochondrial integrity

Signs of early membrane destabilization, sperm viability, and mitochondrial integrity were analysed using a Gallios™ flow cytometer (Beckman Coulter, Bromma, Sweden) equipped with standard optics – violet laser (405 nm), argon laser (488 nm), and HeNe-laser (633 nm) – and filter configuration – Violet, FL9 480SP 450/50 (Hoescht 33342), FL10 550/40; Blue, FL1 550SP 525BP (YO-PRO-1 iodide), FL2 595SP 575BP, FL3 655SP 620/30 (propidium iodide), FL4 730SP 695/30 - alt 675BP, FL5 755LP; Red, FL6 710SP 660BP (MitoTracker Deep Red), FL7 750SP 725/20, FL8 755LP. The instrument was controlled using Navios software (Beckman Coulter, Bromma, Sweden). Analyses of acquired data were performed using Kaluza software (Beckman Coulter, Bromma, Sweden). In all cases, 25,000 events were assessed per sample, with a flow rate of 500 cells/sec.

The following fluorophores were used: YO-PRO-1 iodide (Invitrogen), propidium iodide (Invitrogen), MitoTracker Deep Red (Invitrogen), and Hoescht 33342 (Sigma-Aldrich). Stock solutions of the fluorochromophores were prepared in MilliQ water to 2.4 mM of propidium iodide and 8.9 mM for H33342, and in dimethyl sulfoxide (Sigma-Aldrich) to 75 µM of YO-PRO-1 and at 100 µM of MitoTracker Deep Red. Stock solutions were kept at -20°C for YO-PRO-1, propidium iodide, and MitoTracker Deep Red and at +4°C for H33342. Each solution was brought to room temperature immediately before diluting them in Beltsville-Thawing Solution for use.

For analysis, a 25- μ L aliquot of treated sperm from a specific time point was mixed with 175 μ L of Beltsville-Thawing Solution containing 0.2 μ M of YO-PRO-1, 2.4 μ M of PI, 100 nM of MitoTracker Deep Red, and 4.5 μ M of H33342. Flow cytometric analysis was used to identify the following parameters: sperm viability (YO-PRO-1 negative/ propidium iodide negative), signs of early membrane destabilization (YO-PRO-1 positive/ propidium iodide negative), and mitochondrial integrity (MitoTracker Deep Red positive/ propidium iodide negative).

Sperm kinematic analysis

Sperm motility, progressive motility, and sperm velocity were assessed using the QualispermTM software (Biophos SA, Lausanne, Switzerland) connected via a CMOS camera (UEye, IDS Imaging Development Systems GmbH, Ubersulm, Germany) to an upright Zeiss Axio Scope A1 light microscope using a 10x phase contrast objective (Carl Zeiss). The semen samples were placed on a thermal plate (Temp Controller 2000-2, Pecon GmbH, Erbach, Germany) kept at 38°C.

QualispermTM technology is based on correlation analysis of single particles (spermatozoa) in confocal volume elements, yielding a regression fluctuation algorithm (a statistical analysis of fluctuation, comparable with fluorescence correlation spectroscopy) of sperm numbers and translation classes. Individual spermatozoa are projected on a pixel grid of a CMOS camera. The algorithm calculates the number of fluctuations in each pixel by correlation function, which is used to calculate the speed (velocity) distribution. This system runs in high throughput mode (usually 4 fields per minute), analyzing >2,000

spermatozoa/field, and has been validated for several species, including porcine (Tejerina et al., 2008; 2009; Rodriguez-Martinez et al 2008; Johannisson et al., 2009; Siqueira et al., 2011; Vicente-Carrillo et al., 2015).

Statistics

The results obtained by challenging boar spermatozoa with opioid receptor agonists and antagonists were tested for normality with the Shapiro-Wilk test, and analyzed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics 23 (IBM Corporation, Armonk, NY, USA) to compare the effect of the different concentrations (dose-response analysis) and the effect of the different incubation times (time-effect analysis). An independent samples *t*-test was applied to determine when the differences observed became significant at $P < 0.05$.

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Figure Legends

Figure 1. Detection of μ - (**A**), δ - (**B**), and κ - (**C**) opioid receptor proteins in human and boar spermatozoa by Western blot. Human samples were used as a positive control.

Figure 2. Localization of μ - and δ -opioid receptors in human and boar spermatozoa by laser confocal microscopy. **A**: μ -opioid receptor, human spermatozoa. **B**: δ -opioid receptor, human spermatozoa. **C**: κ -opioid receptor, human spermatozoa. **D**: negative control (primary antibody excluded), human spermatozoa. **E**: μ -opioid receptor, boar spermatozoa. **F**: δ -opioid receptor, boar spermatozoa. **G**: κ -opioid receptor, boar spermatozoa. **H**: negative control (primary antibody excluded), boar spermatozoa. Scale bar, 10 μm .