# Delta and Kappa opioid receptors on mouse sperm cells: expression, localization and involvement on *in vitro* fertilization

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**Running head:** Delta and Kappa opioid receptors on mouse sperm and IVF

## Abstract

The endogenous opioid peptides have been reported to be involved in the regulation of reproductive physiology. Many of the studies conclude with sentences around the harmful effect of opioids in male fertility but, actually, there is only one study regarding the real fertility potential of spermatozoa that have been exposed to mu specific opioids. The aim of the present study was to see if the modulation of delta (OPRD1) and kappa (OPRK1) opioid receptors in mouse sperm during capacitation was able to vary the embryo production after *in vitro* fertilization (IVF). The presence of OPRD1 and OPRK1 in mouse mature spermatozoa was analyzed by RT-PCR and immunofluorescence. Incubating the sperm with, on one hand, the delta specific agonist DPDPE and/or antagonist naltrindole, and, on the other hand, the kappa specific agonist U-50488 and antagonist nor-binaltorphimine, we analyzed the involvement of OPRD1 and OPRK1 on IVF and preimplantational embryo development. We verified the presence of OPRD1 and OPRK1 in mouse mature spermatozoa, not only at the mRNA level but also at protein level. Moreover, the sperm incubation with DPDPE, before the IVF, had an effect on the fertilization rate of sperm and reduced the number of reached blastocysts, which was reverted by naltrindole. Instead, the use of the kappa agonist U-50488 and the antagonist nor-binaltophimine did not have any effect on the amount and the quality of the achieved blastocysts. Although nowadays the pure delta or kappa opioid ligands are not used for the clinic, clinical trials are being conducted to be used in the near future, so it would be interesting to know if the modulation of these receptors in sperm would generate any consequence in relation to fertilization capacity.

**Key words:** Sperm, Opioid, Embryo, IVF

## 1. Introduction

The δ-opioid receptor (OPRD1) and κ-opioid receptor (OPRK1), along with the μ-opioid receptor (OPRM1), are G-protein-coupled receptors that bind the endogenous opioid peptides (EOPs) to activate and exert their effects. EOPs are substances derived from proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) precursors and are involved in intercellular communication [1]. They have been widely studied in neuronal action, but they also participate in other mechanisms like the regulation of reproductive physiology at multiple sites [2]. In fact, the opioid system was described many years ago in many of the male and female reproductive tissues [3,4] and it appears to be increasingly important in sperm cell function [5].

When it was discovered that opium users had reduced sperm motility (asthenozoospermia), it began the study of the effect of opioids in that tissue [4]. Its relevance increased since opiates are widely used in the clinic to treat moderate to severe pain and the side effects that could have on reproduction are not yet completely known [6]. Firstly, very high concentrations of endorphins and enkephalins cells were found in human semen and sperm [7–9]. Later, it was described the presence of the three opioid receptors in human spermatozoa [10,11] and the presence of mu and delta receptors in equine sperm cells [12,13]. In experiments where sperm were incubated with opioids, mu specific opioids like morphine and naloxone were able to modify the sperm motility and capacitation [10,12] and beta-endorphin stimulated the acrosome reaction in human sperm [14]. On the other hand, delta specific opioids like DPDPE and naltrindole showed that delta opioid receptor was actively involved in modulating the kinetic of human spermatozoa [10] and also the acrosome reaction in equine spermatozoa [13]. Furthermore, it seems that the opioid degrading enzymes also are involved in the control of sperm motility [15–17].

In recent years we have learnt about the role of the opioid system in sperm physiology but, there is only one study related to the real fertility potential of spermatozoa that have been in contact with opioids, more exactly, with morphine and naloxone, which are mu specific opioids [18]. Therefore, to continue understanding the function of the opioid system in the reproductive role of sperm, the aim of the present study is to determine whether the modulation of delta and kappa opioid receptors in mouse sperm during capacitation is able to vary the embryo production after *in vitro* fertilization (IVF).

## 2. Materials and methods

### *2.1. Experimental animals*

Approval for this study was obtained from the Animal Research Ethical Committee of the University of the Basque Country (UPV/EHU CEEA reference number: M20-2015-016-173/M20-2018-247-249) and all the experiments were performed according to the Guide for Care and Use of Laboratory Animals, endorsed by the Society for the Study of Reproduction and European legislation. The specific pathogen free (SPF)-grade hybrid adult mice used in this study (C57BL6/J x DBA/2J) were housed in the animal facility of the University of the Basque Country. Animals were kept in an animal house (6-8 animals per cage) under controlled conditions of temperature (23 ± 1°C) and photoperiod (light/dark cycle 14 h:10 h). Animals were given free access to water and food. Their health status was monitoring two days a week. Hybrid mice were commonly used because their high fertility rates (putatively because of hybrid vigor) make them good models for general reproductive studies [19]. The use of two-month old mice was because they reach the sexual maturity around 28 and 60 days [20].

### *2.2. Reverse-transcription PCR*

Two month old male mice of reproductive age were euthanized through cervical dislocation and dissected to remove the vasa deferentia and caudae epididymides.. Sperm were removed from epididymis with the aid of a surgical forceps in PBS 1X. For this experiment sperm from 3 males were used in each of the 3 replicate trials. After 10 min centrifugation, RNA from spermatozoa was isolated using the Dynabeads mRNA Direct Micro Kit (Ambion, Life Technologies AS, Oslo, Norway). Immediately after extraction, the procedure for obtaining the cDNA was performed using the GoScript Reverse Transcription system (Promega, Madison, WI, USA), according to manufacturer`s instructions. Positive control was performed using RNA from mouse cerebral cortex.

Primers used for PCR were as follows: OPRD1, 5´-TTGGCATCGTCCGGTACAC-3´ and 5´-GCACACCGTGATGATGAGGA-3´ (482-bp product); OPRK1, 5`-CCGATACACGAAGATGAAGAC-3` and 5`-GTGCCTCCAAGGACTATCGC-3` (342-bp product) and mouse B-actin (ACTB), 5´-GCTTCTTTGCAGCTCCTTCG-3´ and 5´-ACGGTTGGCCTTAGGGTTCA-3´ (390-bp product) used as endogenous control. The primers were located on different exon of each respective gene (i.e., they span introns). Even so, we verified the possible carryover of genomic DNA during the extraction process by performing PCR in the absence of reverse transcriptase. If genomic DNA were present, it would be amplified in subsequent PCR. PCRs were performed using the following parameters: 95ºC for 2 min, 40 cycles at 95ºC for 30 s, 58ºC for 30 s and 72ºC for 1 min, followed by a final extension step at 72ºC for 5 min. The cycles used to perform the PCR were in the linear range for all transcripts evaluated. The mixture was electrophoretically separated on a 2% agarose gel.

*2.3. Immunofluorescence*

Isolated spermatozoa were suspended in PBS and smeared onto a slide coated with poly-l-lysine. Sperm from 3 males were also used in each of the 3 replication trials for this experiment. Samples were washed in PBS and fixed in 4% paraformaldehyde (Panreac, Barcelona, Spain) for 10 min at room temperature. The OPRD1 and OPRK1 receptors were inmunocytochemically detected in cells that had been treated in the presence or absence of 1% Triton X-100, in PBS with 10% fetal calf serum (FCS) for 1 h at room temperature, to detect the presence of intracellular receptor and surface expressed receptor, respectively. Afterwards, spermatozoa were incubated overnight at 4ºC in PBS containing 10% FCS and 1:200 rabbit polyclonal antibody anti-OPRD1 (Millipore, UK) and anti-OPRK1 (Abcam, UK). Spermatozoa were washed 3 times in PBS and then incubated in PBS supplemented with 5% FCS serum and 1:500 goat polyclonal secondary antibody Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Next, spermatozoa were washed 3 times in PBS. In all cases, nuclei were stained with Hoechst 33342 (5 mg/ml) and finally, spermatozoa were mounted in microdrops with Fluoromount G (EMS, Hatfield, United Kingdom) and examined by confocal microscopy (LSM 800; Zeiss).

*2.4. Isolation and in vivo maturation of oocytes*

*In vivo* matured oocytes, in metaphase II stage (MII), were collected from 8- to 10-wk-old oviducts of female mice superovulated by intraperitoneal injections of 7.5 IU of equine chorionic gonadotropin (eCG; Folligon, Intervet, Castle Hill, Australia), followed, 48h later, by 7.5 IU of human chorionic gonadotropin (hCG; Veterin Corion; Divasa-Farmavic S.A., Spain). Briefly, at 14 h after hCG administration, oviducts were removed from superovulated female mice and placed in a Petri dish containing M2 medium at room temperature. After washing, collected oviducts were placed in fresh M2 medium

and cumulus-oocyte complexes (COCs) were released from the ampulla with the aid of a needle and washed in new M2 medium until fertilization.

*2.5. In vitro fertilization of oocytes*

Two-month-old male mice (C57BL6/J x DBA/2J) of proven fertility were euthanized by cervical dislocation and dissected to remove the vasa deferentia and caudae epididymides. Sperm were extracted from the epididymis with the aid of surgical forceps in a drop of 500 µl of human tubal fluid (HTF) medium which was coated with mineral oil. For these experiments sperm from 4 males were used in each of the 5 replicate trials. Immediately after extraction, sperm were incubated for 1 h in the HTF medium under 5% $CO_2$ at 37ºC for capacitation. During capacitation, sperm were incubated with increased concentration of the main OPRD1 selective agonist DPDPE (1 nM, 10 nM or 100 nM) and/or the main antagonist naltrindole (100 nM) ($K_i$ for OPRD1: 1.4 and 0.13 nM , respectively [21,22]). In the same way, sperm were incubated with increased concentration of the main OPRK1 selective agonist U-50488 (1 nM, 10 nM or 100 nM) or the main antagonist Nor-binaltorphimine (1 nM, 10 nM or 100 nM) ($K_i$ for OPRK1: 0.72 and 0.038 nM, respectively [23,24]). All drugs stock solutions were prepared in PBS-PVA 0.1%. Sperm containing the same amount of PBS-PVA 0.1% were used as an incubation control. After capacitation, sperm were centrifuged 3 min at 13000g and washed in HTF before performing the IVF, to eliminate the drug residue in the medium. After *in vivo* maturation, isolated COCs were transferred to 500µl equilibrated HTF drops and were overlaid with mineral oil and a concentration of 2 x $10^6$ spermatozoa/mL was used to carry out the IVF (in each condition).

Five hours after *in vitro* fertilization, presumptive zygotes were washed in HTF medium and cultured in 25µl drops of equilibrated culture medium KSOM overlaid with mineral

oil at 37°C under an atmosphere of 5% $CO_2$ in air with maximum humidity (n=25 for each condition). Embryos were cultured for 5 days, and cleavage rates were assessed on day 1 (24 h after fertilization) and blastocysts on day 4 (96–100 h after fertilization).

To assess whether adding OPRD1 or OPRK1 agonist and/or antagonist affected rates of oocyte nucleus maturation and fertilization, the presence of the second polar body and pronuclear formation were identified respectively at 24 h after fertilization. The presumptive zygote that had not divided into 2 cells were fixed in 4% paraformaldehyde for 10 min and then stained with Hoechst 33342 for observation with immunofluorescence microscope (Axioskop, Zeiss) under UV light.

*2.6. Measure of embryo quality*

Embryos reaching the blastocyst stage (day 4 after IVF) were stained to check the cell number. They were washed in PBS-PVA 0.1% and fixed in 4% paraformaldehyde for 10 min. Immediately, they were washed again in PBS-PVA 0.1% and stained with Hoechst 33342 during 15 min for observation with immunofluorescence microscope (Axioskop, Zeiss) under UV light. Embryo quality was measured counting the number of cells from each blastocyst using the ImageJ software.

*2.7. Statistical analysis*

All statistical tests were performed by Graph Pad Prism 5 software and IBM SPSS Statistics 21.0. Cleavage rates and blastocyst yields between the different conditions were tested for normality of the distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). The data were normally distributed and compared by 1-way ANOVA. Data from blastocyst count cell were normalized as a fold over control group from each experiment and in this case, it was compared by 1-way ANOVA. Bonferroni was used as a post hoc test. Significance was set at $P<0.05$.

## 3. Results

### *3.1. Oprd1 and Oprk1 mRNA expression in mouse spermatozoa*

The presence of the *Oprd1* and *Oprk1* was evaluated by RT-PCR. We studied the presence of the transcript for *Oprd1* and *Oprk1* gene in mouse spermatozoa and we compared it with the transcript for *Oprd1* and *Oprk1* of mouse cerebral cortex as a positive control. The expected 482 bp fragment for *Oprd1* and 342 bp fragment for *Oprk1* were detected in the cerebral cortex and in the sperm cells of mouse (Fig 1). To verify that the RNA extraction and the cDNA generation were correct, the housekeeping gene *Actb* was detected in both tissues at the expected 398 bp (Fig 1). The primers used were located on different exons to avoid the amplification of genomic DNA and the retrotranscriptase negative controls were performed to assure the absence of that genomic DNA (Data not shown).

### *3.2. Immunolocalization of OPRD1 and OPRK1 in mouse spermatozoa*

Immunofluorescence analysis revealed that OPRD1 protein was present in mouse sperm cells, with less intensity under non-permeabilized than permeabilized conditions (Fig 2A). Specifically, OPRD1 labeling was observed in the sperm head with more intensity at the anterior acrosomal region, above all, when the cell was permeabilized. In addition, the tail was marked weakly (Fig 2A).

Regarding OPRK1, the immunofluorescence analysis revealed that it was present only on the tip of the hook of the sperm head, both in non-permeabilized and permeabilized cells (Fig. 2B).

### *3.3. Effect of opioid agonist DPDPE during sperm capacitation on fertility and subsequent embryo development*

We incubated the mouse spermatozoa with increased concentration of delta opioid specific agonist DPDPE (0 nM, 1 nM, 10 nM and 100 nM) during the capacitation time, before IVF process, and we used this sperm to fertilize oocytes *in vitro*. We observed that when sperm were incubated with DPDPE, the IVF process and the embryo development were impaired. The percentage of obtained zygotes was significant less ($p<0.05$) in the experiments where the sperm had been capacitated in presence of DPDPE (100 nM) in comparison of control group (without DPDPE). In addition, we observed a significant difference ($p<0.05$) between the blastocyst rate reached from sperm capacitated in absence or in presence of DPDPE at any used concentration, obtaining almost half of blastocysts when sperm had been incubated with DPDPE compared to the control group. (Table 1). This deleterious effect did not show a marked dose-dependent behavior although there does appear to be a trend in the data for at least the IVF rates (Fig 3). The AC50 in the conditions where a significant effect of the DPDPE was seen was 0.78 nM for zygotes and 0.85 nM for blastocysts (Fig 3). Moreover, the cell number of the blastocysts derived from sperm incubated with DPDPE was lower compared to the control (Fig 4A).

*3.4. Effect of opioid antagonist naltrindole during sperm capacitation on fertility and subsequent embryo development*

We next examined whether the observed effect of DPDPE, specifically that produced by adding DPDPE 10 nM (for being the concentration that generated the most harmful effect), during sperm capacitation, could be blocked by the delta opioid specific antagonist naltrindole. The procedure was the same as in experiments with agonists but, in this case, we also incubated the sperm with naltrindole (100nM). The incubation of sperm only with naltrindole did not generate any significant change in comparison with the control (Table 2). However, the sperm incubation with naltrindole along with DPDPE

before IVF, blocked significantly ($p<0.05$) the deleterious effect observed on the rate of blastocysts derived from sperm incubated with DPDPE (Table 2). This blocking effect was also observed in relation to the cell number of blastocysts achieved (Fig 4B).

### *3.5. Effect of opioid agonist U-50488 during sperm capacitation on fertility and subsequent embryo development*

When we incubated the mouse spermatozoa with increased concentration of kappa opioid specific agonist U-50488 (0 nM, 1 nM, 10 nM and 100 nM) during the capacitation time, before IVF process, and using this sperm to fertilize oocytes *in vitro,* we did not observe any significant variation between treatments regarding the IVF process or the embryo development (Table 3). The cell number of the blastocysts derived from sperm incubated with U-50488 did not show any difference compared to the control (Fig 4C).

### *3.6. Effect of opioid antagonist nor-binaltorphimine during sperm capacitation on fertility and subsequent embryo development*

As we did not observed any difference with the kappa specific agonist U-50488, instead of conducting an experiment to block the effect of the agonist U-50488 by the antagonist nor-binaltorphimine, we observed if there was any dose-response action using the antagonist nor-binaltorphimine alone. Thus, we incubated the mouse spermatozoa with increased concentration of kappa opioid specific antagonist nor-binaltorphimine (0 nM, 1 nM, 10 nM and 100 nM) during the capacitation time, before IVF process, and we used this sperm to fertilize oocytes *in vitro*. In this case, we also did not find any significant difference between treatments regarding the IVF process or the embryo development (Table 4). In this case, the cell number of the blastocysts derived from sperm incubated with nor-binaltorphimine did also not show any difference compared to the control (Fig 4D).

## 4. Discussion

The majority of the physiological roles suggested for opioids regarding male fertility have been harmful effects [25]. However, there is only one study regarding the real fertility potential of spermatozoa that have been exposed to opioids [18]. In our present work, we have verified the presence of the OPRD1 and OPRK1 in mouse mature spermatozoa, not only at the mRNA level but also at protein level. Moreover, we have elucidated that the sperm incubation with the delta specific opioid agonist DPDPE, during the capacitation process, affects the fertilization rate of sperm and decreases the number and the quality of embryos that reached blastocyst stage. On the other hand, the presence of the kappa specific agonist U-50488, during the capacitation, did not generate these adverse effects in the subsequent generation of blastocysts.

RT-PCR revealed the presence of *Oprd1* and *Oprk1* mRNA in the mouse spermatozoa. The quantity of mRNA is lower in spermatozoa than in the cerebral cortex and, we also found less amount of transcript for *Oprd1* than *Oprk1*. Even so, although the presence of a specific mRNA in the mature sperm can indicate the expression of a specific protein during spermatogenesis, its amount in the mature sperm is not very relevant, *a priori*, for that mature sperm, because it is widely accepted that the mature spermatozoa do not perform transcription and translation processes due to the scarcity of cytoplasm capable of supporting translation [26] and, moreover, in the final stages of spermatogenesis, the spermatozoa lose most of the cytoplasm, including most of the mRNA [27]. Something that could be interesting in relation to the amount of mRNA found is that there is a limited pool of RNAs stored from spermatogenesis that may have a role in early zygote development [28]. The mRNA of *Oprd1* has been also previously detected in human and equine mature spermatozoa [10,13], as well as, in the mouse spermatogenic cells [29], which supports our findings.

Immunofluorescence analysis revealed the presence of the OPRD1 protein in the mouse sperm head with more intensity at the anterior acrosomal region, as it was described previously [29], above all, when the cell was permeabilized, but also weakly in the tail. The differences in the OPRD1 staining pattern between non-permeabilized and permeabilized spermatozoa indicate that this receptor could be present in internal structures [30], like the acrosome membrane. The OPRD1 protein has been previously detected in other species: in the mid piece of the tail of equine sperm [13], in the acrosomal region of the head of boar spermatozoa and weakly in the tail [31], and, in human sperm, in the plasma membrane at the front part of the sperm head (over the acrosomal region), in the middle region, and uniformly distributed along the tail [10]. Due to the highly polarized structure and function of spermatozoa, they require the compartmentalization of particular metabolic and signaling pathways to specific regions [32] and that was the reason why firstly, the OPRD1 was related to a possible function in the acrosome reaction and motility. Nevertheless, nowadays, we already know that, certainly, both the acrosome reaction and motility can be modulated by OPRD1 [10,13,31]. On the contrary, this is not the case of the OPRK1 receptor which, although it has been located in the sperm, no action has been seen in relation to motility, acrosome reaction or other physiologic processes of the sperm. We found its location limited to the plasma membrane of the tip of the hook of the sperm head in mouse sperm, while a previous study located OPRK1 throughout the head [29]. Finally, the OPRK1 has also been observed in the plasma membrane of the sperm head, the middle region and the tail of human sperm [10], although it was not detected in boar sperm [31].

As we have described in the introduction, to date, all studies that have analyzed the opioid system in sperm have been limited to show the presence of this system and to analyze how the incubation of sperm with opioids modulates motility, capacitation and acrosome

312 reaction [18]. Thanks to these studies, we know that there is an opioid component in the
313 functioning of sperm, but, if opioids modulate sperm physiological processes, what
314 happens if sperm are in the presence of exogenous opioids previous or during
315 capacitation? Something that could occur due to the presence of opiates in women
316 reproductive tissues [33,34] as well as in men [35] after the use of this medicine/drug.

317 In a previous work, we demonstrated that the sperm incubation with morphine, before the
318 IVF, had an effect on the fertilization rate of sperm reducing the number of reached
319 blastocysts, which was reverted by naloxone, so it seems that it was due to the mu opioid
320 receptor [18]. Nowadays, the OPRD1 and the OPRK1 are the other members of the opioid
321 receptor family that are under intense investigation. OPRD1 agonists are being studied
322 with the aim to ease the pain and to avoid OPRM1-induced side effects, in fact, there are
323 some drugs in phase I and II clinical development [36]. In the case of OPRK1, they are
324 the antagonists that are currently being considered for the treatment of a variety of
325 neuropsychiatric conditions, including depressive, anxiety, substance abuse disorders or
326 emotional component of chronic pain [37,38] and there are also drugs in phase I clinical
327 trial [39]. We believe that, even if pure delta- or kappa-opioid ligands are not used for the
328 clinic, everything indicates that this will be done in the near future, so it is interesting to
329 know if the modulation of these receptors in sperm would generate any consequence in
330 relation to fertilization capacity. Therefore, as a first step, we examined the consequence
331 of incubating the spermatozoa with increased concentration of DPDPE (0 nM, 1 nM, 10
332 nM and 100 nM) before IVF (during the capacitation process) and we observed that all
333 doses produced a significant decrease in the blastocyst rate as well as a decrease in the
334 blastocyst cell count. In addition, the sperm co-incubation with the delta opioid antagonist
335 naltrindole (100 nM) along with DPDPE, blocked the deleterious effect of DPDPE (10
336 nM) in blastocyst rate and quality. As it has been suggested elsewhere, the quality of early

337 embryos determines the pregnancy outcome and it is essential for a successful delivery
338 [40], so the effect observed in this study by DPDPE on blastocyst production and quality
339 could have poor consequences at later stages. On the other hand, the use of the kappa
340 agonist U-50488 and the antagonist nor-binaltophimine had no effect on the amount and
341 the quality of the achieved blastocysts.

342 In summary, our data lead us to hypothesize that OPRD1, modulating some sperm
343 functions that occur during capacitation, could be able to regulate the fertilization process
344 and embryo early development; something that looks like the OPRK1 is not able to do.
345 Thus, the decrease in sperm motility described by the activation of the OPRD1 by DPDPE
346 in equine [13] and boar [31] sperm, as well as the blockade of acrosome reaction
347 described by the activation of the same receptor [13], could lead to a loss of the fertilizing
348 capacity of the sperm. Even so, there are also studies where DPDPE did not generate any
349 changes [41] and where the use of the naltrindole antagonist generated decreases in
350 motility at high concentrations and increases of that at lower concentrations [10,13]. In
351 the same way, there are clinical studies carried out with opioids that affect mu and delta
352 receptors that did not generate changes in sperm motility [42]. Even so, based on our
353 results, the activation of OPRD1 could modulate other sperm functions, apart from
354 motility and acrosome reaction, because, even though the fertilization rate falls due to the
355 action of DPDPE, the ratio of blastocysts achieved with sperm incubated with that delta
356 agonist falls even further and that lower capacity to generate quality blastocysts cannot
357 be attributed to a deregulation in mobility or acrosomic reaction. On the other hand, there
358 are studies were the modulation of OPRK1 by U-50488 (agonist) or nor-binaltorphimine
359 (antagonist) did not generate any change in human [10] and boar [31] sperm mobility;
360 results that would be in line with our discoveries in the present work. Even so, a recent
361 work by Urizar-Arenaza and coworkers [43] describes that OPRK1 could regulate human

sperm fertility by inhibiting the hyperactive motility and acrosome reaction through phosphorylation changes in sperm-specific proteins. That is why it is not recommended to link changes seen in motility, capacitation and acrosome reaction with direct effects on the fertilizing potential of sperm and, therefore, it is always worth conducting IVF experiments to see if changes are actually generated in the production of embryos.

In conclusion, we have reported the presence of OPRD1 and OPRK1 in mouse mature sperm. Moreover, the OPRD1 activation with the delta specific opioid agonist DPDPE, during the capacitation process, decreases the number and the quality of embryos that reached blastocyst stage. Conversely, the presence of the kappa specific agonist U-50488, during the capacitation, did not generate these adverse effects in the subsequent generation of blastocysts.

## Conflict of interest

The authors declare no conflicts of interest.


## Funding

This work received financial support from the University of the Basque Country (Grant PPGA18/01). E.O. has carried out all the experimentation without any public or private financial help. L.T. and J.R acknowledge the financial support given by University of the Basque Country (PIF15/149 and ESPDOC17/33, respectively).

## Acknowledgements

The authors thank SGiker (UPV/EHU) for personal and technical support and particularly Ricardo Andrade and Alejandro Díez for their assistance with the confocal microscopy

analysis. Finally, we would like to thank Maxime Reverchon, Ainhoa Lapitz and Ane Portillo for help when we started the present work.

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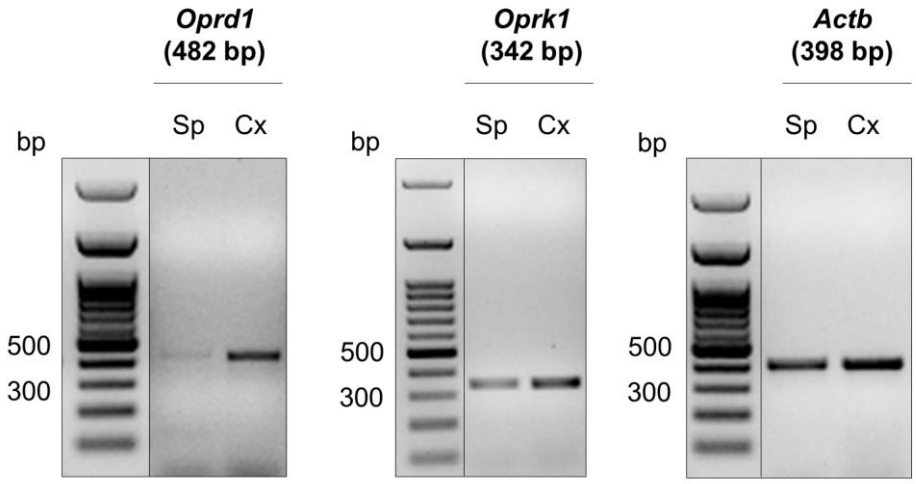
**Figure legends:**

**Fig. 1.** Expression of *Oprd1* and *Oprk1* in mouse spermatozoa. Reverse transcription PCR products for *Oprd1, Oprk1* and ß-actin (*Actb*) in mouse spermatozoa (Sp) and cerebral cortex (Cx), used as positive control. Base pairs (bp) are indicated on the left side, representative RT-PCR experiment is shown (n=3).

**Fig. 2.** Immunolocalization of OPRD1 (A) and OPRK1 (B) in mouse spermatozoa under non-permeabilized and permeabilized conditions. Representative microphotography showing the distribution of OPRD1 and OPRK1, in green, and hoechst-labelled DNA, in blue. It is also shown the phase-contrast image. n=3. Scale bar represents 25 µm.

**Fig. 3.** Concentration-response curve to determine the AC50 of the agonist DPDPE in each stage of embryo development. Different points represent normalized data between 0 and 100 % (defining 0 % as the smallest value in each data set and 100 % as the largest value in each data set) vs transformed data from treatments (Log [Agonist], nM). Different lines represent the dose-response curve from previously explained points in MII oocytes recovered from mice, fertilized oocytes (zygote), 2 cell-stage embryos and blastocysts after IVF. AC50 was 1.15 nM, 0.77 nM, 2.32 nM and 0.85 nM, respectively, for the different stages of embryo development represented.

**Fig. 4.** Blastocyst cell count at day 4 after IVF using mouse sperm incubated with (A) increased concentration of DPDPE (0nM, 1nM, 10nM and 100nM); (B) DPDPE (10 nM), naltrindole (100 nM) and both together (for blocking experiment); (C) increased concentration of U-50488 (0nM, 1nM, 10nM and 100nM); (D) increased concentration of Nor-Binaltorphimine (0nM, 1nM, 10nM and 100nM). Results are the means ± S.E.M. of 5 independent experiments. Significant differences between groups are indicated with different letters; $p<0.05$ in all cases. Representative microphotography from reached blastocysts of each treatment are shown; scale bar represents 25 µm.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Oprk1
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Actb
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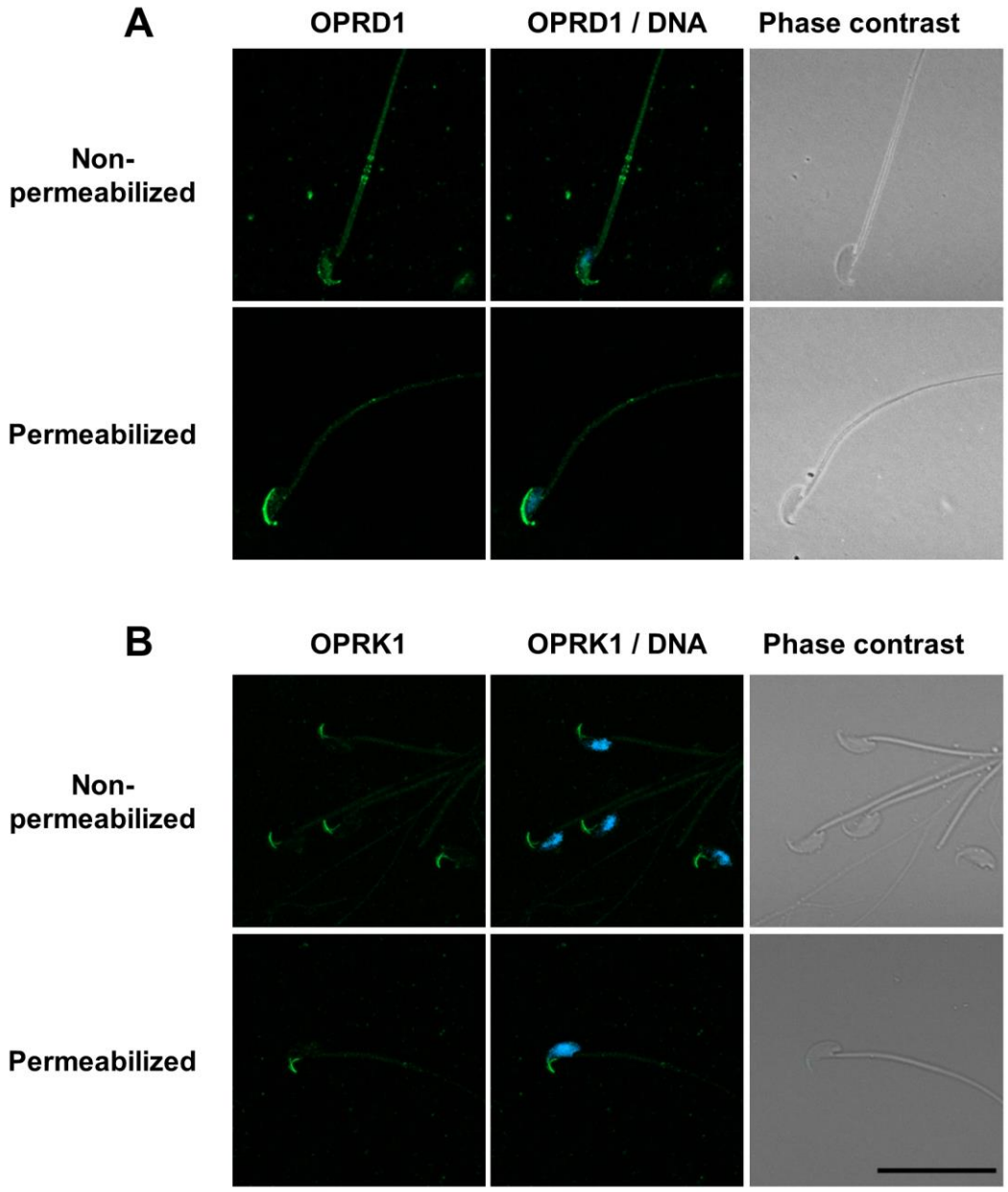
Sp
Cx
bp
500
300

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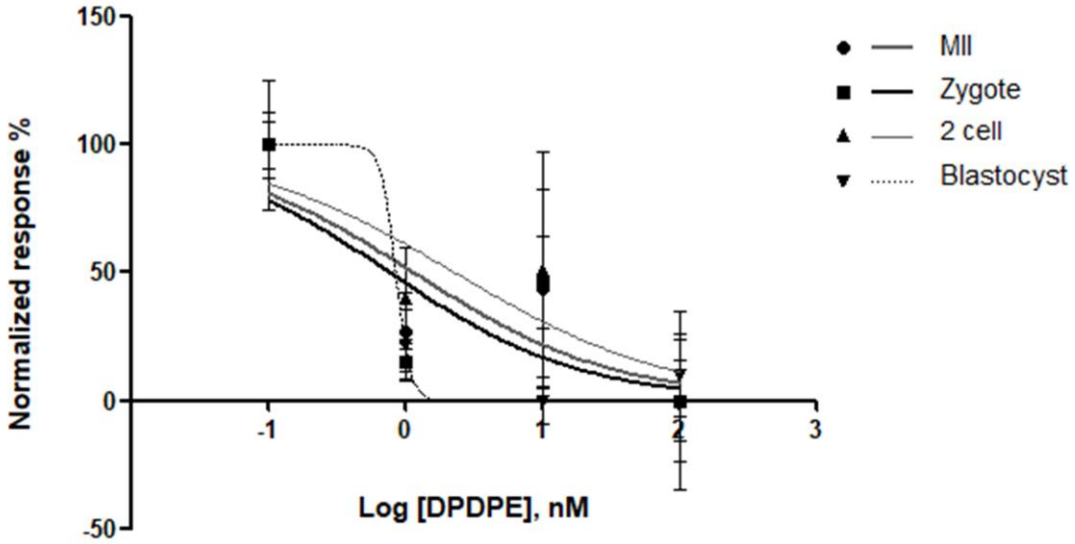
150
100
50
0
-50
-1
0
1
2
3
Normalized response %
Log [DPDPE], nM
MII
Zygote
2 cell
Blastocyst

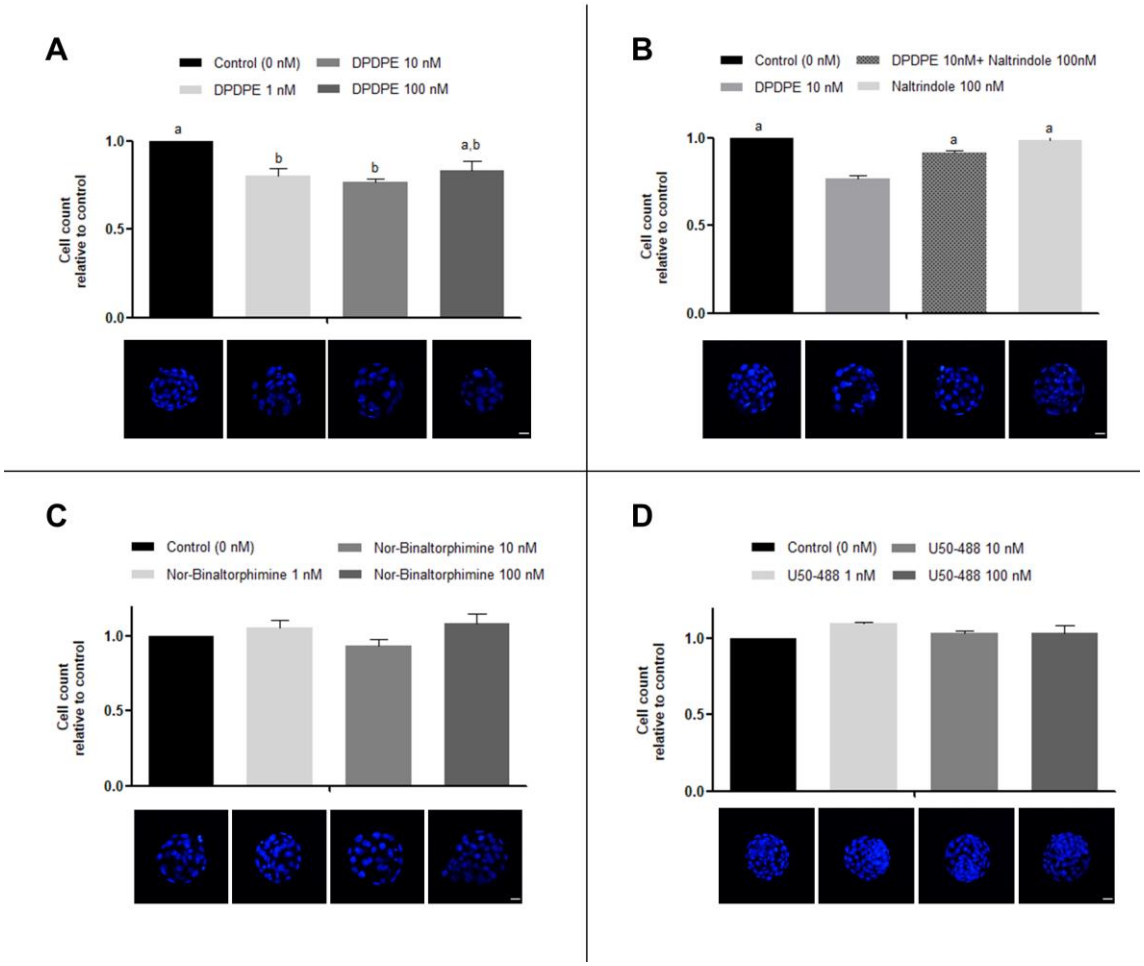
A
Control (0 nM)
DPDPE 10 nM
DPDPE 1 nM
DPDPE 100 nM
Cell count relative to control
1.0
0.5
0.0
a
b
b
a,b
B
Control (0 nM)
DPDPE 10nM+ Naltrindole 100nM
DPDPE 10 nM
Naltrindole 100 nM
Cell count relative to control
1.0
0.5
0.0
a
a
a
C
Control (0 nM)
Nor-Binaltorphimine 10 nM
Nor-Binaltorphimine 1 nM
Nor-Binaltorphimine 100 nM
Cell count relative to control
1.0
0.5
0.0
D
Control (0 nM)
U50-488 10 nM
U50-488 1 nM
U50-488 100 nM
Cell count relative to control
1.0
0.5
0.0

**Table 1**. Embryo development from metaphase II to blastocyst in presence of OPRD1 agonist DPDPE. Percentages of MII oocytes recovered from mice, fertilized oocytes (zygote), 2 cell-stage embryos and blastocysts after IVF using mouse sperm incubated with increased concentration of DPDPE (0nM, 1nM, 10nM and 100nM). Results are the means ± S.E.M. of 5 independent experiments. The different combinations of letters indicate significant differences between groups; $p<0.05$ in all cases.

| | MII | Zygote | 2-cell | Blastocyst |
|---|---|---|---|---|
| **Control (0 nM)** | 97.12 ± 1.3 | 91.17 ± 2.0[a] | 83.49 ± 4.1 | 76.42 ± 6.9[a] |
| **DPDPE 1 nM** | 92.00 ± 1.3 | 75.29 ± 3.8[a,b] | 72.26 ± 3.2 | 51.70 ± 4.5 |
| **DPDPE 10 nM** | 93.51 ± 2.3 | 77.62 ± 5.7[a,b] | 70.33 ± 4.3 | 46.94 ± 5.3 |
| **DPDPE 100 nM** | 86.35 ± 2.4 | 71.02 ± 5.7[b] | 65.98 ± 5.4 | 52.91 ± 5.5 |

**Table 2.** Embryo development from metaphase II to blastocyst in presence of OPRD1 agonist and/or antagonist. Percentages of MII oocytes recovered from mice, fertilized oocytes (zygote), 2 cell-stage embryos and blastocysts after IVF using mouse sperm incubated with DPDPE (10 nM), naltrindole (100 nM) and both together. Results are the means ± S.E.M. of 5 independent experiments. The different combinations of letters indicate significant differences between groups; $p<0.05$ in all cases.

| | MII | Zygote | 2-cell | Blastocyst |
|---|---|---|---|---|
| **Control (0 nM)** | 97.12 ± 1.3 | 91.17 ± 2.0 | 83.49 ± 4.1$^{a,b}$ | 76.42 ± 6.9$^{a}$ |
| **DPDPE 10 nM** | 93.51± 2.3 | 77.62 ± 5.7 | 70.33 ± 4.3$^{a}$ | 46.94 ± 5.3 |
| **DPDPE 10 nM + Naltrindole 100 nM** | 95.27 ± 1.9 | 87.82 ± 5.0 | 83.24 ± 5.9$^{a,b}$ | 72.43 ± 11.1$^{a}$ |
| **Naltrindole 100 nM** | 100.00 ± 0.0 | 96.04 ± 2.4 | 91.82 ± 4.1$^{b}$ | 70.54 ± 15.9$^{a}$ |

**Table 3.** Embryo development from metaphase II to blastocyst in presence of OPRK1 agonist U-50488. Percentages of MII oocytes recovered from mice, fertilized oocytes (zygote), 2 cell-stage embryos and blastocysts after IVF using mouse sperm incubated with increased concentration (0nM, 1nM, 10nM and 100nM). Results are the means ± S.E.M. of 5 independent experiments.

| | MII | Zygote | 2-cell | Blastocyst |
|---|---|---|---|---|
| **Control (0 nM)** | 90.90 ± 7.4 | 81.81 ± 14.8 | 81.81 ± 14.8 | 76.81 ± 10.7 |
| **U50488 1 nM** | 98.88 ± 1.1 | 98.88 ± 1.1 | 95.07 ± 2.8 | 93.96 ± 3.9 |
| **U50488 10 nM** | 86.07 ± 9.1 | 86.07 ± 9.0 | 86.07 ± 9.1 | 75.53 ± 10.6 |
| **U50488 100 nM** | 100.00 ± 0.0 | 97.69 ± 1.1 | 95.39 ± 2.3 | 89.44 ± 2.2 |

**Table 4.** Embryo development from metaphase II to blastocyst in presence of OPRK1 antagonist nor-binaltorphimine. Percentages of MII oocytes recovered from mice, fertilized oocytes (zygote), 2 cell-stage embryos and blastocysts after IVF using mouse sperm incubated with increased concentration of nor-binaltorphimine (0nM, 1nM, 10nM and 100nM). Results are the means ± S.E.M. of 5 independent experiments.

| | MII | Zygote | 2-cell | Blastocyst |
|---|---|---|---|---|
| **Control (0 nM)** | 95.00 ± 2.1 | 87.00 ± 1.0 | 84.00 ± 4.1 | 82.00 ± 7.1 |
| **Nor-Binaltorphimine 1 nM** | 94.00 ± 2.0 | 86.00 ± 1.1 | 85.00 ± 3.2 | 84.00 ± 4.1 |
| **Nor-Binaltorphimine 10 nM** | 95.00 ± 2.2 | 88.00 ± 1.3 | 84.00 ± 5.0 | 81.00 ± 4.8 |
| **Nor-Binaltorphimine 100 nM** | 94.00 ± 1.9 | 87.00 ± 0.9 | 85.00 ± 4.3 | 84.00 ± 5.0 |