NOVEL MODULATORS OF HUMAN SPERM MOTILITY AND MIGRATION

by

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ABSTRACT

Overall, signaling events in human sperm, together with the identity of many of the molecules stimulating them, remain very poorly characterised. A two-pronged approach involving characterisation of sperm receptors in a model-cell system, followed by single cell $[Ca^{2+}]_i$ imaging of large numbers of capacitated human spermatozoa, was employed to start to unravel some of these possibilities. Data were compared to responsiveness of the best-characterised physiological ligand of sperm – the steroid hormone progesterone.

Proteomic data strongly indicates the presence of OR2AE1, an olfactory receptor, in mature human sperm. OR2AE1 was cloned into the mammalian expression vector pcDNA3 and expressed in HEK293 cells. Functional expression of OR2AE1 (5.6±1.1% of cells showing correct localisation of FLAG-tagged recombinant receptor at the plasma membrane level) was demonstrated. Despite this relatively low level of functional expression, this system still allowed identification of a potent volatile agonist for OR2AE1 – omega (Ω) – via an elimination-panel system used alongside $[Ca^{2+}]_i$ imaging. Around 3% of cells in the heterologous system generated a response to the agonist, indicating correct functional expression.

When sperm were exposed to 50 µM Ω, $[Ca^{2+}]_i$ imaging revealed a transient response which occurred in 30-40% of capacitated cells. 10 µM NNC 55-0396, an antagonist of the CatSper channels, inhibited around 80% of these responses. Similar inhibition levels were demonstrated by inhibitors of the cAMP pathway (100 µM SQ22536 and 25 µM cAMPS-Rp), but no substantial effect was observed by inhibition of PLC by 5 µM U73122. Chemotactic

assays indicated that 50 μM Ω had an optimal chemoattractive effect, with 500 μM Ω significantly stimulating acrosome reaction.

An unrelated family of chemicals, the boronic acids, were also examined for their ability to affect sperm motility, migration and $[Ca^{2+}]_i$ dynamics. A significant effect on the number of sperm which migrated from semen when exposed to these chemicals was observed, with pathway inhibition distinctly indicating a bifurcating signal propagating mainly via IP_3 but also involving the cAMP pathway.

Manipulation of the signalling events described, with inherent modulation of sperm motility may underlie the ability to develop future novel therapy, including contraceptive and diagnostic treatment regimes for the human male.

To those who are my all: - my family

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular free calcium concentration
ACIII	Adenylyl cyclase III
AOI	Area of interest
AR	Acrosome reaction
BSA	Bovine serum albumin
CaCC	Calcium activated chloride channel
cAMP	Cyclic adenosine monophosphate
CASA	Computer assisted semen analysis
CNGC	Cyclic nucleotide-gated channel
CRHR1	Corticotropin releasing hormone receptor 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Estrogen receptor
FRT	Female reproductive tract
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
G_{olf}	Olfactory specific G-protein α subunit
GPCR	G-protein coupled receptor
HFEA	Human Fertilisation and Embryology Authority
HMPBA	2-(Hydroxymethyl)phenylboronic acid cyclic monoester
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptors
LH	Luteinizing hormone
mAC	Membrane-associated adenylyl cyclase

MOE	Main olfactory epithelium
NO	Nitric oxide
OR	Olfactory receptor
ORF	Open reading frame
OSN	Olfactory sensory neuron
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PEBA	Phenethylboronic acid
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
ROI	Region of interest
ROS	Reactive oxygen species
sAC	Soluble adenylyl cyclase
sEBSS	Supplemented Earl's balanced salt solution
VNO	Vomeronasal organ
ZP	Zona pelucida

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1. INTRODUCTION

1.1. Olfaction

Smell, otherwise known as olfaction, is a deep-rooted sense shared by vertebrates and other animals and forms an essential part of the set of chemosensory tools that allows them to perceive the environment around them. Olfaction identifies essential cues important for the organisation of feeding, mating and social behaviours that are strongly linked to learning and memory. Among higher eukaryotes, from flies through to mammals, there is a striking evolutionary convergence towards a conserved organisation of signaling pathways in olfactory systems (Hildebrand and Shepherd, 1997).

For air breathing organisms such as humans, odorants must be sufficiently volatile to be detected by the nose. A large fraction of volatile chemicals have a discernible odour, placing the number of 'smellable' chemicals in the range of the thousands, if not millions (Mombaerts, 2004). These odorants are mostly organic compounds, usually low molecular mass aliphatic or aromatic molecules, with varied carbon backbones and diverse functional groups, including aldehydes, esters, ketones, alcohols, alkenes, carboxylic acids, amines, imines, thiols, halides, nitriles, sulphides and ethers (Firestein, 2001). Most of the time these chemicals are present in the environment at very low concentrations, and therefore processing these stimuli requires a very sensitive system backed up by a massive repertoire of receptors to match the chemical heterogeneity.

1.1.1. Mammalian olfactory system

The mammalian nasal cavity can include several olfactory subsystems (Figure 1.1), each of which serving different distinct functions. Normally two systems are distinguished: the main olfactory epithelium (MOE), responsible for sorting through the immense array of odorous molecules providing information about the external environment (food, predators, prey and territory), and the vomeronasal organ (VNO), which processes species specific airborne olfactory signals known as pheromones, conveying information about location and reproductive availability.

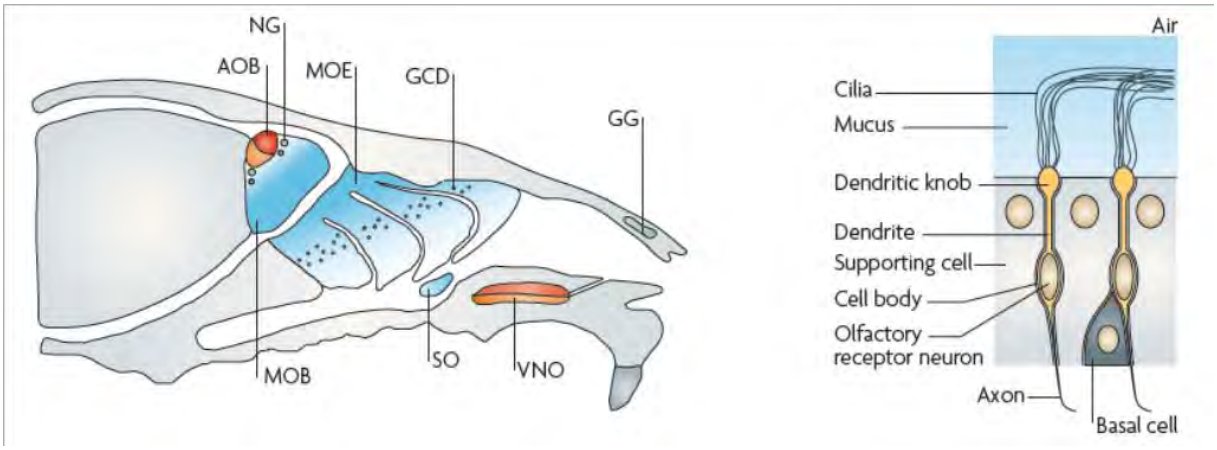


Figure 1.1 – Olfactory subsystems in vertebrates
The vertebrate nasal cavity (left) can include several olfactory subsystems: the main olfactory epithelium (MOE), the Vomeronasal organ (VNO), the Grüneberg ganglion (GG), the septal organ (SO) and guanylate cyclase D-containing cells (GCDs) in the MOE. Axons of sensory neurons in the MOE and SO project to glomeruli of the main olfactory bulb (MOB), sensory cells of the GG and GCDs send their axons to the necklace glomeruli (NG) and sensory neurons on the VNO project to the accessory olfactory bulb (AOB). Olfactory sensory neurons (OSNs) in the MOE are bordered by supporting cells and constantly regenerated from basal cells. Each OSN projects one dendrite that ends in a dendritic knob. From each dendritic knob cilia extend into the nasal mucus. Adapted from Kaupp, 2010.

The MOE covers a series of cartilaginous outcroppings in the posterior nasal cavity, called turbinates, comprising 6-10 million olfactory sensory neurons (OSNs) (Firestein, 2001). Each of these bipolar sensory cells extend an axon from the proximal pole of the cell into a region

of the forebrain known as the olfactory bulb, and a single dendrite to the surface of the tissue, that ends in a knob-like structure projecting numerous long and very fine cilia into the layer of mucus covering the tissue. These chemosensoric cilia include both receptor proteins and elements of the olfactory signal transduction machinery and provide an extensive receptive surface for the interaction of odours with the cell.

The VNO, located at the base of the nasal cavity, is enclosed in a cartilaginous or bony structure and divided by the nasal septum. The vomeronasal epithelium is isolated from the airstream in the nasal cavity, and the system relies on a pumplike action mediated by the surrounding vascular system to deliver stimuli to the VNO (Keverne, 1999). Receptor neurons interact with pheromones through apical microvilli and their axons form nerves converging at the accessory olfactory bulb (AOB) (Keverne, 1999).

1.1.2. The olfactory receptor

Olfactory receptors (Figure 1.2) form part of the G-protein coupled receptor (GPCR) superfamily and are characteristically encoded by intronless genes, sharing amino acid motifs that distinguish them from other non-OR seven-transmembrane proteins (Glusman et al., 2001). Their seven-transmembrane topology consists of seven transmembrane α-helices interconnected by extra and intracellular loops. Additionally, there is an extracellular N-terminal chain and an intracellular C-terminal chain (Buck and Axel, 1991; Pilpel and Lancet, 1999).

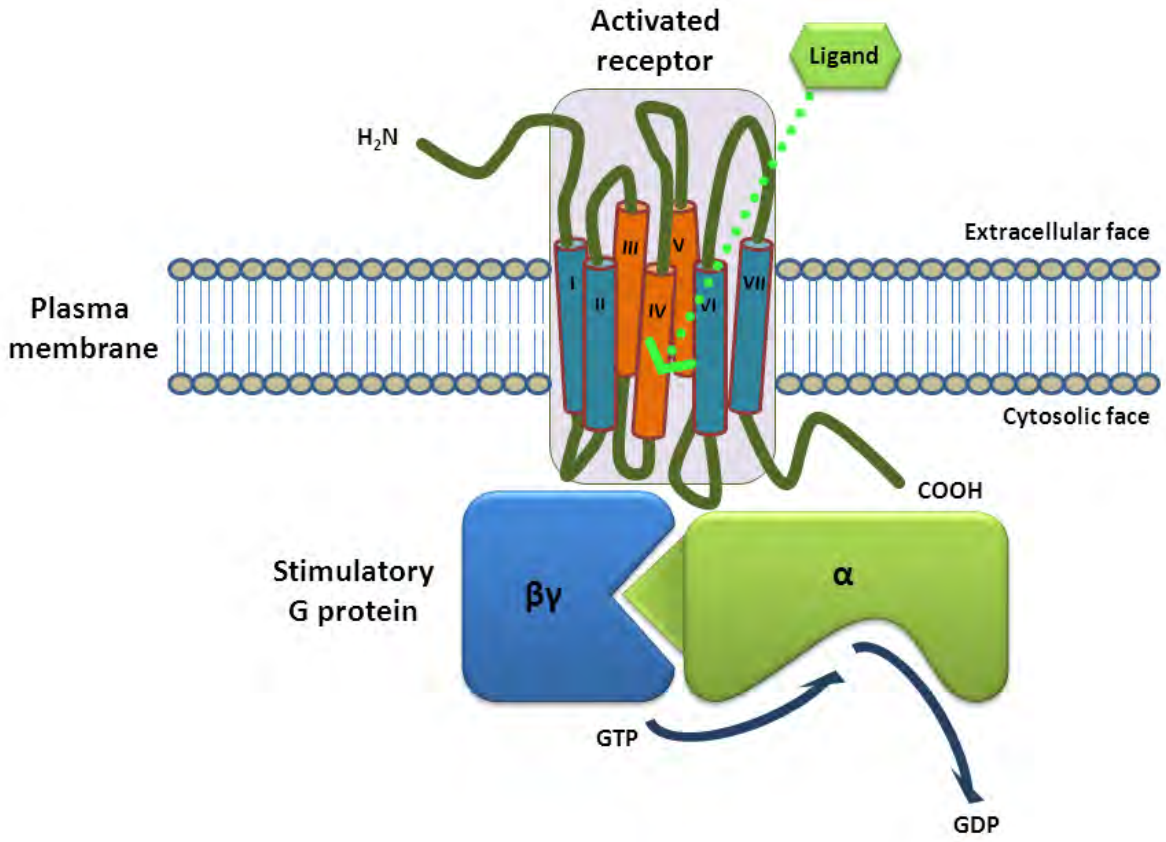


Figure 1.2 – Olfactory receptors
Diagram of the three-dimensional structure of the olfactory receptor, based on the structure of rhodopsin. Like all GPCRs, ORs couple to G proteins. Transmembrane domains III-V (in orange) display the highest residue variability and are thought to include the odorant binding pocket.

The number of ORs present in different mammalian species varies considerably (Table 1).

Table 1 – Olfactory receptor repertoire in complete mammalian genomes

Organism	Species	Number of OR genes		
		Intact	Pseudogenes	Total
Human	*Homo sapiens*	384	467	851
Chimp	*Pan troglodytes*	353	546	899
Dog	*Canis familiaris*	713	258	971
Mouse	*Mus musculus*	1,194	181	1,375
Rat	*Rattus norvegicus*	1,284	292	1,576
Opossum	*Monodelphis domestica*	899	617	1,516

adapted from Zhang and Firestein, 2009

Humans have 851 olfactory receptor genes, but 55% of these are listed as pseudogenes. Numbers in chimps are similar to human, whereas mice have a larger gene pool (1,375 ORs) with a much lower rate of pseudogenisation (13%) (Aloni et al., 2006; Glusman et al., 2001), probably relating to an evolutionary reduction in the sense of smell. Surprisingly, this reduction in functional ORs in primates has been correlated with the acquisition of trichromatic vision, suggesting that, at least in primates, better vision might have caused a partial redundancy in olfaction (Gilad et al., 2004).

1.1.3. Olfactory signal transduction pathway

Binding of the odour molecule (ligand) to the olfactory receptor triggers a cascade of events that converts chemical energy into a neural signal, and this is a generally well understood and comprehensively characterised process in mammalian chemosensoric systems.

As reviewed by (Kaupp, 2010), activation of most olfactory receptor neurons by binding of an odorant to a specific olfactory receptor (Figure 1.1 and Figure 1.3a) stimulates the rapid synthesis of cAMP by adenylyl cyclase III (ACIII) via a process mediated by the olfaction-specific G protein α subunit (G_{olf}). This rise in cAMP causes cyclic nucleotide-gated channels (CNGCs) to open, which increases intracellular calcium concentration $[Ca^{2+}]_{i.}$ This increase in calcium levels triggers the opening of Ca^{2+}-activated chloride channels (CaCCs), and because OSNs accumulate rather than export chloride ions, the opening of CaCCs leads to chloride efflux that further depolarises the cell. Recovery and adaptation of the odorant-induced response involves several Ca^{2+}-dependant and Ca^{2+}-independent pathways: OSNs display both short and long term adaptation to brief or sustained stimulation respectively, and both modes of adaptation appear to be controlled by changes in $[Ca^{2+}]_i$ (Zufall and Leinders-Zufall,

2000) and response termination may occur at all stages of the OR signaling pathway (Figure 1.3b).

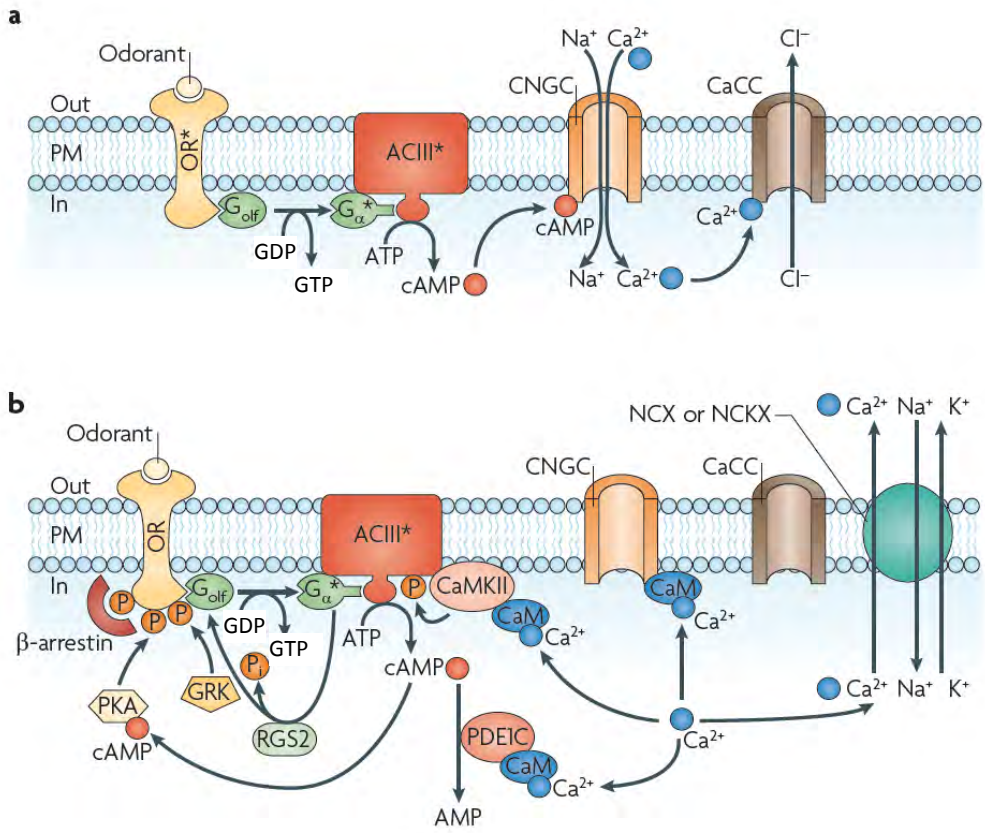


Figure 1.3 – Signal transduction in mammalian olfactory sensory neurons
a. The odour-induced signal transduction pathway; **b**. Recovery and adaptation of the odorant-induced response; adapted from Kaupp, 2010

Recognition of odorants by olfactory receptors is widely accepted to follow the "lock-and-key" molecular interaction model, where receptors identify particular molecular shapes in odorant molecules (Axel, 2005). More than a decade ago, however, Luca Turin (Turin, 1996) has proposed an alternative mechanism describing how ORs could "read" odorants, where molecular vibrations of either the whole odorant molecule or particular functional groups, instead of their shape, would be the "signature" contributing for odour recognition. This

work suggested that olfaction, together with colour vision and hearing, is in fact a spectral sense. A recent study by the same group (Franco et al., 2011), using deuteration of odorant molecules, shows that *Drosophila melanogaster* flies can differentiate between the deuterated and non-deuterated isotopes, odorant molecules with the same conformation but different vibrational modes. This cannot be explained by the "lock-and-key" model, and although this subject still remains controversial, the vibrational model has been gaining momentum.

1.1.4. Ectopic expression of ORs

Although ORs are predominantly expressed in the olfactory epithelium, there is ample evidence of these receptors being transcribed in non-olfactory tissues in several mammalian species, mainly in testis and germ cells (Asai et al., 1996; Goto et al., 2001; Linardopoulou et al., 2001; Parmentier et al., 1992; Spehr et al., 2003; Vanderhaeghen et al., 1997a; Vanderhaeghen et al., 1997b; Volz et al., 2003) but also in erythroid cells (Feingold et al., 1999), tongue (Durzynski et al., 2005; Gaudin et al., 2001), prostate (Xia et al., 2001; Xu et al., 2000) and other tissues (Conzelmann et al., 2000; Itakura et al., 2006; Otaki et al., 2004; Weber et al., 2002; Yuan et al., 2001). Interestingly, ORs have also been reported in canine sperm (Vanderhaeghen et al., 1993) and, through proteomic approaches, in human mature sperm (Baker et al., 2007; Lefievre et al., 2007; Spehr et al., 2004). Furthermore, one of these receptors, OR1D2, previously identified in the testis, has been described as mediating the chemotactic response elicited by bourgeonal in human sperm (Spehr et al., 2003). To date OR1D2, together with the recently characterised OR7A5 and OR4D1 (Veitinger et al.,

2011) remain the only ORs ectopically expressed in human with both a known ligand and a potential physiological role.

1.1.5. Heterologous expression of ORs

Since Buck and Axels' discovery almost 2 decades ago (Buck and Axel, 1991), ORs have been the focus of intense research interest. However, lack of adequate heterologous systems for expressing and assaying odorant responses (McClintock and Sammeta, 2003) has hampered significant advances in the field, with very few receptor-ligand relationships for mammalian ORs having so far been identified (Mombaerts, 2004). Transfected ORs exhibit low levels of plasma membrane expression (functional expression), due to endoplasmic reticulum (ER) retention, leading to subsequent degradation by the proteosome (Gimelbrant et al., 2001; Lu et al., 2003; McClintock et al., 1997). Several strategies have been tested to circumvent this deficiency: adding an N-terminal leading sequence from another GPCR led to success in characterizing a limited number of ORs (Kajiya et al., 2001; Krautwurst et al., 1998; Wetzel et al., 1999), but currently the scope is on providing these systems with the molecular machinery necessary for correct translocation of these receptors to the plasma membrane on the host cell, as they would normally do in OSNs. In line with this, RTP1S and RTP2, together with a receptor expression-enhancing protein REEP1 have been reported to play important roles in receptor protein translocation to the plasma membrane of HEK293T cells (Saito et al., 2004; Zhuang and Matsunami, 2007), thus significantly enhancing OR functional expression. The co-expression of these accessory proteins with the olfactory-specific G-protein α subunit G_{olf} and a putative guanine nucleotide exchange factor Ric8b (Von Dannecker et al., 2005; Von Dannecker et al., 2006), and combination with N-terminal

tagging of receptors has promised success in high throughput de-orphaning of mammalian ORs (Zhuang and Matsunami, 2007). Furthermore, a testis-enriched variant of the Hsc70 heat shock protein family (Hsc70t) has also been reported to facilitate OR expression in HEK293 (Neuhaus et al., 2006) and more recently the type 3 muscarinic acetylcholine receptor (M3-R), another GPCR found in mammalian OSNs, has been shown to interact with and enhance the responses to cognate odorants of several ORs expressed in HEK293T cells (Li and Matsunami, 2011). On the other hand, studies looking at OR trafficking in heterologous expression systems have shown that these receptors undergo constitutive internalization via a clathrin-dependant mechanism (Jacquier et al., 2006; Mashukova et al., 2006) with subsequent endosomal recycling, suggesting a similar mechanism in OSNs could be instrumental to allow rapid recovery of odour perception (Jacquier et al., 2006).

1.2. Sperm

1.2.1. Spermatogenesis and sperm structure

In humans, the production of mature male gametes from spermatogonial sperm cells – spermatogenesis – is initiated with puberty and constitutes a highly ordered and complex process that maintains a constant supply of male gametes throughout adult life.

Sperm production occurs in the testis, within the seminiferous epithelium, which consists of Sertoli cells (somatic cells) and several types of germ cells. The whole spermatogenesis takes around 64 days (Heller and Clermont, 1963) and is the totality of four sequential processes (Figure 1.4): spermatogonial development (stem cell and subsequent cell mitotic divisions – Figure 1.4, cell types 1-3), meiosis (DNA synthesis and two meiotic divisions to yield haploid spermatids – Figure 1.4, cell types 3-5), spermiogenesis (spermatid development involving

differentiation of head and tail structures – Figure 1.4, cell types 5-6), and spermiation (the process of release of mature sperm into the tubule lumen – Figure 1.4, cell type 7) (McLachlan et al., 2002).

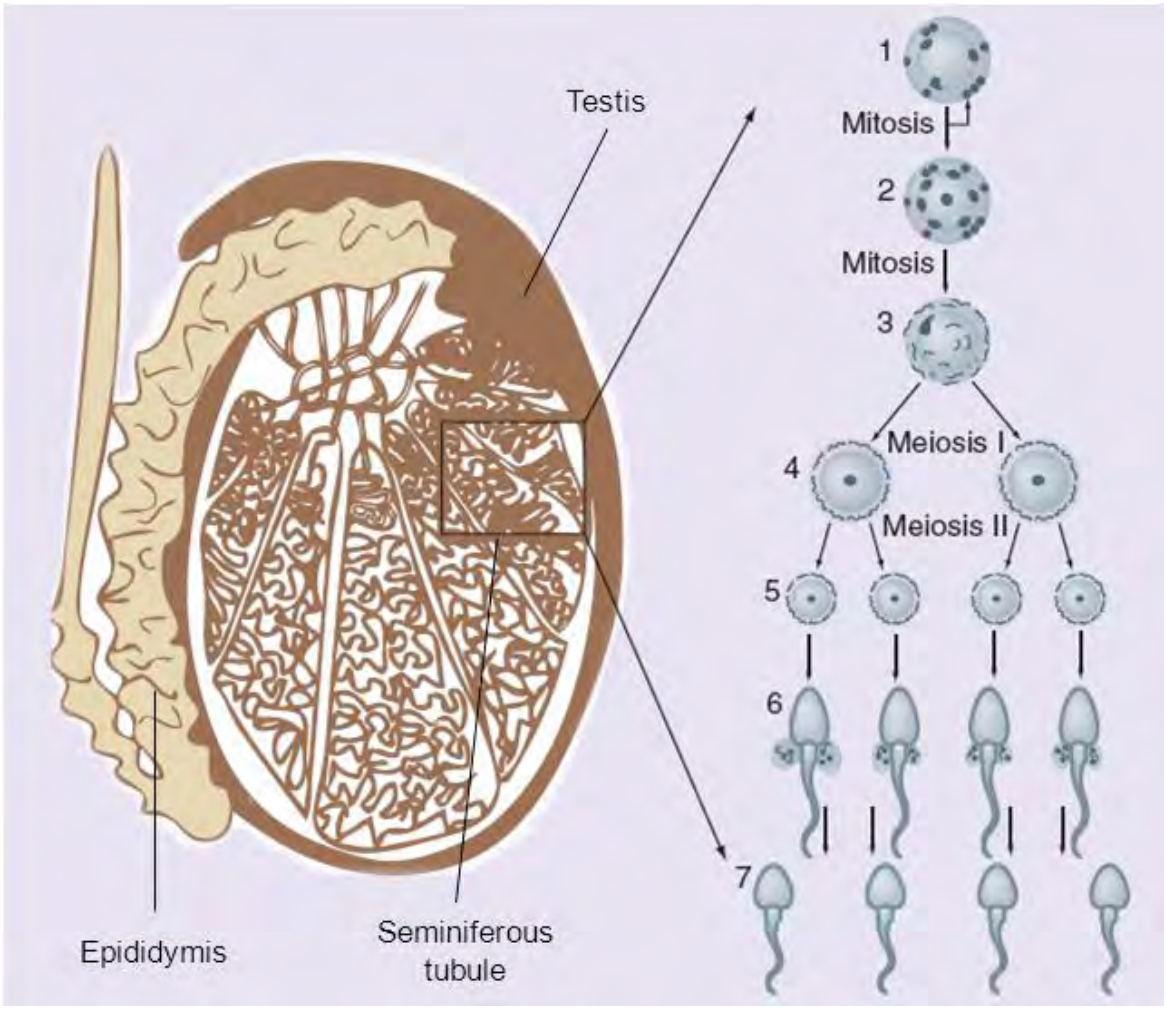


Figure 1.4 – Schematic representation of human spermatogenesis
Adapted from Phillips and Tanphaichitr, 2010

Regulation of spermatogenesis involves both intrinsic and extrinsic components. Intrinsic regulation relies on the effects of testosterone, growth factors and other neurocrine substances secreted by Leydig and Sertoli cells to control an intricate circle of regulation of

cell functions and development of germ cells, but also other functions like peristaltic movement of the seminiferous tubules (sperm transport) and blood flow in the itertubular vasculature (Middendorff et al., 1997). This local regulation is itself controlled by external stimuli: follicle stimulating hormone (FSH) secreted by the anterior pituitary gland stimulates Sertoli cells and is essential for the maturation of germ cells, whereas gonadotropin releasing hormone (GnRH) secreted by the hypothalamus triggers release of luteinizing hormone (LH) by the hypophysis, which in turn promotes testosterone production by Leydig cells. Testosterone levels will influence not only local events but also provide feedback to the hypophysis about the secretory activity of Leydig cells (Holstein et al., 2003).

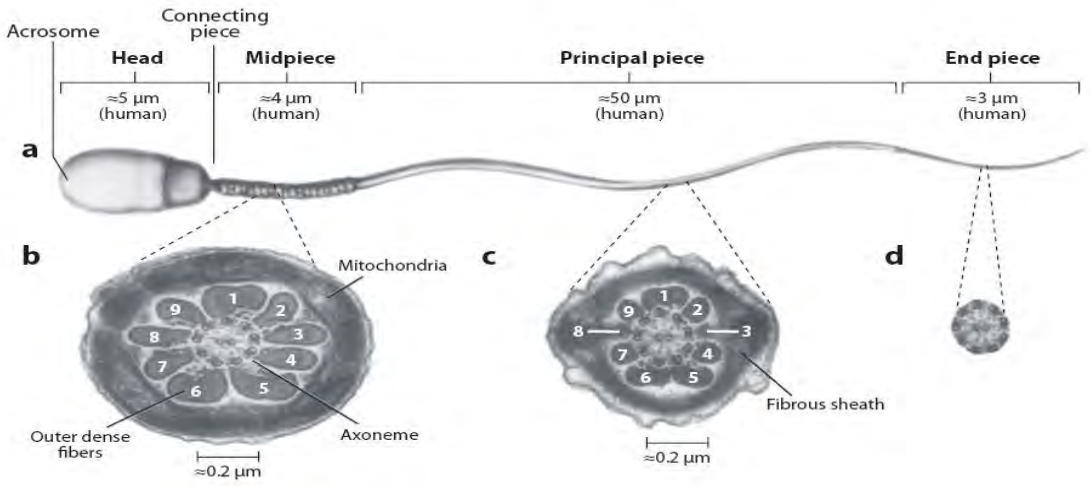


Figure 1.5 – Human sperm cell structure
a. diagrammatic view of a human spermatozoon; **b, c and d.** flagellum cross-sections at midpiece, principal piece and end piece, respectively. Adapted from Gaffney et al. In Press

The mature human spermatozoon is a relatively small and highly polarised haploid cell (Figure 1.5) comprising two major regions: the tail, used for energy production and initiation and maintenance of motility to drive the gamete to the fertilisation site, and the head,

crucially carrying all the genetic material and mechanisms necessary for zona recognition and sperm-egg fusion (Curry and Watson, 1995). In terms of organelles, the sperm cell is very efficient, including only the absolutely indispensable for its journey: the head consists of a nucleus, where the DNA is very tightly packed, and a single large secretory vesicle – the acrosome, storing a number of different hydrolytic enzymes crucial for penetrating the zona pellucida. The flagellum includes a mitochondria powerhouse in the midpice, providing all the energy required for motility and other cellular processes, and an axoneme, which stretches the full length of flagellum and constitutes the motor apparatus of the sperm tail. The whole cell is contained by the plasma membrane but, characteristically, spermatozoa lack other major cytoplasmic organelles such as the Golgi apparatus and endoplasmic reticulum. Despite recent and controversial evidence supporting nuclear gene expression in mammalian sperm involving 55S mitochondrial ribosomes (Gur and Breitbart, 2006), the general view, supported by earlier work looking at incorporation of radiolabeled amino acids (Engel et al., 1973; Hernandez-Perez et al., 1983), is that spermatozoa are devoid of any major transcriptional and translational activity (Baker et al., 2005; Blaquier et al., 1988a; Blaquier et al., 1988b; Naz and Rajesh, 2004; Ross et al., 1990).

1.2.2. Maturation and capacitation

When spermatozoa are released from the seminiferous epithelium of the testis they have already undergone a lengthy development, from undifferentiated germ cells to highly compartmentalised and specialised ones. However their developmental process is not completed yet: their journey out of the testis, through the seminiferous tubules, epididymis and vas deferens, before eventual ejaculation, can take several weeks. This process will add

functional aptitudes to cells that would otherwise lack motility and therefore fertilising ability: during their transit through the epididymis, in a process referred to as maturation, sperm will undergo a series of changes that culminate in the acquisition of progressive motility and the ability to capacitate (Cooper, 1995). Importantly, these events include addition of crucial function-modulating non-sperm expressed (epididymal) proteins into the maturing sperm proteome, such as the cysteine-rich secretory proteins (Ellerman et al., 2006; Gibbs et al., 2006; Nolan et al., 2006). At the time of ejaculation sperm are further mixed with seminal vesicle and prostate secretions during deposition at the cervix, and again at this stage, additional proteins and signaling modulators may be added to the sperm from so-called prostasomes – membrane-bound vesicles derived from prostate epithelial cells (Arienti et al., 2002; Arienti et al., 2004; Palmerini et al., 2003). A recent study by Park et al., 2011 has found evidence of several molecules involved in Ca^{2+} signaling like progesterone (PR) and ryanodine receptors (RyRs), secretory pathway Ca^{2+} ATPases (SPCAs) and cyclic adenosine diphosphoribose (cADPR)-enzymes being transferred from prostasomes to the sperm midpiece, correlating interference with these molecules to reduced sperm motility and /or infertility.

Before they are fully equipped for their ultimate purpose, however, freshly ejaculated spermatozoa must undergo a series of parallel functional and structural changes imposed by the female reproductive tract, in a complex series of events known as capacitation, which eventually grant them their fertilising capacity (Austin, 1952; Chang, 1952; Chang, 1984).

Transition from uncapacitated to capacitated state is thought to involve removal of decapacitation factors (DFs) from the external surface of decapacitation factor receptors (DF-R) (Fraser et al., 2006). Attempts to characterise these molecules in mouse sperm have

identified an anionic protein of about 40kDa associated with fucose residues important for function (Fraser, 1998). More recently Bi and colleagues (Bi et al., 2009) have identified a new PLCζ1 isoform - NYD-SP27, which belongs to the PLCζ family but lacks the EF domains in its N terminus. This work reports NYD-SP27 localises to the acrosome in human and mouse sperm, and detaches from sperm as they go through capacitation and AR, therefore suggesting NYD-SP27 as a physiological inhibitor of PLC that acts as an intrinsic decapacitation factor, regulating capacitation and AR. Further work by Fraser's group also revealed that DF binds to a second protein (DF-R) which shows high homology with phosphatidylethanolamine-binding protein 1 (PEBP 1) and attaches to the sperm surface via a glycosylphosphatidylinositol (GPI) anchor (Gibbons et al., 2005). Whether DF is bound to DF-R or not is hypothesized to alter sperm plasma membrane architecture, together with the conformation and function of membrane-associated proteins like Ca^{2+}-ATPase and stimulatory and inhibitory GPCRs, controlling cytosolic levels of second messenger cAMP via membrane-associated adenylyl cyclase mAC, and therefore regulating downstream tyrosine phosphorylation and sperm function (Fraser, 2010).

In human *in vitro* work, capacitation is marked by tyrosine phosphorylation events (Ficarro et al., 2003; Luconi et al., 1995) or assessed as an ability to fuse with the oocyte. Understandably, *in vivo* work is a lot sparser, and signals undeniably more complex. These signals are mediated by a diverse molecular array, where players like calcium, bicarbonate, serum albumin, glycodelins and other endogenous factors (Bjorndahl et al., 2006; De Jonge, 2005; de Lamirande et al., 1997; Ford, 2004; Fraser et al., 2006; Yanagimachi, 1994; Yeung et al., 2009) seem to have centre stage roles. These molecules are present at different levels in different female tract fluids, but detailed molecular mechanisms and chain of events leading

to sperm capacitation are yet to be unravelled. One of the major changes sperm are exposed to is a significant shift in ion fluxes, which in turn determines subsequent plasma membrane potential: prior to ejaculation, caudal epididymal sperm are stored in a high potassium, low sodium and very low bicarbonate environment. These ion concentrations, however, change dramatically on ejaculation, first in the seminal fluid and afterwards in the female reproductive tract, with both bicarbonate and sodium increasing considerably and potassium being significantly reduced (Brooks, 1983; Yanagimachi, 1994). *In vitro* work with boar spermatozoa has demonstrated an initial rapid PKA-dependant protein phosphorylation via activation of sAC by bicarbonate-containing medium, along with a later increase in tyrosine phosphorylation (Harrison, 2004). These events are thought to activate a phospholipid scramblase, resulting in significant reduction in membrane stability (Gadella and Harrison, 2002), crucially allowing cholesterol removal from the plasma membrane. This cholesterol loss, facilitated by extracellular albumin acting as cholesterol acceptor (Visconti et al., 1999), can directly or indirectly affect the cell's capacitation state, via modulation of intracellular pH, changes in membrane fluidity and fusibility, and potential exposure of membrane receptors important in sperm-zona interaction and acrosome reaction (Cross, 1998), therefore representing a major event in controlling subsequent sperm function.

Another stepping stone in sperm capacitation is hyperactivation. This physiological state, first described in mammalian sperm by Yanagimachi, 1970, is characterised by cells displaying very active thrusting, together with high amplitude, asymmetrical flagellar bending. As reviewed by Suarez, 2008, hyperactivated motility is set off by a rise in flagellar calcium levels, primarily mediated by plasma membrane CatSper channels, and appears to be required for successful zona pellucida penetration. Additionally, in many species

hyperactivation has also been suggested to play an important role in facilitating sperm release from the oviductal storage reservoir, as well as their progression through the mucus present in the oviductal lumen and the cumulus oophorus.

1.2.3. Acrosome reaction

Sperm's acrosome is a sizeable membrane-limited secretory organelle derived from the Golgi that houses a complex of enzymes and overlies the nucleus in the apical region of the sperm head (Yanagimachi, 1994). This vesicle is the key player in a major exocytotic event essential for fertilisation, leading to the release of acrosomal enzymes and exposure of molecules in the inner acrosomal membrane surface known as acrosome reaction (AR) (Figure 1.6). The release of the acrosome's contents starts with point fusions between outer acrosomal membrane and overlying plasma membrane. This releases the acrosome's lytic content, thus facilitating the carving of a path through the oocyte's vestments.

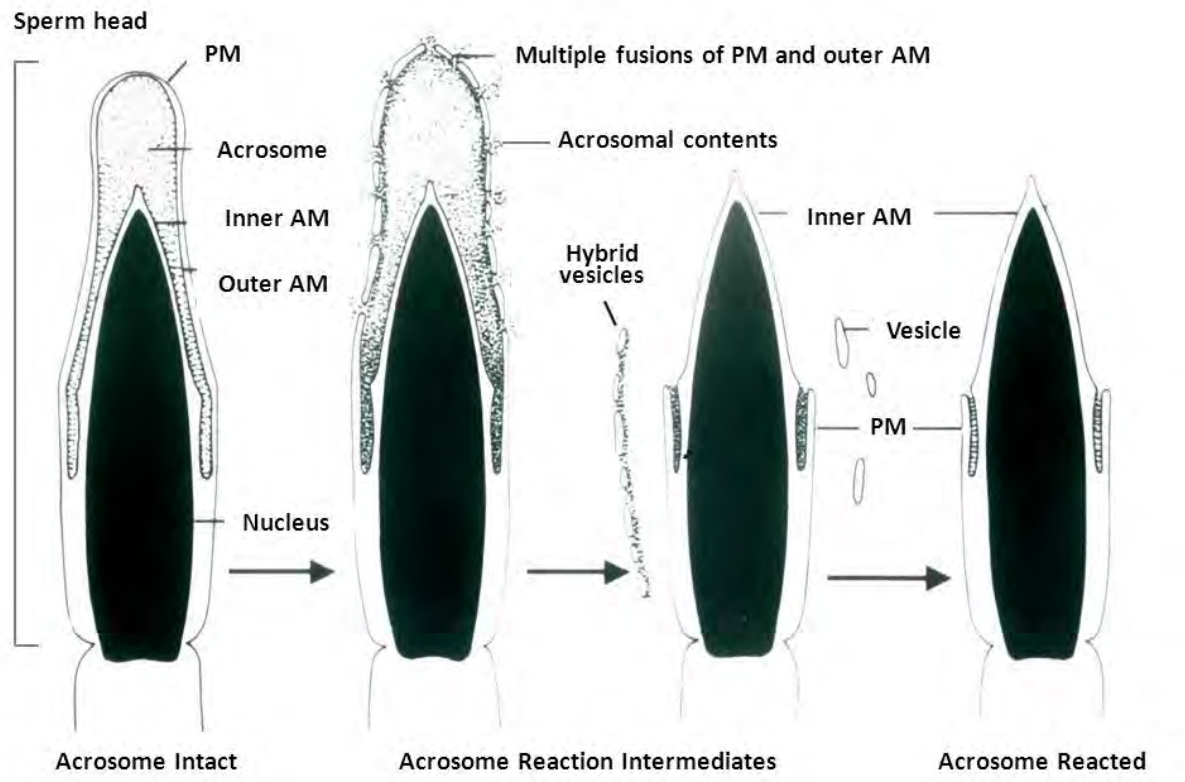


Figure 1.6 – Acrosome reaction in human sperm
General features of the human sperm acrosome before (acrosome intact), during (acrosome reaction intermediates) and after (acrosome reacted) acrosome reaction. PM – plasma membrane; AM – acrosomal membrane. (Adapted from Wassarman and Litscher, 2008)

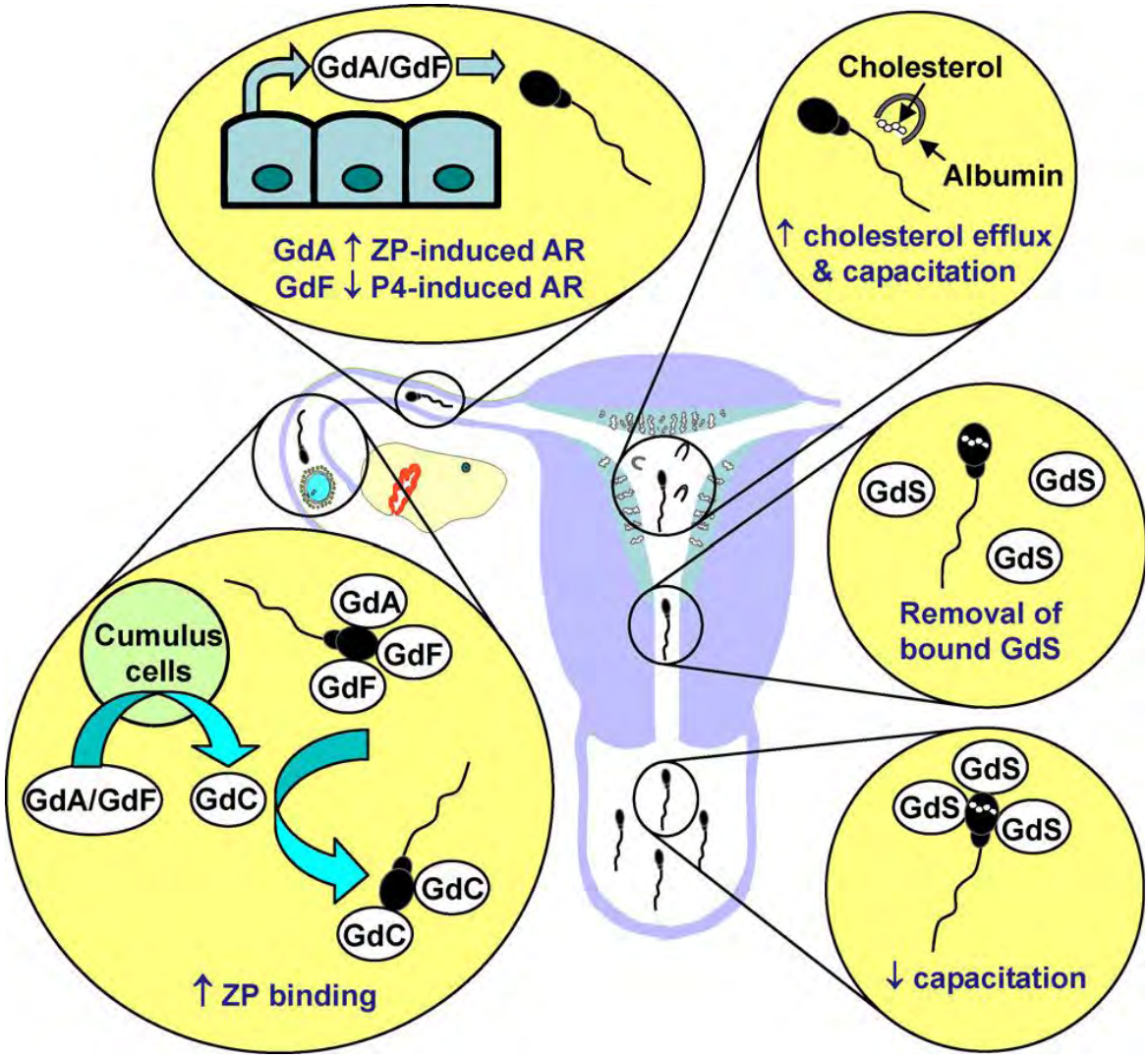
In human, the first barrier between the spermatozoon and the oolema is the cumulus oophorus, a hyaluronic acid-rich layer of cumulus cells surrounding the ovulated oocyte, consisting of an outer cell layer (cumulus cells) and an inner cell layer (cells of the corona radiata). The passage through the cumulus is thought to prime sperm cells for AR (Tesarik et al., 1988), and those that successfully negotiate it will then encounter the next vestment: the zona pellucida (ZP). ZP is a specialised matrix of the egg, made up of different glycoproteins (ZP1-ZP4), that serves roles as important as species-restricted recognition between gametes, prevention of polyspermy and protection of the embryo before implantation (Yanagimachi, 1994). The well-studied mouse ZP model consists of filaments of repeated ZP2-ZP3

heterodimers occasionally cross-linked by ZP1, forming a mesh-like structure (Dean, 2007), whereas in the human an additional protein (ZP4) similar to ZP1 (both in function and structurally) has also been identified (Lefievre et al., 2004). Mouse ZP3 has been found to be crucial for fertilisation by acting as a receptor for sperm (Wassarman and Litscher, 2008) and inducer of AR (Beebe et al., 1992) and this is thought to be the case for other mammalian species including human (van Duin et al., 1994).

In order to undergo AR in response to physiological stimuli spermatozoa must be capacitated: physiological AR is dependent on many of the same effectors of capacitation, and intracellular signaling involved in AR starts to be tuned during capacitation (de Lamirande et al., 1997). The dogma states that in most species the fertilising spermatozoon undergoes AR very close to, or on the zona pellucida (Cummins, 1995). However, recent evidence suggests that, at least in the mouse, this might not be the case: Jin and colleagues (Jin et al., 2011) demonstrated that fertilising mouse spermatozoa begin the acrosome reaction before reaching the zona pellucida of cumulus-enclosed oocytes. Furthermore, this work has also shown that the incidence of in vitro fertilisation of cumulus-stripped oocytes is increased when these are incubated with cumulus cells, pointing to an important role of the cumulus matrix *in vivo*. As reviewed by Kirkman-Brown et al., 2002, the effects of cumulus cells on spermatozoa *in vivo* are still largely unknown, but the fact that the cumulus secretes progesterone at levels sufficient to trigger AR in capacitated human sperm (De Jonge et al., 1988; Osman et al., 1989) could relate to Jin's recent findings in mouse (Jin et al., 2011) and support the idea of cumulus as AR inducer *in vivo*.

1.2.4. Motility, transport and interactions with the female reproductive tract

For fertilisation to occur, spermatozoa must be able to navigate their way through the female reproductive tract (FRT) and reach the fertilisation site. In the human this translates into a journey several thousand times the spermatozoon's body length through a viscous and constantly changing environment. Sperm need to penetrate the cervix through the cervical mucus, an FRT's secretion whose viscosity is regulated by endocrine control (Kremer and Jager, 1988), therefore constricting its permeability to sperm to the female's fertile period. After penetrating the cervix spermatozoa need to traverse the uterus, find the correct oviductal opening (20 -50 µm in diameter) and make their way to the fertilisation site through the ciliated and highly convoluted surface of the oviduct, to which a series of transient binding events may occur, although a site of true sperm storage has yet to be demonstrated (Baillie et al., 1997; Morales et al., 1996; Pacey et al., 1995; Williams et al., 1993). Ultimately, sperm's journey through the FRT, culminating in fertilisation, constitutes a strict selection process through which a population of millions is reduced to a single cell, and success is conditional to exhibiting the right motility at the right moments. Certainly, the FRT plays a crucial role in the regulation of the spermatozoon's different motilities and correct synchronisation of crucial events like capacitation, hyperactivation and AR (Suarez and Pacey, 2006).



from Yeung et al., 2009

Figure 1.7 - Glycodelin glycoforms modulate sperm function in the female reproductive tract

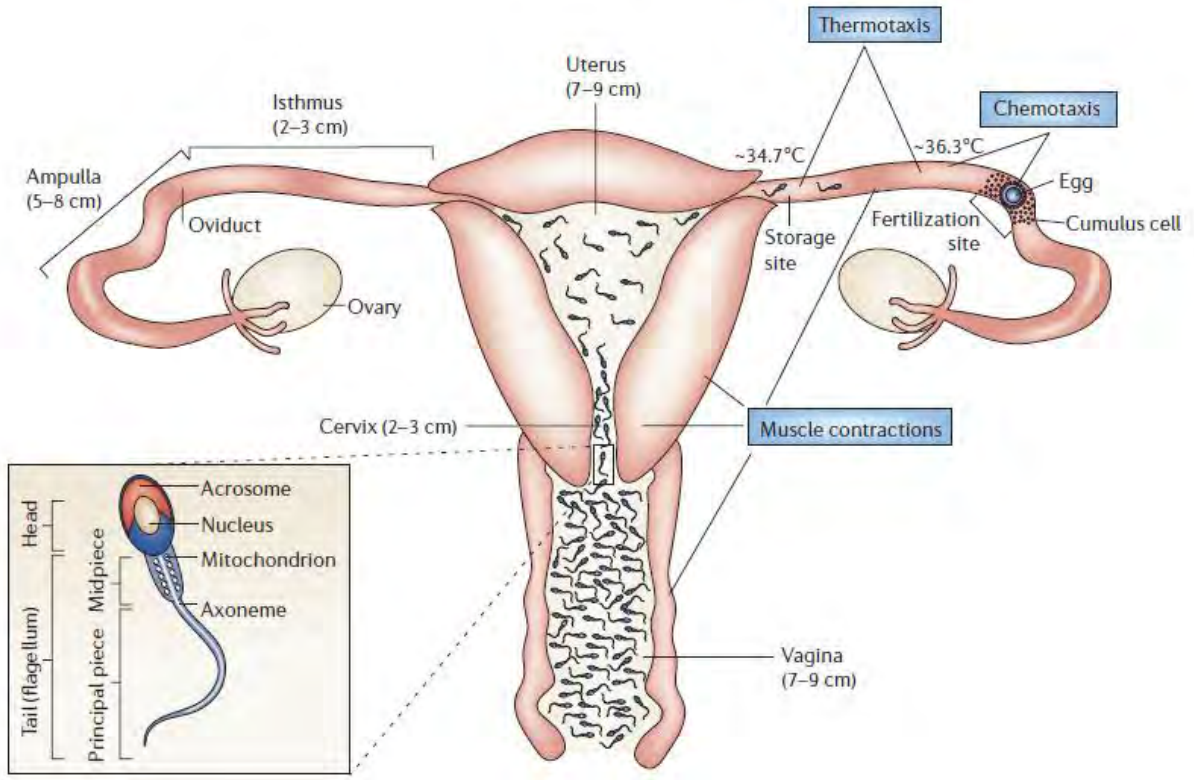
Throughout their journey, spermatozoa may follow several signals presented by the FRT, including temperature, fluid flow, and chemical cues (Eisenbach and Giojalas, 2006), and at the same time their function will be delicately modulated by most of those signals. One of the molecules mediating this modulation is glycodelin, a glycoprotein with four known glycoforms, differing only in their glycosylation. As recently reviewed by Yeung et al., 2009, during their journey to the oocyte, sperm are exposed to these different glycoforms in a

sequential manner (Figure 1.7). Glycodelin-S, isolated from seminal plasma, is the only male glycoform identified to date and inhibits albumin-induced cholesterol efflux from the plasma membrane. This prevents sperm from capacitating before their passage through the cervix, where glycodelin-S is removed from the cell surface by interaction with the cervical mucus (Chiu et al., 2005). Oviduct-produced Glycodelin-A (isolated from amniotic fluid) and glycodelin-F (isolated from follicular fluid) have similar activity, both inhibiting spermatozoa-zona pellucida binding (Chiu et al., 2003b; Oehninger et al., 1995). More importantly, glycodelin-F prevents premature acrosome reaction induced by progesterone secreted by the granulosa and cumulus cells (Chiu et al., 2003a), whereas glycodelin-A primes sperm for zona pellucida-induced AR. Their combined actions allow for a timely start of AR, essential for successful fertilisation. Glycoforms F and A are likely to be converted later to glycoform C by the cumulus cells (Chiu et al., 2007; Tse et al., 2002), resulting in the displacement of glycodelins F and A and enhancement of sperm-zona pellucida binding (Chiu et al., 2007).

1.2.5. Possible guidance mechanisms in the mammalian female reproductive tract

In mammals, the chance for interaction between male and female gametes is mostly dependent on the male gamete, and fertilisation is only possible if sperm prove able to successfully overcome the series of difficulties presented by a hostile and long journey through the female reproductive tract. However, in this context, it is widely accepted that all along this journey spermatozoa are being presented with a series of cues by the FRT, which are used in a set of distinct guidance mechanisms that allow sperm to navigate their way to the oocyte. Two active guidance mechanisms – chemotaxis and thermotaxis – have been described as significant in allowing sperm to reach the fertilisation site (reviewed in

Eisenbach and Giojalas, 2006 - see Figure 1.8), although passive sperm transport by muscular activity of the vagina, cervix and uterus, together with ciliar movement by oviductal epithelial cells, is also thought to be important in sperm migration in the FRT.



from Eisenbach and Giojalas, 2006

Figure 1.8 – Sperm guidance mechanisms within the mammalian female reproductive tract
Diagrammatic representation of the mammalian female reproductive tract, illustrating possible sperm guidance mechanisms. Organ dimensions presented are derived from studies in human (Harper, 1982) and temperatures were measured in rabbits (Bahat et al., 2005).

Chemotaxis, the directional movement of cells along a chemical gradient, has been thoroughly investigated in marine invertebrates (Kaupp et al., 2006), but the overall understanding of this phenomenon in mammals if much more limited. Nevertheless, the evidence that chemotaxis indeed has a significant role in human and other mammals has been building for some time now, with several groups reporting mammalian spermatozoa as

chemotactically active towards follicular fluid (Fabro et al., 2002; Giojalas and Rovasio, 1998; Oliveira et al., 1999; Ralt et al., 1991; Villanueva-Diaz et al., 1990), substantiating chemotaxis as a physiologically relevant and widespread guidance mechanism also in organisms with internal fertilisation. Unlike in marine invertebrates, however, only a subpopulation of capacitated mammalian spermatozoa is considered to be chemotactically active at any given time (Cohen-Dayag et al., 1994; Cohen-Dayag et al., 1995; Fabro et al., 2002; Oliveira et al., 1999). The identity of the molecule/s responsible for physiological chemotaxis in human sperm remains contentious: progesterone has been suggested as the chemoattractant in follicular fluid, with recent work by Teves et al., 2009 reporting pM concentrations of the steroid as capable of triggering chemotaxis in human sperm, whereas earlier work (Jaiswal et al., 1999; Ralt et al., 1991) describes progesterone as chemotactically inactive at the concentration range sperm are most likely to encounter in the female reproductive tract (Munuce et al., 2006). A different line of research suggests sperm could be attracted towards chemicals secreted by either by the egg-cumulus complex or the FRT itself. Evidence of human and mouse sperm being chemotactically attracted towards synthetic odorous molecules in vitro (Fukuda et al., 2004; Spehr et al., 2003) have prompted the authors to propose the receptors involved in the chemotactic events observed in vitro could be stimulated by molecules similar to the synthetic odorants tested.

Chemotactic effects from any potential chemoattractant within the FRT will certainly be limited to a length scale of around 1cm from the source of the chemoattractant molecule, so a more elaborate multi-step chemotaxis phenomenon involving different chemoattractants exerting their effects at different stages of the journey to the fertilisation site is considered

to be a more likely mechanism allowing sperm guidance towards the egg (Eisenbach and Giojalas, 2006).

The molecular mechanisms ruling mammalian sperm chemotaxis are still poorly understood, mostly due to the uncertainty over the molecules involved and the difficulty in designing relevant experimental methods with physiological significance, however, in vitro observations have provided a common basis for the mammalian chemotactic response where the importance of cyclic nucleotides and subsequent Ca^{2+} transients seem to be a commonality between marine species and mammals (Eisenbach and Giojalas, 2006; Kaupp et al., 2006; Spehr et al., 2006).

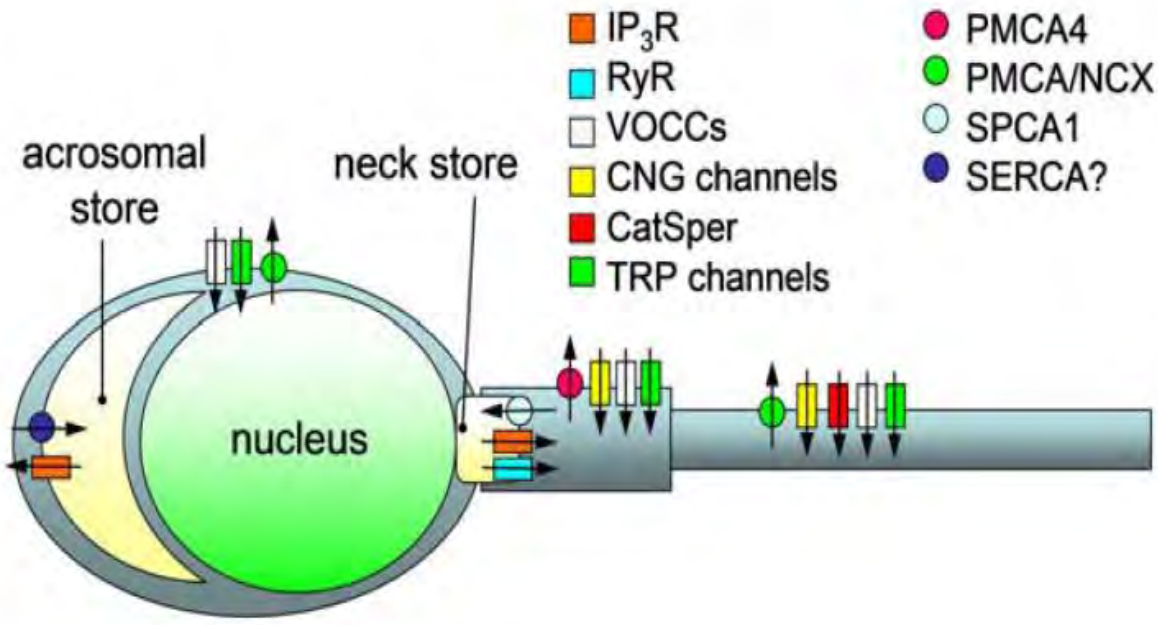
Thermotaxis is another guidance mechanism which some mammalian sperm have been reported to use to navigate from the isthmic region of the fallopian tube (where sperm storage is thought to occur in species like the bovine, swine and ovine) to the site where fertilization eventually occurs. Temperature measurements within pig fallopian tubes have revealed a temperature gradient to be present between the caudal isthmus and the cranial portion of the ampulla during early estrus of mated animals. The former proved cooler that the later by an average of 0.7°C, and this difference was no longer present shortly after ovulation (Hunter and Nichol, 1986). These differences in temperature are thought to reflect the differential activities of the vascular and lymphatic beds, as well as muscular activity related to peristaltic contractions by the oviduct on different phases of the menstrual cycle. Another possible temperature modulator could be the chemical microenvironment within the in the caudal isthmus, where sperm are stored for relatively long periods before fertilisation. This area might be the stage of endothermic reactions generated in the viscous glycoprotein secretions that accumulate before ovulation. During and after ovulation, a

temperature increase in the storage region would facilitate activation and release of maturing spermatozoa, thus providing adequate number of spermatozoa for successful fertilization to occur (Hunter, 2009). Furthermore, both rabbit and human sperm have also been shown to sense temperature differences as low as 0.5°C and swim from the cooler to the warmer temperature (Bahat et al., 2003) in a thermotactic response which, similar to the chemotactic responses in mammalian sperm, is only observed in capacitated cells, therefore suggesting thermotaxis could play a role in sperm navigation within mammalian species.

1.2.6. Cell signaling in mammalian spermatozoa

Spermatozoa are highly specialised cells and are generally accepted as translationally / transcriptionally inactive. Unlike somatic cells, spermatozoa are largely dependent on post-translational modification of "inherited" proteins for the regulation of their cellular processes. This non-genomic regulation involves a fine balance between levels of intracellular messengers like cAMP, cGMP, nitric oxide and calcium. All these messengers play important roles in crucial cell processes like capacitation (Breitbart, 2002; Hess et al., 2005), but as reviewed by Publicover et al., 2007, calcium takes centre stage in sperm cell signaling. Free calcium levels in the cytoplasm are maintained very low, and signaling involves modulating membrane channels that control Ca^{2+} flux between the cytosol and either the extracellular milieu or intracellular calcium stores, where Ca^{2+} levels are up to four orders of magnitude higher (Costello et al., 2009). $[Ca^{2+}]_i$ is pivotal for sperm function, regulating crucial events like AR (Evans and Florman, 2002; Kirkman-Brown et al., 2002), hyperactivation (Harper et al., 2004; Suarez, 2008) and potentially chemotaxis (Spehr et al., 2003), being also important in sperm capacitation (Breitbart, 2002; Fraser, 2010). Although

spermatozoa lack a functional endoplasmic reticulum, the main, but by no means the only Ca^{2+} storage organelle in somatic cells (Michelangeli et al., 2005), any or several of the intracellular membranous sperm structures can potentially work as Ca^{2+} store, provided they include the relevant molecular machinery (recently reviewed by Costello et al., 2009), therefore allowing sperm to maintain high levels of non-cytosolic intracellular Ca^{2+}.



from Publicover et al., 2008

Figure 1.9 – $[Ca^{2+}]_i$ regulation mechanisms in sperm
Distribution of the molecular machinery involved in the regulation of $[Ca^{2+}]_i$ in mammalian sperm. Channels are represented as rectangles, pumps as circles and arrows represent normal Ca^{2+} flow direction.

The molecular machinery involved in the regulation of $[Ca^{2+}]_i$ includes several classes of membrane receptors and ion pumps (Figure 1.9). These molecules are unevenly distributed in different regions of the plasma membrane and intracellular stores, and allow sperm to generate complex and apparently localized $[Ca^{2+}]_i$ signals (Publicover et al., 2008). Present at the plasma membrane we can find voltage-operated Ca^{2+} channels (VOCCs), transient

receptor potential channels (TRPCs), cyclic nucleotide-gated channels (CNG) and cation channels of sperm (CatSper), whereas intracellular calcium stores regulate the release of Ca^{2+} to the cytosol via ryanodine receptors (RyR) and inositol triphosphate (IP_3) receptors (IP_3Rs). These Ca^{2+} -permeable channels open in response to extra or intracellular stimuli allowing ions to flow down their electrochemical gradient, and therefore initiating a $[Ca^{2+}]_i$ signal with virtually no energy consumption. On the other hand, the maintenance of a low $[Ca^{2+}]_i$ level is mainly dependent on the action of energy-driven ion pumps that clear calcium from the cytoplasm to intracellular Ca^{2+} stores or to the extracellular space. In mammalian sperm there are three types of Ca^{2+} pumps: the plasma membrane calcium ATPases (PMCAs), the sarcoplasmic-endoplasmic Ca^{2+} ATPases (SERCAs) and the secretory pathway Ca^{2+} ATPases (SPCAs) (Michelangeli et al., 2005). Furthermore, both the sodium/calcium exchanger (NCX) and mitochondrial Ca^{2+} uptake can also contribute to maintain low cytosolic Ca^{2+} levels (Jimenez-Gonzalez et al., 2006).

Another crucial signaling mechanism in mammalian sperm is protein phosphorylation. As these cells are unable to synthesise new protein, post-translational modification of the existent protein array plays a fundamental role in regulating phenomena like capacitation and AR. Protein phosphorylation/dephosphorylation is reguated by the activity of kinases and phosphatases, and their counteracting activities provide cells with an effective switch that can turn the funtion of various effector proteins on or off, depending on their phorphorylation state.

Although phosphorylation of other protein residues like serine and threonine have been reported in spermatozoa, tyrosine phosphorylation has proved to be the main

phosphorylation event, being implicated in the regulation of major events like capacitation, hyperactivated motility, binding to the zona pellucida and AR (Naz and Rajesh, 2004).

An increase in protein tyrosine phosphorylation has been observed during capacitation of many mammalian species' sperm, and this increase is reported to be mediated by a cAMP-mediated pathway (Naz and Rajesh, 2004). Sperm's flagellum seems to be the major component of sperm to undergo a specific sequence of phosphorylation in most mammalian species. In human spermatozoa, protein A-kinase anchoring proteins (AKAPs), localised to the fibrous sheath, are the most prominent protein group undergoing tyrosine phosphorylation during capacitation (Carrera et al, 1996).

Tyrosine phosphorylation events were shown to be related to the acquisition of hyperactivated motility, and interestingly, the level of tyrosine phosphorylation in capacitating sperm was shown to be modulated by interaction with oviductal epithelial cells, indicating these interactions might be important to help synchronize sperm function with the time of ovulation (Petrunkina et al, 2003).

Adding to the already mentioned signaling mechanisms, sperm function can also be regulated by reactive oxygen species (ROS), which include reduced forms of oxygen and their reaction products with other molecules. As reviewed by Ford, 2004, ROS have been reported to promote capacitation in sperm from several mammalian species, with the in vitro addition of ROS generally causing increase in capacitation levels across most of the studies reviewed. Furthermore, ROS have also been implicated in sperm maturation, by facilitating nuclear condensation (Aitken and Vernet, 1998), and motility initiation, by enhancing cAMP synthesis and protein phosphorylation (Aitken, 2000). One of the best studied ROS in sperm is nitric oxide (NO): since the discovery that this gas could act as an intracellular messenger

(Palmer et al., 1987), and as recently reviewed by Lefievre et al., 2009, research has suggested an important role for NO in the communication between the FRT and sperm approaching the fertilisation site, since NO is actively synthesised both the tract epithelium and the cumulus-oocyte complex (Ekerhovd et al., 1999; Lapointe et al., 2006; Tao et al., 2004), and at low concentrations, to cause motility changes in sperm and possibly act as a chemoattractant (Herrero et al., 1994; Miraglia et al., 2007; Zhang and Zheng, 1996). The signaling mechanisms involved are not fully understood, but NO mode of action involves activation of soluble guanylate cyclase, leading to kinase activation and ion channel gating (Cary et al., 2006), but also protein nitrosylation, which acts as a switch to protein phosphorylation (Mannick and Schonhoff, 2004).

1.2.7. Progesterone and intracellular calcium

The signaling events that occur upon exposure of sperm to progesterone are complex and at present still confusing. However, a universally reported finding is that treatment with progesterone causes a transient elevation of $[Ca^{2+}]_i$, often followed by a sustained elevation (Aitken et al., 1996; Baldi et al., 1991; Blackmore et al., 1990; Bonaccorsi et al., 1995; Clapham, 1995; Kirkman-Brown et al., 2000; Tesarik et al., 1996). Importantly, induction of a rapid elevation in $[Ca^{2+}]_i$ upon in vitro exposure to a bolus of progesterone in the 0.3 nM to 3 µM leads to AR in humans (Harper et al., 2003; Kirkman-Brown et al., 2002), and this concentration range does relate to physiological progesterone levels sperm might encounter along the oviduct and near the fertilisation site (Munuce et al., 2006; Oehninger et al., 1994). Additionally, progesterone has been implicated in sperm guidance (Eisenbach and Giojalas, 2006; Oren-Benaroya et al., 2008; Teves et al., 2009), mobilisation of intracellular calcium

stores leading to complex calcium signaling (Publicover et al., 2007), and also in potentiating ZP-induced AR in human sperm (Morales et al., 2002; Schuffner et al., 2002), indicating a role for this steroid in initiating a stable but intermediate stage of "AR-readiness" before crucial ZP-induced acrosomal exocytosis.

When previously reviewed (Correia et al., 2007), the identity of receptor molecules involved in progesterone-evoked signaling events was still not clear, although evidence seemed to indicate a SERBP1/PGRMC1 complex as the most likely functional progesterone receptor in sperm. However, recent evidence by two independent labs (Lishko et al., 2011; Strunker et al., 2011) applying patch-clamp to mature human sperm has revealed CatSper, a pH-dependent Ca^{2+} channel of the sperm flagellum, as the most likely receptor mediating non-genomic actions of progesterone, although effects via other steroid binding receptors and ligand-gated ion channels cannot be excluded. Both reports demonstrate a rapid activation of CatSper by progesterone, incompatible with signal transduction involving metabotropic receptors and second messengers. Whether progesterone binds directly to the channel or to a different protein which associates with CatSper forming a channel/receptor complex is still uncertain, but the recent findings by Lishko and Strunker constitute a major advance in the understanding of the non-genomic actions progesterone evokes in sperm.

1.3. Aims of the research

The current work aimed to characterise the most prevalent OR in mature human sperm (OR2AE1) and investigate potential roles it might play in sperm biology. The strategy involved cloning the receptor and the expression on a heterologous system which would allow the screening of a large odorant library for potential receptor agonists. These were

then to be tested with human sperm to identify and characterise possible physiological and / or functional effects on these cells, with the ultimate purpose of enriching our understanding of a research area which is still on its infancy. In a different approach, and relating to other lines of research within our lab, a similar characterisation was anticipated for unrelated compounds (boronic acids) which had previously showed promise in enhancing sperm motility.

2. CHARACTERISATION OF OLFACTORY RECEPTORS EXPRESSED IN HUMAN SPERMATOZOA

2.1. Introduction

From the discovery of ORs two decades ago (Buck and Axel, 1991), evidence of these receptors being expressed in tissues unrelated to olfaction is copious (see section 1.1.4). The finding that ORs are also expressed in mammalian mature sperm (Baker et al., 2007; Lefievre et al., 2007; Spehr et al., 2004; Vanderhaeghen et al., 1993) was of particular interest for reproductive biologists as it opens new perspectives for a possible functional role these receptors might be playing in reproduction. Given the particularities of mature sperm and that these cells are translationally and transcriptionally inactive, the fact that during spermiogenesis resources are invested in arming them with OR proteins, increases the likelihood that these receptors must serve a purpose within the general reproductive marathon.

Following on pioneering work in which Wetzel and colleagues were able to match the first human olfactory receptor (OR17-40) to its agonist helional (Wetzel et al., 1999), the same group later published a landmark paper implicating a testicular OR (OR1D2) in the chemotactic response elicited by the odorant bourgeonal in mature human sperm (Spehr et al., 2003). These and subsequent works (Spehr et al., 2004; Veitinger et al., 2011) combined screening of odorant libraries and heterologous expression of ORs in HEK293 cells (Graham et al., 1977) to successfully identify agonists for orphan human ORs via single-cell Ca^{2+} imaging.

As reviewed by Mombaerts, 2004, other groups have taken different approaches to match odorants to cloned ORs, but the few success stories for human ORs were achieved using variations of Wetzel's strategy. The current work applied the same core strategy to effectively identify an agonist for OR2AE1, an olfactory receptor previously identified in mature human sperm (Baker et al., 2007; Lefievre et al., 2007).

2.2. Material and Methods

2.2.1. OR cloning and vector design

Both OR genes featured in this study were amplified from genomic DNA by PCR and initially cloned into pGEM-T Easy vector (Promega Corporation, USA). Primers used for OR2Y1 (Gene ID 134083) were: *forward* = ATGGGAAGTTTCAACACC; *reverse* = CTACCCTGAGTCCCTGC. Primers for OR2AE1 (Gene ID: 81392) were: *forward* = ATGTGGCAGAAGAATCAGACCTC; *reverse* = CTACACTCGGGGCAACCACAAT. Resulting clones were then sub-cloned into mammalian expression vectors after assessment for the correct sequence by at least four sequencing runs. OR2AE1's sequence, including the final stop codon, was inserted into pEGFP-N2 (Clontech – Becton Dickinson Biosciences, USA), out of frame with the EGFP gene so no chimeric expression of the OR was possible with EGFP (Figure 2.1), whereas OR2Y1's sequence was sub-cloned into pcDNA3 (Invitrogen Life Technologies, USA) (Figure 2.2).

Figure 2.1 – FLAG-OR2AE1 plasmid.
OR2AE1 sub-cloned into mammalian expression vector pEGFP-N2 between SpeI and BamHI restriction sites. Construct coding for CRHR1 and FLAG inserted between SacI and SpeI.

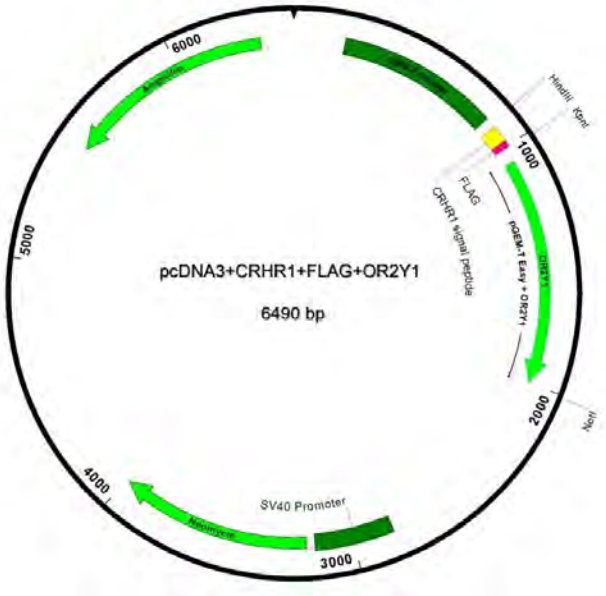


Figure 2.2 – FLAG-OR2Y1 plasmid.
OR2Y1 sub-cloned into mammalian expression vector pcDNA3 between KpnI and NotI restriction sites. Construct coding for CRHR1 and FLAG inserted between HindIII and KpnI.

Both mammalian expression vectors used were subsequently modified to include a single ORF expressing the ORs as N-terminal fusions with: 1) signaling peptide of human corticotropin releasing hormone receptor 1 (CRHR1), which is removed in the endoplasmic reticulum, and has been reported to enhance GPCR plasma membrane trafficking in HEK293 cells (Ladds, 2009); 2) FLAG tag, which, by being adjacent to the N-terminal chain of the OR, localises extracellularly, therefore allowing to check for receptor expression at plasma membrane level. All clones used where screened for correct restriction enzyme profile and sequence.

2.2.2. Cell culture and transfection

Human Embryonic Kidney 293 cells (HEK293) (Graham et al., 1977) were maintained at 37 °C, 95% air – 5% CO2 in high glucoseDulbecco's Modified Eagle Medium (D-MEM - DULBECCO and FREEMAN, 1959) (Gibco/Invitrogen, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Biosera, Ringmer, UK).

Initially, transient transfection conditions were assessed using pEGFP-N2 with no insert, which expresses a red-shifted variant of wild-type GFP, allowing for transfection rate quantification by detection of fluorescence within the green range. GeneJuice (Novagen, USA) was used to transiently transfect semiconfluent cells (50-80%) and conditions were optimized to transfection of 2 µg of plasmid DNA per 35 mm dish (Falcon 353001, Becton Dickinson, NJ, USA), 24 h after plating and 24 h before single cell calcium imaging experiments.

For each calcium imaging experiment, two parallel sets of cells were produced, one transfected with OR-containing plasmid (receptor-transfected cells) and another transfected with the plasmid without an insert (mock-transfected cells).

2.2.3. Heterologous OR expression – plasma membrane localisation

In order to assess whether transfected ORs were being expressed at the plasma membrane of HEK293 cells, and as described in 2.2.1, recombinant ORs included an N-terminus FLAG tag. ORs correctly localized to the membrane would therefore include FLAG on extracellular N-terminus chain, allowing for immunodetection with an anti-FLAG antibody (Webster et al., 2005).

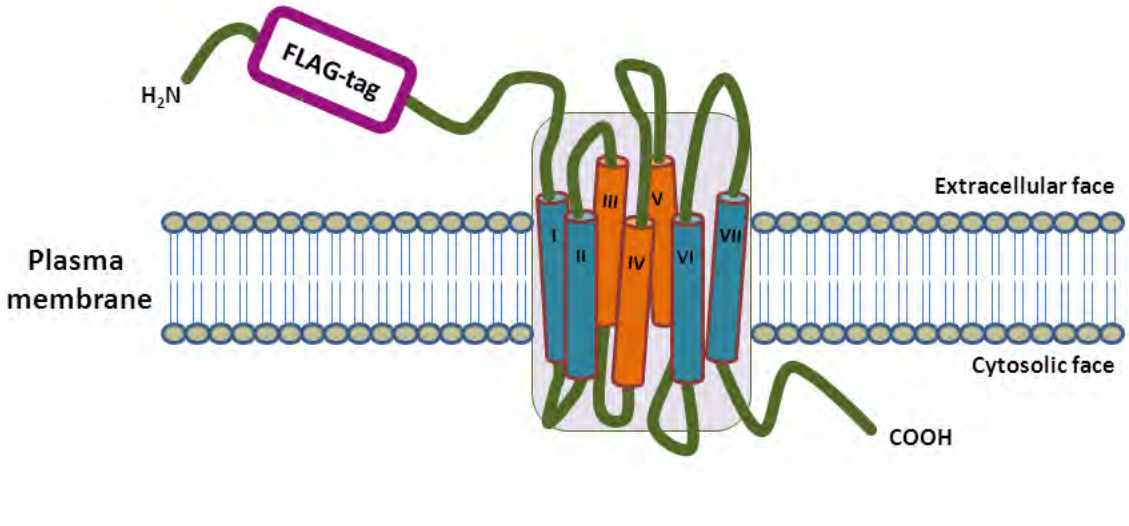


Figure 2.3 – Recombinant FLAG-OR2AE1
Functional expression of recombinant OR2AE1 incorporating FLAG tag on the extracellular N-terminus chain.

HEK293 cells were transiently transfected with FLAG-OR2AE1 plasmid as described (2.2.2), fixed with 4% paraformaldehyde (37 ^{o}C, 30 minutes), blocked with 1% BSA (37 ^{o}C, 30 minutes) and incubated at room temperature for one hour with primary antibody (5µg/ml mouse monoclonal FLAG Tag antibody – autogenbioclear, UK). Cells were the incubated with FITC-conjugate anti-mouse IgG antibody (F0257 – Sigma, UK) for one hour at room temperature and detection of fluorescence was done at 40X magnification using a Nikon TE300 inverted microscope illuminated with a blue LED – 470 nm (CAIRN Research Ltd, UK) coupled to a Photometrics Quant-Em 512 CCD camera.

2.2.4. Single cell calcium imaging and odorant exposure

Ligand screening for receptors expressed in the heterologous system consisted of 3 consecutive steps, having Wetzel's odorant screening strategy (Wetzel et al., 1999) as base. An odorant library including one hundred odorous chemicals (Henkel 100; Henkel, Dusseldorf, Germany) from several classes and groups (aromatics, aliphatics, alcohols, aldehydes, esters, ethers, ketones, amines, alkanes, heterocyclics and others) was subdivided into submixtures, each with increasingly smaller numbers of odorants (Figure 2.4). The first step of the screening strategy involved dividing Henkel 100 into two mixtures with 50 odorants each and screens these for the ability of triggering calcium responses in receptor-transfected cells. These two mixtures were further divided into 10 mixtures with 10 odorants each, and tested on screening step two. Finally, on screening step three, individual odorants making up the mixture identified on step two were assessed and, ultimately, agonists/antagonists identified. Odorant mixtures were stored as stock solutions (all odours at 10 mM) and diluted just before use in Tyrode's solution (see Appendix I: Media).

Before imaging experiments, D-MEM medium was replaced by Tyrode's solution. Cells were then loaded with a cell-permeant Ca^{2+} indicator (7.5 µM Calcium Green-1 AM, 0.1% (w/v) Pluronic F-127, both sourced from Invitrogen, USA). Calcium Green-1 was chosen over other calcium indicators on the basis of its adequate Ca^{2+} affinity (dynamic range from 10nM to 40 µM; K_d of 190 nM) and its relatively low cytotoxicity. Cells were incubated with the fluorescent dye for 45 min at 37 °C under 5% CO2 to allow for dye internalisation. Washing-off of excess dye and ensuring remaining dye de-esterification before the start of the imaging experiment was achieved under standard perfusion with Tyrode's (≈ 1 ml/min) for 10 min at room temperature.

Solutions were delivered through a RC-37 cell culture dish perfusion chamber insert (Harvard Apparatus, USA), fed by a gravity-driven eight-valve perfusion system (Harvard Apparatus, USA).

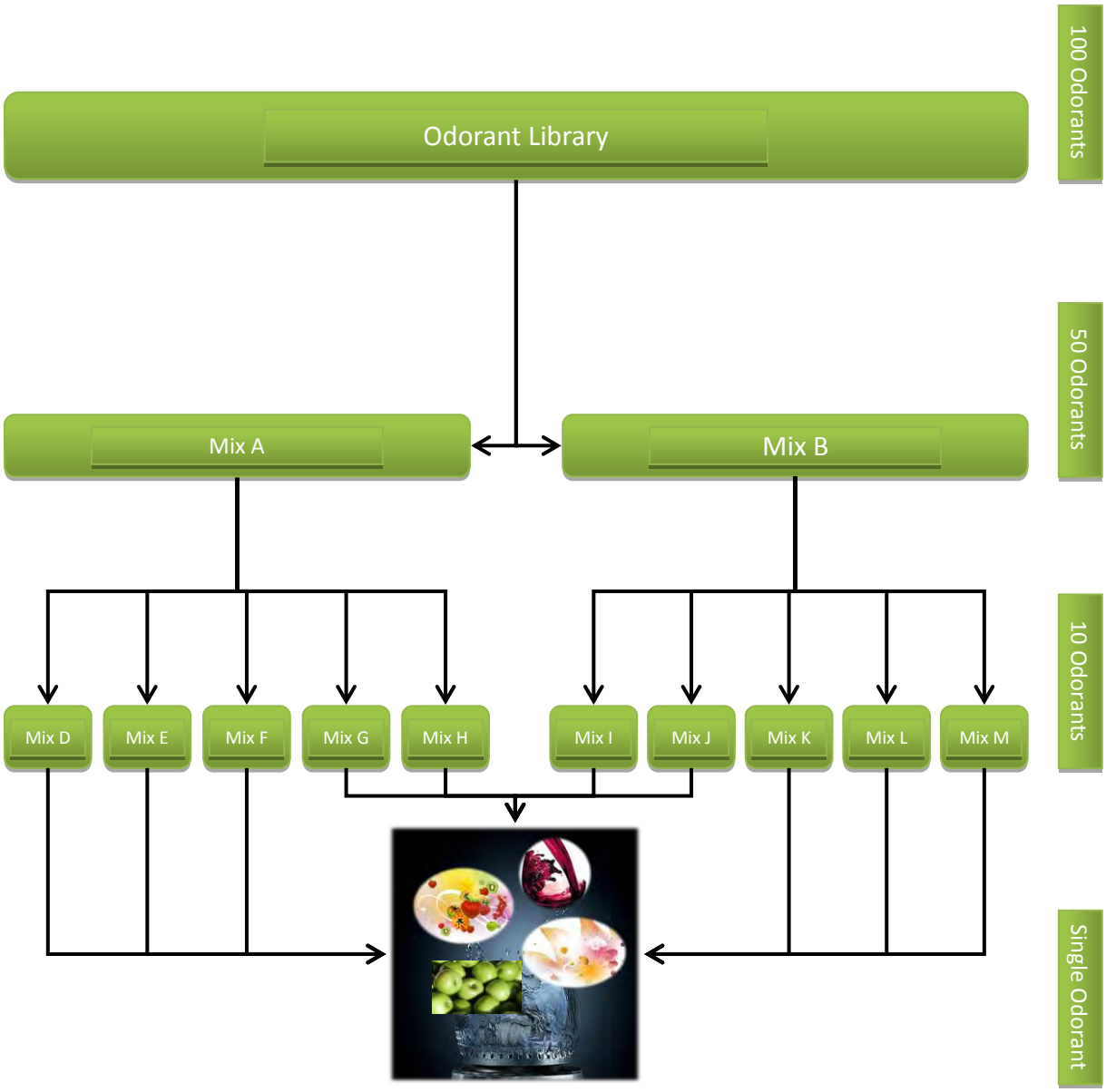


Figure 2.4 – Odorant screening strategy
To identify active components in the Henkel 100 odorant library, the 100 odorant mixture has been subdivided into fractions with increasingly smaller numbers of odorants and these were sequentially tested for activity in single cell calcium imaging assays.

Typical experiments consisted of 20 second applications of different odorant mixtures, diluted in Tyrode's solution to 50 µM. Odorant applications were separated by 60 second periods in which cells were continuously perfused with Tyrode's. HEK293 cells` physiological

state and their ability to produce PLC-mediated Ca^{2+} signals (Hansen et al., 1993) were monitored by application of 200 µM ATP (Sigma, UK) at the end of each experiment.

Fluorescence recordings, indicating $[Ca^{2+}]_i$, were taken at room temperature (≈ 25 °C) under continuous perfusion using a Nikon TE300 inverted microscope illuminated with a blue LED – 470 nm (CAIRN Research Ltd, UK). Images were acquired at 0.5 Hz 20X magnification using a Photometrics Quant-Em 512 CCD camera. Perfusion switching (Warner Instruments VC8 valve controller), image acquisition and storage were controlled by a PC running MetaMorph acquisition software (Molecular Devices, USA).

Data were processed offline using image analysis software (Image-Pro Analyser, Media Cybernetics, USA). Briefly, regions of interest (ROI) were drawn around single cells and mean intensity values extracted for each time point. All ROIs were individually assessed for correct placing around the cell at each time point and excluded from later analysis if cells had moved. As previously described (Kirkman-Brown et al., 2000), raw intensity values were exported to Microsoft Excel and normalized using the equation

$$R = \left[\frac{F - F_{rest}}{F_{rest}}\right] \times 100\%$$

where *R* is normalized fluorescence intensity, *F* is fluorescence intensity at time t and F_{rest} is the mean of 10 determinations of *F* taken during the control period. When looking at calcium responses evoked by odorant mixtures in HEK293 cells, responses were classed as positive only when the intensity change from baseline was at least 1/3 of that evoked by ATP for each individual cell.

2.3. Results

2.3.1. Heterologous expression of OR2AE1

Because olfactory receptor proteins, like all GPCRs, are structurally characterised by an extracellular N-terminus, and as described in 2.2.1, ORs featured on this work were expressed as N-terminal fusions with a short tag (FLAG) to allow for later receptor localisation and assess whether recombinant proteins were correctly localising to the plasma membrane. Figure 2.5 illustrates functional expression of OR2AE1 on HEK293 cells at plasma membrane level. As described in 2.2.2, cells were transfected with 2 µg of plasmid DNA (FLAG-OR2AE1) 24 hours after plating, fixed and immunostained with an anti-FLAG antibody to assess plasma membrane expression. Incubation with secondary FITC-conjugate anti-mouse antibody revealed a sparse labelling pattern at the plasma membrane level, on 5.6±1.1% of the cell population (Figure 2.5c), indicating a low level of functional expression of the receptor at the plasma membrane level.

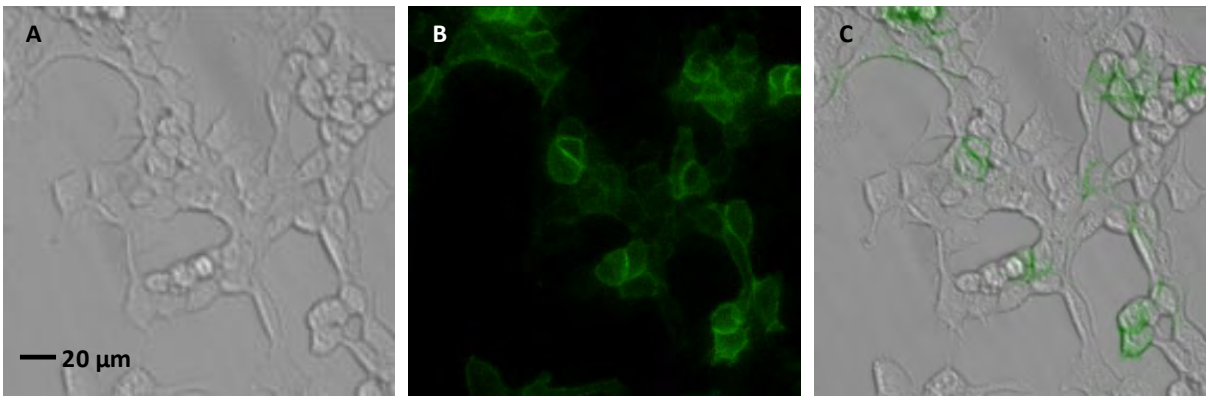


Figure 2.5 – Heterologous expression of OR2AE1
HEK293 cells were transfected with 2 µg of plasmid DNA (FLAG-OR2AE1) 24 hours after plating and immunostained with an anti-FLAG antibody. Detection done by incubation with a secondary FITC-conjugate anti-mouse antibody and assessed by confocal microscopy at 40X magnification (**a.** Bright field; **b.** FITC fluorescence; **c.** Composite image = Bright field + FITC fluorescence).

2.3.2. Odorant library screening – 50 odorant mixtures

In line with the screening strategy adopted, and as described in 2.2.3, the initial screening step consisted of pinpointing activity to one of the two 50 odorant mixtures. HEK293 cells transfected with 2 µg of plasmid DNA were tested via single cell calcium imaging and percentages of responding cells for OR2AE1 or mock-transfected cells were compared (Figure 2.6). Responses on OR2AE1 transfected cells were notoriously higher for mixture A (0.9±0.5%) than for Mixture B (0.6±0.3%). Furthermore, there was a substantial difference between responses evoked in mock and receptor-transfected cells for mixture A, with levels in receptor-transfected cells (0.9±0.5%) being nearly four times higher than on mock-transfected ones (0.2±0.2%). This, however, was not the case for mixture B, where mock-transfected cells show a higher level of positives (0.8±0.4%) than receptor-transfected ones (0.6±0.3%), with the difference between them being a lot lower than the one observed for mixture A. Despite statistical analysis revealing no significant differences between the mean values described above (paired T test, $t(40)$, $P > 0.05$), data points towards a higher level of responses in receptor-transfected cells when these were exposed to odorant mixture A.

Although pointing towards activity in mixture A, the initial screening step for OR2Y1 did not comprise sufficient number of replicate experiments to allow for a comprehensive decision on which of the 50 odorant mixtures should be tested on further screening steps. Investigations for this receptor were abandoned at this stage due to lack of time to complete further odorant screening experiments necessary and therefore data is presented as preliminary in appendix (please see Appendix II: Supplementary Data, Supp. Figure 1)

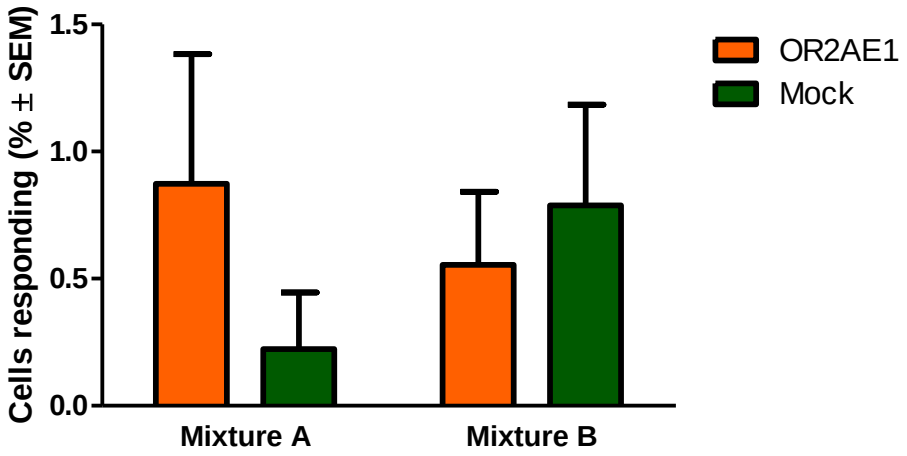


Figure 2.6 – Calcium responses to mixtures A and B
Mixtures include 50 odorants each at 50 µM. HEK293 cells were transfected with 2 µg of plasmid DNA (OR2AE1 or mock) 24 hours after plating and labelled with 7.5 µM Ca2+ Green-1 24 hours after transfection. Single cell calcium imaging readings taken at 2 s intervals and processed data, plotted as percentage of responding cells ± SEM, consists of 21 experiments, each including an average of 250 cells. Differences between mean values of receptor and mock transfected cells responding are not statistically significant (Paired T test, *t*(20), P > 0.05)

2.3.3. Odorant library screening – 10 odorant mixtures

The next step involved screening mixtures D to H, 10 odorants each (Figure 2.7, Figure 2.8). As described above (2.2.3), and taking in to account the relatively high level of unspecific responses observed with the 50 odorant mixtures, a refinement of the technique was introduced, whereby in each experiment cells would be exposed to two consecutive applications of each odorant mixture (see second step, 2.2.4). This approach allowed for further discrimination between cell populations in each experiment in terms of resolution of calcium responses. This is based on the assumption that if a given cell responds to both applications of a particular odorant mixture (re-evoked response), this is more likely to be a specific response to the components of that mixture than if a cell responds to only one of the applications. When looking at total responses (percentage of cells within the observed

population that respond to either of the two applications of any given mixture) evoked on receptor-transfected cells, and although differences between means proved non-significant (paired T test, $t_{(40)}$, $P > 0.05$), mixtures F (2.3±1.8%) and G (1.7±1.2%) stand out from the rest, with levels of responses being higher (more than 4 fold) than the ones evoked by any of the other mixtures (Figure 2.7).

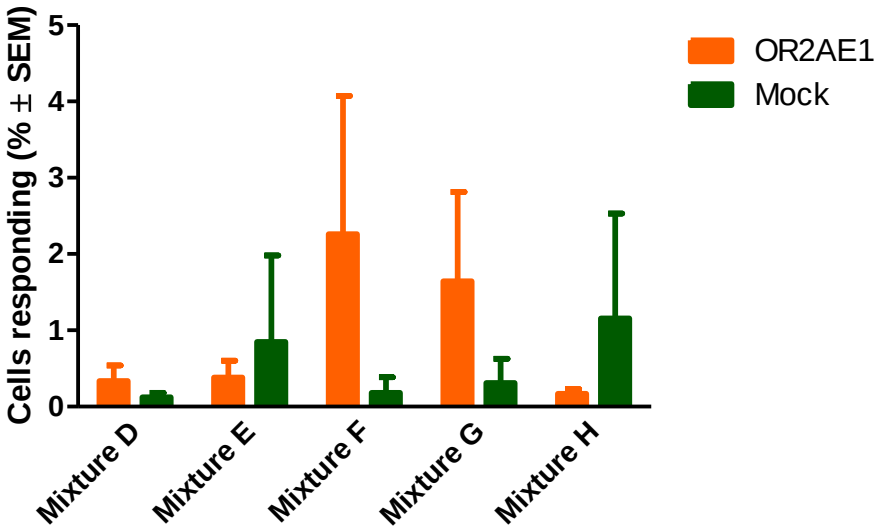


Figure 2.7 – Total calcium responses to mixtures D to H
Mixtures include 10 odorants each at 50 µM. HEK293 cells were transfected with 2 µg of plasmid DNA (OR2AE1 or mock) 24 hours after plating and labelled with 7.5 µM Ca^{2+} Green-1 24 hours after transfection. Single cell calcium imaging readings taken at 2 s intervals and processed data, plotted as percentage of responding cells ± SEM, consists of 41experiments, each including an average of 250 cells. Differences between mean values of receptor and mock transfected cells responding are not statistically significant (paired T test, $t_{(40)}$, $P > 0.05$).

However, when looking at levels of cells that respond to both applications of presented mixtures (re-evoked responses, Figure 2.8), it is obvious that, although overall level of responses is about one order of magnitude lower than the one observed for total responses (Figure 2.7), the ratio between specific (triggered on receptor-transfected cells) and unspecific responses (triggered on mock-transfected cells) is a lot higher, indicative of a better resolution between cells that functionally express the receptor and cells that don't. By

analysing the data set in this way, mixture G (0.4±0.2%) alone stands out, being the only mixture triggering significantly more responses in OR2AE1 transfected cells than in mock transfected ones (paired samples T test, $t_{(40)} = 2.24$, $P < 0.031$).

Again, as the second screening step for OR2Y1 for the 10-odorant sub-mixtures included in mixture A did not result in conclusive data, probably due to an insufficient number of replicate experiments, a comprehensive decision on which of the 10 odorant mixtures should be tested in the following screening step could not be made, and data is therefore presented as preliminary (please see Appendix II: Supplementary Data, Supp. Figure 2).

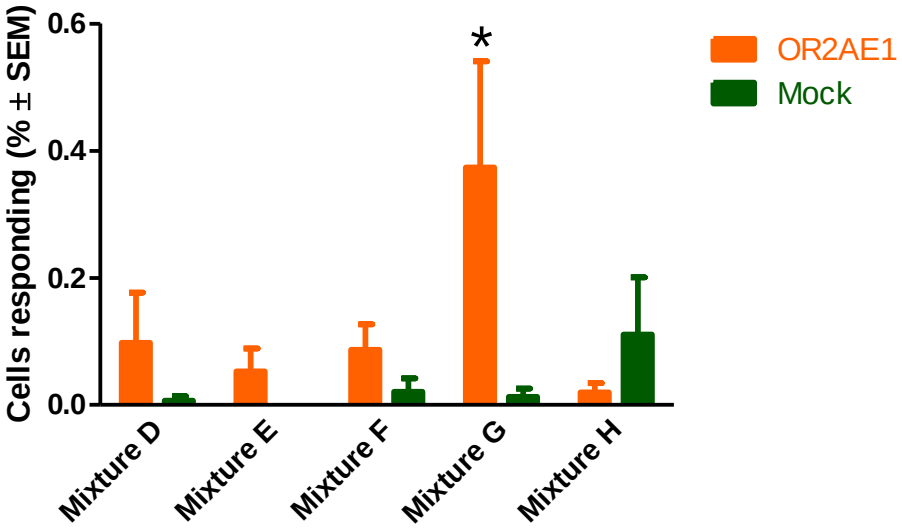


Figure 2.8 – Re-evoked calcium responses to mixtures D to H
Mixtures include 10 odorants each at 50 µM. HEK293 cells were transfected with 2 µg of plasmid DNA (OR2AE1 or mock) 24 hours after plating and labelled with 7.5 µM Ca2+ Green-1 24 hours after transfection. Single cell calcium imaging readings taken at 2 s intervals and processed data, plotted as percentage of responding cells ± SEM, consists of 41 experiments, each including an average of 250 cells; * indicates significant difference between responses in OR2AE1 and mock transfected cells (paired samples T test, $t_{(40)} = 2.24$, $P < 0.031$).

2.3.4. Odorant library screening – individual odorants

The second screening step identified mixture G as the one to be tested in the final screening step, and as discussed below (2.4), this involved identifying which of the individual components of mixture G was responsible for the increased excitability observed. Exposure of OR2AE1-transfected cells to the odorants making up mixture G has ultimately revealed ethyl 3,5,5-trimethylhexanoate, hereafter designated as Ω, as the agonist for the receptor in study ().

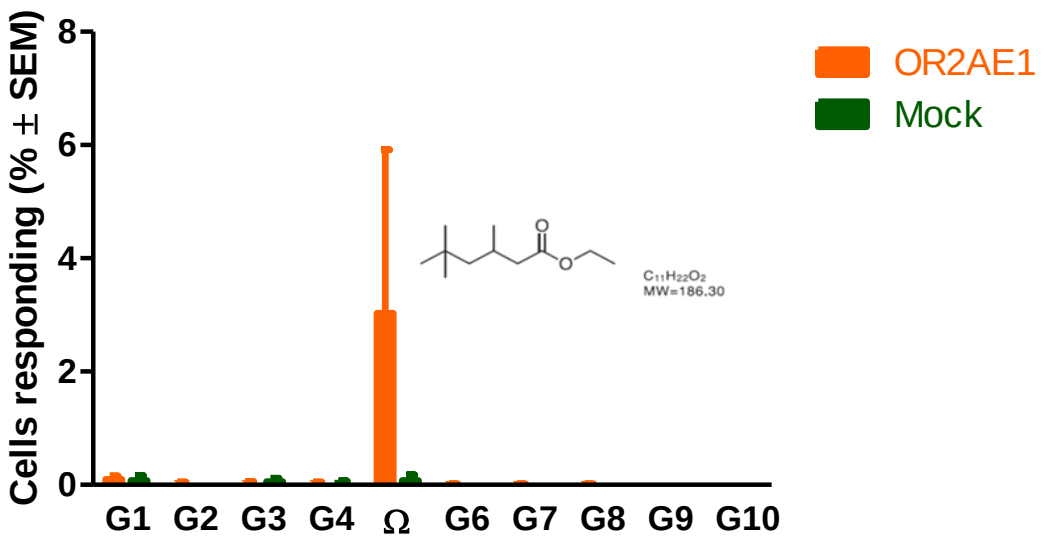


Figure 2.9 – Re-evoked calcium responses to individual odorants in mixture G
Odorants presented at 50 µM. HEK293 cells were transfected with 2 µg of plasmid DNA (OR2AE1 or mock) 24 hours after plating and labelled with 7.5 µM Ca2+ Green-1 24 hours after transfection. Single cell calcium imaging readings taken at 2 s intervals and processed data, plotted as percentage of responding cells ± SEM, consists of 3 experiments, each including an average of 300 cells; Differences between mean values of receptor and mock transfected cells responding are not statistically significant (paired T test, $t_{(2)}$, $P > 0.05$)

This odorant triggered re-evocable responses in 3.0±2.8% of receptor-transfected cells and caused negligible stimulation (0.1±0.1%) in mock-transfected cells. Similar to what was observed for G1-G4, and when compared with stimulation levels caused by Ω, the remainder

odorants in mixture G (G6-G10) also failed to induce noticeable levels of responses in both mock and receptor-transfected cells.

2.4. Discussion

Olfactory receptors, together with a huge array of other GPCRs, have had huge interest from the scientific community as potential pharmacological targets. Particularly in olfaction, substantial research effort has been directed to identify ligand-receptor pairs, to which a major roadblock preventing further and more expedite advances has been the absence of an adequate heterologous expression system allowing good functional expression levels for ORs. Mainly because heterologous cells lack some of the specific molecular machinery used by native cells to correctly process and translocate these receptors to the plasma membrane, levels of functional expression in heterologous systems tend to be very low (Gimelbrant et al., 2001; Lu et al., 2003; McClintock et al., 1997). Adding to this shortfall, ORs, like other GPCRs, are known to undergo a desensitisation process (Gainetdinov et al., 2004), where proteins expressed at plasma membrane are internalised via a clathrin-dependant pathway after ligand binding (Jacquier et al., 2006; Mashukova et al., 2006; Rankin et al., 1999). In addition, single-molecule OR trafficking work revealed that in fact these receptors could also undergo constitutive internalisation even in the absence of ligand (Jacquier et al., 2006), through a process of continuous recycling of receptor molecules present at plasma membrane level. This, however, is not an absolute limitation for studying ORs in heterologous systems: data obtained using HEK293 cells as expression system, although made up of low levels of responses reflecting the low levels of functional expression, proved unequivocal in differentiating between mock and receptor-transfected

cells, therefore allowing adequate screening of the odorant library for OR2AE1 agonists. The low levels of responses observed, however, meant that the number of individual cells observed had to be maximized in order to increase the chance of including cells functionally expressing the OR in each observation. As reviewed by Mombaerts, 2004, this combination of calcium imaging and heterologous expression on HEK293 cells has been used before to successfully identify olfactory receptor – agonist / antagonist pairs in mouse and rat (Gaillard et al., 2002; Kajiya et al., 2001; Krautwurst et al., 1998; Oka et al., 2004), but more importantly also in human (Neuhaus et al., 2006; Spehr et al., 2003; Wetzel et al., 1999). Pervious success stories and relative resemblance of HEK293 cells with neurons (Shaw et al., 2002) determined the choice of this system to study ORs featured in this project.

In line with the pre-defined screening strategy to identify ligands for the olfactory receptors in this study, and looking at OR2AE1 in particular, the initial screening of the 50 odorant mixtures (Figure 2.6) pinpointed activity to mixture A. As expected, levels of responses in receptor-transfected cells to both mixtures tested were low. Mixture A evoked a higher level of responses in OR2AE1 transfected cells than mixture B, although this difference was not enough to take as an indication of what odorant subset to screen next. However, by taking into account the level of responses triggered by those mixtures in mock-transfected cells (responses to mix B twice as frequent than responses to mix A), the decision on further screening mix A components was substantiated both by a lower level of responses in mock-transfected cells and also a higher level of responses in receptor-transfected cells. At this point of the screening, and because of the relatively complex nature of the stimuli involved, a complex response pattern was also expected – mixtures tested were made up of 50 distinct odorants, each one with its own chemical identity. It's not unreasonable to assume

that these mixtures could potentially activate distinct cellular responses via the cells' set of native receptors, therefore explaining the differences observed for different mixtures in mock-transfected cells. Furthermore, it has recently come to light that a small group of natural and synthetic odorants, commonly used by the fragrance industry, can have a dual activity by activating both ORs at the plasma membrane as well as the nuclear estrogen receptor α (αER) in OSNs (Pick et al., 2009). Although any potential effects mediated by αER on HEK293s would hardly fall within the time frame of odorant screening experiments featured in this work, future work should consider including transcription / translation blockers to minimise possible interaction between OR and αER signaling cascades. On the other hand, reports about rapid non-genomic actions of estrogens in various cell types are abundant, together with evidence favouring the existence of plasma membrane receptors for 17β-estradiol (Stratton et al., 2010), and therefore the possibility of these mediating calcium signaling events triggered by odorants present in the mixtures tested should not be discarded. None of possible interactions mentioned above has been investigated in detail, as such investigations would fall out of the scope of the current work.

Following on the results obtained for the initial step of the screening process, the second step consisted of testing components of mixture A for their capacity to evoke calcium signals in receptor-transfected cells. This was done by subdividing the 50 odorant subset into 5 mixtures of 10 odorants each (mixtures D to H, Figure 2.7, Figure 2.8). At this stage however, and because as discussed above results for the 50 odorant mixtures showed relatively high levels of unspecific responses in mock-transfected cells, the experiment design was changed to allow for two consecutive applications of each mixture. This approach was based on the assumption that if a given cell responds to both applications of the same odorant mixture,

this is more likely to be an OR-mediated response than if a cell responds to only one of the applications. By looking at number of cells that respond to both applications of the same odorant mixture (re-evoked calcium responses - Figure 2.8) as opposed to total number of responses to either the first or second applications (total calcium responses - Figure 2.7), it becomes evident that, although the number of events recorded are substantially lower for re-evoked responses, the difference between events observed on receptor and mock-transfected cells is much higher, indicating better resolution between specific events, mediated by OR2AE1 expressed at the plasma membrane in receptor-transfected cells, and unspecific responses, unrelated to the olfactory receptor, in mock-transfected cells.

As such, and substantiated by re-evoked responses triggered by mixture G in OR2AE1-transfected cells being at least three times higher than any of the other 10 odorant mixtures tested, the final screening step consisted on testing individual odorants making up mixture G. As evidenced in , only one of the odorants present in mixture G was able to evoke a noticeable level of responses in cells expressing the receptor, therefore revealing Ω as a strong agonist for OR2AE1.

Interestingly, the level of re-evoked responses in receptor-transfected cells evoked by Ω was substantially higher (about one order of magnitude) than responses observed for mixture G, pointing towards the presence of an inhibitory effect within mixture G. This could possibly relate to a direct competition for the receptor's binding site by one or more odorants in the mixture, if we accept the "lock-and-key" model to explain the molecular recognition between odorant and receptor molecules, or to the existence of one or more odorants in the mixture with the same vibrational profile, if we assume the vibrational model.

Overall, the screening strategy adopted, in conjunction with use of HEK293 cells as an expression system, allowed for good resolution of responses, despite the small numbers observed. This system, although demanding considerable number of repetitions due to the low levels of functional expression, has ultimately delivered a strong agonist for OR2AE1, which was subsequently tested in human sperm (see chapter 3).

3. FUNCTIONAL INVESTIGATION OF OR2AE1 IN HUMAN SPERMATOZOA

3.1. Introduction

It has long been observed that a substantial subset of the OR gene pool is not restricted to olfactory organs, also being expressed ectopically in a variety of other tissues, with particular incidence in the male reproductive system, including testis and male germ cell line (Asai et al., 1996; Goto et al., 2001; Linardopoulou et al., 2001; Parmentier et al., 1992; Spehr et al., 2003; Vanderhaeghen et al., 1997a; Vanderhaeghen et al., 1997b; Volz et al., 2003). Interestingly, OR expression has also been reported in mature sperm of several mammalian species (Baker et al., 2007; Lefievre et al., 2007; Parmentier et al., 1992; Spehr et al., 2004; Vanderhaeghen et al., 1993; Vanderhaeghen et al., 1997b). The big question arising from OR expression in mammalian sperm is whether these proteins play a role in reproduction or if their presence in sperm is only a stroke of chance. Recent microarray work looking at OR expression in non-olfactory tissues (De la Cruz et al., 2009) reveals that ectopic expression of OR orthologous genes in human and chimpanzee co-localise in the same non-olfactory tissues more frequently than expected by chance alone. Importantly this work also exposes a stronger evolutionary constraint for the ectopically expressed OR subset when compared to genes expressed in the olfactory epithelium alone, which is certainly consistent with the notion that these genes have additional functions in non-olfactory tissues.

The proposal of such functions for ectopically expressed ORs was first published in a landmark paper by Marc Spehr and colleagues (Spehr et al., 2003). This work matched a testicular OR (OR1D2, also known as hOR17-4), later found to be present also in mature

human sperm (Spehr et al., 2004), to its agonist odorant bourgeonal, but most importantly, this work showed that this small aldehyde molecule triggers a robust Ca^{2+} signal in human sperm, resulting in a positive chemotactic response towards the odorant. Shortly after, Fukuda et al., 2004 have also linked a mouse testicular OR (Olfr16, also known as mOR23) and activating odorant lyral, to a similar chemotactic effect and corresponding increase in calcium signaling on mouse spermatozoa. More recently, and following on Spehr's previous work, Veitinger et al., 2011 have further matched two human testicular ORs (OR7A5 and OR4D1) to their activating molecules (myrac aldehyde and PI-23472, respectively), and characterised specific calcium signaling events evoked by these molecules, associating them with distinct stimulus-specific motility patterns in human sperm. Although based on sperm behaviour observations made in *in vitro* conditions– which, as reviewed by Gaffney et al., 2011, are far from correctly modelling the environment spermatozoa find *in vivo* – evidence on the mentioned chemotactic and chemokinetic effects observed strongly suggests that OR-controlled sperm guidance and swimming behaviour could be present within the mammalian female reproductive tract and play an important role in reproduction. The fact that sperm display the appropriate molecular tools for OR signal transduction (Baxendale and Fraser, 2003 Spehr et al., 2004) further substantiates this possibility.

Following on the characterization of OR2AE1 in the heterologous expression system and matching of the receptor with its newly found agonist (see section 2), the experimental work within this chapter aimed to investigate and characterise potential physiological effects of Ω in mature human sperm.

3.2. Material and Methods

3.2.1. Sperm preparation / capacitation

Sperm samples were obtained from a panel of volunteer healthy donors recruited at the Birmingham Women's Hospital (HFEA centre 0119) in accordance with the Human Fertilisation and Embryology Authority (HFEA) Code of Practice. Ejaculates were obtained by masturbation and semen allowed to liquefy for 30 minutes. As described by Mortimer, 1994, and depending on whether further experiments required capacitated or uncapacitated cells, motile spermatozoa were then isolated by swim-up into either capacitating (sEBSS - supplemented Earl's Balanced Salt Solution) or non-capacitating (Tyrode) medium (Appendix I: Media). Briefly, 1 ml of media was under-layered with 0.3 ml of semen in 4 ml round-bottomed tubes (Falcon 2054) and set-up was incubated for one hour at 37^{o} C 6% CO_2. The motile fraction was then recovered from the top layer of the swim-up set-up and cell concentration assessed and adjusted to 6x10^{6} cells/ml. For capacitating conditions, sperm were further incubated for 4 hours at 37^{o} C 6% CO_2 to allow capacitation to occur.

3.2.2. Single cell calcium imaging

Capacitated sperm cell suspensions (see 3.2.1) were split into 200 µL aliquots (6x10^{6} cells/ml) and incubated in labelling solution (7.5 µM Calcium Green-1 AM (Invitrogen, USA) 0.1% (w/v) Pluronic F-127 (Invitrogen, USA) in sEBSS) for 45 minutes at 37^{o} C 6% CO_2. Cell suspensions were washed from the labelling media (300 RCF, 7.5 minutes), pellet re-suspended in dye-free sEBSS and cells left to rest for at least 10 minutes (37^{o} C 6% CO_2) to allow for washing-off of excess dye and completion of any remaining dye de-esterification before the start of the experiment. An aliquot (110 µL) of the cell suspension was then

loaded into a purpose-built perfusable imaging chamber where the lower glass coverslip had previously been coated with 0.1% Poly-D-lysine (BD, USA). The loaded imaging chamber was incubated at 37^{o} C 6% CO_2 for a further 10 minutes, to allow cells to attach to the Poly-D-Lysine-coated surface, and then linked to a gravity-driven eight-valve perfusion system (Harvard Apparatus, USA) adjusted to a 1 ml/min flow rate.

In standard single-cell calcium imaging experiments, cells were kept under continuous sEBSS perfusion, allowing for an initial 10 minute control period before any test solution being presented. Stock solutions of the different odorants tested were initially prepared in dimethyl sulfoxide (DMSO) and dispersed in sEBSS on the day of the experiment to their final working concentration ([DMSO] < 0.001%). Odorants were delivered as four sequential 20 seconds applications interleaved with 5 minute rest periods, where cells were perfused with sEBSS alone, and 3.2 µM progesterone was applied at the end of each experiment to assess cell's capacity to develop calcium transients. Fluorescence recordings, indicating $[Ca^{2+}]_i$, were taken at room temperature (≈ 25° C) using an inverted microscope (Nikon TE300) illuminated with a blue LED – 488 nm (CAIRN Research Ltd, Kent, UK). Images were acquired at 40X magnification using a CCD camera (Quant-Em 512 – Photometrics, USA) running at 0.4 Hz. Perfusion switching (Warner Instruments VC8 valve controller), data acquisition and storage were controlled by a PC running MetaMorph acquisition software (Molecular Devices, USA). Data were processed offline using image analysis software (Image-Pro Analyser, Media Cybernetics, MD, USA). Briefly, a single region of interest (ROI) was drawn for each sperm cell (to include head and midpiece) and mean intensity values were extracted for each time point. All ROIs were individually assessed for correct placing around the cell at each time point and excluded from later analysis if cells had moved. As previously described (Kirkman-

Brown et al., 2000), raw intensity values were exported to Microsoft Excel and normalized using the equation

$$R = \left[\frac{F - F_{rest}}{F_{rest}}\right] \times 100\%$$

where *R* is normalized fluorescence intensity, *F* is fluorescence intensity at time *t* and F_{rest} is the mean of 20 determinations of *F* taken during the control period (rest period before first application of agonist). A cell was considered to have developed a measurable calcium transient if the condition

$$M_p - CI_{98\%}(M_p) > M_c + CI_{98\%}(M_c)$$

was satisfied, where M_p is the arithmetic mean of the five highest values within the 50 seconds period immediately after odorant application, M_c is the arithmetic mean of the five highest values within the 50 seconds period immediately before odorant application, and $CI_{98\%}$ is the mean's 98% confidence interval (α = 0.02, normal distribution), indicating the reliability of the estimate. This approach to identify calcium transients is a minor variation of the previously described method (Kirkman-Brown et al., 2000) and results from an adaptation of the former to this work's experimental conditions.

For the high-speed single-cell calcium imaging experiments, designed to investigate sub-cellular onset of Ω-evoked calcium signals, cells were prepared / capacitated as described (3.2.1) and, unless otherwise stated, set-up was identical to standard experiment described above. Cells were exposed to a single 20 second application of 50 µM Ω and florescence recordings were taken at 60X magnification for 70 seconds from agonist application, at 12.5 Hz. Response delays for individual cells were defined as the time (s) between Ω application (start of fluorescence recording – T0) and the time point where average fluorescence

variation from control period (20 time points from T0) exceeded 30% of the amplitude of the corresponding peak.

For experiments aiming to elucidate which pathways are involved in the transduction of the Ω-evoked calcium signal, set-up was the same as described above for the standard single-cell calcium imaging experiment. Inhibitors tested were: 10 µM SQ 22536 (mAC inhibitor – cAMP pathway), 25 µM cAMPS-Rp (cAMP analogue that acts as a competitive antagonist of the cAMP-induced activation of PKA – cAMP pathway), 5 µM U 73122 (PLC inhibitor – IP_3 pathway) and 10 µM NNC 55-0396 (T-type calcium channel blocker). As described above for the standard experiment, cells were exposed to four 20 seconds applications of Ω at 50 µM, but in this case the time lapse included an extra 10 minutes perfusion period between the second and third applications of Ω, where cells were exposed to the appropriate concentration of the various inhibitors tested, and also, the third application of Ω was done in presence of inhibitor. Inhibition was defined as the ratio of cells that, responding to Ω applications 2 and 4, did not respond to application of Ω together with inhibitor.

3.2.3. Chemotaxis assay

In order to probe odorant molecules for potential chemotactic effects, a simple experimental design similar to the sperm accumulation in a capillary assay described by Ralt et al., 1994, was applied whereby aliquots of motile spermatozoa suspension (3.2.1) were exposed to either Ω (5, 50 or 500 µM; 0.01, 0.001 or 0.0001% DMSO respectively), bourgeonal (5, 50 or 500 µM; 0.01, 0.001 or 0.0001% DMSO respectively) or sEBSS + 0.01, 0.001 or 0.0001% DMSO (as control solutions for 5, 50 or 500 µM odorant concentrations,

respectively). Briefly, spermatozoa pre-labelled with 3 µg/ml Hoechst 33342 (Invitrogen, USA) were divided into 12 20 µL aliquots in 0.2 ml reaction tubes.

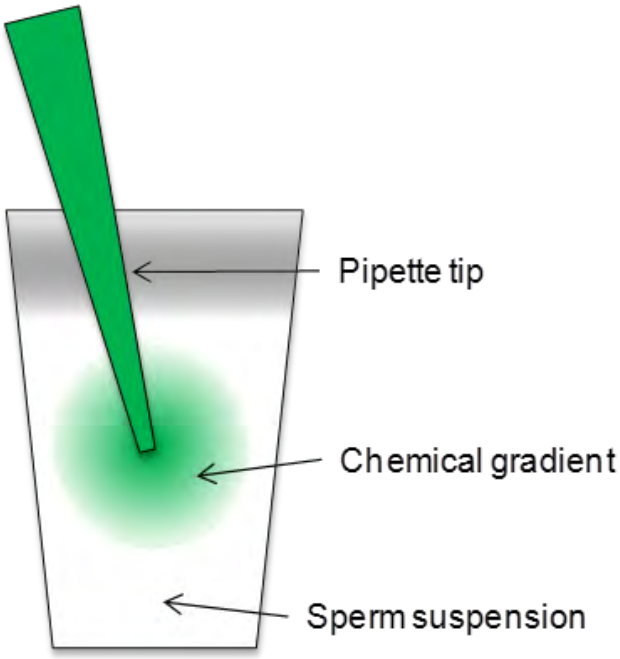


Figure 3.1 – Chemotaxis assay set-up

A multi-channel micro pipette was loaded with the different test solutions and controls (20 µL in each pipette tip) and tips dipped into the 20 µL sperm suspensions (Figure 3.1).

The gradient formation for the different odorant concentrations (Figure 3.2) was calculated according to the formula

$$T = \frac{L^2}{D}$$

where *T* is time (seconds), *L* is length (cm) and *D* is diffusion coefficient (cm^2/s). The formula describes diffusion of a chemical in a still fluid, from a 'blob' source which has constant chemical concentration, and calculations assume a constant diffusion coefficient of 5.7×10^{-6} cm^2/s, calculated according to Hayduk-Laudie method (Hayduk and Laudie, 1974), appropriate for modelling diffusion of low charge molecules in aqueous solutions.

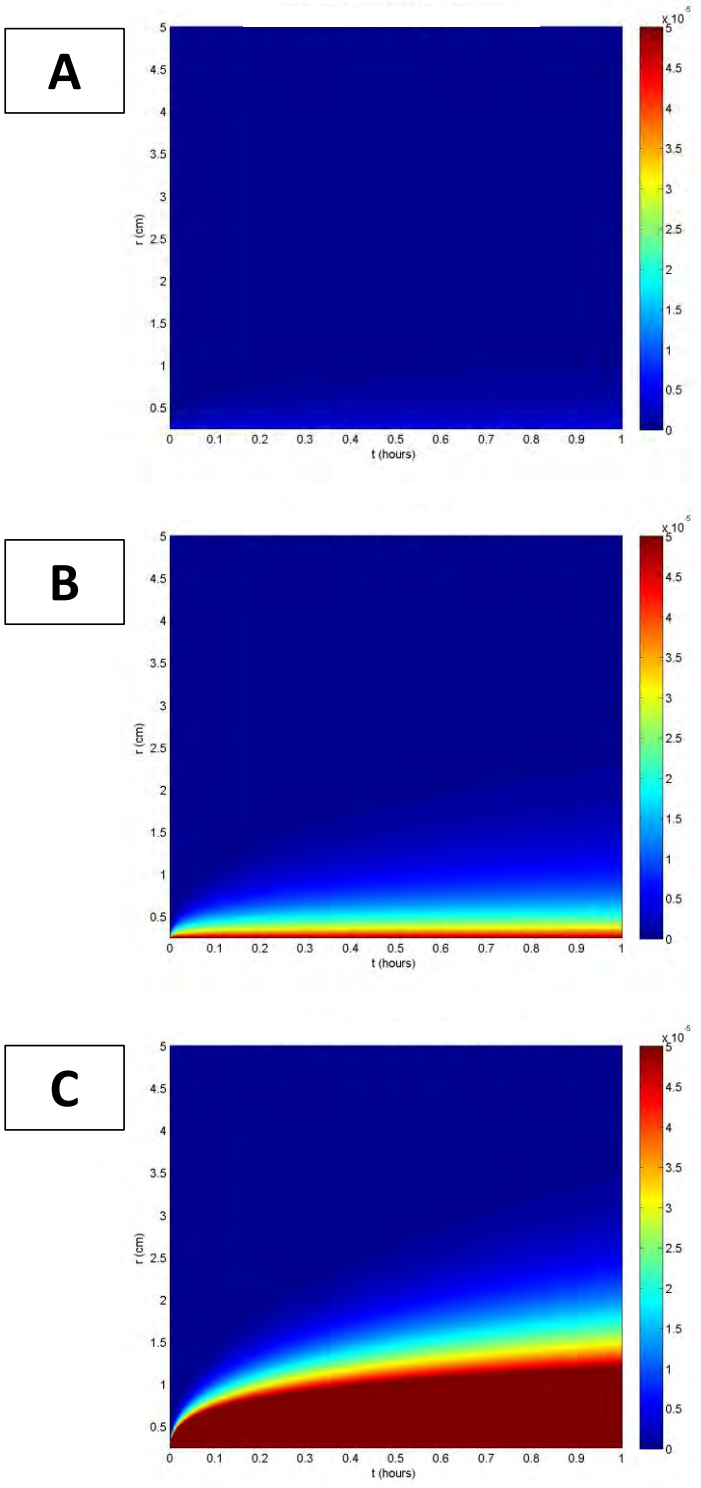


Figure 3.2 – Gradient formation in chemotaxis assay set-up for different odorant concentrations
Gradient formation for **A.** 5 µM, **B.** 50 µM and **C.** 500 µM concentrations of Ω and bourgeonal.

Different solutions (odorants and controls) were tested in duplicate and set-up was incubated for one hour at 37^{o} C 6% CO_2. Individual volumes in the pipette tips were then transferred to a clean 0.2 ml tube, incubated at 65^{o} C for 10 minutes and centrifuged at 4000 RCF for 10 minutes. Supernatant was discarded and cell pellet re-suspended in 10 µL H_2O and loaded into 20 µm CASA slides (Leja, Netherlands). The whole volume on the CASA slide was scanned at 10X magnification on a Nikon TE300, coupled to a CCD camera (Quant-Em 512 – Photometrics, USA) and illuminated with a UV LED – 365 nm (CAIRN Research Ltd, Kent, UK). Fluorescently labelled cells were scored offline on a PC running MetaMorph imaging software (Molecular Devices, USA).

3.2.4. Acrosome reaction

For the assessment of Ω-induced acrosome reaction, cells were prepared / capacitated as described in section 3.2.1. After capacitation period, sperm suspension was centrifuged at 300 RCF for 7 minutes (2X) and cell pellet re-suspended in phosphate buffered saline (PBS) to remove any excess BSA. Cell suspension was adjusted to 6X10^{6} cells/ml and divided into 100 µL aliquots. Spermatozoa were stimulated with either Ω (5, 50 or 500 µM; 0.01, 0.001 or 0.0001% DMSO respectively), calcium ionophore (10 µM A23187 – positive control) or sEBSS + 0.01, 0.001 or 0.0001% DMSO (as negative control for 5, 50 and 500 µM Ω, respectively). The reaction mixtures were incubated for one hour at 37^{o} C 6% CO_2 and 3 µM propidium iodide was added 10 minutes before the end of the incubation period to distinguish non-viable spermatozoa. Mixtures were then centrifuged at 300 RCF for 7 minutes and cell pellets re-suspended in 40 µL PBS. Cells were smeared onto microscope slides pre-coated

with 20 µg/ml poly-L-lysine and air-dried. Smears were then permeabilised in 100% methanol for one minute, air-dried and stained with 1 µg/ml FITC-PSA (Sigma-Aldrich, USA) for one hour at 37º C. Slides were washed three times with PBS, air-dried and mounted using Fluoromount (Sigma-Aldrich, USA). Slides were left overnight at 4º C for mounting to set, and acrosome reaction scored under fluorescent microscopy as described by (Mendoza et al., 1992). Each result is an average of 2 replicate slides, with >200 cells being scored in each slide, and Ω-induced AR was expressed as AR stimulation, according to the formula

$$AR_{stim} = \frac{R - C}{I - C} \times 100$$

where *R* is the percentage of acrosome reacted spermatozoa (AR) in the test mixture, *C* is percentage AR in control mixture and *I* is the percentage AR in suspension treated with calcium ionophore A23187.

3.2.5. Migration assay

Effect of Ω on sperm migration was investigated using a modified Kremer test (hyaluronate migration test – HMT) (Kremer, 1965). Briefly, 50 x 4 x 0.4 mm glass capillary tubes (CM Scientific, UK) were filled by capillary action with 1% methylcellulose (4000cp viscosity on a 2% solution at 20°C – Sigma-Aldrich, UK) made up in sEBSS, sealed at one end with Cristaseal (Hawksley, UK) and equilibrated at 37º C 6% CO_2 on a humidified chamber. Fresh semen samples were allowed to liquefy at room temperature for 30 minutes, divided into 50 µL aliquots in BEEM capsules (Biopoint, UK) and treated in duplicate with either 50 µM Ω or sEBSS + 0.001% DMSO. The capillaries were then lowered into each BEEM capsule and test was incubated at 37º C 6% CO_2 for one hour, after which number of spermatozoa at 1, 2, 3

and 4cm migration distances (total of four fields of view per distance) were assessed microscopically using a 20X phase contrast objective.

3.2.6. Motility

Computer assisted semen analysis (CASA) was used in order to investigate the effects of Ω in sperm motility. Sperm suspensions were prepared as described in section 3.2.1, treated either with 50 µM Ω (0.001% DMSO) or sEBSS + 0.001% DMSO, and loaded into 50 µm depth CASA slides (Conception Technologies, USA). Motility parameters were assessed at times 0, 5, 15, 30 and 60 minutes after exposure to the test solution by CASA (Hamilton Thorne, USA). Cells were considered to exhibit hyperactivated motility if their track speed (VCL) was > 150 µm/s, their linearity (LIN) was < 50% and their lateral head displacement (ALH) was either > 5 µm (cells classed as sort 5) or > 7 µm (cells classed as sort 7).

3.3. Results

3.3.1. Ω evokes Ca^{2+} signaling events in human spermatozoa

Based on firm proteomics evidence substantiating ectopic expression of OR2AE1 in mature human sperm (Baker et al., 2007; Lefievre et al., 2007), investigations of potential functional roles this particular receptor might play on these cells was started by assessing whether, similarly to what was observed in the heterologous expression system, its newly found agonist Ω (see section 2) would also trigger Ca^{2+}-mediated signaling events in human sperm. Figure 3.3 illustrates a typical single-cell Ca^{2+} imaging experiment in which, as described in section 3.2.2, sperm cells were sequentially exposed to four 20 second applications of 50 µM

Ω. In this particular example, one of the cells (cell 15) shows no noticeable variation in fluorescence after all Ω applications, whilst still capable of generating calcium signals as evidenced by response to 3.2 µM progesterone. Contrastingly, cell 254 responds to three of the four 50 µM Ω applications (App 2, 3 and 4), with fluorescence increases of around 25-30%.

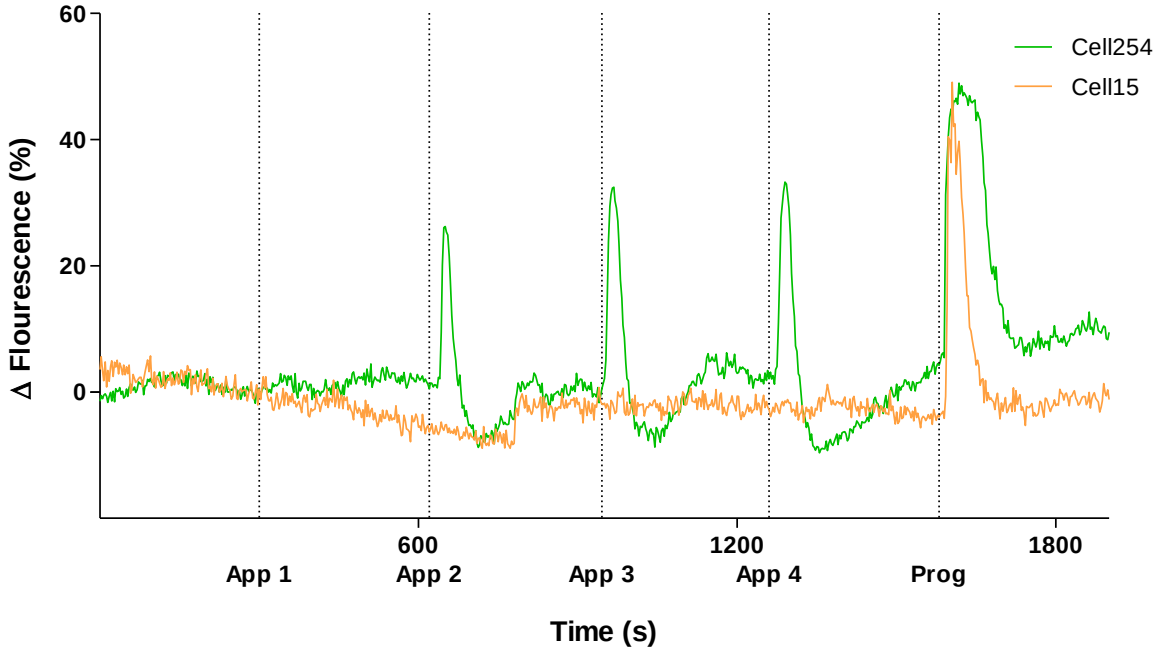


Figure 3.3 – Ca^{2+} responses to sequential applications of 50 µM Ω
Typical $[Ca^{2+}]_i$ recording: traces show normalized fluorescence plot for 2 sperm cells labelled with 7.5µM Ca^{2+} Green and perfused 4 x 20s with 50µM Ω. 3.2µM progesterone applied at the end of the experiment to verify physiological state and ability to generate Ca^{2+} signals.

As 50 µM Ω caused measurable Ca^{2+} transients in sperm, the next set of experiments aimed to preliminarily delimit an effective concentration range of the OR agonist capable of triggering the observed signaling events.

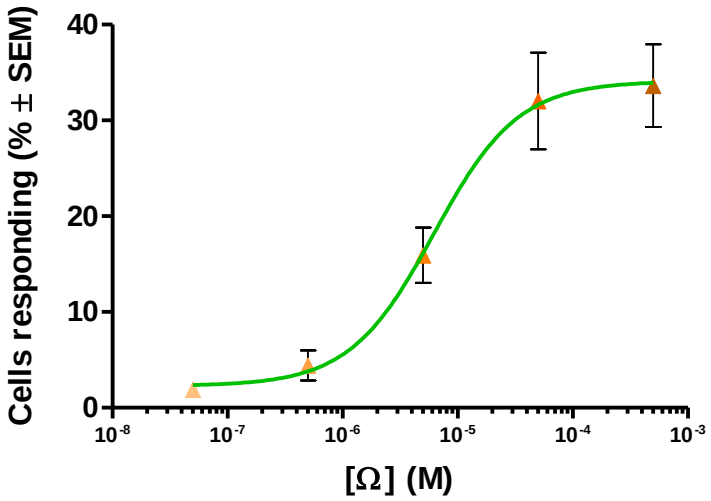


Figure 3.4 – Dose-response curve for Ca^{2+} responses evoked in mature sperm by Ω
Sperm cells were allowed to capacitate for 5 hours, labelled with 7.5µM Ca^{2+} Green and exposed to 20s applications of increasing Ω concentrations ($5x10^{-8}$, $5x10^{-7}$, $5x10^{-6}$, $5x10^{-5}$and $5x10^{-4}$ M). Data, plotted as percentage of responding cells ± SEM, consists of 4 experiments, each including an average of 280 cells. Best-fit values derived from variable curve fitting model, with a Hill coefficient of 1.2.

Exposure of sperm to increasing Ω concentrations resulted in an increase in the number of cells initiating calcium signals (Figure 3.4). The percentage of the cell population responding to Ω increases from 1.9±0.5% for 50 nM, to 33.6±4.3% for 500 µM. This increase was more evident between 500 nM and 5 µM (11.5%) and between 5 µM and 50 µM (16.1%), with the nonlinear variable curve fitting resulting in a typical sigmoidal shaped curve (R^2 = 0.842, *df* = 26) with an EC_{50} of $6.2X10^{-6}$ M and a Hill coefficient of 1.2.

3.3.2. Variability of Ω-evoked signaling

Further testing of 50 µM Ω across different donors revealed a substantial degree of variability in the percentage of cells responding to the agonist, both within and across donors (Figure 3.5). Amplitude in the percentage of cells responding to the third application

of agonist within sperm donors ranged from 6.6% for D700, to 32.2% for D600. Pooled results ranged from a minimum of 9.4% to a maximum of 46.6%, with a median value of 21.2%.

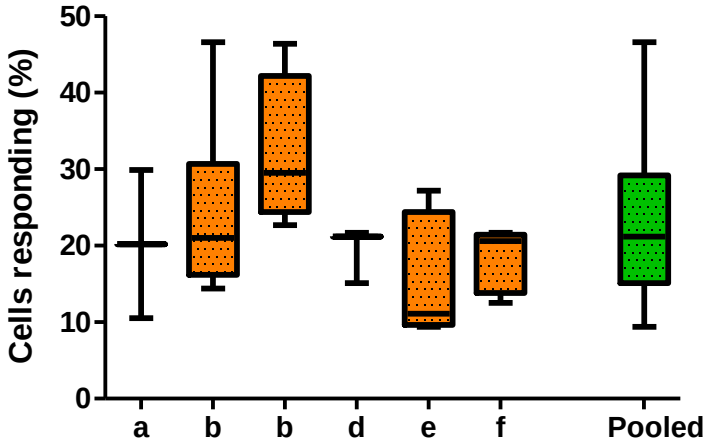


Figure 3.5 – Variability of Ω-evoked Ca^{2+} responses in capacitated sperm from different donors
Sperm cells were allowed to capacitate for 5 hours, labelled with 7.5µM Ca^{2+} Green and exposed to four sequential applications of 50 µM Ω, each lasting 20s. Ω applications were intercalated with 5 minute rest periods where cells were perfused with media alone. Data consists of 27 experiments across 6 donors, each including an average of 280 cells. Boxplots show response distribution to the third application of Ω within individuals (individual donors, in orange) and within donor population (pooled results for all donors, in green); whiskers represent minimum and maximum percentage cells responding to agonist.

Experimental data gathered to this stage pointed to a complex response pattern within the sperm cell population, with some cells developing Ω-dependant calcium transients from the first exposure to the agonist, and others only responding to Ω after one or more previous applications. A subset of experiments were conducted to further investigate this variability. As described in section 3.2.2, capacitated spermatozoa were exposed to four 20 second applications of 50 µM Ω, intercalated with 5 minute rest periods, where cells were maintained under continuous perfusion with sEBSS. The average percentage of cells developing calcium transients in response to agonist exposure increases from 11.7±1.8% for

App 1, to 29.1±4.5% for App 4 (Figure 3.6). The percentage of cells responding to second, third and fourth applications was found to be significantly higher than cells responding to the first application (one-way ANOVA, $F_{(1,11)}$ = 9.822, p<0.01), indicating that the first exposure to Ω has a priming effect on the cells' responsiveness to the odorant.

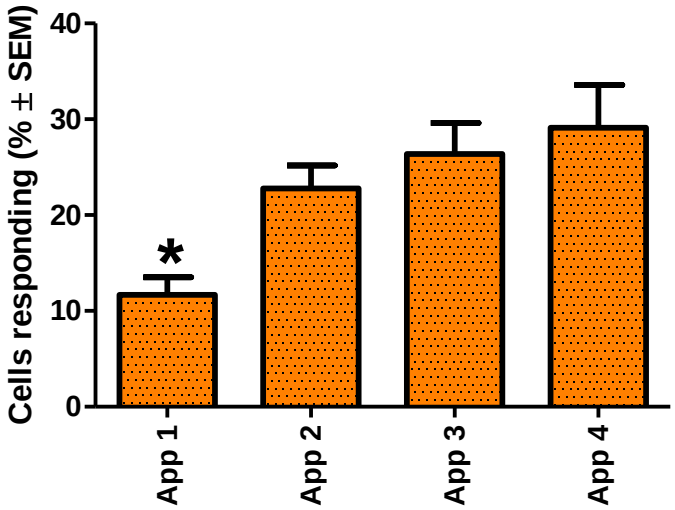


Figure 3.6 – Distribution of human sperm population responding to successive applications of 50 µM Ω
Sperm cells were allowed to capacitate for 5 hours, labelled with 7.5µM Ca^{2+} Green and exposed to four sequential applications of 50 µM Ω, each lasting 20s. Ω applications were intercalated with 5 minute rest periods where cells were perfused with media alone. Data, plotted as percentage of responding cells ± SEM, consists of 13 experiments, each including an average of 290 cells. * indicates significant difference between groups (one-way ANOVA, $F_{(1,11)}$ = 9.822, p<0.01).

3.3.3. Ω-evoked Ca^{2+} signaling in sperm is capacitation dependent

Another indication arising from preliminary experiments testing Ω's ability to evoke calcium signaling in sperm was that this effect appeared to be dependent on the time cells were allowed to capacitate. Results for percentage of cells responding to the third application of Ω (results collected from 36 experiments with identical format – four sequential agonist applications spaced by 5 minute rest intervals) were compiled and analysis of the association

between capacitation time and the percentage of cells responding to Ω shows a high positive correlation between both variables (Figure 3.7), illustrated by the two-tailed Pearson's correlation coefficient (r=0.57, p<0.0003).

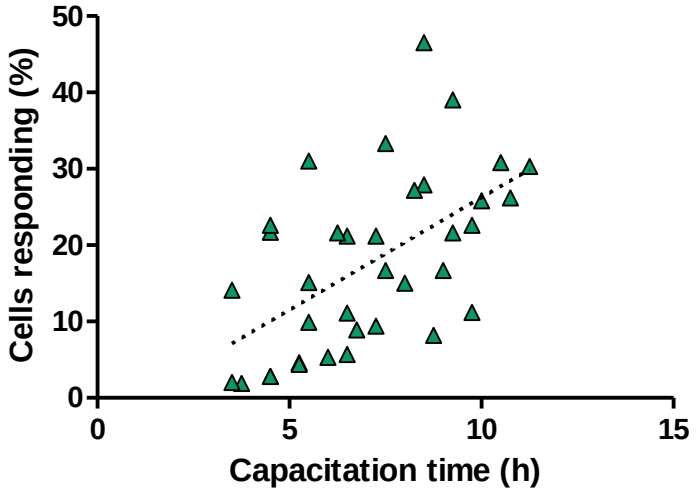


Figure 3.7 – Association between Ca^{2+} responses evoked by Ω in mature sperm and sperm cell capacitation
Dashed line represents linear regression between capacitation time and percentage of cells responding to third application of 50 µM Ω (two-tailed Pearson's correlation coefficient, r=0.57, p<0.0003). Data compiled from 36 experiments, each including an average of 250 cells.

3.3.4. Ω triggers chemotactic responses in sperm

Following on previous results revealing Ω as capable of eliciting signaling events in human sperm, the next step in this research involved investigating whether these events actually translated to any measurable changes in sperm cell function. To this end, an experimental method was designed to test odorant molecules for potential chemotactic effects in motile sperm (see section 3.2.3). Ω, an odorant molecule identified in the current work as capable of activating the olfactory receptor OR2AE1, was tested alongside bourgeonal, another

odorant previously reported to exhibit a potent chemotactic effect in human sperm (Spehr et al., 2003) (Figure 3.8).

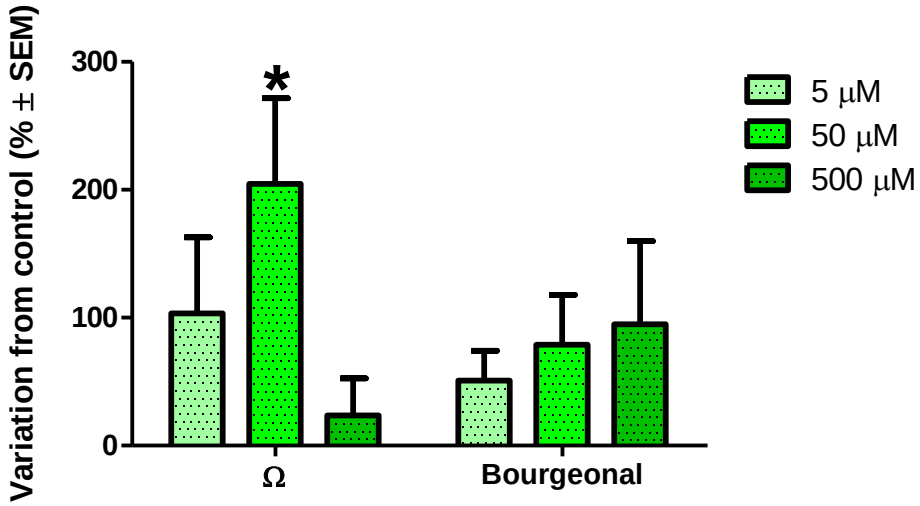


Figure 3.8 – Effect of Ω and bourgeonal in human sperm chemotaxis
Chemotactic effect evoked by Ω and bourgeonal in motile sperm. Data, plotted as percentage variation from control ± SEM includes 13 replicates across 6 donors; * indicates significant difference between control and test stimuli (2-tailed Wilcoxon signed ranks test, Z=-3.110, p<0.02).

5, 50 and 500 μM solutions of both odorants all caused a measurable increase in the number of cells in the pipette tip volume, but this increase was only significantly different from control (2-tailed Wilcoxon signed ranks test, Z=-3.110, p<0.02) for 50 μM Ω, with an average of 204.6±67.0% more cells travelling into the pipette volume than in control. Interestingly, for bourgeonal the chemotactic effect increases steadily with increasing concentrations (50.9±23.2, 78.7±39.1 and 94±64.9% for 5, 50 and 500 μM respectively), whereas for Ω this effect increases from 103±59% (for 5 μM) to 204.6±67% (for 50 μM) but for 500 μM Ω the chemotactic effect drops radically to 23.5±29.1%.

3.3.5. Effect of Ω on acrosome reaction

Another sperm function investigated was the ability to undergo the acrosome reaction. As described in section 3.2.4, acrosome reaction was measured as AR stimulation, which excludes spontaneous AR in the cell population and indexes maximum stimulation to AR levels observed in cells exposed to calcium ionophore. Concentrations of 5, 50 and 500 µM Ω were tested for their ability to trigger the acrosome reaction in motile spermatozoa (Figure 3.9). Both 5 and 50 µM Ω failed to cause a significant variation in the acrosome reaction rate (2-tailed Wilcoxon signed ranks test, Z=-1.992, p<0.046), with AR stimulations of 1.2±2.1% and 0.1±2.8% for 5 and 50 µM Ω respectively. However, when exposed to 500 µM Ω, sperm cells displayed a significantly higher acrosome reaction rate than control (Appendix II: Supplementary Data, Supp. Figure 3), as illustrated by a stimulation rate of 24.6±15.7%.

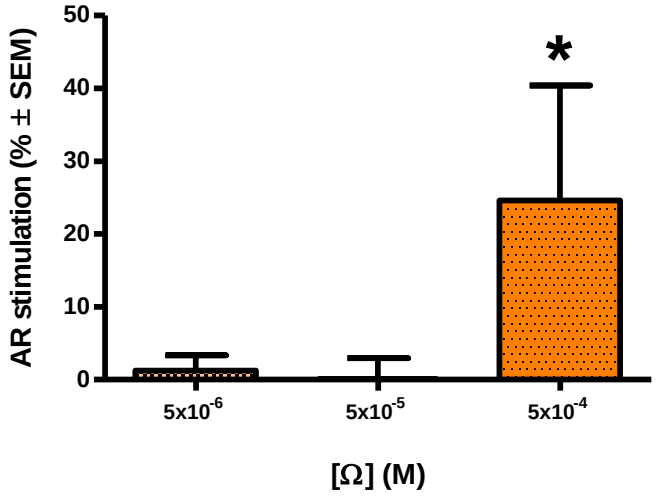


Figure 3.9 – Effect of Ω on acrosome reaction
Acrosome reaction triggered by 5, 50 and 500 µM Ω in mature human sperm. Data, plotted as percentage AR stimulation ± SEM, includes 6 experiments across 6 donors. * indicates a significant difference between control and test stimuli (2-tailed Wilcoxon signed ranks test, Z=-1.992, p<0.046).

3.3.6. Effect of Ω on sperm migration and motility

The effect of Ω on sperm migration was also assessed (). As described in section 3.2.5, spermatozoa were exposed to 50 µM Ω and allowed one hour to travel up a microcapillary pre-filled with a 1% methylcellulose solution (modified Kremer test – Kremer, 1965). The choice of this assay was based on its approximation to the conditions sperm might encounter in vivo, with the solution's viscosity closely mimicking that of cervical mucus, and on balance, the absence of a suitable positive control represented a minor caveat.

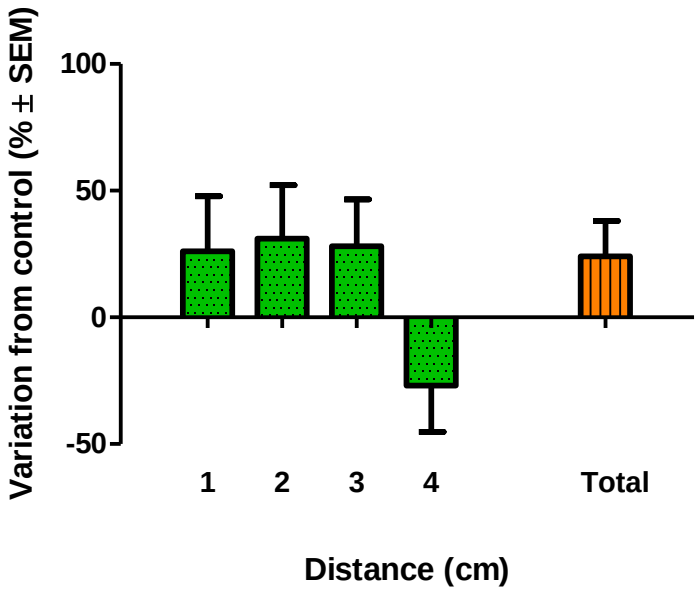


Figure 3.10 – Effect of Ω on sperm migration
Migration was scored for four distances along the capillary (1, 2, 3 and 4 cm, in green; sum of cells scored at all distances, in orange). Data, plotted as percentage variation from control ± SEM, consists of 6 experiments across 6 donors.

The number of cells scored at 1, 2 and 3 cm was on average 28% higher for samples exposed to the odorant than in control, whereas at 4 cm there were 27% less cells than in control.

Overall, number of cells scored at all distances was 24% higher for samples exposed to 50 µM Ω than for control samples, but these differences did not prove statistically significant (one-way ANOVA).

Motility changes evoked by 50 µM Ω were also investigated by computer assisted sperm analysis (CASA). As described in section 3.2.6, motile cells were isolated and incubated in capacitating medium for 5 hours. Spermatozoa were then exposed to either control solution (sEBSS) or 50 µM Ω and their motility assessed at times 0, 5, 15, 30 and 60 minutes after exposure to the test solution (Figure 3.11).

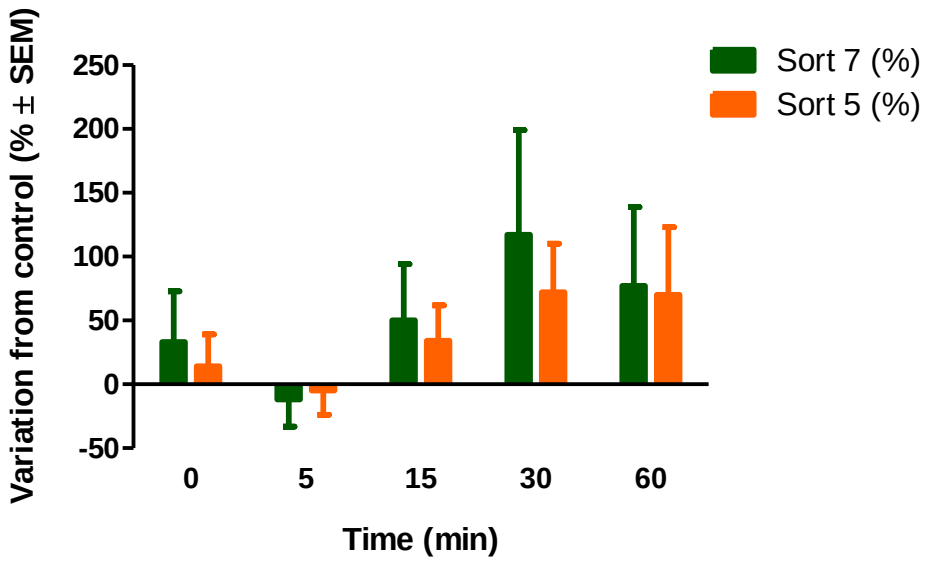


Figure 3.11 – Effects of Ω on sperm hyperactivation
Motile sperm were exposed to 50 µM Ω and motility changes accessed by CASA at 0, 5, 15, 30 and 60 minutes. Data, plotted as percentage variation from control ± SEM, includes 7 experiments across 6 donors. Differences presented proved non-significant (one-way ANOVA, $P > 0.053$).

The cell population exposed to Ω appear to include a higher ratio of sperm exhibiting hyperactive motility, as defined by CASA sorts 5 and 7 (see section 3.2.6). With the exception of time 5 min, for all the other times observed the level of hyperactive cells is higher in

populations exposed to the odorant than in cells treated with the control solution. This is particularly evident at time 30 min, where sort 7 and sort 5 are respectively 117±82% and 72±38% higher in cells treated with Ω than in the control population. These differences, however, did not prove statistically significant (one-way ANOVA). Other motility parameters were also assessed by CASA on cells exposed to 50 µM Ω, but treatment did not cause significant changes when compared with control (Supp. Figure 4 and Supp. Table 1 - Appendix II: Supplementary Data).

3.3.7. Characterisation of Ω-evoked Ca^{2+} signaling

After characterising functional effects evoked by Ω in human sperm, and as described in section 3.2.2, a series of high speed single cell calcium imaging experiments was carried out aiming to gain an insight on the origin of the inherent Ca^{2+} signal within the sperm cell (Figure 3.12). Briefly, motile sperm cells were allowed to capacitate for 5 hours, exposed to a 20 seconds application of 50 µM Ω and intracellular calcium levels monitored at 12.5 Hz for 70 seconds. For each cell included in the analysis two areas of interest (AOIs) were drawn (one including the whole head and the other including the whole midpiece) and onset of the calcium signal was recorded. Delay observations in individual cells ranged from -2.88 s (signal initiated in the head and 2.88 seconds later propagated to the midpiece) to +9.36 s (signal initiated in the midpiece and 9.36 seconds later propagated to the head). Mean delay across cell population was +1.36±0.51 seconds, and a one-sample *t* test revealed a statistically reliable difference between the hypothetical delay of zero and the mean delay reported ($t_{(30)}$=2.678, p<0.012).

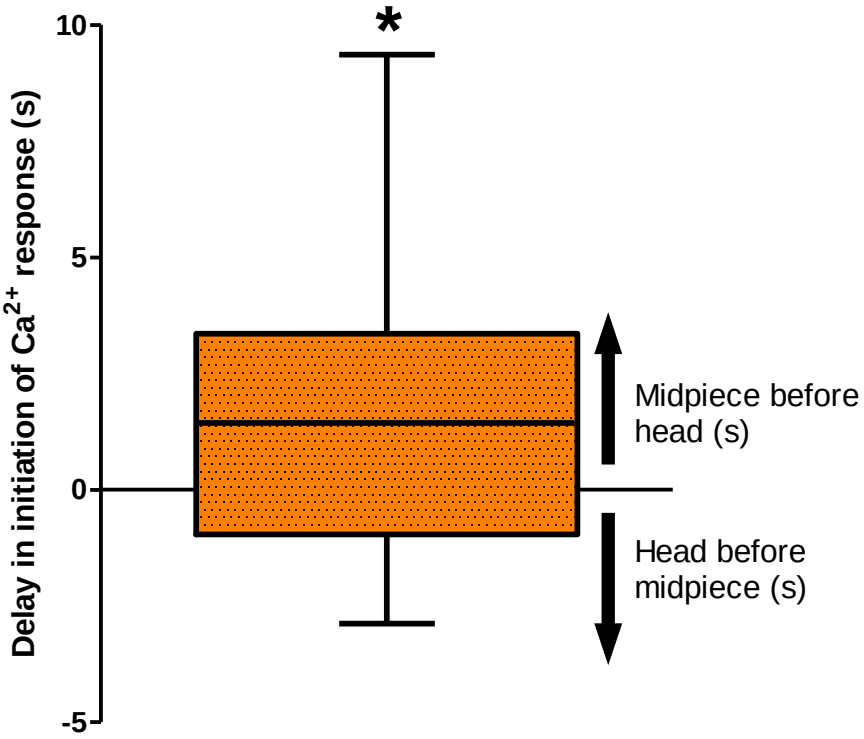


Figure 3.12 – Subcellular onset of Ca^{2+}responses triggered by 50 µM Ω in human spermatozoa
Boxplot illustrates distribution of the Ca^{2+} response onset delay (s) across sperm head and midpiece. Data includes 31 cells and whiskers represent minimum and maximum values. * indicates a significant difference between the hypothetical delay of zero and the mean delay observed (one-sample *t* test, $t_{(30)}$=2.678, p<0.012).

Further to the investigation of the Ω-evoked calcium signal onset, a further set of single-cell calcium imaging experiments were performed, aiming to elucidate which pathways might be involved in the signal transduction observed. Briefly, and as described in section 3.2.2, a series of inhibitors of key players in pathways known to transduce calcium signals were tested. Motile fractions of the semen samples were isolated and incubated in capacitating medium for 5 hours. Cells were initially exposed to two applications of 50 µM Ω on its own and then perfused with appropriate concentration of inhibitor for 10 minutes. A third application of Ω was done in the presence of inhibitor, followed by a fourth application without inhibitor. As described in 3.2.2 (discussed below – section 3.4), inhibition was

defined as the ratio of cells that, responding to Ω applications 2 and 4, did not respond to application of Ω together with inhibitor. Inhibitors tested were: SQ 22536 (mAC inhibitor – cAMP pathway), cAMPS-Rp (cAMP analogue that acts as a competitive antagonist of the cAMP-induced activation of PKA – cAMP pathway), U 73122 (PLC inhibitor – IP_3 pathway) and NNC 55-0396 (T-type calcium channel blocker) ().

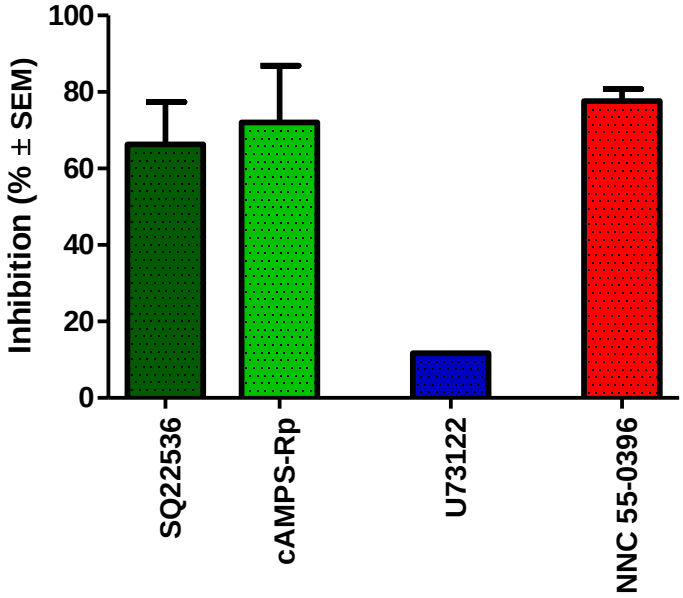


Figure 3.13 – Inhibition of calcium signaling events triggered by 50 µM Ω in human sperm
Inhibitors tested were: SQ 22536 (100 µM), cAMPS-Rp (25 µM), U 73122 (5 µM) and NNC 55-0396 (10 µM). Sperm cells were allowed to capacitate for 5 hours, labelled with 7.5µM Ca^{2+} Green and exposed to three applications of odorant alone and one application of odorant + inhibitor. Data, plotted as percentage inhibition ± SEM (see section 3.2.2 for inhibition definition), consists of 7 (SQ 22536 100), 2 (cAMPS-Rp), 1 (U 73122) and 11 (NNC 55-0396) experiments, each including an average of 290 cells.

Inhibitor concentrations used were chosen in line with published results by other groups where the inhibitor has been tested in human (NNC 55-0396 - Strunker et al., 2011; SQ 22536 – Spehr et al., 2004; U 73122 – Bahat et al., 2010) or bovine sperm (cAMPS-Rp – Galantino-Homer et al., 1997).

Both inhibitors acting on the cAMP pathway (100 µM SQ 22536 and 25 µM cAMPS-Rp) were able to inhibit most of the cells from developing calcium transients in response to 50 µM Ω (66.3±11.1% and 72±14.9%, respectively). Interference on the IP_3 pathway with 5 µM U 73122 resulted in a much lower level of inhibition (11.7%), whereas, blocking T-type calcium channels with 10 µM NNC 55-0396 caused the highest level of inhibition observed (77.6±3.2%).

Importantly, all inhibitors tested had themselves effects on $[Ca^{2+}]_i$ (Figure 3.14). The most dramatic effect was observed when spermatozoa were exposed to 10 µM NNC 55-0396: cells developed an almost instantaneous (10 seconds from application) Ca^{2+} transient, reflected by an increase in fluorescence peaking at around 40%. This transient lasted around 200 seconds, and $[Ca^{2+}]_i$ levels were maintained around 10% above baseline level after the transient for as long as the cells were exposed to the inhibitor. 5 µM U73122 had a similar effect, although the transient's amplitude was lower (peaking at around 10% above baseline), slightly shorter (lasting around 150 seconds) and with a longer onset (20 seconds). In this case, however, $[Ca^{2+}]_i$ levels after the transient tended to raise steadily, from 200 to 600 seconds, to around 10% above baseline. Unlike the previous inhibitors, the rise in $[Ca^{2+}]_i$ triggered by 25 µM cAMPS-Rp was not "transient-like", rather a more or less continuous increase up to around 20% above baseline, with a slower onset (50 seconds). The effect of 100 µM SQ22536 on $[Ca^{2+}]_i$ was not as evident as for the other inhibitors tested, with majority of cells failing to develop Ca^{2+} transients and $[Ca^{2+}]_i$ staying raised, but within 5% from base level.

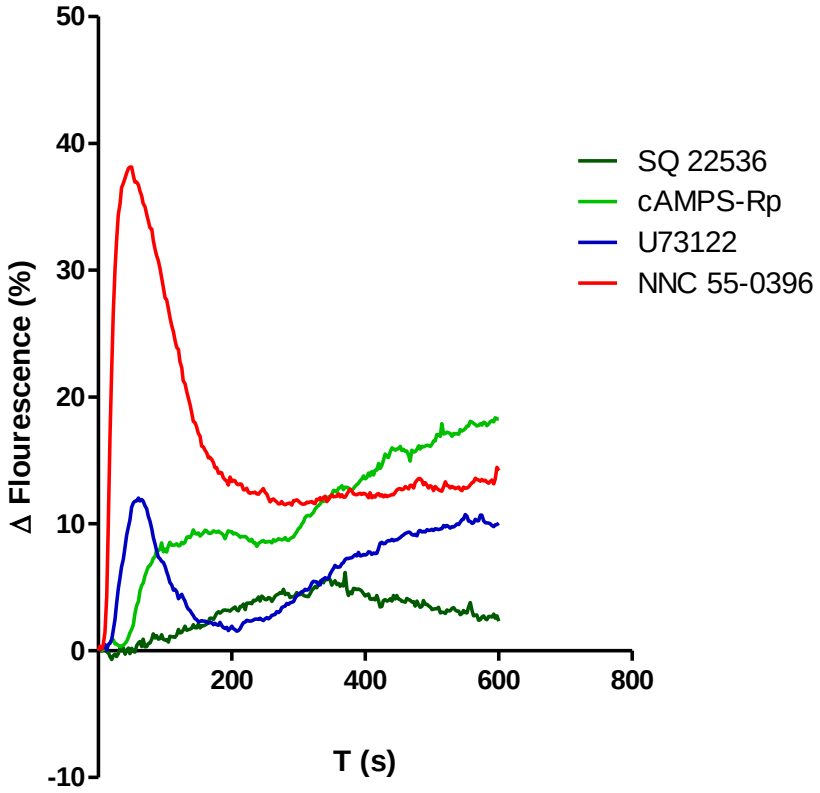


Figure 3.14 – Ca^{2+} transients developed by human spermatozoa when exposed to different inhibitors
Inhibitors tested were: SQ 22536 (100 µM), cAMPS-Rp (25 µM), U 73122 (5 µM) and NNC 55-0396 (10 µM). Sperm cells were allowed to capacitate for 5 hours, labelled with 7.5µM Ca^{2+} Green and exposed to inhibitor for 600 seconds under constant perfusion. Data collected from one representative experiment for each inhibitor and traces, normalised to the start of the application, represent average fluorescence variation from control period (see section 3.2.2) for the whole cell population monitored (average of 290 cells).

Figure 3.15 is a particular example of a typical Ca^{2+} imaging experiment where the aim was to elucidate the pathways involved in the transduction of calcium signals elicited by Ω. This experiment tested the ability of T-type Ca^{2+} channel inhibitor NNC 55-0396 at 10 µM to inhibit Ω-evoked calcium transients. As described in section 3.2.2, cells were exposed to the agonist twice (Ω_1 and Ω_2, for 20 seconds each, intercalated with a 4 minute rest period where cells were perfused with media alone) before being perfused with inhibitor (NNC – 10 µM NNC 55-0396 for 6 minutes). Immediately following perfusion with the inhibitor (no rest

period), cells were exposed to a solution including both agonist and inhibitor (Ω+NNC, 20 seconds). Cells were then exposed to a third application of Ω alone (Ω_3) and a final application of 3.2 μM progesterone (P4), both preceded by 4 minute rest periods.

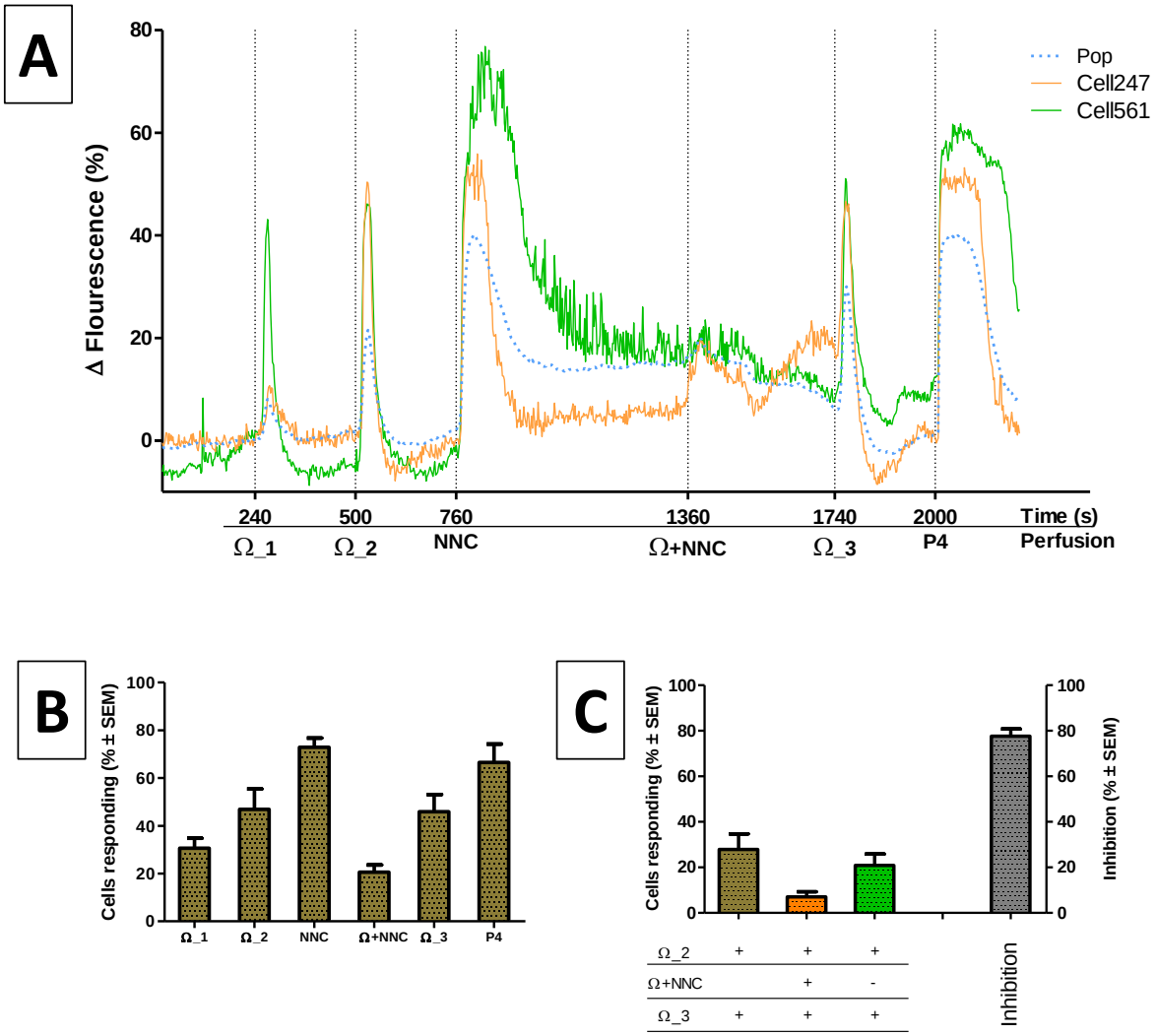


Figure 3.15 – Typical single-cell Ca^{2+} imaging experiment testing inhibition of Ω-evoked Ca^{2+} responses by NNC 55-0396 **A.** Typical $[Ca^{2+}]_i$ recording: traces show normalized fluorescence plot for the whole cell population (blue dashed trace – Pop); cell 247 (orange trace), showing a Ca^{2+} transient when presented with 50 µM Ω in the presence of 10 µM NNC 55-0396; and cell 561 (green trace), showing Ca^{2+} responses to all applications of the agonist on its own but not when this is presented together with the inhibitor. **B and C.** Inhibition of Ω-evoked Ca^{2+} signaling by 10 µM NNC 55-0396; data, plotted as percentage of cells responding ± SEM (B. and C. – left Y axis) or inhibition ± SEM (C. – right Y axis; see section 3.2.2 for inhibition definition), consists of 11 experiments, each including an average of 290 cells. Spermatozoa were labelled with 7.5µM Ca^{2+} Green and 3.2µM progesterone (P4) was applied at the end of the experiment to verify cell's physiological state and ability to generate Ca^{2+} signals.

Traces in Figure 3.15 A. show averaged $[Ca^{2+}]_i$ recordings for the whole cell population observed (dashed blue trace – Pop) and $[Ca^{2+}]_i$ recordings for two individual cells, one which was able to develop a Ca^{2+} transient when co-applied odorant and inhibitor (Cell247 – orange trace), and another which, although responding to all applications of Ω alone, failed to develop a transient when co-applied odorant and inhibitor (Cell561 – green trace),

pointing to an inhibition of the Ω-evoked signal. This was true across samples (Figure 3.15 B. and C.), with 77.6±3.2% of the cells that responded to both Ω_2 and Ω_3 (27.9±6.8% of all the cells analysed) failing to respond when Ω was co-applied with NNC 55-0396. Oddly, exposure to the inhibitor on its own caused measurable Ca^{2+} transients in 72.9±3.8% of the cells (Figure 3.15 A. and B.).

A detailed analysis of single-cell calcium experiments where spermatozoa were exposed to 50 µM Ω in absence/presence of 10 µM NNC-550396, and limiting the analysis to cells that responded to all applications of the odorant alone (Ω1, Ω2 and Ω3 -), revealed two distinct subpopulations: subpopulation A (A), where cells failed to develop measurable calcium transients when odorant was co-applied with inhibitor (Ω + NNC 55-0396), and subpopulation B (B), where cells developed a transient even in the presence of inhibitor (A, red trace), although with much lower amplitudes than transients evoked by agonist alone in the same population.

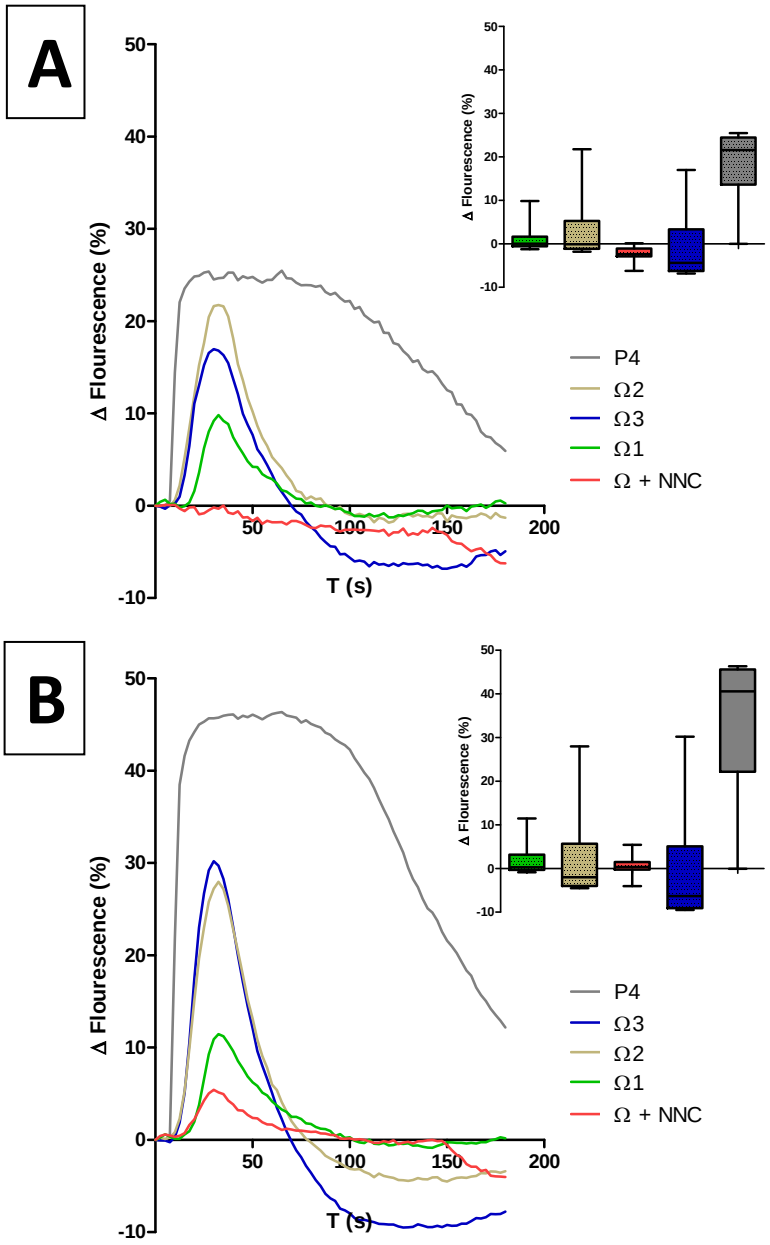


Figure 3.16 – Kinetics of Ω-evoked Ca^{2+}-transients
Data represent fluorescence variations averaged from: **A.** 65 cells responding to applications of agonist alone (Ω1, Ω2 and Ω3 – green, brown and blue traces, respectively; Ω at 50 µM), but show no significant calcium transients when Ω was presented together with T-type calcium channel blocker NNC-550396 at 10 µM (Ω + NNC – red trace); **B.** 407 cells showing significant calcium transients for all agonist applications, both in absence (Ω1, Ω2 and Ω3) and presence (Ω + NNC) of T-type calcium channel blocker NNC-550396 (10 µM). In both A and B cells where exposed to a final application of 3.2 µM progesterone (P4 – grey trace) to verify physiological state and ability to generate Ca^{2+} signals. Inset boxplots show response distribution within cell populations observed (whiskers represent minimum and maximum fluorescence variation).

Table 2 summarises the differences in the response kinetics parameters from both populations. Response amplitudes were generally higher in subpopulation B, and this was particularly evident for the second and third applications of agonist alone (Ω2 and Ω3), together with progesterone, for which amplitude in population B is almost double the one observed for population A. On the other hand, transient durations for the first Ω application (Ω1) are higher in subpopulation B, whereas the delay between the application of the agonist and the onset of the transient is slightly lower than the one observed in subpopulation A.

Table 2 – Kinetics parameters for Ω-evoked calcium signals in sperm cell subpopulations A and B in absence/presence of T-type calcium channel inhibitor NNC 55-0396.

Parameter	*Ω1*		*Ω2*		*Ω+NNC 55-0396*		*Ω3*		*P4*	
	A	B	A	B	A	B	A	B	A	B
Amplitude (%)	10.4	11.5	22.5	30.9	-	5.4	22.2	37.8	25.5	46.4
Duration (s)	55	75	62.5	57.5	-	75	50	50	170	170
Delay (s)	20	15	12.5	12.5	-	15	15	15	7.5	10

Additional to experiments above looking at potential effects by a range of inhibitors on Ω-evoked Ca^{2+} transients, and because undecanal has previously been reported as a potent OR antagonist, suppressing signaling events elicited by bourgeonal and related compounds via OR1D2 (Spehr et al., 2003), a set of experiments was conducted to investigate if this chemical would also prove capable of antagonising Ω-evoked signaling (). There was a dramatic difference in the number of cells developing Ca^{2+} transients in response to both bourgeonal and Ω between capacitating and non-capacitating conditions: uncapacitated

cells responded to both agonists at a much lower rate than cells maintained under capacitating conditions (A).

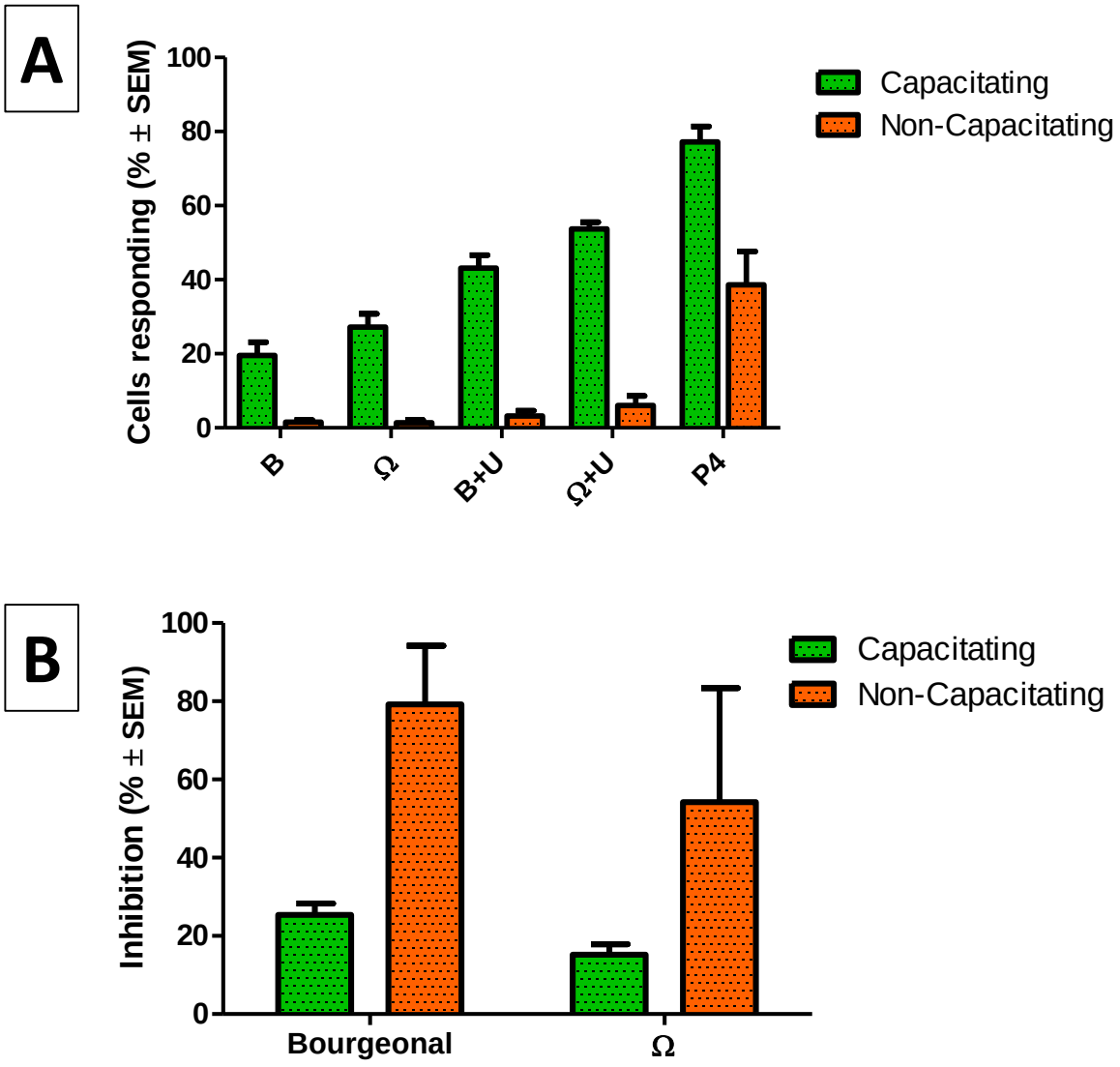


Figure 3.17 – Antagonistic effect of undecanal on Ω and bourgeonal-evoked Ca^{2+} signaling in capacitated and uncapacitated spermatozoa

A. Ca^{2+} signaling evoked by 50 µM bourgeonal and 50 µM Ω in presence/absence of equimolar undecanal, and **B.** corresponding inhibition (see section 3.2.2 for inhibition definition) of these signaling events by undecanal, both in capacitating and non-capacitating media. Motile spermatozoa were isolated by swim-up into sEBSS (capacitating media) or tyrode (non-capacitating media). Cells where exposed to a final application of 3.2 µM progesterone to verify physiological state and ability to generate Ca^{2+} signals. Data consists of 5 (capacitating conditions) and 3 (non-capacitating conditions) experiments, averaging 280 cells each.

As expected from previous observations, 50 µM Ω elicited Ca^{2+} signals in 27.2±3.6% of the cell population, a value about 20 times higher than the one obtained under non-capacitating

conditions (1.4±0.7). Similarly, whereas under capacitating conditions 50 µM bourgeonal triggered calcium responses in 19.5±3.6% of cells, in non-capacitating media this value was a lot lower (1.5±0.6%).

When co-presented with equimolar undecanal under capacitating conditions, 50 µM Ω caused Ca^{2+} transients in a larger number of cells (53.7±3.8%) than when presented on its own. The same happened under non-capacitating conditions, with 6.0±2.6% of cells developing Ca^{2+} transients to the Ω-undecanal mixture. Similarly, the level of responses to co-application of bourgeonal and undecanal was found to be higher than when bourgeonal was presented alone, both under capacitating (43.1±3.5%) and non-capacitating conditions (3.2±1.4%). The antagonistic effect of undecanal was assessed by looking only at the cells that responded to the initial application of either agonist and account for the ones that fail to develop measurable transients when the agonists were co-presented with undecanal (B). Undecanal inhibition of Ω and bourgeonal-evoked signaling was found to be much higher under non-capacitating conditions than when cells were allowed to capacitate (54.2±29.2% versus 15.2±2.7% for Ω, and 79.2±15% versus 25.4±2.9% for bourgeonal).

3.4. Discussion

OR2AE1, an olfactory receptor with no identified agonists before the current work, is known to be ectopically expressed in mature human spermatozoa (Baker et al., 2007; Lefievre et al., 2007). In chapter 2 a volatile organic compound, designated Ω, was identified as a potent agonist of the OR2AE1 receptor in a heterologous expression system via measurement of evoked calcium transients. As the starting information was native receptor protein

expression in sperm the current chapter has focused upon potential functional effects mediated by OR2AE1 in mature human sperm.

Initial assessments were performed to confirm that Ω was able to trigger calcium signaling events in mature human spermatozoa. Capacitated sperm cells were exposed to 50 µM Ω, a concentration which proved effective in evoking calcium transients in the heterologous expression system (Figure 3.3), this induced calcium transients in a subpopulation of spermatozoa, with fluorescence variations of around 30%. These transients lasted around 60 seconds () and proved to be re-evocable within minutes of previous stimulation.

As 50 µM Ω was effective in induction of calcium signaling an experimental series was undertaken testing Ω concentrations between 50 nM and 500 µM to define the effective concentration range. The dose-response curve obtained resulted in a typical sigmoid curve with an EC_{50} of $6.2X10^{-6}$ M (nonlinear curve fitting, Figure 3.4), indicating a response saturation between 50 and 500 µM, with the maximum percentage of cells responding to the agonist (33.6±4.3%) being observed at 500 µM. This was not an exhaustive dose-response study for the drug-receptor pair and therefore should not be interpreted as such. The curve was drawn from data obtained across 4 experiments from a single sperm sample; detailed data with at least three samples within each molar denomination, as opposed to one, would be required to generate an accurate graph. The purpose of this subset of experiments was to determine a suitable concentration of agonist to be used in further investigations. Based on these results, 50 µM Ω was used in further experimentation as a reference concentration.

Further testing of 50 µM Ω across a larger donor population (27 experiments across 6 sperm donors) revealed a substantial degree of variability in the percentage of spermatozoa within

each sample capable of developing measurable calcium signaling events in response to agonist application, both within and across sperm donors (Figure 3.5). Boxplots were used to illustrate the distribution of the percentage of cells responding to the third application of 50µM Ω as these display differences between samples without the need to assume any particular statistical distribution (non-parametric), with the relative positioning of the bars and sizes of the box providing a good indication of the spread and skew in the data. The percentage of cells responding to Ω across sperm donors ranged from 9.4% to 46.6%, with a median value of 21.2%, representing a much more variable response pattern than what was observed for progesterone, which triggered responses in 79.0% of the cells, with a much narrower variation, as illustrated by and SEM value of 2.7%. Thus, Ω shows a broad range of responsiveness, which could be explained by heterogeneity of the sperm samples in terms of numbers of cells expressing the receptor, allied to the complexity of potential signaling events occurring within the sperm cell (Publicover et al., 2007). As discussed below, the signaling events evoked by Ω appear themselves to comprise a certain degree of variability and complexity, proving to be dependent on capacitation state of the cell population and also appearing to be sensitive to a priming effect by the initial exposure to the agonist with it triggering more cells to respond to subsequent Ω applications.

Data gathered from experiments where cells were sequentially exposed to four applications of 50 µM Ω indicated a complex response pattern within the cell population, with some cells proving able to develop transients from the first exposure to the agonist whereas others would only respond to the agonist after one or more previous exposures (Figure 3.6). The percentage of cells responding to Ω rose from the first application (11.7±1.8%) to the fourth (29.1±4.5%), but this rise was only found to be statistically significant from application one

to application two, possibly indicating that the first exposure to Ω has a priming effect on a fraction of the cell population, increasing these cell's responsiveness to the odorant. This was not observed in the heterologous expression system, where the number of cells responding to the first application of Ω was generally higher than cells responding to the second agonist application (Figure 2.7 and Figure 2.8). This difference in re-evocability between spermatozoa and HEK 293 cells could partially be explained by the low rate of functional expression of OR2AE1 in the heterologous system, in which odorant receptors tend to be internalised after odorant stimulation (Mashukova et al., 2006).

As mentioned above, the ability of Ω to trigger calcium signaling events in human sperm was also found to be strongly dependent on the capacitation state of the sperm population (Figure 3.7). The experimental conditions were designed to favour capacitation, by maintaining cells in medium including adequate levels of calcium, bicarbonate and albumin (Cross, 1998; Gadella and Harrison, 2002; Harrison, 2004; Visconti et al., 1999). The association between the percentage of cells responding to the third application of agonist and the time they were allowed to capacitate resulted in a high positive correlation between both variables illustrated by the two-tailed Pearson's correlation coefficient (r=0.57, p<0.0003). This high linear dependence between variables was only found to be true for capacitation times between 3 and 12 hours, with the linear model failing to fit the association between variables for incubation times above 12 hours. These data unequivocally show that the subpopulation of spermatozoa capable of developing Ca^{2+} signaling when exposed to Ω increases for higher capacitation times, suggesting that the signaling mechanism could be kept more or less dormant before capacitation occurs,

coherent with a possible integration of these signaling events with a potential guidance mechanism.

The idea that ORs could be playing a significant role in a possible *in vivo* sperm guidance mechanism in human has been gaining increasing support since the landmark publication by Spehr et al., 2003, showing that in vitro, human sperm are chemotacticaly attracted to bourgeonal, an odorous molecule responsible for the smell of the lily-of-the-valley. Similarly, the current work has also given rise to the question of whether Ω would trigger identical behavioural effects in human sperm. To this effect, a new experimental method was designed to investigate potential chemotactic effects of odorants in human spermatozoa. As described in section 3.2.3, motile spermatozoa were maintained under capacitating conditions and exposed to odorant gradients formed by 5, 50 and 500 µM solutions of both Ω and bourgeonal. A simple set-up was used whereby micropipette tips filled with odorant (or control) solution were dipped in a suspension of motile spermatozoa (Figure 3.1) and then incubated for 1 hour to allow for gradient formation, and potential cell response to the gradient. The molecules tested are relatively similar in terms of overall molecular charge and size, and therefore a constant diffusion coefficient of 5.7×10^{-6} cm^2/s, calculated according to Hayduk-Laudie method (appropriate for modelling diffusion of low concentrations of low charge molecules in aqueous solutions – Hayduk and Laudie, 1974), was assumed for the modelling of gradients established by the different solutions of Ω and bourgeonal (see section 3.2.3 for modelling method). Figure 3.2 shows a graphical representation of the theoretically modelled gradient formation for the three odorant concentrations used: 5, 50 and 500 µM odorant concentrations form a stable gradient after just a few minutes, allowing sperm cells to be exposed to the chemical, “read” the gradient, and potentially change

direction in response to it. As expected, bourgeonal, reported as a potent chemoattractant towards human sperm (Spehr et al., 2003), has caused more cells to travel up the pipette tip than control (Figure 3.8), and this effect proved dose-dependent, ranging from 50.9±23.2% (for 5 µM bourgeonal) to 94.9±64.9% (for 500 µM bourgeonal). Excitingly, in these conditions, 50 µM Ω was significantly more potent (approximately 2X) than the best performing concentration of bourgeonal, causing 204.6±7% more cells to accumulate in the pipette tip than the control solution. However, unlike what was observed for bourgeonal, the accumulation effect caused by Ω was not dose-dependent, dropping sharply for 500 µM Ω (23.5±29.1%). This drop could possibly relate to the increased AR rates observed when sperm were exposed to the same Ω concentration (Figure 3.9). At this level the odorant causes a significant increase in AR stimulation (24.6±15.8%), much higher than what was observed for 5 and 50 µM (1.2±2.1% and 0.1±2.8%, respectively). The fact that 500µM but not lower concentrations of Ω trigger AR shows that at this level, the odorant has a distinct physiological effect, possibly evoked via a different mechanism, which translates into lower concentrations tested causing a much higher accumulation effect than 500µM Ω. Although the method used did not allow the distinction of whether the accumulation effect observed was due to a chemotactic or a chemokinetic phenomenon, it nevertheless allowed a robust and expedite method to test the effect of different odorants on sperm accumulation on the pipette tip by screening a large number of samples, which would be a lot more time-consuming with alternative methods. On the other hand, and as further discussed below, a more detailed investigation on the effect of 50 µM Ω on sperm motility carried out both by CASA (Supp. Figure 4 and Supp. Table 1 - Appendix II: Supplementary Data) and modified Kremer test () failed to identify significant differences in motility exhibited by spermatozoa

exposed to the odorant and their parallel controls for all the parameters analysed. If 50 µM Ω would indeed trigger a significant chemokinetic effect in capacitated human sperm, CASA would arguably be the best method to identify it, but as this was not the case, the possibility of the accumulation effect discussed above being due to chemokinesis and not chemotaxis is fairly remote.

The chemotactic assay design differs in complexity from those carried out by other workers (Eisenbach and Tur-Kaspa, 1999; Spehr et al., 2003; Teves et al., 2009) with one particular difference being that direct observation of the sperm moving towards or away from the source was not possible. However, an advantage of an 'accumulation at maximum concentration' assay was the substantial number of sperm that could be counted (> 500 cells per replicate) to provide a robust result for statistical analysis. It is possible to argue within this system that the sperm inside the pipette tip may have become 'trapped' there due to hyperactivated motility at the maximum concentration, however, as discussed later, although 50 µM Ω did induce changes in the number of hyperactivated cells, these proved non-significant, and the very low actual numbers of these (generally under 8%) within the cell population observed would be unlikely to account for the large variations observed in the chemotaxis assay. In any case, within the non-physiological situation of low viscosity media (Kirkman-Brown and Smith, 2011) this observation is as accurate as any other.

The method used to test for chemotaxis did not, however, intend to model or correlate to a possible chemotactically-driven guidance mechanism in the human FRT. As discussed by Gadelha et al., 2010, the viscosity sperm are exposed to will affect cell trajectory and movement, so for a chemotaxis assay to mimic in vivo environment, and as reviewed by Kirkman-Brown and Smith, 2011, such method should approximate the media viscosity to

the levels sperm experience in the FRT. Here they navigate their way to the ovum by traversing mucus coating the tract surfaces and they experience viscosities much higher than the ones in standard media used in in vitro studies. However the increased times to set up a gradient by diffusion in such a system (described in Kirkman-Brown and Smith, 2011) precluded these experiments. In future, development of photo-labile versions of the Ω molecule as derived for progesterone by Ben Kaupp's group (Kilic et al., 2009), may make these experiments feasible.

Early observations by Cohen-Dayag et al., 1994 show than only 2-12% of the sperm population is chemotactically responsive to active factors in follicular fluid at any given time, and, as later reviewed by Eisenbach and Tur-Kaspa, 1999, it is likely that only the capacitated fraction of the sperm population is capable of chemotaxis, so the effect this may have needs to be considered when interpreting results obtained with the chemotaxis assay described in the current work. As discussed for Figure 3.7, capacitation time itself has an effect on Ω induction of calcium signaling, but at this stage it is unclear whether there is in fact a further-rarefied chemotactically-active sub-population within motile spermatozoa, and whether those cells are all in the same capacitation stage. In order to even begin to answer this question, detailed observation of sperm motility in gradients alongside calcium imaging, probably by photo-labile compounds as discussed above, would be required.

A secondary question to the drop-off in chemotaxis observed at 500 µM Ω was whether other non-motility signaling pathways may have been activated, hence acrosome reaction rates were examined. It was clearly shown that 5 and 50 µM Ω had no effect on acrosome reaction, however 500 µM Ω did cause a significant rate of induction (Figure 3.9). Interestingly, no difference was observed in response kinetics for 50 and 500 µM Ω (Supp.

Figure 5) which, together with the effect in acrosome reaction, could indicate that at 500 µM Ω begins to stimulate other pathways beyond those involved in motility modulation, without noticeable effects in $[Ca^{2+}]_i$. For three other recently characterised receptor systems (Veitinger et al., 2011) the agonists were only assayed up to 10^{-5} levels for acrosome reaction effects and no differences in spontaneous AR rates were observed, so this data is in agreement over that range. Veitinger et al., 2011, did report that at these lower levels the sperm obtained within the microcapillary system they employ were more responsive to a non-physiological inducer of acrosome reaction (phorbol 12-myristate 13-acetate). Future examination of the responsiveness of the sperm population collected in our chemotaxis/accumulation assay both in calcium imaging and acrosome reaction experiments would be very interesting, though use of the physiological agonist progesterone may give a more meaningful result. It is not clear whether investigation of the 500 µM Ω AR-inducing effect is worthwhile: if a potential physiological agonist of OR2AE1 is secreted or synthesised within the cumulus egg complex, it is entirely possible that at the last stage of their journey sperm may encounter the agonist at this concentration range. This differing signal/action at different stages of sperm migration would be a very effective and efficient use of the sperm's signaling toolkit by possibly integrating this and other agonist signals, such as those of the ZP, in the ultimate accomplishment of the gamete's objective - fertilisation. However, until a physiological agonist is defined this remains pure speculation.

As the exact location of any physiological OR2AE1 agonist within the female tract is unknown, the effects of semen exposure to 50 µM Ω - the equivalent of sperm seeing the agonist in the vaginal environment - and subsequent migration effects were examined (). In this situation no significant effects on the percentage of sperm migrating up the capillary

were observed. It is worth noting that there were slightly fewer (NS) sperm observed at 4 cm versus control: this could be consistent with a potential small number of chemotactically-active cells being attracted back towards the semen which contained the chemoattractant Ω. Additionally, potential effects of 50 µM Ω in sperm motility were investigated by computer assisted semen analysis (CASA). The results showed no significant differences between stimulated and control spermatozoa (Supp. Figure 4 and Supp. Table 1 - Appendix II: Supplementary Data) although a clear trend is present when looking at hyperactivation markers (sorts 5 and 7 - Figure 3.11). Samples stimulated with the odorant tend to exhibit higher number of cells classed as sort 5 and 7 (see section 3.2.6 for sort definition), and this is particularly evident 30 minutes after stimulation: when compared to control, 72±38% and 117±82.2 more cells were sort 5 and sort 7, respectively. Although these differences did not prove statistically significant (results for sort 7 and sort 5 at T30 are borderline – ANOVA, $p = 0.053$ and 0.078 respectively) they hint to the possibility of Ω having a hyperactivation effect on a subpopulation of human sperm. The results could have failed to show this effect in a more robust manner possibly due to the high variability observed, both across and within sperm donors. The data set could therefore benefit from being amplified across a larger number of donors, so that the referred variability would have a smaller weight on the observation.

It would also be interesting to know if cells remain hyperactive for as long as the odorant is present or if they go in and out of hyperactivation, with different cell subsets being hyperactive at any given time, and maybe by different odorant concentration ranges. If the latter is observed to be true, it could be that a potential OR2AE1 agonist within the FRT would have multiple effects in modulating sperm motility, be it, as discussed earlier, through

a more direct chemotactic guidance mechanism, or, through a less directional effect, causing cells to randomly change direction when going in and out of different hyperactive states, therefore maximizing the chance of "selected" sperm to find the oocyte and possibly providing them with the right motility to penetrate cumulus when they do. In any case, further and more detailed observations of the effects of Ω on sperm motility and hyperactivation under the right physiological conditions (the right viscosity being paramount, as mentioned earlier), would be essential to clarify this question.

In a preliminary attempt to localise the subcellular onset of calcium signals observed when sperm were stimulated with 50 µM Ω, a set of experiments was carried out where immobilised spermatozoa were exposed to the agonist for a short period (20 seconds) and $[Ca^{2+}]_i$ was monitored at 12.5 Hz for 70 seconds, from the beginning of the application, by florescence microscopy (Figure 3.12). For this set of experiments, a good compromise between excessive light exposure (if a ratiometric dye was to be used) and adequate temporal resolution allowing for distinction of signal propagation was achieved by the use of a single-wavelength Ca^{2+} indicator (Calcium Green-1 AM). Also, given the preliminary character of these investigations and the difficulty to obtain reliable data for other areas of a fast moving flagellum, only sperm head and midpiece were included in the analysis as main general areas of the sperm cell.

For the majority of the cells observed, the signal tended to initiate in the midpiece, with a mean delay of 1.36±0.51 seconds before it would propagate to the head. The delay observed proved statistically different (one-sample *t* test; $t_{(30)}$=2.678, p<0.012) from a hypothetical zero delay (simultaneous signal onset in head and midpiece), indicating OR2AE1 receptor, together with immediate elements involved in the signal transduction, will probably localise

to the midpiece rather than the head. Although from a less detailed analysis, these results are consistent with what was previously reported by Spehr et al., 2004 (localisation and signal transduction via OR1D2) and more recently by Veitinger et al., 2011 (signal transduction via OR7A5 and OR4D1) for other ORs found in human sperm, showing that calcium transients evoked by OR agonists tend to initiate in either the midpiece or proximal flagellum and propagate to the sperm head within < 4 seconds.

Further characterisation of the signaling evoked by Ω in human sperm involved the use of a series of inhibitors of key molecules crucial for the transduction of calcium signals (). Spehr et al., 2004 have shown that, similar to OR signaling in sensory neurons, the calcium signals evoked in human sperm by OR1D2's agonist bourgeonal involve an increase in cAMP catalysed by the membrane-associated adenylyl cyclase (mAC), but other authors acknowledge a significant role for the IP_3 pathway in vertebrate olfactory signal transduction (Ko and Park, 2006). More recently two independent groups (Strunker et al., 2011 and Lishko et al., 2011) have identified the sperm-specific Ca^{2+} channel CatSper as the main molecule mediating the Ca^{2+} influx triggered by progesterone in human sperm. This subset of experiments, therefore, aimed at using the pharmacological tools available to gain some insight on what molecules are involved in the transduction of the Ca^{2+} signal observed when human sperm were exposed to Ω. To this end, inhibitors acting on the cAMP pathway (SQ 22536 and cAMPS-Rp), IP_3 pathway (U 73122) or blocking Ca^{2+} influx via CatSper (NNC 55-0396 - T-type calcium channel blocker) were tested for their ability to block the Ω-evoked Ca^{2+} signals (). Both SQ 22536 (a specific P-site known to block all 9 isoforms of mAC - Hurley, 1999) and cAMPS-Rp (cAMP analogue that acts as a competitive antagonist of the cAMP-induced activation of PKA - Van Haastert et al., 1984) caused a substantial reduction in the

number of cells responding to Ω, with inhibitions of 66.3±11.1% and 72.0±14.9% respectively. SQ 22536, a P-site inhibitor, is known to block all isoforms of mAC without interfering with sAC activity (Sunahara and Taussig, 2002 and Hurley, 1999). Interestingly, the inhibitory effect of SQ 22536, over Ω signals was achieved with a much lower dose (10 μM) than the one required to block bourgeonal-evoked signals in human sperm (10 mM), as reported by Spehr et al., 2004. This could indicate a more specific action of the inhibitor, meaning the Ω-evoked Ca^{2+} signal is therefore more dependent on mAC activity than the similar response reported for bourgeonal.

Interfering with the IP_3 pathway by blocking PLC (U73122 - Bleasdale et al., 1990), on the other hand, resulted in a much lower inhibition of the agonist-evoked response (11.7%), whereas blocking T-type Ca^{2+} channels yielded the highest level of inhibition (77.6±3.2%).

At this stage, however, it is essential to consider the effects of the biochemical tools used in these experiments. A particular problem when employing any inhibitor are the immediate effects that they may induce. These could be specific via their action, or non-specific and unrelated. Immediate inhibitor-related responses in baseline calcium were observed across the four inhibitors employed and some were found to be substantial (Figure 3.14).

SQ 22536 had the least effect on baseline calcium; presuming the effect was specific, this would indicate that a resting cell had little mAC activity that was related to resting $[Ca^{2+}]_i$ homeostasis. This contrasts to the more dramatic effects of cAMPS-Rp, which if specific, may indicate that there is PKA activity in 'resting' sperm, the implication being that PKA inhibition causes a steady increase in cAMP, which apparently, is not being recycled at a fast enough rate by the phosphodiesterases (PDEs). As discussed below, the mechanism by which this rise in cAMP originates the elevation in $[Ca^{2+}]_i$ observed is unknown, but it seems likely that

CatSpers are being activated either directly or indirectly by the elevated cAMP resulting from blocking PKA. However, further experiments combining the use of cAMPS-Rp with SQ 22536 and NNC 55-0396 would be required to reveal whether these hypotheses are likely, or effects are more non-specific and artefactual.

Interference with the IP_3 pathway by inhibiting PLC has also resulted on an elevation in $[Ca^{2+}]_i$: if specific, the effect of U73122 on baseline calcium would be via switching-off of resting PLC activity, the later sustained effects on $[Ca^{2+}]_i$ could then likely relate to accrual of earlier phosphoinositides in the pathway, which may have a myriad of other signaling effects as discussed by Michell, 2009. Furthermore, effects other than the inhibition of PLC have previously been described for U73122 in other cellular systems: Mogami et al., 1997 have described U73122 as capable of directly activating ion channels and triggering Ca^{2+} release from intracellular stores in mouse pancreatic acinar cells.

The observed responses to NNC 55-0396, also recently reported by Strunker et al., 2011, are more difficult to understand: at present these would appear to be a non-specific response, however, a role of the CatSpers in a baseline-level resting cell divalent cation leak – which when inhibited caused sperm to generate a further large response through a different system, cannot at this time be ruled out. The inhibition of the Ω response by NNC 55-0396 was the subject of a more detailed analysis, looking not only at variations in the cell populations observed (Figure 3.15), but also at the kinetics of the Ca^{2+} transients evoked by Ω in presence or absence of the inhibitor (). The majority of cells (72.9±3.8%) developed transients when presented with 10 µM NNC 55-0396 (Figure 3.15 A and B). However, the response pattern displayed by the cell population when looking at inhibition (see section 3.2.2 for definition) of Ω-evoked Ca^{2+} transients (Figure 3.15 C) is somewhat harder to

interpret. Only 27.9±6.8% of cells respond to applications 2 and 3 of the agonist (cell population used for inhibition calculation purposes); of these, 20.9±5.0% do not develop transients when agonist is co-presented with inhibitor (population A - A), resulting in 77.6±3.2% inhibition, but 7.0±2.3% of cells will still show measurable transients (population B - B). The differences in kinetics for these two spermatozoa subpopulations (, summarised in Table 2) show that population B is overall more responsive that population A, with higher amplitude transients. This is particularly evident for the second and third applications of agonist alone (Ω2 and Ω3), but also progesterone, for which amplitude in population B is almost double the one observed for population A. Consistent with earlier results showing Ω stimulation as capacitation dependent, these differences could certainly relate to cells in different capacitation stages, with some cells further ahead in the capacitation process and therefore ready to respond to potential stimuli, and others taking longer to capacitate and only being responsive later in the process. If a chemotactically active agonist for OR2AE1 indeed exists in the FRT, such populational heterogeneity would probably result in an increased fertility potential, since the period in which chemotactically-capable cells would be available for stimulation would consequently be extended, allowing for a better synchronism with the female fertility window.

In any event, on the subject of the effects triggered by the inhibitors themselves, the data for the mAC – cAMP – PKA pathway obtained with SQ 22536 would seem the most robust and least likely to be totally at fault, as it results on a lower resting $[Ca^{2+}]_i$ and still evidencing effective inhibition of the Ω response, but interpretation of Ca^{2+} signals triggered by all these compounds, but particularly NNC 55-0396, U73122 and cAMPS-Rp is complicated for their many implications. When interpreting agonist + inhibitor responses, these signals could

result in a number of unknowns: perhaps the higher levels of calcium would mean that a receptor or channel involved in Ca^{2+} intake, if voltage sensitive, would not be able to depolarise and therefore partially or completely stop working; another possibility would be that the raised Ca^{2+} would be affecting other components down the signaling cascade. One of such molecules could be sAC, whose activity is modulated by different cytosolic calcium levels, as reviewed in Kamenetsky et al., 2006.

Taken together, results obtained for the different inhibitors show that the Ca^{2+} signal evoked by Ω is dependent on the conversion of ATP to cAMP by one or more membrane associated isoforms of adenylyl cyclase. The involvement of the IP_3 pathway in this response appears minimal, but importantly, CatSper proved a major player, mediating a significant Ca^{2+} influx after stimulation with Ω, observed as a large increase in $[Ca^{2+}]_i$.

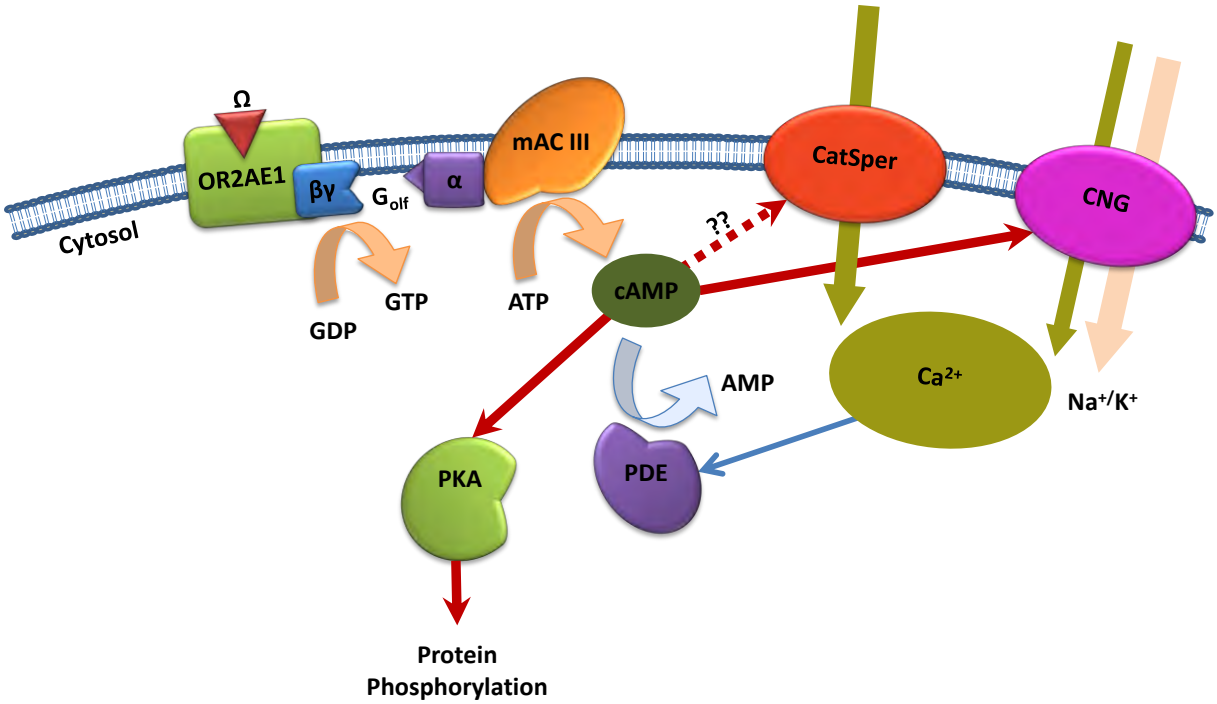


Figure 3.18 – Proposed transduction mechanism for the Ω-evoked signals in human spermatozoa

The information available at the moment is insufficient to provide an absolute model of how the Ω-evoked signal is transduced (Figure 3.18). Nonetheless, it seems likely that, similar to chemosensory transduction on OSNs, stimulation of the OR2AE1 in human sperm causes activation of mAC, via the alpha subunit of the receptor complex G_{olf}, previously shown to co-localise with isoform III of the membrane-bound adenylyl cyclase (mAC III) in human sperm's flagellum and midpiece (Spehr et al., 2004). As observed by others (Aoki et al., 1999; Ren et al., 2001; Wiesner et al., 1998), the rise in cAMP levels leads to increased $[Ca^{2+}]_i$. It seems likely that the elevation in $[Ca^{2+}]_i$ results mostly from CatSper stimulation (or arguably other T-type Ca^{2+} channels), since the Ω response is also largely blocked by NNC 55-0396, but Ca^{2+} influx via CNG may also play a role in $[Ca^{2+}]_i$ observed after stimulation, as a CNG is known to be present in mammalian sperm (Parmentier et al., 1992). However, the way the cAMP rise results in CatSper stimulation is still elusive and a current subject of discussion (see review by Ren and Xia, 2010). The possibilities of Ω acting directly on CatSper, or indirectly stimulating the channel by association of the OR complex or some of its components with CatSper, similar to what is seen insect OSNs, where a receptor/channel complex exists (Kaupp, 2010; Sato et al., 2008; Wicher et al., 2008), cannot be excluded, but the results obtained by interfering with the cAMP pathway suggest that most of the stimulation is channelled through mAC. Furthermore, the differences when comparing kinetics of Ω and progesterone-evoked Ca^{2+} signals (and Table 2) strongly suggest an indirect activation of CatSper by the rise in second messenger cAMP caused by Ω stimulation, with the Ω-evoked transients taking longer to develop than the ones evoked by progesterone, which according to recent evidence (Lishko et al., 2011 and Strunker et al., 2011), causes

Ca^{2+} influx by directly activating CatSper. In any case, independent of which mechanism causes CatSper activation after Ω stimulation, the resulting rise in $[Ca^{2+}]_i$ is likely to be crucial in the regulation of capacitation and likely hyperactivation, as evidenced by the increased number of hyperactivated cells observed in sperm samples exposed to the agonist (Figure 3.11).

To examine the possibility of Ω stimulating other sperm ORs other than OR2AE1, we took advantage of the only agonist/antagonist pair characterised so far for human ORs (OR1D2, whose activation by bourgeonal is reported to be inhibited by undecanal - Spehr et al., 2003), a set of experiments were conducted to investigate the effect of undecanal on the Ω-evoked response (). Our standard capacitating media system was employed alongside a non-capacitating media which did not contain bicarbonate or serum, as this mirrors that employed by Spehr et al., 2003 for their work. Effects of both bourgeonal and Ω on $[Ca^{2+}]_i$ proved fundamentally different depending on the capacitation state of the sperm population, with spermatozoa maintained under capacitating conditions responding in much greater numbers to both agonists than cells kept in non-capacitating media. Similarly to what was seen for the Ω-evoked response, sequential exposure to bourgeonal resulted in an overall increase in the number of cells responding to the odorant. The responsive population proved dynamic, with the population responding at any given application not including all which had previously responded.

The presence of Undecanal with bourgeonal or Ω, in capacitating or non-capacitating conditions, revealed an increase in the number of cells responding. It was unclear whether this increase in the responsive population was due to undecanal or simply a further exposure of cells to the other agonist. To investigate the classification of individual cells previously

responding to bourgeonal was investigated to see how many subsequently responded. Surprisingly, even in this situation the inclusion of bourgeonal on the experimental set-up as a positive control for undecanal inhibition revealed a relatively low level of inhibition (25.4±2.9%) compared to the total inhibition of Ca^{2+} responses reported for sperm presented with equimolar doses of bourgeonal and undecanal reported by Spehr et al., 2003.

The results reported by Spehr et al., 2003 apparently refer to observation of spermatozoa kept under non-capacitating conditions, but interestingly, the antagonistic effect of undecanal observed on our system over both bourgeonal and Ω was much higher on non-capacitated cells (79.2±15% and 54.2±29.2%, respectively) than when cells were kept in capacitating media (25.4±2.9% and 15.2±2.7%, respectively). This would suggest that the molecular mechanism responsible for the relatively high antagonistic properties of undecanal under non-capacitating conditions is to a big extent abolished under capacitating conditions. Whether this results from a metabolic change inherent to the complex capacitation process or if the different media used cause the odorants to interact differently with the receptors is speculative. However, a possible factor allowing for the striking differences in number of cells responding to the agonists in capacitating and non-capacitating media could be the major reconfiguration of the sperm's plasma membrane during the capacitation process itself, recognised to radically alter the cell's function.

4. FUNCTIONAL INVESTIGATION OF BORONIC ACIDS IN HUMAN SPERMATOZOA

4.1. Introduction

One in six couples in the developed world experience subfertility (Hull et al., 1985) and male factor infertility explains over half of all cases, with the motility of the sperm being the major problem – not the more often publicised sperm count.

Sperm motility is crucial to natural conception and its improvement or modulation is arguably the safest and least invasive way to improve fecundity levels. A key but less well-known action of the female hormonal contraception pill is to thicken the cervical mucus and hence prevent sperm migration (Benagiano et al., 2009), hence there is significant potential for interventions which may affect sperm's ability to migrate.

Migration of sperm inside the human female reproductive tract is fraught with many challenges including, but not limited to, dramatic changes in viscosity (Wolf et al., 1977); osmolarity (Yeung et al., 2003); many surrounding chemical modulators, including progesterone (reviewed in Correia et al., 2007); and glycodelin proteins (Seppala et al., 2007); nitric oxide (Machado-Oliveira et al., 2008); and potential phagocytotic attack from leucocytes (Pandya and Cohen, 1985). These challenges mean that in just a few hours the many millions of sperm deposited in the vagina are reduced to hundreds in the fallopian tubes (Williams et al., 1993) or even as few as ten reaching the oocyte (Nottola et al., 2001). Many of these factors and their interplay are excellently reviewed in Suarez and Pacey, 2006. In section 3 the rapidly evolving world of odorant receptor research is discussed and data for characterisation of the effects of one of these (OR2AE1) and associated chemotaxis thought

to occur higher within the female tract is discussed. However, all of these studies have failed to take into account the fundamental biological differences in viscosity and how that may affect the observations (Kirkman-Brown and Smith, 2011). A different question is whether sperm in semen can be 'improved', or 'more' sperm can be made to migrate from semen and colonise the cervical mucus by affecting their motility. As to date, there is no specific drug treatment available to improve the function of the sperm cell. Treatments for male problems are mainly restricted to IVF, implying consequent risky procedures for the female, and therefore there is a potential to develop drug regimes to stimulate sperm function.

Previous methods aimed at assisting sperm function have been limited to the use of cellular signaling pathway inhibitors (IVF), or adding a natural ligand, Platelet Activating Factor (PAF) (Roudebush et al., 2004) to prepared sperm for artificial insemination (IUI). Both of these methods have significant drawbacks as they are invasive, costly and cause patient embarrassment.

Ongoing research into human sperm motility regulation in our laboratory identified a novel boronic acid compound which showed promise in significantly increasing the number of sperm that migrate through a cervical mucus analogue – this is a test which mimics what sperm have to undergo in-vivo and hence a strong predictor that more sperm in an ejaculate mixed with this compound would manage to get through the female reproductive tract.

This section of the current work sets out to validate whether sperm enhancing capabilities of the compound and characterise the method of boronic compound action in human spermatozoa, hypothesising that it may either be through olfactory g-protein coupled receptors or via a direct effect of binding saccharide moieties on the cell-surface.

4.2. Material and Methods

4.2.1. Sperm preparation / capacitation

Sperm were prepared and capacitated according to previously described methods, see section 3.2.1.

4.2.2. Single cell calcium imaging

The core set of single-cell imaging experiments were performed according to previously described methods (see Section 3.2.2), with the adjustment that instead of odorants, phenethylboronic acid (PEBA) at a final concentration of 10 nM was perfused as the agonist. Initial 5M stocks of PEBA (Sigma UK Ltd) were made up in 1:1 DMSO:npH_2O. These were serial diluted in H_2O to intermediate stocks, which were then freshly diluted (1:100 in sEBSS) to their final working concentration on the day when experiments took place. High speed imaging and experiments with inhibitors were as described in section 3.2.2, but employing 10 nM PEBA as the agonist.

4.2.3. Migration assay

The ability of boronic acids to affect sperm migration were assessed according to previously described methods, see Section 3.2.5, by modified Kremer assay. Migration tests were performed in duplicate: two capsules (BEEM capsule, G360-2, Agar Scientific, Stansted, UK) were filled with 50 µl of semen pre-mixed with either test (2 different boronic acids at 40 nM in PBS pH 7.4 with 1:50,000,000 DMSO) or control (PBS pH 7.4 with 1:50,000,000 DMSO) solutions on a 3:1 ratio. One capillary was placed vertically into each capsule so that the

hyaluronic acid was in direct contact with the semen. Assembled setups were then incubated (37°C, 5% CO2) for 1 h, after which cell number along the capillary (10, 20, 30 and 40 mm from the lower end) was assessed. Assessment was done at 200X total magnification using phase-contrast microscopy on an upright Olympus BX50 microscope.

For experiments testing sperm motility / migration enhancers in conjunction with key pathway inhibitors, an extra boronic acid (boric acid -BA) and caffeine were included (final concentrations of 10 nM and 1mM, respectively) in the assay described above, and tested together with 5 µM U 73122 and 25 µM cAMPS-Rp.

4.2.4. Acrosome reaction

Acrosome reaction induction was performed and assessed according to previously described methods (see 3.2.4), but in this case employing a range of PEBA concentrations, diluted (1:100) from appropriate stock solutions (see section 4.2.2).

4.3. Results

4.3.1. PEBA evokes Ca^{2+} signaling events in human spermatozoa

Using a different approach to that described in chapters 2 and 3, previous research on our lab has identified phenethylboronic acid (PEBA) as a potential enhancer of sperm migration in vitro. The effects of this compound on Ca^{2+} signaling were assessed by single-cell calcium imaging, as described in section 4.2.2. A range of PEBA concentrations was tested for their ability to cause Ca^{2+} transients in capacitated human spermatozoa. Figure 4.1 represents a typical experiment where sperm were exposed to subsequent applications of increasing

concentrations of PEBA (3 pM to 100 nM). The green trace shows an individual cell (cell2) developing Ca^{2+} transients when exposed to PEBA in the pM (30 pM) and nM (1, 10 and 100 nM) ranges, whereas cell26 (orange trace) only responds to 100 nM PEBA.

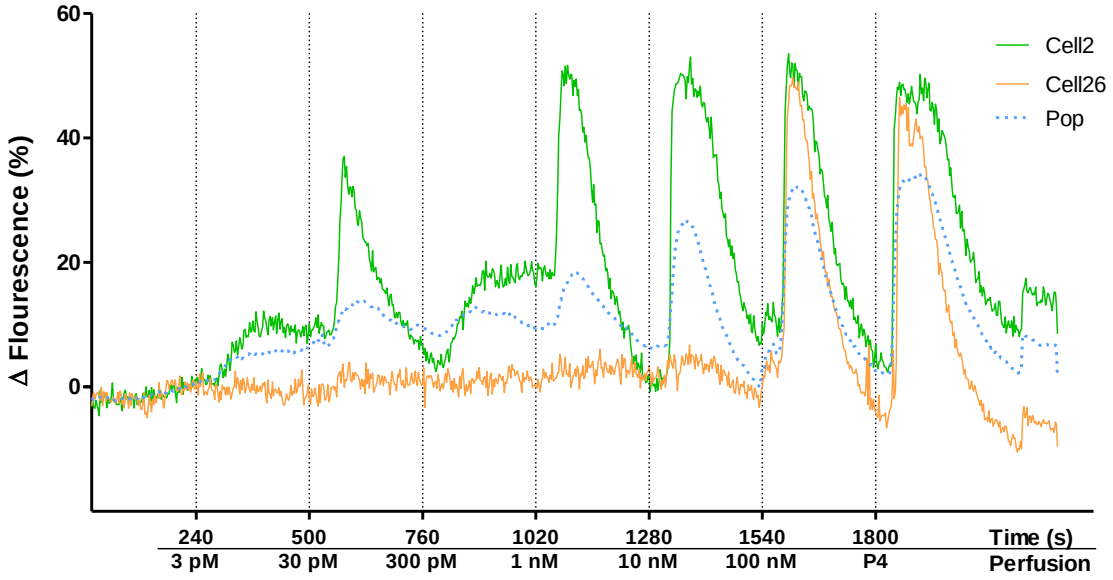


Figure 4.1 - Ca^{2+} responses to increasing concentrations of PEBA
Typical $[Ca^{2+}]_i$ recording: traces show normalized fluorescence plot for the whole cell population (blue dashed trace – Pop) and 2 individual sperm cells (cell 2 – green trace; cell 26 – orange trace). Spermatozoa were labelled with 7.5µM Ca^{2+} Green and sequentially perfused for 20 seconds with increasing concentrations of PEBA (3 pM, 30 pM, 300 pM, 1 nM, 10 nM and 100 nM). 3.2µM progesterone (P4) was applied at the end of the experiment to verify physiological state and ability to generate Ca^{2+} signals.

There was variability in the number of cell responding to each PEBA concentration, and this is reflected on the average fluorescence variation of the whole cell population observed (Pop - dashed blue trace), indicating that the number of cells responding to PEBA increases for higher concentrations of the chemical.

Following on initial assessment on whether PEBA was capable of triggering Ca^{2+} signaling events in human sperm, a broader range of concentrations was tested to define the optimal concentration to be used in subsequent experiments. The dose-response curve (Figure 4.2)

drawn for PEBA concentrations ranging from 3 fM to 100 µM shows an obvious trend where higher levels of the chemical cause more cells to develop Ca^{2+} transients. However, unlike typical dose-response curves, the non-linear fit of the curve proved unsuitable to describe the trend observed (dashed green line). In this case, a linear regression analysis provided a better fit for the values observed, reporting a moderate correlation between percentage of cells responding and the logarithm of PEBA concentration (R^2 = 0.31). The fact that PEBA concentrations in the nM range are sufficient to cause 80% of cells to respond can be indicative of a high potency agonist acting on a system characterised by the receptor-reserve phenomenon, where the maximum response is achieved with only partial occupancy of the receptor pool.

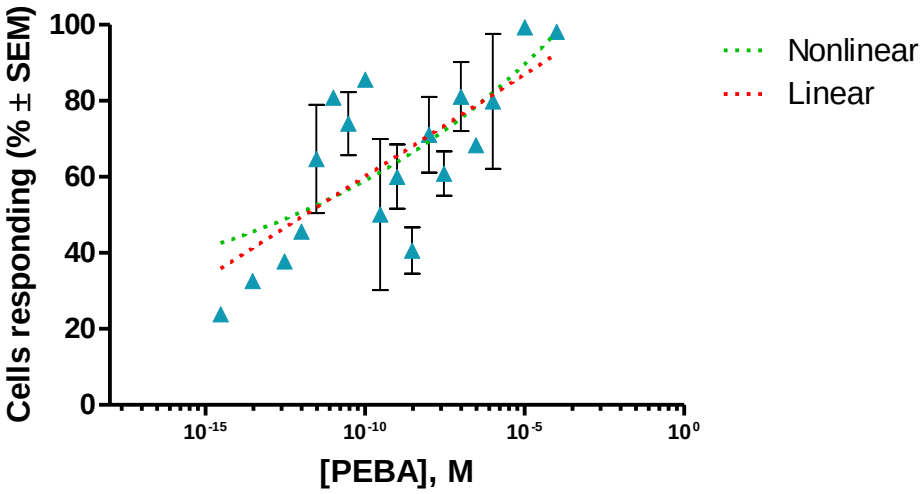


Figure 4.2 – Dose-response for Ca^{2+} responses evoked in mature sperm by PEBA
Sperm cells were allowed to capacitate for at least 5 hours, labelled with 7.5µM Ca^{2+} Green and exposed to 20s applications of increasing PEBA concentrations (from 3 fM to 100 µM). Data, plotted as percentage of responding cells ± SEM, consists of 8 experiments, each including an average of 280 cells.

4.3.2. Effect of PEBA on sperm migration

In order to further validate previous results indicating PEBA was capable of enhancing in vitro sperm migration, human spermatozoa were exposed to 10 nM PEBA in a modified Kremer test (section 4.2.3), alongside the same concentration of another boronic acid (HMPBA - 2-(Hydroxymethyl)phenylboronic acid cyclic monoester) that also proved effective in in previous testing in our laboratory (Figure 4.3). At this concentration, PEBA caused a significant increase in sperm numbers at all distances scored along the capillary, ranging from 43.1±14.3% (1 cm) to 77.0±16.2% (2 cm) when compared to control.

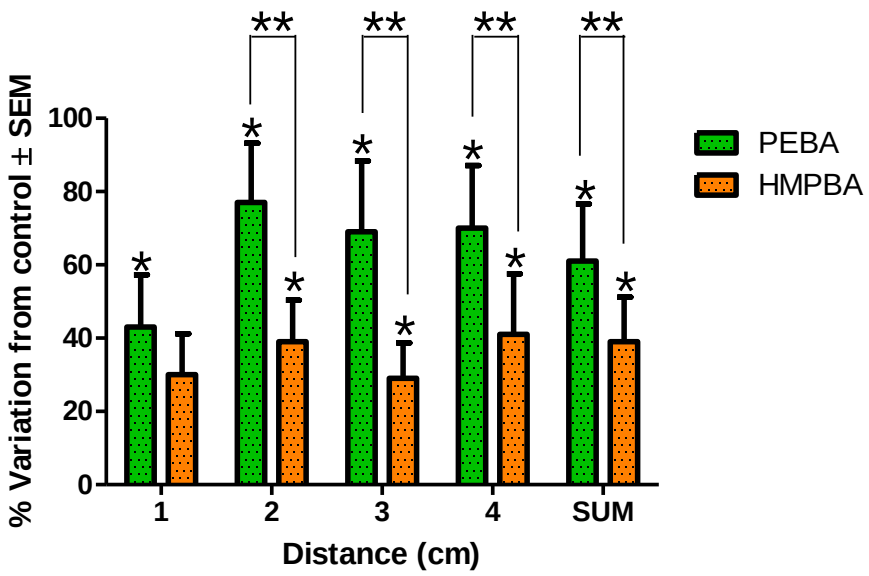


Figure 4.3 – Effect of PEBA and HMPBA on sperm migration
Both PEBA (green) and HMPBA (orange) were tested at 10 nM. Migration was scored at 1, 2, 3 and 4 cm from the open end of the capillary (SUM is the variation from control for the total number of cells scored for the 4 distances). Data, plotted as percentage variation from control ± SEM, derives from the screening of 72 sperm donors. * indicates a significant variation from control and ** indicates a significant difference between test solutions (PEBA and HMPBA) (paired *t* test, $p<0.05$).

Similarly, 10 nM HMPBA has caused more cells to migrate along capillary tube, although the positive effect observed for this boronic acid was lower than PEBA for all distances, ranging from 29.1±9.7% (3 cm) to 41.3±16.6% (4 cm). Overall, the total number of cells scored along

the capillary was on average 61.0±15.6% and 38.6±12.2% higher than control, respectively for PEBA and HMPBA.

4.3.3. Effect of PEBA on acrosome reaction

Further investigation of potential functional effects of PEBA in human sperm included a preliminary assessment of its effect on acrosome reaction. As described in section 4.2.4, motile spermatozoa were allowed to capacitate and exposed to a range of PEBA concentrations (3 nM – 300 µM; Figure 4.4). At concentrations lower than 300 µM, PEBA does not seem to cause a significant difference in the incidence of AR, with mean AR stimulation across both experiments (see section 4.2.4 for AR stimulation definition) varying between -2.2±1.3% for 3 nM PEBA, and 4.3±1.8% for 300 nM PEBA. However, 300 µM PEBA did cause a noticeable increase in number of cells undergoing AR, with a mean AR stimulation of 7.4±2.1%.

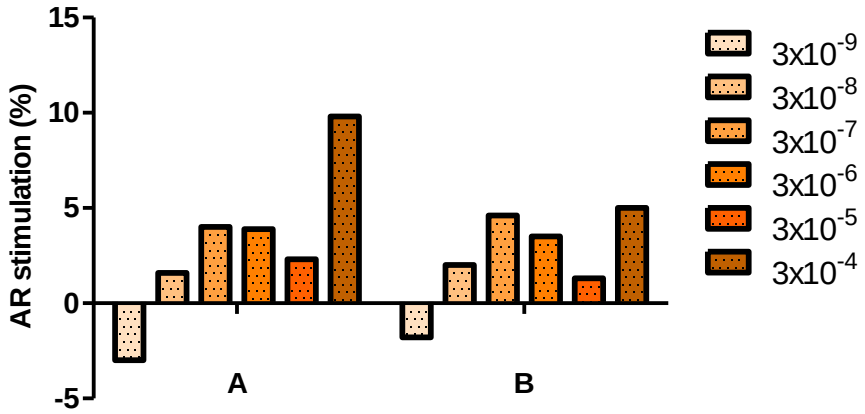


Figure 4.4 – Effect of PEBA on acrosome reaction
Acrosome reaction triggered by increasing concentrations of PEBA ($3x10^{-9}$ to $3x10^{-4}$ M) in mature human sperm. Data includes 2 experiments across 2 donors (donor A and donor B).

4.3.4. Characterisation of PEBA-evoked Ca^{2+} signaling

As part of the characterisation of the Ca^{2+} signaling events observed when human sperm are exposed to PEBA, a series of high speed single-cell Ca^{2+} imaging experiments was conducted aiming to define the subcellular onset of the calcium signaling (Figure 4.5). Briefly, and as described in section 4.2.2, motile spermatozoa were isolated and allowed to capacitate for 5 hours. After labelling with the appropriate calcium indicator (Ca^{2+} Green-1) cells were kept under constant perfusion and exposed to a 20 second application of 10 nM PEBA. $[Ca^{2+}]_i$ was monitored at 12.5 Hz for 70 seconds from start of PEBA application and raw intensities extracted. For each cell analysed two areas of interest (AOIs) were drawn (one including the whole head and the other including the whole midpiece) and onset of the calcium signal was recorded. The delays observed ranged from -3.44 seconds (negative delay describes a signal initiated on the sperm head and propagated to the midpiece 3.44 seconds later) to +3.52 seconds (positive value describes a signal initiated on the midpiece and 3.52 seconds later propagated to the sperm head). Overall, signals tended to start in the midpiece rather than the head, with a mean delay across the cell population observed of +0.44±0.38 seconds. These differences, however, proved non-significant on a one-sample t test ($t_{(18)}$=1.154, p<0.264).

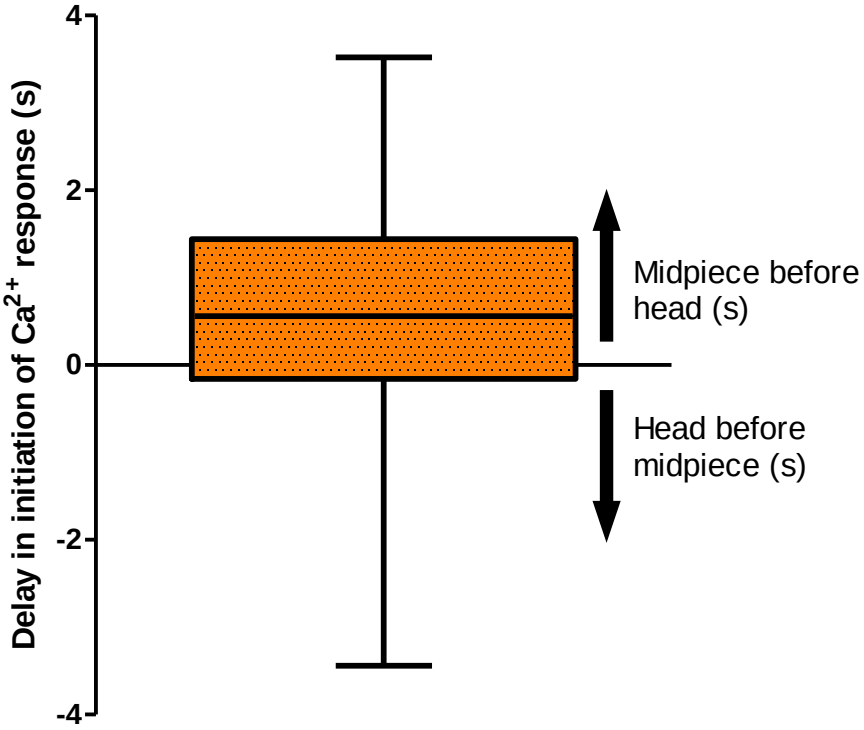


Figure 4.5 – Subcellular onset of Ca^{2+}responses triggered by 10 nM PEBA in human spermatozoa
Boxplot illustrates distribution of the Ca^{2+} response onset delay (s) across sperm head and midpiece. Data includes 19 cells and whiskers represent minimum and maximum values.

Further investigations on the PEBA-evoked signal characterisation involved the use of inhibitors of key molecules in Ca^{2+} signal transduction to have an insight over which pathways might be involved (Figure 4.6). To this effect, a series of experiments was carried out (as described in section 4.2.2) where motile sperm were allowed to capacitate and exposed to 10 nM PEBA both in absence and presence of each of the three inhibitors tested (25 µM cAMPS-Rp, 5 µM U 73122 and 10 µM NNC 55-0396) whilst $[Ca^{2+}]_i$ was being monitored. Inhibition (defined as the number of cells that, after responding to application of PEBA on its own, fail to respond when PEBA is co-presented with the inhibitor) was highest for NNC 55-0396 (73.7±2.0%), closely followed by U 73122 (67.3±6.3%). cAMPS-Rp, however, caused a much lower level of inhibition than the other two inhibitors, with only 33.9±10.1% of the cells failing to display Ca^{2+} transients when PEBA was co-applied with the inhibitor.

Regarding the results above, however, it is worth noting that, as shown earlier (section 3.3.7), all the inhibitors tested had themselves more or less dramatic effects in $[Ca^{2+}]_i$ (Figure 3.14).

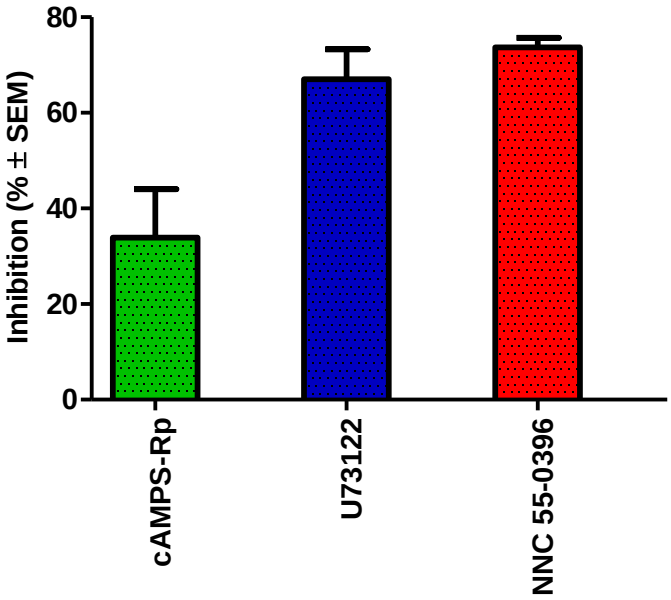


Figure 4.6 – Inhibition of calcium signaling events triggered by 10 nM PEBA in human sperm
Inhibitors tested were: cAMPS-Rp (25 μM), U 73122 (5 μM) and NNC 55-0396 (10 μM). Sperm cells were allowed to capacitate for at least 5 hours, labelled with 7.5μM Ca^{2+} Green and exposed to three applications of PEBA alone and one application of PEBA + inhibitor. Data, plotted as percentage inhibition ± SEM (see section 3.2.2 for inhibition definition), consists of 2 experiments for each inhibitor, each including an average of 290 cells.

A final set of experiments was undertaken investigating combined effects on human sperm migration when spermatozoa were exposed to both boronic acids tested in section 4.3.2 (HMPBA and PEBA, both at 10 nM) in conjunction with the inhibitors tested above in this section. Boric acid (10 nM BA) was included as an extra test compound, and additionally, caffeine (1 mM), as a known stimulant of sperm motility (Lardy et al., 1971), was included as a positive control (Figure 4.7). Results from the modified Kremer test, expressed as

percentage variation from control of the total number of cells scored along the capillary ± SEM, show all sperm enhancers causing a positive effect on sperm migration (Figure 4.7 A), although none of these variations proved statistically significant in this small dataset when compared to control (paired *t* test, $p>0.05$). PEBA caused the highest increase in total number of cells migrating into the capillary, causing 42.7±19.7% more cells to migrate into the capillary, followed by BA, which caused a slightly lower increase of 28.4±19.9%, whereas the effect observed for HMPBA and caffeine was less than half of PEBA's (11.5±13.6 and 15.2±15.6%, respectively).

When enhancers were tested in conjunction with 25 µM cAMPS-Rp (cAMP analogue that acts as a competitive antagonist of the cAMP-induced activation of PKA), the positive effect mentioned above was largely abolished (Figure 4.7 B) for all enhancers tested, with caffeine showing the biggest drop (65.5±15.8%) in number of sperm penetrating the capillary, followed by BA, with a decrease of 53.7±20.6%. Similar results were obtained for 5 µM U 73122, albeit slightly less dramatic, with reductions around 35% for the boronic acids and slightly higher (53.6±14.5%) for caffeine.

Under control conditions (no enhancer added to the sperm suspension; Figure 4.7 B – Control + Inhibitor), both inhibitors tested showed themselves a detrimental influence on sperm migration, with 5 µM U 73122 causing a more dramatic effect in the number of cells travelling up the capillary than 25µM cAMPS-Rp (reductions of 58.0±17.2% and 33.2±19.0%, respectively), although differences from control were found to be non-significant (paired *t* test, $p>0.05$).

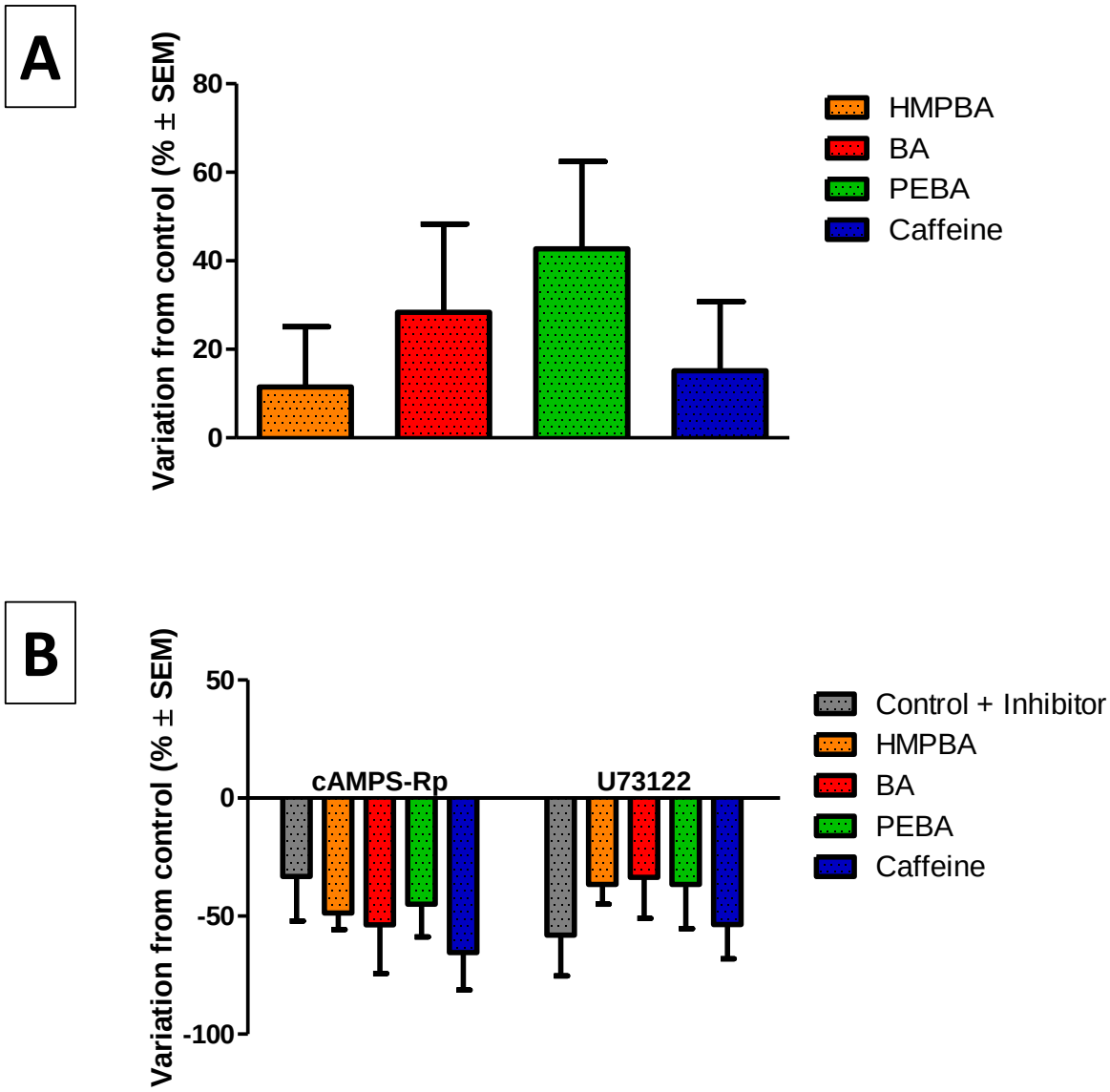


Figure 4.7 – Effects of sperm motility enhancers and key pathway inhbitors on sperm migration
A. Effect of boronic acids (HMPBA, BA and PEBA, all at 10 nM) and caffeine (1 mM) on human sperm migration; **B.** Combined effect of motility / migration enhancers and inhibitors acting on IP_3 (5 µM U73122) and cAMP (25 µM cAMPS-Rp) pahways on human sperm migration. Data, ploted as variation from control ± SEM, consists of four experiments across four different donors.

4.4. Discussion

Ongoing research within our lab has identified a compound (phenethylboronic acid – PEBA) apparently capable of enhancing human sperm migration on a test mimicking in vivo cervical

mucus penetration (modified Kremer test – Kremer, 1965). The purpose of the work discussed below has been both to validate the enhancing capabilities PEBA has previously shown over human sperm, but also to attempt to characterise the mechanisms involved in possible physiological and functional effects PEBA triggers in sperm. With this in mind, and because of calcium's central role in most cellular events in human sperm (Publicover et al., 2007), the effect of PEBA on $[Ca^{2+}]_i$ was investigated (Figure 4.1). The boronic acid caused Ca^{2+} transients on capacitated human sperm over a range of concentrations, and these consistently proved re-evocable on most cells observed, with responses to higher PEBA concentrations (10 – 100 nM) displaying, at least first glance, similar kinetics to the traces evoked by 3.2 µM progesterone. Because transients were observed for concentrations as low as 3 pM, a broader concentration range was tested (Figure 4.2), aiming to define a reasonable PEBA concentration to be used in subsequent studies. Unlike what was seen for Ω (section 3.3.1), the relation between percentage of cells developing transients and the logarithm of the PEBA concentration tested has not resulted in the typical sigmoid dose-response curve when the non-linear fit was tested. This looks likely to result from the chemical being active over a broader concentration range than the one tested, which is in itself surprising, as 3 fM PEBA still triggered transients in just under a fourth of the cell population (24%). Further testing would have been necessary to draw a more complete and explicit dose-response curve, featuring even higher dilutions of the chemical. Nevertheless, the current results did provide a good indication to decide on the further testing of PEBA within the lower nM range, which proved active in 60-70% of the cells observed.

To contextualise the Ca^{2+} signals observed, and test for functional effects these might relate to, a large dataset was gathered by testing PEBA, alongside another boronic acid (2-

(Hydroxymethyl)phenylboronic acid – HMPBA) which also showed promise in enhancing sperm migration, in the standard penetration test used in our lab (modified Kremer test – see section 4.2.3). The dataset included the screening of 72 sperm donors for the effects of both boronic acids on human sperm migration (Figure 4.3). It is worth highlighting that this test intends to mimic conditions encountered by freshly ejaculated sperm migrating from the vagina, through the cervix and into the uterus: both the distances presented, but importantly also the viscosity of the media, are closely related to what sperm would encounter in vivo around ovulation. Sperm migration was significantly enhanced when cells were exposed to either of the chemicals. Moreover, this enhancement was significantly higher for PEBA than for HMPBA, with 61.0±15.6% more cells scored in the microcapillary than in control, as opposed to 38.6±12.2% observed for HMPBA. These results strongly suggest both chemicals are triggering signaling cascades in sperm that ultimately result in one or more behavioural effects responsible by the functional effect observed. These could certainly be due to a chemokinetic effect on sperm motility, which would explain the increased number of cells migrating into the capillary, but trapping in the capillary by increased hyperactivation rates or even a negative chemotactic cannot be ruled out at present. Investigation over effects on motility parameters and hyperactivation like the one described for Ω (section 3.3.6) will be crucial in providing a more educated answer to this question, and will surely be underway in the near future.

Because a relatively high percentage of cells developed Ca^{2+} transients in response to PEBA, an additional set of experiments was conducted to investigate the effect of this chemical on acrosome reaction (Figure 4.4). Although reported as preliminary (dataset consists of two observations across two donors), the results indicate that PEBA concentrations below 30 µM

do not cause major effects in AR, with AR stimulation sitting below 5%. 300 µM, however, caused a relatively higher AR stimulation (7.4±2.1%), which could point to a significant effect of the chemical for concentrations in the mM range. A larger dataset would certainly benefit any definite conclusions to be taken, but it seems likely that the effect of PEBA in AR is negligible, at least for the concentration used in the migration studies (10 nM). At the lowest concentration tested (3 nM), the chemical causes a negative AR stimulation (-2.2±1.3%); it would be interesting to know if, on an extended dataset, this and lower concentrations would maintain the trend, resulting in a measurable reduction on the spontaneous AR rate.

In order to gain some understanding on the characteristics of the calcium response caused by PEBA in capacitated spermatozoa, a preliminary attempt to localise the signal onset was undertaken (Figure 4.5), similar to what was done for Ω (see section 3.3.7). Unlike the Ω-evoked transients, whose signals clearly start in the midpiece before they propagate to the head (on average 1.36±0.51 seconds later), onset of PEBA signals could not be significantly identified as starting in the midpiece: around two thirds of the cell population observed tended to initiate transients in the midpiece, with signal propagating to the sperm head 0.44±0.38 seconds later. This delay, however, did not prove significantly different from a hypothetical zero delay, as shown by a one sample *t* test ($t_{(18)}$=1.154, p<0.264). This indicates a considerably faster signal propagation between midpiece and head than what was previously observed for the Ω response, probably relating to the two chemicals signaling through distinct pathways.

The indication that Ω and PEBA are in fact signaling through different pathways is indeed further substantiated by the different inhibition pattern observed for both signals. Where Ω-response is largely unaffected by exposure to PLC inhibitor U73122 (), interfering with the IP_3

pathway in the same way causes a much higher inhibition of the PEBA response (Figure 4.6). On the other hand, PKA inhibitor cAMPS-Rp, which when tested with Ω resulted in a 72±14.9% inhibition, has a much lesser effect on the PEBA-evoked response (33.9±10.1% inhibition). Interstingly, inhibition levels obtained with NNC 55-0396 are very similar over both responses (77.6±3.2% and 73.7±2.0% for Ω and PEBA responses, respectively).

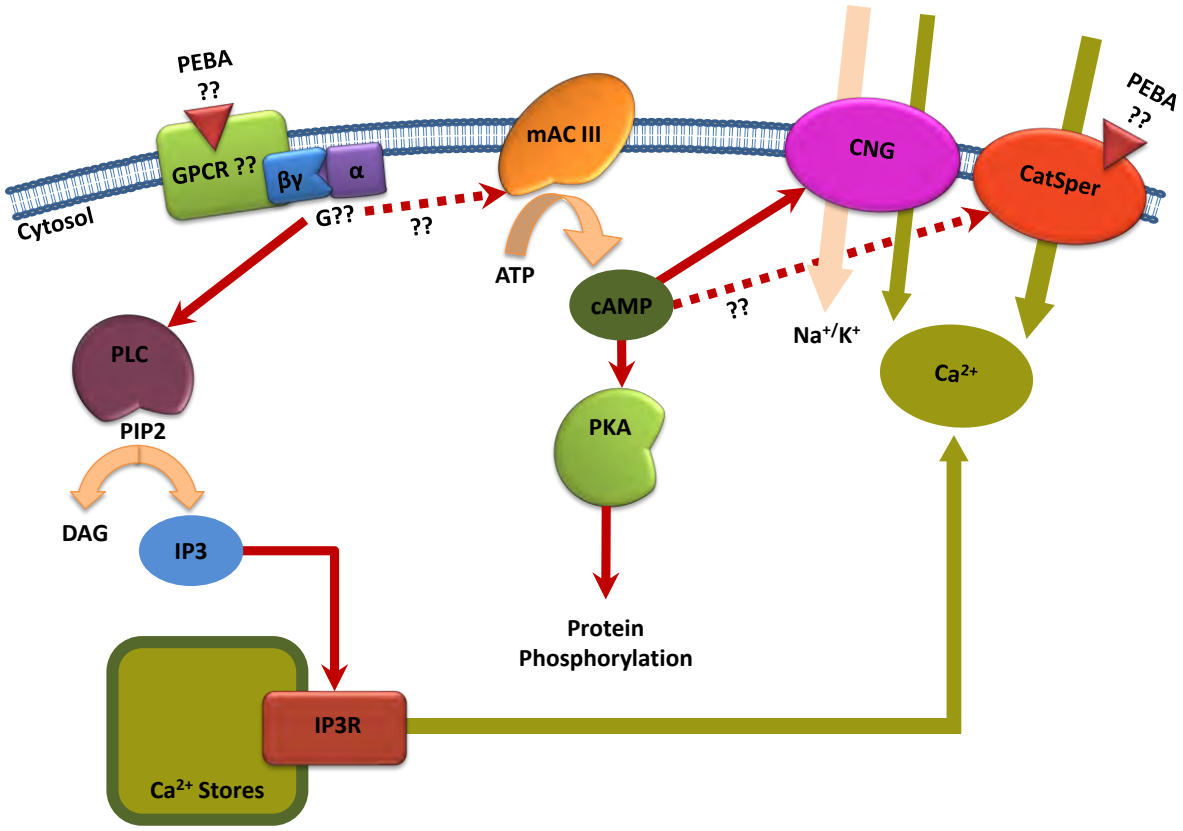


Figure 4.8 – Proposed transduction mechanism for the PEBA-evoked signals in human spermatozoa

Although, as discussed in section 3.4, inhibitors tested had themselves significant effects on $[Ca^{2+}]_i$, which should not be disregarded, the current results on PEBA signaling point toward a bifurcating signal which is highly dependent on both CatSper and PLC, but is still considerably affected by PKA blockage. It could be that IP_3 and cAMP pathways are both

involved in the transduction of the PEBA signal. Such a signal has been described for a particular rat OR (rat olfactory receptor I7), whose agonist octanal causes the receptor to signal via cAMP at concentrations lower that 10^{-4} M, but concentrations above that cause the receptor to trigger both IP_3 and cAMP pathways (Ko and Park, 2006). The model proposed in Figure 4.8 intends to summarise how PEBA signal transduction might relate to the observed changes in $[Ca^{2+}]_i$. Apart from the involvement of CatSper, PKA and PLC, current results provide no solid evidence of the participation of any of the other molecules included, but these are known to be present in sperm and are likely to interact in a more or less similar manner as described for better studied signalling cascades. As PEBA is shown to be signaling mainly via PLC, the possibility of the boronic acid activating a GPCR on the cell's plasma membrane is likely, similarly to what is seen for GPCR activation by certain hormones and neurotransmitters in other cell types; however, the possibility of the boronic acid stimulating other type of receptor directly or indirectly interacting with PLC cannot be discarded. Furthermore, and as recently reviewed by Costello et al., 2009, mammalian sperm display all the molecular entities of a functional IP_3 signaling pathway, including many of the known PLC isoforms (Saunders et al., 2002; Tomes et al., 1996; Walensky et al., 1995), but also IP_3 receptors (IP_3R) (Dragileva et al., 1999; Ho and Suarez, 2001; Ho and Suarez, 2003; Kuroda et al., 1999; Naaby-Hansen et al., 2001) and the G-protein alpha subunit ($G\alpha_{q/11}$) known to activate PLC (Walensky et al., 1995). Ca^{2+} release from intracellular stores via IP_3R in the acrosomal membrane and anterior midpiece could explain, at least in part, the rise in intracellular calcium levels, and is coherent with the signal's subcellular onset. On the other hand, the involvement of CatSper seems unquestionable, given the level of inhibition observed for NNC 55-0396. However, how this channel is being activated is still elusive, as

discussed in section 3.4, and direct activation by PEBA remains a possibility. The pathway characterisation is therefore still lacking important information and further investigation, in particular on the identity of the receptor/s molecule/s involved will be essential in future work, but the strong effect of PEBA on sperm motility and the fact that this chemical causes functional effects and also Ca^{2+} responses in human sperm at very low concentrations is indicative of a highly sensitive system.

A final set of experiments was undertaken aiming to investigate if the inhibition effects observed on PEBA-evoked Ca^{2+} signals had a measurable functional effect on sperm migration, particularly on the migration enhancement caused by the boronic acid (Figure 4.7). The migration enhancing effects observed on this subset of experiments for both PEBA and HMPBA (Figure 4.7 A) were consistent with what was previously reported (see section 4.3.2). Additionally, another boronic acid (boric acid - BA) was tested, alongside caffeine, a known stimulant of sperm motility (Lardy et al., 1971). As this was a preliminary investigation, and the dataset comprised a total of four experiments across four donors, the differences in migration were not statistically significant from control for any of the enhancers, although previous data gathered for PEBA and HMPBA proved otherwise. In terms of effects of the inhibitors tested (Figure 4.7 B), both cAMPS-Rp and U73122 caused a substantial reduction on sperm migration themselves: in control cells exposed only to the inhibitor, cAMPS-Rp has caused a 33.2±19.0% reduction in the number of cells scored in the capillary, whereas U73122 had an even higher detrimental effect (53.6±14.5%). This clearly indicates a negative effect on sperm motility, but interpretation of similar results obtained for cells exposed to the enhancers in presence of either inhibitor is therefore less straightforward, as it isn't clear if any of the effects observed for the different enhancers

have a direct causal link to the enhancer itself or if they represent combined effects of both inhibitor and enhancer. Nevertheless, it seems that migration is further reduced when enhancers were tested in presence of cAMPS-Rp, whereas in presence of U73122, all boronic acids, but not caffeine seem to cause sperm to slightly recover from the detrimental effect the inhibitor has on migration.

5. GENERAL DISCUSSION

The data presented has assisted in characterising human olfactory receptor (OR2AE1) believed to be prevalent on sperm surface from proteomic evidence (Baker et al., 2007; Lefievre et al., 2007). A potent agonist (Ω) was identified for the receptor and its effects as a motility and acrosomal status modulating agent, but importantly also as sperm chemoattractant were examined. Contrastingly, a novel modulator of sperm motility, phenethylboronic acid (PEBA), has also been examined and the second messenger systems identified, allowing comparison of the data, though as yet we have no identified receptor for this action.

The agonist identified for OR2AE1 within the heterologous expression system proved effective in triggering calcium signals in human sperm, which we believe, are likely to be mediated by the native receptor expressed in sperm. These signals were largely abolished by the T-type Ca^{2+} channel inhibitor NNC 55-0396, probably by its action on CatSper, but also by blocking mAC activity with SQ22536. The inhibition pattern observed for the Ω response, together with the observation that its onset is slower than the one observed for the progesterone-mediated signal substantiate that, unlike progesterone-mediated signals, reported to result from direct activation of CatSper (Lishko et al., 2011; Strunker et al., 2011), calcium entry via CatSper or another channel on the Ω-evoked signal is via a second-messenger activation, likely related to raised levels of cAMP resulting from OR2AE1 stimulation.

The inhibition pattern observed for PEBA indicates a different signal transduction pathway to that for Ω: the stimulation of a receptor whose identity is not yet known causes activation of

PLC, but also seems to relate to PKA activity, whose blockage causes a substantial reduction of the response, although not as dramatic as when PLC is inhibited. Similar to the Ω signals, most of the calcium influx may be mediated by CatSper as indicated by its NNC 55-0396 sensitivity.

Both the heterologous system and sperm showed responses to other molecules in the odorant mixtures tested, and similarly, sperm also responded not only to PEBA but also to some other boronic acids (though to a much lesser extent), both functionally and via Ca^{2+} transients. If these responses are specific, this could be indicative of a more broadly tuned receptor which can be stimulated by closely related molecules, be it because these share the same vibrational pattern (according to the vibrational model - Franco et al., 2011) or a particular molecular shape (in tune with the "lock-and-key" model). Such a characteristic is not unusual amongst ORs, with some receptors being activated by a range of structurally (or spectrally) similar odorants instead of showing a strict specificity towards a single molecule (Malnic et al., 1999; Mombaerts, 2004; Veitinger et al., 2011). Alternatively, sperm could be responding to different stimuli via other receptors yet to be identified.

Further work on olfactory receptors in sperm would be advanced if specific targeted antibodies were available. If time allowed it would be interesting to screen the Ω responses I observed with a wider array of inhibitors. This could include trying to find a competing volatile, such as undecanal was suggested to be for bourgeonal, though my finding that this was not occurring may make such a discovery unlikely. Only when ongoing research into the contents of female tract secretions identifies a panel of chemicals that can be tested on the ORs are we likely to know the form of the true physiological agonist.

In the intracellular calcium imaging experiments performed, the single-wavelength dye Calcium Green-1 AM was employed as it has an established history in our research of low toxicity to sperm over prolonged imaging periods. A potential disadvantage of this strategy versus using alternative ratiometric dyes like Fura-2 AM is that calibration of calcium concentrations could not be performed. However, because most of the experiments required long exposure sequences, the benefit of the extra data from ratiometric measurements did not outweigh the increased cytotoxicity likely to occur due to long exposure to UV light.

The mechanisms behind this complexity are something about which we still have no knowledge – both PEBA and Ω induce sperm $[Ca^{2+}]_i$ signaling which to the observer is similar, yet across dosage ranges and chemicals causes totally different migration phenotypes to be displayed. It is clear that a greater understanding of the sperm flagellar beat will be crucial to comprehending this (Gaffney et al., 2010) alongside assessment in the correct relative viscosity (Kirkman-Brown and Smith, 2011) and higher speed and resolution calcium imaging. The calcium signalling in the oocyte and the contribution of the sperm is probably better understood now (reviewed in Whitaker, 2006) and whether certain components, such as PLCzeta may have a role in both sperm signalling and later in the oocyte is an intriguing possibility (Saunders et al., 2002).

It is tempting to speculate that a substantial portion of the people with 'unexplained infertility' may have functional sperm issues that could be diagnosed with development of tests such as those based around effects of motility-modulating agents, but again this would mean using the agent in the relevant context to find the result which may add unnecessary

complexity to standard semen analysis. One tempting idea being that men could sniff a 'smell panel' which could confirm whether they had functioning olfactory receptors of the relevant kind being expressed – as a non-invasive assay.

In summary, within the current work I was able to functionally express OR2AE1 in HEK293 cells and used this system to identify a potent agonist (Ω) for the receptor from a library of odorant compounds. Testing this agonist in human sperm, where OR2AE1 is known to be ectopically expressed, I have shown that 50 μM Ω triggers re-evocable Ca^{2+} responses in capacitated spermatozoa and that the same concentration of agonist acts as a chemoattractant. On a parallel approach, I have also tested a related odorant (PEBA) which proved capable of triggering calcium signals associated with a significant increase in sperm migration.

Functionally the ability to derive a treatment based upon my data may be some distance away, however the ability of PEBA to increase migration of sperm from semen is a useful property with a potential that, if harnessed in a vaginal lubricant, could assist natural fecundity.

As knowledge around the agonists and antagonists of these motility systems emerges there may be further therapeutic or contraceptive developments that become possible and use of sperm motility as a truly affordable way to help people with their fertility problem is an inspiring target that I hope to have made incremental progress towards.

6. APPENDIX I: MEDIA

Dulbecco's Modified Eagle Medium (D-MEM) (1X) liquid (high glucose)

COMPONENTS	Molecular Weight	Concentration	
		(mg/L)	mM
Glycine	75	30	0.4
L-Arginine hydrochloride	211	84	0.398
L-Cystine 2HCl	313	63	0.201
L-Glutamine	146	580	3.97
L-Histidine hydrochloride-H_2O	210	42	0.2
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.4
L-Serine	105	42	0.4
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine	181	72	0.398
L-Valine	117	94	0.803
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.00839
Folic Acid	441	4	0.00907
Niacinamide	122	4	0.0328
Pyridoxine hydrochloride	204	4	0.0196
Riboflavin	376	0.4	0.00106
Thiamine hydrochloride	337	4	0.0119
i-Inositol	180	7.2	0.04
Calcium Chloride ($CaCl_2 \cdot 2H_2O$)	147	264	1.8
Ferric Nitrate ($Fe(NO_3)_3 \cdot 9H_2O$)	404	0.1	0.000248
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	246	200	0.813
Potassium Chloride (KCl)	75	400	5.33
Sodium Bicarbonate ($NaHCO_3$)	84	3700	44.05
Sodium Chloride (NaCl)	58	6400	110.34
Sodium Phosphate monobasic ($NaH_2PO_4 \cdot 2H_2O$)	154	141	0.916
D-Glucose (Dextrose)	180	4500	25
Phenol Red	376.4	15	0.0399
Sodium Pyruvate	110	110	1

Suplemented Earls Balanced Salt Solution (sEBSS)

COMPONENTS	Molecular Weight	Concentration	
		(g/L)	mM
Sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$)	156.01	0.158	1.01
Potassium chloride (KCl)	74.55	0.4	5.37
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	246.48	0.2	0.81
Dextrose ($C_6H_{12}O_6$)	180.16	1	5.55
Sodium pyruvate ($C_3H_3NaO_3$)	110.05	0.3	2.73
dl-lactic acid ($C_3H_6O_3$)	112.06	4.68	41.76
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	147.02	0.264	1.80
Sodium bicarbonate ($NaHCO_3$)	84.01	2.2	26.19
Sodium chloride (NaCl)	58.44	6.8	116.36

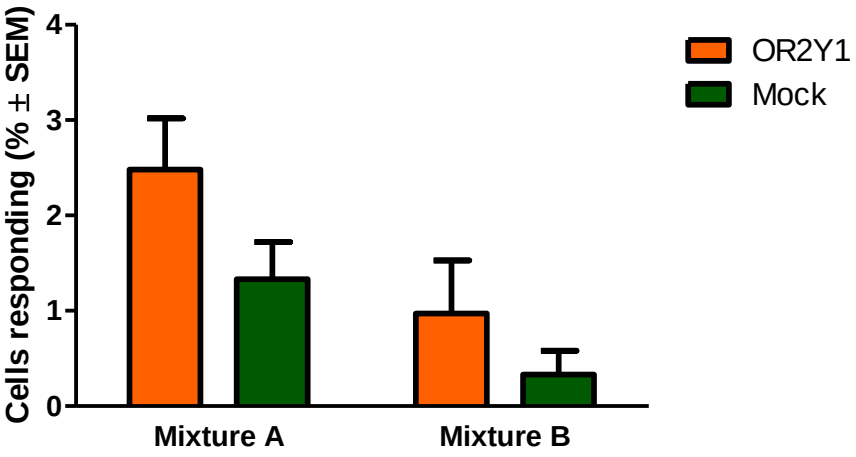
285 <mOsm< 295; 7.15 <pH< 7.45; *I* (Ionic strength) ~ 0.361; 0.3% BSA added before use for final capacitating formulation (sEBSS)

Tyrodes Solution

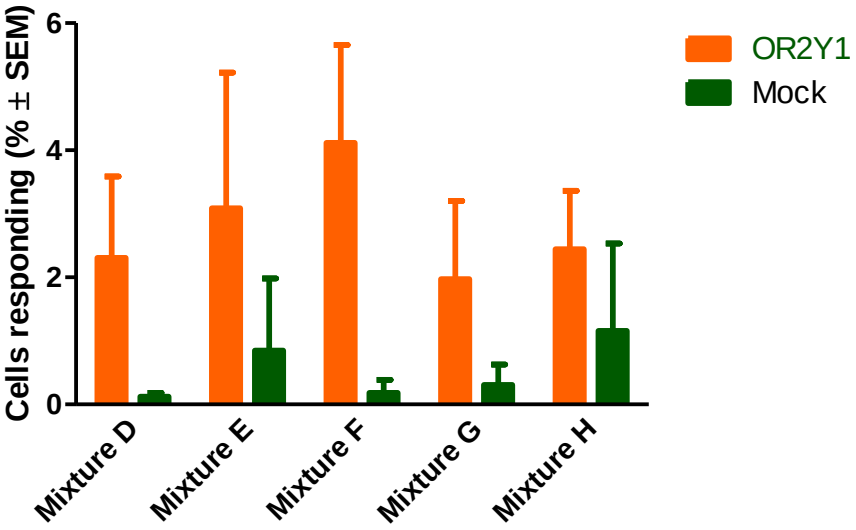
140 mM NaCl
10 mM HEPES
10mM Glucose
10 mM Pyruvic acid
5 mM KCl
1 mM $MgCl_2.6H_2O$
1 mM $CaCl_2$

pH ~ 7.4
Osm ~ 300 mOsm

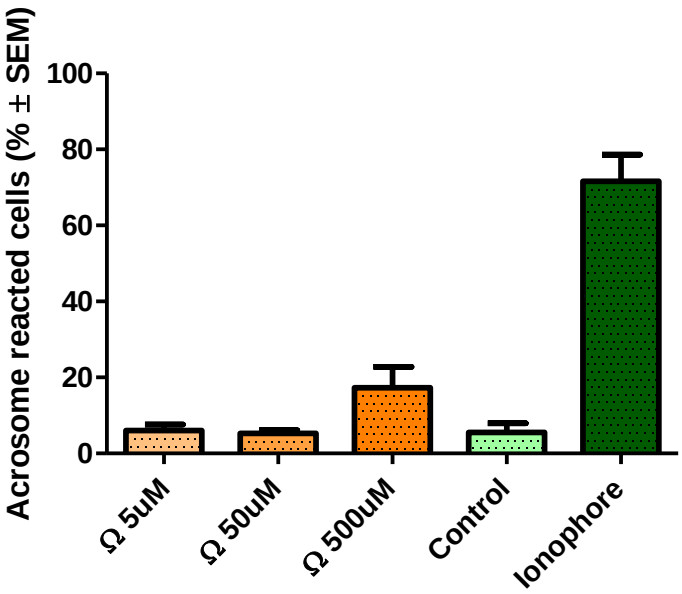
7. APPENDIX II: SUPPLEMENTARY DATA



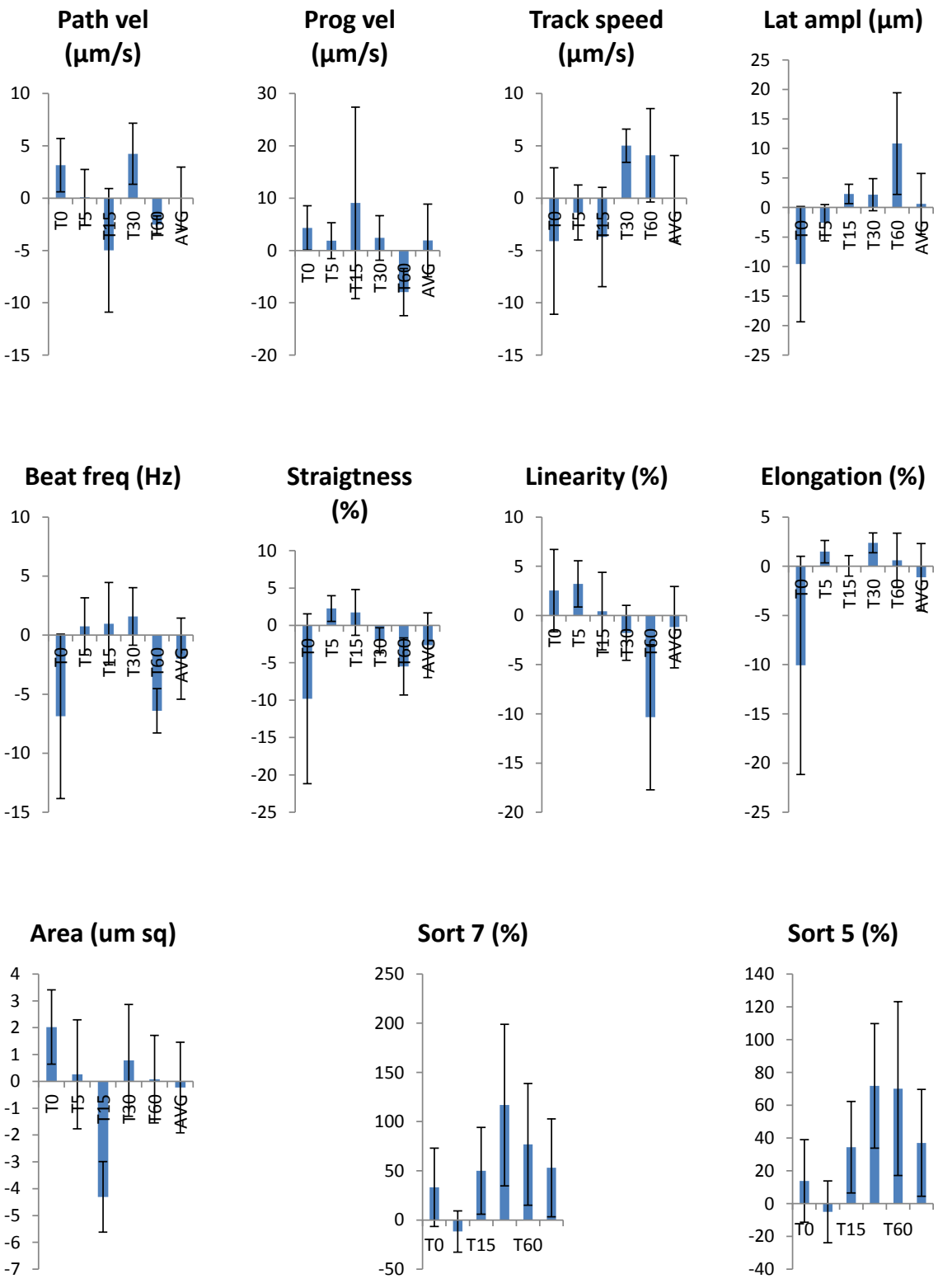
Supp. Figure 1 - Calcium responses to mixtures A and B
Mixtures include 50 odorants each at 50 µM. HEK293 cells were transfected with 2 µg of plasmid DNA (OR2Y1 or mock) 24 hours after plating and labelled with 7.5 µM Ca2+ Green-1 24 hours after transfection. Single cell calcium imaging readings taken at 2 s intervals and processed data, plotted as percentage of responding cells ± SEM, consists of 7 experiments, each including an average of 230 cells; Differences between mean values of receptor and mock transfected cells responding are not statistically significant (paired T test, $t_{(6)}$, $P > 0.05$).



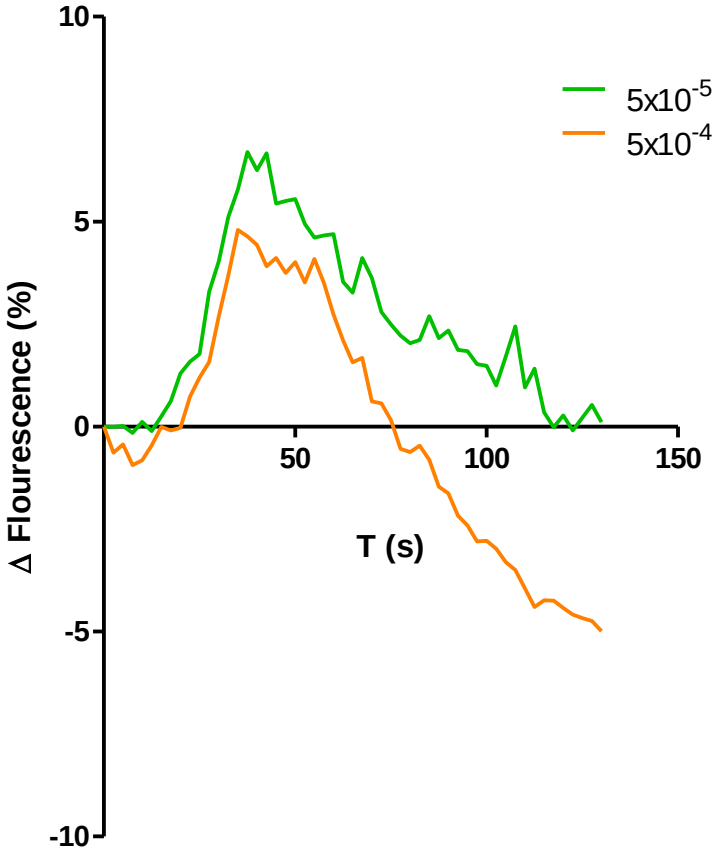
Supp. Figure 2 - Total calcium responses to mixtures D to H
Mixtures include 10 odorants each at 50 µM. HEK293 cells were transfected with 2 µg of plasmid DNA (OR2Y1 or mock) 24 hours after plating and labelled with 7.5 µM Ca2+ Green-1 24 hours after transfection. Single cell calcium imaging readings taken at 2 s intervals and processed data, plotted as percentage of responding cells ± SEM, consists of 7 experiments, each including an average of 230 cells; Differences between mean values of receptor and mock transfected cells responding are not statistically significant (paired T test, $t_{(6)}$, $P > 0.05$).



Supp. Figure 3 - Effect of Ω on acrosome reaction
Acrosome reaction triggered by 5, 50 and 500 µM Ω in mature human sperm. Data, plotted as percentage of acrosome reacted cells ± SEM, includes 6 experiments across 6 donors.



Supp. Figure 4 – Effects of 50 µM Ω on human sperm motility observed by CASA
For each variable, results represent variation from control ± SEM over one hour (T0 to T60 minutes).



Supp. Figure 5 – Kinetics of Ca^{2+} transients evoked by 50 and 500 µM Ω
Traces (50 µM – green trace; 500 µM – orange trace) represent the average of the normalised fluorescence variations of all the cells observed (n=273 cells) in a single experiment where 50 and 500 uM Ω were presented sequentially as 20 second applications, intercalated with a 5 minute resting period.

Supp. Table 1 – Effects of 50 µM Ω on motility exhibited by capacitated human spermatozoa
(Values are mean variation from control)

	Path vel (µm/s)			Prog vel (µm/s)			Track speed (µm/s)			Lat ampl (µm)			Beat freq (Hz)			Straigtness (%)			Linearity (%)			Elongation (%)			Area (µm sq)			Sort 7 (%)			Sort 5 (%)		
Time (min)	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
0	3	2.54	7	4	4.23	7	-4	7	7	-10	9.77	7	-7	6.97	7	-10	11.36	7	3	4.18	7	-10	11.1	7	2	1.38	7	33	39.77	7	14	25.16	7
5	0	2.66	7	2	3.43	7	-1	2.63	7	-3	3.06	7	1	2.42	7	2	1.72	7	3	2.36	7	1	1.13	7	0	2.03	7	-12	21.06	7	-5	18.84	7
15	-5	5.9	4	9	18.31	4	-4	4.75	4	2	1.62	4	1	3.49	4	2	3.06	4	0	3.94	4	0	1.05	4	-4	1.32	4	50	44.1	4	34	27.91	4
30	4	2.92	7	2	4.24	7	5	1.59	7	2	2.7	7	2	2.43	7	-2	1.67	7	-2	2.8	7	2	1.01	7	1	2.08	7	117	82.19	7	72	38.01	7
60	-3	0.85	3	-8	4.51	3	4	4.47	3	11	8.62	3	-6	1.88	3	-5	3.81	3	-10	7.37	3	1	2.76	3	0	1.63	3	77	61.95	3	70	53.04	3

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