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5 Regulation and roles of Ca<sup>2+</sup> stores in human sperm.

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10

11 Abstract.

12  $[Ca^{2+}]_i$  signalling is a key regulatory mechanism in sperm function. In mammalian sperm the  $Ca^{2+}$ -  
13 permeable, plasma membrane ion channel CatSper is central to  $[Ca^{2+}]_i$  signalling but there is good  
14 evidence that  $Ca^{2+}$  stored in intracellular organelles is also functionally important. Here we briefly  
15 review current understanding of the diversity of  $Ca^{2+}$  stores and the mechanisms for the regulation  
16 of their activity. We then consider the evidence for the involvement of these stores in  $[Ca^{2+}]_i$   
17 signalling in mammalian (primarily human) sperm, the agonists that may activate these stores and  
18 their role in control of sperm function. Finally we consider the evidence that membrane  $Ca^{2+}$   
19 channels and stored  $Ca^{2+}$  may play discrete roles in the regulation of sperm activities and propose a  
20 mechanism by which these different components of the sperm  $Ca^{2+}$ -signalling apparatus may  
21 interact to generate complex and spatially diverse  $[Ca^{2+}]_i$  signals.

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23 1. Ca<sup>2+</sup> signalling in sperm

24 Cellular activity is constantly regulated by environmental cues and signals from other cells. Long  
25 term regulation of cell function is normally achieved by control of gene expression, changing the  
26 complement and levels of proteins in the cell, but rapid or short-term changes are achieved by ‘post-  
27 translational’ protein modification, such as phosphorylation, sumoylation and nitrosylation, which  
28 alter the function/activity of proteins already present. Ca<sup>2+</sup>-signalling is a key regulator of such post-  
29 translational modifications, with changes in cytoplasmic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) controlling the  
30 activities of key enzymes and proteins. Large changes in [Ca<sup>2+</sup>]<sub>i</sub> can be achieved ‘instantaneously’ by  
31 flux of Ca<sup>2+</sup> into the cytoplasm from the extracellular fluid or from storage organelles (primarily the  
32 endoplasmic reticulum) within the cell (fig 1a). The rapidity with which [Ca<sup>2+</sup>]<sub>i</sub>-signals can be  
33 generated is crucial for ‘instantaneous’ cellular responses such as activation of muscle contraction  
34 and secretion of neurotransmitter, that are achieved by rapid post-translational-modification of  
35 protein function.

36 The highly-condensed nucleus of sperm is transcriptionally silent (Miller and Ostermeier 2006,  
37 Miller, et al. 2005) and translational activity is also negligible [though evidence has been presented  
38 for translation occurring at mitochondrial ribosomes (Chandrashekan, et al. 2014a,  
39 Chandrashekan, et al. 2014b, Gur and Breitbart 2007, Zhao, et al. 2009)]. Regulation of sperm  
40 function is therefore dependent primarily on post-translational processes. [Ca<sup>2+</sup>]<sub>i</sub> signalling is pivotal  
41 to this regulation and in mammalian sperm it plays a central role in controlling the cell’s behaviour  
42 (motility type and potentially chemotaxis), the induction of acrosome reaction and the process of  
43 capacitation (Darszon, et al. 2011, Darszon, et al. 2007, Publicover, et al. 2007). The importance for  
44 sperm function of membrane Ca<sup>2+</sup>-channels and Ca<sup>2+</sup>-influx is well-established (Darszon, et al. 2011)  
45 but there is also good evidence for the existence and functional importance of intracellular Ca<sup>2+</sup>-  
46 storage organelles in sperm (Darszon, et al. 2007, Publicover, et al. 2007). Previously we reviewed  
47 the identities and functions of Ca<sup>2+</sup> stores in sperm, focussing on the evidence for the existence of

48 such stores, their components (pumps and channels) and their possible roles in the regulation of  
49 function in the mature sperm cell (Costello, et al. 2009). Since then considerable progress has been  
50 made in understanding the central role of  $\text{Ca}^{2+}$  signalling in the regulation of mammalian and non-  
51 mammalian sperm function and the mechanisms by which sperm  $[\text{Ca}^{2+}]_i$  signals are generated. In  
52 particular successful application of whole cell patch clamp technique, in human as well as mouse  
53 sperm, has revealed the central importance of  $\text{Ca}^{2+}$  influx through CatSper, a sperm specific,  $\text{Ca}^{2+}$ -  
54 permeable channel in the membrane of the flagellar principal piece. Male mice null for CatSper are  
55 infertile (Ren, et al. 2001) and their sperm show defective motility (Carlson, et al. 2003). Here we  
56 review recent progress in understanding the diversity of mechanisms for the regulation of  $\text{Ca}^{2+}$  store  
57 activity and the evidence for their involvement in controlling sperm function.

## 58 2. $\text{Ca}^{2+}$ stores and their regulation

59 The importance of  $\text{Ca}^{2+}$  stores in generating complex  $\text{Ca}^{2+}$  signals in somatic cells has long been  
60 recognized. Until relatively recently the endoplasmic reticulum  $\text{Ca}^{2+}$  store has been the major focus  
61 for research as this was the first organelle to show controllable mobilization of  $\text{Ca}^{2+}$  through second  
62 messengers acting upon intracellular  $\text{Ca}^{2+}$  channels, as well as being able to be refilled via  $\text{Ca}^{2+}$   
63 pumps. Additionally, these  $\text{Ca}^{2+}$  signals could also be re-modelled through the regulation of these  
64  $\text{Ca}^{2+}$  transporters to generate complex spatial and temporal  $\text{Ca}^{2+}$  transients (Berridge, et al. 2003). It  
65 has now become clear that many other organelles such as mitochondria, endosomes, lysosomes and  
66 Golgi complexes also contribute to the generation and propagation of these complex  $\text{Ca}^{2+}$  signals  
67 within cells (Michelangeli, et al. 2005). Furthermore, novel  $\text{Ca}^{2+}$  transporters have also been  
68 identified within these other organelles and several have recently been identified in sperm (Costello,  
69 et al. 2009).

### 70 (i) Intracellular $\text{Ca}^{2+}$ Channels

71 The major intracellular  $\text{Ca}^{2+}$  channels that have been identified and appear to be almost ubiquitously  
72 distributed within mammalian cells, especially on the endoplasmic reticulum, include the inositol-  
73 1,4,5-trisphosphate-( $\text{IP}_3$ )-sensitive  $\text{Ca}^{2+}$  channel (or  $\text{IP}_3$  receptor;  $\text{IP}_3\text{R}$ ) and the ryanodine receptor  
74 (RyR) (Michelangeli, et al. 2005) (fig 1a). The  $\text{IP}_3$  receptor, as the name implies, is activated by the  
75 second messenger  $\text{IP}_3$  that is generated through the hydrolysis of phosphatidylinositol-4,5-  
76 bisphosphate. This channel has a specific  $\text{IP}_3$  binding site that is located towards the N-terminus of  
77 the protein (Seo, et al. 2012) and also has a requirement for  $\text{Ca}^{2+}$  which acts as a co-agonist in order  
78 for the channel to open (Bezprozvanny, et al. 1991). The activation of RyR is likely to be through a  
79 mechanism involving  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release (CICR) and by the action of the putative second  
80 messenger cyclic-adenosine diphospho-ribose (cADPR) (Ogunbayo, et al. 2011) (fig 1a). cADPR is  
81 made from nicotinamide-adenine-dinucleotide (NAD) by the action of an ADP-ribosyl cyclase enzyme  
82 such as CD38 (Cosker, et al. 2010), although other as yet unidentified enzymes may also be involved  
83 in catalysing this reaction (Guse 2014). It is as yet unclear whether, unlike the  $\text{IP}_3\text{R}$ , cADPR binds  
84 directly to RyR or whether it binds to accessory proteins such as calmodulin or FK506-binding  
85 protein, that then interact with the RyR (Guse 2014).

86 Another metabolite of NAD which is believed to have  $\text{Ca}^{2+}$  mobilizing ability is nicotinic acid adenine  
87 dinucleotide phosphate (NAADP) (Genazzani, et al. 1997). NAADP is made from NADP through the  
88 action of either CD38 acting as a base-exchanger, swapping the nicotinamide group for nicotinic acid  
89 or via an unidentified NADP-deaminase (Guse 2014). NAADP is believed specifically to mobilize  $\text{Ca}^{2+}$   
90 from acidic stores such as lysosomes (Churchill, et al. 2002, Menteyne, et al. 2006 ), which can then  
91 induce CICR at RyRs and  $\text{IP}_3\text{Rs}$  in mammalian cells (Cancela et 1999) (fig 1a). Results initially  
92 presented by Calcraft and colleagues (Calcraft, et al. 2009), indicated that NAADP specifically  
93 activates  $\text{Ca}^{2+}$  specific 'two-pore' channels (TPC) within the acidic organelles, these channels being  
94 first described in plants (Peiter, et al. 2005). However, in kinetic studies there is a prominent lag  
95 between addition of NAADP and  $\text{Ca}^{2+}$  mobilization (Genazzani, et al. 1997). Combined with the  
96 observation that photo-affinity labelling with azido-NAADP (Lin-Moshier, et al. 2012) showed

97 labelling of only low molecular weight proteins, not consistent with TPCs, this suggests that NAADP  
98 might function by binding to accessory proteins rather than directly to the channel. More recently  
99 two studies (Cang, et al. 2013, Wang, et al. 2012) have raised considerable controversy as whether  
100 the NAADP-sensitive  $\text{Ca}^{2+}$  channel is a TPC. Both studies suggest that TPCs are in fact  $\text{Na}^{2+}$ -specific  
101 channels with very low  $\text{Ca}^{2+}$  selectivity that are activated by phosphoinositide lipids and modulated  
102 by mTOR, but not by NAADP. More work is currently being undertaken to clarify this and a number  
103 of possible theories as to what role TPCs play in NAADP-induced  $\text{Ca}^{2+}$  mobilization are being explored  
104 (see Morgan and Galione (2014)).

105 Numerous kinases have been shown to modulate the activity of both the  $\text{IP}_3\text{Rs}$  and  $\text{RyRs}$ , these  
106 include several ubiquitous ser/thr kinases such as PKA, PKG and CaMKII (Camors and Valdivia 2014,  
107 Yule, et al. 2010). Indeed some of these kinases such as PKA appear to have both stimulatory and  
108 inhibitory effects on the  $\text{IP}_3\text{R}$  dependent upon isoform subtype and the presence of multiple kinase-  
109 dependent phosphorylation sites on the same receptor (Dyer, et al. 2003). Less ubiquitous ser / thr  
110 kinases such as Akt and polo kinases as well as tyrosine kinases such as fyn kinase have also been  
111 shown to affect these channels (Camors and Valdivia 2014, Yule, et al. 2010).

112 Both the  $\text{RyRs}$  and the  $\text{IP}_3\text{Rs}$  are modulated by changes in their oxidation states caused by reactive  
113 oxygen species (ROS) and reactive nitrogen species (RNS) and this occurs mainly through  
114 modification of specific cysteine (cys) amino acid residues. Oxidation of these cys residues in  $\text{RyRs}$   
115 occurs both by s-glutathionylation as well as s-nitrosylation by the second messenger nitric oxide  
116 (NO) (Csordas and Hajnoczky 2009) and promotes the activity of the channel by enhancing  $\text{RyR}$   
117 subunit interactions and also by reducing the efficacy of inhibitory modulators (Hamilton and Reid  
118 2000). In  $\text{IP}_3\text{Rs}$  the effects of oxidative stress are complex: low levels of cys oxidation caused by low  
119 concentrations of thimerosal (a cys-modifying mercuric compound) and naturally generated ROS  
120 cause sensitization of this channel, while higher concentrations of thimerosal inhibit channel activity

121 (Missiaen, et al. 1991, Sayers, et al. 1993). Currently, however, there is little evidence that NO can  
122 affect the activity of the IP<sub>3</sub>Rs.

123 (ii) Intracellular Ca<sup>2+</sup> pumps

124 The major transporter involved in refilling Ca<sup>2+</sup> stores within the endoplasmic reticulum is the  
125 sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA) (fig 1a) and these pumps occur abundantly  
126 in all somatic cells. Their role is to pump Ca<sup>2+</sup> back into the storage organelles to help terminate Ca<sup>2+</sup>  
127 signals (Michelangeli and East 2011, Michelangeli, et al. 2005). There are 3 isoforms of this  
128 Ca<sup>2+</sup>ATPase, each encoded by a different gene and each isoform can exist in a variety of spliced  
129 variants that differ in size and regulatory properties (Michelangeli and East 2011). SERCA1 is mainly  
130 confined to skeletal muscle, while SERCA2 is widely distributed in most other tissues and organs and  
131 type 3 has a limited expression. Another related Ca<sup>2+</sup>ATPase that is also found ubiquitously within  
132 somatic cells is the secretory pathway Ca<sup>2+</sup> ATPase (SPCA) which is localized to the Golgi apparatus  
133 (Wootton, et al. 2004). SPCA exists in 2 isoforms with the expression of type 1 being far more  
134 widespread than type 2, which appears to be mainly located within glandular tissues (Vanoevelen, et  
135 al. 2005). Recently there has been evidence to suggest the SPCA2 can interact with and regulate the  
136 plasma membrane located Orai Ca<sup>2+</sup> channels that are implicated in store-operated Ca<sup>2+</sup> entry (Feng,  
137 et al. 2010), which may indicate a dual function for this Ca<sup>2+</sup> ATPase in cells that express it.

138 There is currently some debate as to which type of intracellular Ca<sup>2+</sup> ATPase is expressed in mature  
139 sperm. We have highlighted that SPCA1 is present in human sperm, where it appears to be mainly  
140 localized to the neck region of the cell where the redundant nuclear envelope (RNE) and calreticulin-  
141 containing vesicles are situated (Harper, et al. 2005). This study also found no evidence for  
142 expression of SERCA in human sperm as no cross-reactivity was observed with a pan-isoform SERCA  
143 antibody and no effects on [Ca<sup>2+</sup>]<sub>i</sub> were observed with specific but saturating concentrations of the  
144 SERCA-inhibitor thapsigargin. However, a more recent study (Lawson, et al. 2007) detected SERCA2,  
145 mainly localized to the acrosome and mid-piece, using a SERCA2-specific antibody.

146 Unlike the intracellular  $\text{Ca}^{2+}$  channels, there is no strong evidence to suggest that either SERCA or  
147 SPCA can be directly phosphorylated and regulated by protein kinases, although some  $\text{Ca}^{2+}$  ATPase  
148 modulatory proteins like phospholamban (that is found almost exclusively in heart) are regulated  
149 through phosphorylation by PKA, PKG and CamKII (Colyer 1998). There is considerable evidence  
150 indicating that oxidative stress can modulate SERCA activity (although no studies have yet been  
151 undertaken on SPCA). Again a number of critical cys residues such as cys674 can be s-  
152 glutathionylated to cause an increase in SERCA pump activity (Adachi, et al. 2004). Modifications of  
153 other cys residues on the  $\text{Ca}^{2+}$  ATPase, however, can have inhibitory effects (Csordas and Hajnoczky  
154 2009, Sayers, et al. 1993, Sharov, et al. 2006).

155

### 156 3. $\text{Ca}^{2+}$ stores, mechanisms for store mobilisation and store-operated $\text{Ca}^{2+}$ channels in sperm

157 During the later stages of their development spermatozoa shed much of their cytoplasm including  
158 intracellular organelles. Thus mammalian sperm contain no organised endoplasmic reticulum.  
159 However, studies on the expression of  $\text{Ca}^{2+}$  store components and on the generation  $[\text{Ca}^{2+}]_i$  signals  
160 suggest that the remaining intracellular organelles function as  $\text{Ca}^{2+}$ -stores and play a significant role  
161 in the regulation of cellular function (Costello, et al. 2009). In particular, the acrosomal vesicle at the  
162 apex of the head and the collection of vesicular membranous structures that occur at the sperm  
163 neck and anterior midpiece (including the cytoplasmic droplet of human sperm) appear to be  
164 functionally important  $\text{Ca}^{2+}$ -stores (fig 1b; shown in green). At both these locations  $\text{IP}_3\text{Rs}$  have been  
165 detected in human and in bovine sperm by immuno-staining (Dragileva, et al. 1999, Ho and Suarez  
166 2001, 2003, Kuroda, et al. 1999, Naaby-Hansen, et al. 2001). Ryanodine receptors (RyRs) have also  
167 been detected in human and rodent sperm (Lefievre, et al. 2007, Trevino, et al. 1998). Staining of  
168 human sperm with anti-RyR1, anti-RyR2, pan-RyR and BODIPY-FLX ryanodine is localised primarily to  
169 the neck region, though some acrosomal staining was also observed (Harper, et al. 2004, Lefievre, et  
170 al. 2007, Park, et al. 2011). In contrast, other authors (Ho and Suarez 2001) have reported no  
171 staining of bovine sperm with BODIPY-FLX ryanodine (see (Costello, et al. 2009) for further



172 discussion). Thus mobilisation of stored  $\text{Ca}^{2+}$  in mammalian sperm may occur in response to  
173 generation of  $\text{IP}_3$  by activity of phospholipase C and by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) at  $\text{IP}_3$ Rs or  
174 RyRs. These processes can be sensitised by effects such as oxidative stress and S-nitrosylation (see  
175 section 2). For instance, exposure of human sperm to  $\text{NO}\cdot$  at levels equivalent to those produced by  
176 explants of reproductive tract lining mobilises store  $\text{Ca}^{2+}$  and modifies flagellar activity (Lefievre, et  
177 al. 2007, Machado-Oliveira, et al. 2008).

178 In addition to generation of  $\text{IP}_3$  in sperm, there is evidence that other  $\text{Ca}^{2+}$  mobilising messengers  
179 (NAADP and cADPR) are synthesised in sperm and/or produced in response to stimulation. Sea  
180 urchin sperm contain significant levels of both cADPR and NAADP, which may contribute to oocyte  
181 activation (Billington, et al. 2002, Chini, et al. 1997). Human sperm have been shown to contain  
182 cADPR at micromolar concentrations but NAADP was not detected (Billington, et al. 2006).  
183 Interestingly, this study also demonstrated synthesis of cADPR by human sperm but the ecto-  
184 enzyme CD38 (an enzyme present on mammalian cells that synthesises both cADPR and NAADP; see  
185 section 2) could not be detected by western blotting. In contrast, Park and colleagues (Park, et al.  
186 2011), reported detection of CD38 in human sperm after co-incubation with prostasomes (prostate-  
187 derived membrane vesicles; see below). Furthermore, the presence of a novel NAADP synthase,  
188 which lacks the cyclase activity of CD38, has been described both in sea urchin (Vasudevan, et al.  
189 2008) and human sperm (Sánchez-Tusie et al, 2014). In sea urchin sperm this enzyme is strongly  
190  $\text{Ca}^{2+}$ -regulated and most active at acid pH whereas the human enzyme shows only weak  $\text{Ca}^{2+}$ -  
191 regulation and activity is maximal at pH 7 to 8 (Sanchez-Tusie, et al. 2014, Vasudevan, et al. 2008).

192 Recent findings have supported the idea that NAADP is functional in human sperm. Sanchez-Tusie et  
193 al. (2014) investigated the effects of cell-permeant (AM-ester) derivatives of NAADP and cADPR. No  
194 effects were observed with cADPR, consistent with previous pharmacological investigation by  
195 Billington et al. (2006), but NAADP caused elevation of  $[\text{Ca}^{2+}]_i$  both in cells incubated under standard  
196 conditions and also when  $[\text{Ca}^{2+}]_o$  was buffered to 100 nM, conditions under which  $\text{Ca}^{2+}$  influx is

197 negligible and  $[Ca^{2+}]_i$  signalling depends solely on mobilisation of stored  $Ca^{2+}$ . Staining of NAADP  
198 receptors using the fluorescent NAADP receptor ligand Ned-19 and identification of acidic organelles  
199 using lysotracker highlighted both an anterior store (potentially the acrosome) and a store at the  
200 sperm neck (fig 1b). Consistent with these findings, Arndt et al. (2014), studying acrosome reaction  
201 (see below), provided evidence for involvement in this process of NAADP and two-pore channels  
202 (TPCs), which have been proposed to be the NAADP receptor/ $Ca^{2+}$  channel of acidic  $Ca^{2+}$  storage  
203 organelles (Calcraft, et al. 2009) (Fig 1a; see section 2).

204 (Park et al. (2011) investigated the incorporation into human sperm of proteins from prostasomes  
205 (prostate-derived vesicles which are normally added to sperm during ejaculation) and their effects  
206 on  $[Ca^{2+}]_i$  signalling. They concluded that CatSper channel proteins were present in the differentiated  
207 sperm, but other  $Ca^{2+}$  signalling 'tools' including RyRs and CD38 were added to the freshly-ejaculated  
208 sperm upon mixing with prostasomes, by fusion with the membrane of the midpiece. They also  
209 examined the effects of stimulation with progesterone on  $[Ca^{2+}]_i$  and motility of sperm exposed to  
210 prostasomes and sperm that had been rapidly removed from semen to minimise mixing with  
211 prostasomes. Their data suggest that the generation of sustained  $[Ca^{2+}]_i$  signals (such as the second  
212 component of the biphasic progesterone-induced  $[Ca^{2+}]_i$  signal) and consequent effects on motility  
213 may depend, at least partly, upon generation of cADPR by prostatesome-derived enzymes.

214 Interestingly, CD38-null mice proved to be fertile, but analysis showed that 20% of normal ADPR  
215 cyclase activity remained in prostasomes from these animals, indicating the presence of a non-CD38  
216 ADPR-cyclase, potentially that described by Sánchez-Tusie et al (2014). Thus both NAADP and cADPR  
217 are potentially synthesised by sperm and involved in regulation of sperm  $Ca^{2+}$  store activity but their  
218 roles are not yet clear.

219 In somatic cells mobilisation of stored  $Ca^{2+}$  induces secondary  $Ca^{2+}$  influx through channels at the cell  
220 membrane (store-operated channels; SOCs) by the process of capacitative  $Ca^{2+}$  entry (CCE) (fig 1a).  
221 CCE both prolongs  $Ca^{2+}$  signals that are induced by store mobilisation and provides  $Ca^{2+}$  for re-

222 charging of the storage organelles. Recently great progress has been made in elucidating the key  
223 players and mechanisms in this process. STIM (stromal interaction molecule) has been identified as  
224 the sensor molecule present in the membrane of the  $\text{Ca}^{2+}$  store. The intraluminal part of STIM  
225 includes a  $\text{Ca}^{2+}$ -binding EF hand that detects depletion of stored  $\text{Ca}^{2+}$ . STIM then redistributes,  
226 moving to a position adjacent to the plasma membrane where it activates channel proteins (Orai  
227 and possibly members of the TRPC [transient receptor potential canonical] family; Cahalan 2009).  
228  $[\text{Ca}^{2+}]_i$  signals in human and other mammalian sperm induced by agonists and by treatments  
229 designed to mobilise stored- $\text{Ca}^{2+}$  show characteristics consistent with the occurrence of CCE  
230 (Blackmore 1993, Dragileva, et al. 1999, Lefievre, et al. 2012, O'Toole, et al. 2000, Park, et al. 2011).  
231 STIM1, Orai and TRPC proteins have been detected in human sperm (Castellano, et al. 2003,  
232 Darszon, et al. 2012, Lefievre, et al. 2012), STIM1 being localised primarily to the neck  
233 region/midpiece and the acrosome where  $\text{Ca}^{2+}$  stores are present (Lefievre, et al. 2012). To date the  
234 application of whole-cell patch clamp has not provided evidence for the occurrence of CCE in human  
235 sperm (Lefievre, et al. 2012) so these findings must be interpreted cautiously, but  $[\text{Ca}^{2+}]_i$  signals  
236 generated by mobilisation of  $\text{Ca}^{2+}$  stores in sperm may be amplified by activation of CCE. Induction of  
237 CCE in somatic cells can have a latency of 10s of seconds due to the need for STIM to migrate to the  
238 peripheral portions of the endoplasmic reticulum where it can interact with SOC proteins (Luik, et al.  
239 2006, Wu, et al. 2006), but in sperm the storage organelles are close to the plasma membrane and  
240 STIM proteins are localised here, such that CCE could be near 'instantaneous'. Pre-treatment of  
241 human sperm with low concentrations of 2-aminoethoxydiphenyl borate (2-APB), which potentiates  
242 CCE by promoting the interaction of STIM with SOCs (Navarro-Borelly, et al. 2008, Wang, et al. 2009,  
243 Yamashita, et al. 2011) significantly enhances the amplitude of the progesterone-induced  $\text{Ca}^{2+}$   
244 transient at the sperm neck (where secondary release of stored  $\text{Ca}^{2+}$  may occur; fig 1b; see section 6)  
245 but does not affect the response in the flagellum, where progesterone activates CatSper channels  
246 (fig 1b), or the kinetics of the signal at either location (Lefievre, et al. 2012). Conversely, when sperm  
247 were pre-treated with a cell-penetrating peptide that mimics part of the key SOAR region of STIM1

248 (potentially preventing auto-inhibitory folding of STIM upon store-refilling) there was a marked  
249 prolongation of the progesterone-induced  $[Ca^{2+}]_i$  transient in a subset of cells (Morris, et al. 2015).

250

251

#### 252 4. Mobilisation of sperm $Ca^{2+}$ stores by agonists

253 In the majority of somatic cells mobilisation of stored  $Ca^{2+}$  is induced by agonist-induced synthesis of  
254  $Ca^{2+}$  mobilising intracellular messengers. Thus agonist-induced synthesis of inositol trisphosphate,  
255 cADPR and NAADP can lead to rapid release of stored  $Ca^{2+}$  and generation of local, global and  
256 complex spatio-temporal signals (fig 1a). Is there evidence that such processes occur and are  
257 functionally significant in responses to agonist stimulation of sperm?

258 The best-characterised agonist-induced  $[Ca^{2+}]_i$  signals in sperm are responses to solubilised zona  
259 pellucida/zona proteins in mouse cells and progesterone in human. Application of patch clamp has  
260 clearly shown that the primary action of progesterone in human sperm is to activate CatSper  
261 channels, leading to  $Ca^{2+}$ -influx (Lishko, et al. 2011, Strunker, et al. 2011). Strunker and colleagues  
262 (Strunker, et al. 2011) investigated the  $[Ca^{2+}]_o$  dependence of progesterone-induced  $[Ca^{2+}]_i$  signals in  
263 rapid-mixing experiments on human sperm and reported that buffering of  $[Ca^{2+}]_o$  to  $\leq 100$  nM  
264 abolished the response (though see Espino et al. (2009)), suggesting that any mobilisation of stored  
265  $Ca^{2+}$  is a secondary response. Synthesis of  $IP_3$  is reported to occur downstream of progesterone-  
266 induced  $Ca^{2+}$  influx (Thomas and Meizel 1989), an important observation that should be pursued.  
267 Stimulation of mouse sperm with zona proteins induces acrosome reaction, which requires elevation  
268 of  $[Ca^{2+}]_i$  in the sperm head (Florman, et al. 2008) and is dependent on mobilisation of  $Ca^{2+}$  from the  
269 acrosomal store ((De Blas, et al. 2002); see below). The nature of the  $Ca^{2+}$  influx following  
270 stimulation is not clear and several channels may be involved (Cohen, et al. 2014, Florman, et al.  
271 2008, Xia and Ren 2009), but  $Ca^{2+}$  signals are sensitive to inhibition of G-protein signalling (using  
272 pertussis toxin) and inhibition of PLC (Florman, et al. 2008, Ren and Xia 2010). Furthermore, in sperm  
273 from mice null for PLC $\delta 4$  (in which males fertility is 'severely impaired') the  $[Ca^{2+}]_i$  response is

274 reduced and zona-induced AR does not occur (Fukami, et al. 2001, Fukami, et al. 2003). Thus  
275 conventional IP<sub>3</sub>-induced mobilisation of stored Ca<sup>2+</sup> is apparently central to this essential aspect of  
276 mammalian sperm physiology.

277 Evidence for the existence of other store-mobilising agonists is largely preliminary, but there are a  
278 number of candidates, of which the best-studied is vitamin D (Blomberg Jensen 2014). Human sperm  
279 have been shown to express vitamin D receptor (VDR) (Aquila, et al. 2009, Blomberg Jensen, et al.  
280 2011, Blomberg Jensen, et al. 2010), the enzymes CYP2R1 and CYP27B (which produce the active  
281 compound (1,25(OH)<sub>2</sub>D<sub>3</sub>) cholcalciferol) and the inactivating enzyme CYP24A1 (Blomberg Jensen, et  
282 al. 2011, Blomberg Jensen, et al. 2010). All are expressed in the neck region of the sperm and  
283 staining of cells for VDR and CYP24A1 shows a strong association. In sub-fertile patients the  
284 proportion of cells expressing CYP24A1 varies greatly and is significantly correlated with semen  
285 quality (sperm count, concentration, morphology and motility) (Blomberg Jensen, et al. 2011,  
286 Blomberg Jensen, et al. 2012). Stimulation of human sperm with 1,25(OH)<sub>2</sub>D<sub>3</sub> (100 pM-1 uM)  
287 induced a [Ca<sup>2+</sup>]<sub>i</sub> response, including a transient and plateau, that was blocked by pre-treatment with  
288 the non-genomic VDR antagonist 1b,25(OH)<sub>2</sub>D<sub>3</sub> but was insensitive to blockade of the nuclear VDR  
289 antagonist ZK159222 (Blomberg Jensen, et al. 2011). This effect was greatly reduced by pre-  
290 treatment with the phospholipase C inhibitor U73122 (2 μM) but was inhibited by incubation in  
291 EGTA-buffered medium for up to 20 min prior to stimulation. Both motility and AR were significantly  
292 increased upon stimulation with 1,25(OH)<sub>2</sub>D<sub>3</sub> (Blomberg Jensen, et al. 2011).

293 Kisspeptin, a peptide agonist of the G-protein coupled receptor GPR54/KISS1R has also been shown  
294 to cause sustained, dose-dependent elevation of [Ca<sup>2+</sup>]<sub>i</sub> in human and in mouse sperm (Hsu, et al.  
295 2014, Pinto, et al. 2012). In neurons binding of kisspeptin to its receptor activates PLC and results in  
296 generation of IP<sub>3</sub> and diacylglycerol, leading to mobilisation of stored Ca<sup>2+</sup> and also depolarisation  
297 (Beltramo, et al. 2014, Liu, et al. 2008, Pielecka-Fortuna, et al. 2008). In human sperm the effect of  
298 kisspeptin on [Ca<sup>2+</sup>]<sub>i</sub> did not occlude the response to stimulation with the CatSper agonist

299 progesterone and was not reduced when applied in the presence of progesterone (Pinto, et al.  
300 2012). Both KISS1R and kisspeptin itself were detected in the head of human sperm, suggesting that  
301 an autocrine action of the peptide may occur. Motility parameters of kisspeptin-treated cells were  
302 significantly altered, including an increase in lateral movement of the head and a decrease in  
303 linearity of the sperm path, characteristics of hyperactivated sperm (Pinto, et al. 2012). Ghrelin,  
304 another peptide hormone which also acts through mobilisation of stored  $\text{Ca}^{2+}$  (Camina, et al. 2003),  
305 has also been detected in human sperm (Moretti, et al. 2014). Micromolar concentrations of ghrelin  
306 have been shown to increase  $[\text{Ca}^{2+}]_i$  and motility in rat sperm (Lukaszyk, et al. 2012) but expression  
307 of ghrelin receptors or effect of ghrelin on human sperm  $[\text{Ca}^{2+}]_i$  have not been investigated.

## 308 5. Functional significance of $\text{Ca}^{2+}$ -stores

### 309 The acrosome

310 Acrosome reaction (AR) is the fusion between the outer acrosomal membrane and the overlying  
311 plasma membrane. Fusion occurs at multiple points, resulting in vesiculation and loss of the fused  
312 outer acrosomal membrane/plasmalemma so that the acrosomal content is released and the inner  
313 acrosomal membrane becomes the new cell surface. Membrane fusion proteins from the SNARE  
314 family are present in the acrosomal region and may be integrated into microdomains that facilitate  
315  $\text{Ca}^{2+}$ -regulated membrane fusion in a manner that has been compared with events at presynaptic  
316 terminals (De Blas, et al. 2005, Mayorga, et al. 2007, Zitanski, et al. 2010). Zona pellucida proteins  
317 interact with sperm surface receptors to activate a signalling cascade leading to AR (Florman, et al.  
318 2008) and release of acrosomal content at the surface of the zona may, in combination with  
319 hyperactivated motility, facilitate zona penetration. However, observation of mouse IVF using sperm  
320 with GFP-labelled acrosomes showed that, in addition to cells that undergo AR at the surface of the  
321 zona, sperm which arrive having already lost their acrosome (probably within the cumulus) may go  
322 on to penetrate the zona and fertilise (Jin, et al. 2011). Physiological inducers of AR that have been  
323 studied (primarily mouse ZP3 and progesterone) induce  $\text{Ca}^{2+}$  influx across the plasma membrane and  
324 a sustained rise in  $[\text{Ca}^{2+}]_i$ . O'Toole, et al (2000) provided pharmacological evidence that ZP3-induced

325 AR in mouse sperm involved activation of store operated  $\text{Ca}^{2+}$  influx downstream of  $\text{Ca}^{2+}$  store  
326 mobilisation and De Blas, et al (2002) showed that in streptolysin-permeabilised human sperm,  
327 mobilisation of the acrosomal  $\text{Ca}^{2+}$  store was a requirement for acrosome reaction even when it was  
328 'directly' induced by introduction of Rab3A into the cytoplasm. Further studies using this  
329 permeabilised sperm model have provided information about the mechanisms by which fusion of  
330 the plasma and outer acrosomal membranes is regulated, resulting in a detailed model in which  
331 mobilisation of the acrosomal store is a central and necessary event (Ruete, et al. 2014). Stimulation  
332 of PLC, leading to generation of  $\text{IP}_3$  and activation of  $\text{IP}_3\text{Rs}$  in the outer acrosomal membrane may be  
333 key to this process (Fukami, et al. 2001, Fukami, et al. 2003), but there is also evidence that the  
334 acrosomal membrane contains the NAADP-sensitive,  $\text{Ca}^{2+}$ -permeable two-pore channels (Calcraft, et  
335 al. 2009) and that NAADP mobilises acrosomal  $\text{Ca}^{2+}$  in mouse sperm (Arndt, et al. 2014).  
336 Interpretation of this finding is complex since the regulation and  $\text{Ca}^{2+}$ -permeability of TPCs have  
337 recently been questioned (Cang, et al. 2013, Wang, et al. 2012) (see section 2).

338

### 339 The redundant nuclear envelope and calreticulin-containing vesicles

340 A second area where  $\text{Ca}^{2+}$  storage organelles have been identified in mammalian sperm is at the  
341 sperm neck and midpiece (fig 1b). Mitochondria have mechanisms for accumulation and release of  
342  $\text{Ca}^{2+}$  (Drago, et al. 2011, Pizzo, et al. 2012) and therefore may contribute to  $\text{Ca}^{2+}$  buffering and  
343 signalling in this part of the sperm. Inhibition of mitochondrial function in sea urchin sperm, using  
344 respiratory inhibitors or uncouplers, causes a rise in  $[\text{Ca}^{2+}]_i$  and leads to activation of  $\text{Ca}^{2+}$  influx that  
345 has characteristics consistent with store-operated channels (Ardon, et al. 2009). Treatment with  
346 mitochondrial uncouplers (2,4 dinitrophenol [2,4 DNP], carbonyl cyanide-4-  
347 (trifluoromethoxy)phenylhydrazone [FCCP]) also increases  $[\text{Ca}^{2+}]_i$  in human sperm (Morris and  
348 Publicover, unpublished). Mitochondria may thus contribute to shaping of  $\text{Ca}^{2+}$  signals in sperm.  
349 However, the primary stimulus-regulated  $\text{Ca}^{2+}$  storage in this part of the sperm is in the redundant  
350 nuclear envelope and/or a second, apparently separate group of calreticulin-containing vesicular

351 structures, both of which are sited at the sperm neck region and cytoplasmic droplet (Ho and Suarez  
352 2001, 2003, Naaby-Hansen, et al. 2001). Mobilisation of  $\text{Ca}^{2+}$  stored in these compartments regulates  
353 flagellar activity and treatment of mouse sperm with thimerosal stimulates hyperactivated motility  
354 by activating  $\text{Ca}^{2+}$  release from these organelles (Ho and Suarez 2001, Marquez, et al. 2007). This  
355 effect occurs in the absence of extracellular  $\text{Ca}^{2+}$  and can be induced in sperm that are null for  
356 CatSper (Marquez, et al. 2007). In mouse sperm the direction of the major, high-amplitude flagellar  
357 bend of hyperactivated sperm can be clearly characterised by reference to the hooked acrosomal  
358 cap (pro-hook or anti-hook). Sperm that became hyperactivated during capacitation *in vitro* (due to  
359 activation of CatSper) show pro-hook bends whereas those activated by store mobilisation (using  
360 thimerosal) show anti-hook bends (Chang and Suarez 2011). When sperm were observed interacting  
361 with the lining of isolated mouse oviducts, most hyperactivated cells showed anti-hook bending of  
362 the type which is elicited by store mobilisation (Chang and Suarez 2012).

363 In human sperm a similar effect of store mobilisation is seen. Thimerosal greatly increases the  
364 proportion of cells showing hyperactivated motility and 4-aminopyridine, which both alkalinises the  
365 cytoplasm (and will thus activate CatSper) and mobilises stored  $\text{Ca}^{2+}$ , is similarly potent (Alasmari, et  
366 al. 2013a, Alasmari, et al. 2013b). In contrast, manipulations that should activate CatSper (elevation  
367 of  $\text{pH}_i$ , stimulation with progesterone or prostaglandin  $\text{E}_1$ ) elevate  $[\text{Ca}^{2+}]_i$  but have only minor  
368 stimulatory effects on the proportion of hyperactivated cells. Instead, these manipulations  
369 significantly increase penetration into viscous media (Alasmari, et al. 2013a, Alasmari, et al. 2013b,  
370 Luo, et al. 2014).

371

## 372 6. Model for interaction of CatSper channels and $\text{Ca}^{2+}$ -stores

373 Patch clamp recordings have provided no evidence that conventional voltage-operated  $\text{Ca}^{2+}$  channels  
374 contribute to  $\text{Ca}^{2+}$  influx in mature mammalian sperm. In mouse sperm null for CatSper1 and the  $\text{K}^+$   
375 channel Slo3 only a small leak current was recorded even at high intracellular pH and strong



376 depolarisation (Zeng, et al. 2013). CatSper channels in mouse and human sperm are pH- and  
377 (weakly) voltage-sensitive but in human sperm the channel is also ligand-sensitive. Established  $\text{Ca}^{2+}$ -  
378 mobilising agonists of human sperm such as progesterone and prostaglandin  $\text{E}_1$  have been shown to  
379 activate CatSper but also a range of other small molecules including environmental pollutants such  
380 as 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane, 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane  
381 (4,4'-DDT), p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) and 4-methylbenzylidene camphor (4-  
382 MBC) are potent agonists (Schiffer, et al. 2014, Tavares, et al. 2013). In addition, agents used to  
383 demonstrate cyclic-nucleotide-activated  $\text{Ca}^{2+}$  influx (such as 8-Br-AMP) have been shown directly to  
384 activate CatSper by binding at the extracellular surface (Brenker, et al. 2012). Thus it is possible that  
385 a significant proportion of the pharmacological data that apparently support the existence of  
386 multiple  $\text{Ca}^{2+}$  influx pathways in sperm are misleading and in fact reflect actions of the drugs on  $\text{Ca}^{2+}$   
387 flux through CatSper channels (Brenker, et al. 2012). Furthermore, experiments using CatSper null  
388 mice provide strong evidence that  $[\text{Ca}^{2+}]_i$  elevation induced by solubilised ZP is dependent on  $\text{Ca}^{2+}$   
389 influx through the CatSper channel in the flagellum, which then propagates to the head (Xia and Ren  
390 2009) (though see (Cohen, et al. 2014)). Interestingly, the ability of solubilised zona to induce  
391 acrosome reaction was not diminished in CatSper-null sperm. These findings not only suggest that  
392 CatSper is the primary  $\text{Ca}^{2+}$  influx pathway in mammalian sperm, but also that in human sperm it  
393 may act as a  $\text{Ca}^{2+}$ -signalling 'hub' or 'node', such that the effects of diverse agonists are  
394 summated/integrated in the rate of  $\text{Ca}^{2+}$  influx into the flagellum (Brenker, et al. 2012). This is an  
395 elegant and simple model for which there is already a significant body of data, but in its basic form it  
396 does not address the question of how a sperm can generate and use diverse  $[\text{Ca}^{2+}]_i$  signals to control  
397 diverse  $\text{Ca}^{2+}$ -sensitive functions.

398 Mouse sperm null for CatSper are unable to hyperactivate (Carlson, et al. 2003) and evidence from  
399 clinical cases suggests that CatSper is also required for normal levels of motility in human sperm  
400 (Avenarius, et al. 2009, Smith, et al. 2013). Why then, is manipulation of  $\text{Ca}^{2+}$  stores more effective in  
401 inducing hyperactivated motility than treatments targeted to CatSper (Alasmari, et al. 2013b)? We

402 have proposed that CatSper activation acts as a trigger and consequent elevation of flagellar  $[Ca^{2+}]_i$   
403 stimulates secondary release of stored  $Ca^{2+}$  at the sperm neck, either by stimulating synthesis of  $IP_3$   
404 or by CICR, leading to hyperactivation (Alasmari, et al. 2013b). Mathematical modelling of the  $Ca^{2+}$   
405 signals induced by CatSper activation in mouse sperm suggests that forward diffusion of  $Ca^{2+}$  from  
406 the flagellum cannot explain the  $[Ca^{2+}]_i$  that occurs at the sperm head upon activation of CatSper and  
407 that such a secondary  $Ca^{2+}$  release at the neck region occurs (Li, et al. 2014, Olson, et al. 2011, Olson,  
408 et al. 2010). Recently we have investigated the occurrence of such secondary responses in human  
409 sperm by uncaging  $Ca^{2+}$  in the principal piece of the flagellum. Uncaging induces a clear  $[Ca^{2+}]_i$   
410 transient in the flagellum that decays within 5-10 s. At the neck region of the sperm the transient is  
411 truncated and rises more slowly, consistent with diffusion of  $Ca^{2+}$  from the uncaged pool, but in a  
412 small proportion of cells ( $\approx 10\%$ ) we have observed a late  $[Ca^{2+}]_i$  response at the neck region, often  
413 including multiple peaks (fig 2). The low incidence of this secondary  $Ca^{2+}$ -mobilisation is consistent  
414 with our observation that, though direct release of stored  $Ca^{2+}$  can induce hyperactivated motility in  
415 the majority of human sperm, only a small proportion of cells hyperactivate upon activation of  
416 CatSper (Alasmari, et al. 2013a, Alasmari, et al. 2013b).

417  $Ca^{2+}$ -store-mediated  $[Ca^{2+}]_i$  oscillations occur more readily in sperm incubated for a prolonged period  
418 ( $>24$  h) under capacitating conditions (Kirkman-Brown, et al. 2004). Capacitation involves generation  
419 of reactive oxygen and reactive nitrogen species (Aitken and Nixon 2013, Herrero, et al. 1999, 2001)  
420 and we have observed that store mobilisation is sensitised and induced by low concentrations of NO  
421 donors, through a mechanism that involves protein S-nitrosylation (Machado-Oliveira, et al. 2008).  
422 RyRs were detected in the human sperm nitrosoproteome (Lefievre, et al. 2007) and it is well-  
423 established that  $IP_3$ Rs and RyRs are sensitised by oxidative stress (Bansaghi, et al. 2014, Bootman, et  
424 al. 1992, Meissner 2004, Sayers, et al. 1993, Stoyanovsky, et al. 1997) (see section 2). We propose  
425 that CICR from the sperm neck  $Ca^{2+}$ -store is regulated during capacitation, perhaps through the  
426 effects of oxidative stress on  $Ca^{2+}$  release channels (Alasmari, et al. 2013b) (fig 3).

427 7. Final remarks

428 The central role of  $[Ca^{2+}]_i$  signalling in the physiology of mammalian sperm and the pivotal  
429 importance of CatSper in this process are well established - mice null for CatSper are infertile (Ren,  
430 et al. 2001) and in men CatSper lesions are associated with impaired sperm function (Avenarius, et  
431 al. 2009, Avidan, et al. 2003, Smith, et al. 2013, Zhang, et al. 2009) . The available evidence suggests  
432 that  $Ca^{2+}$ -stores also play important roles in both acrosome reaction and the regulation of motility.  
433 Future studies should address the mechanisms by which store mobilisation is achieved (both by CICR  
434 and by direct activation by agonist-induced generation of  $Ca^{2+}$ -mobilising 2<sup>nd</sup> messengers) and  
435 regulated, particularly the significance of capacitation in  $Ca^{2+}$ -store filling and in sensitising  $Ca^{2+}$   
436 release mechanisms. Also, similarly to the important species differences in expression and function  
437 of sperm ion channels between human and mouse sperm (Brenker, et al. 2014, Miller, et al. 2014),  
438 there may also be differences in store-regulation and/or function between species. An intriguing  
439 possibility is that, at least in human sperm, it may prove possible to bypass the effects on motility of  
440 lesions in the expression, function or regulation of CatSper channels by pharmacological activation of  
441 store  $Ca^{2+}$  release.

442 Declaration of interest

443

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445 reported

446

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801 Figure legends

802 **Fig.1 a:** Simplified diagrammatic summary of  $[Ca^{2+}]_i$  signalling toolkit in a somatic cell. Ion channels  
803 are shown as rectangles with arrow indicating normal direction of  $Ca^{2+}$  flow (yellow=voltage gated;  
804 green=ligand gated; purple=store-operated; light blue= $IP_3$  receptor; dark blue=ryanodine receptor;  
805 red=NAADP-gated. Pumps are shown as circles with arrows indicating normal direction of  $Ca^{2+}$   
806 movement (red=PMCA'; blue= $Na^+$ - $Ca^{2+}$  exchanger; green=SERCA; blue=SPCA). Activation of  $IP_3$   
807 receptors by membrane receptor activation and phospholipase C is shown in light blue. Generation  
808 of cADPR and NAADP by CD38 and possibly other enzymes (leading to mobilisation of  $Ca^{2+}$  from  
809 intracellular stores) is shown by yellow boxes. **b:** Structure of human sperm showing positions of  
810 CatSper channels (yellow shading around anterior flagellum) and  $Ca^{2+}$  stores in the acrosome and at  
811 the sperm (neck redundant nuclear envelope and calreticulin-containing vesicles) (shown in green).

812 **Fig. 2.**  $Ca^{2+}$  responses evoked in human sperm by uncaging of  $Ca^{2+}$  in the flagellum. Cells were  
813 labelled with fluo-4 and loaded with 'caged'  $Ca^{2+}$  (NP-EGTA), then stimulated by an uncaging flash  
814 (360 nm laser) at the central flagellum (shown by arrow) whilst collecting images at 33 Hz. Changes  
815 in fluorescence, assessed at each of the positions shown by coloured circles in panel 'a', are plotted  
816 (normalised to minimum and maximum) in panel 'b' using the same colour code. Green=neck;  
817 yellow-midpiece; red=proximal flagellum; light blue=mid-distal flagellum; dark blue=distal flagellum.

818 **Fig. 3.** Model for triggering/regulation of CatSper-activated hyperactivation. CatSper channels in the  
819 flagellum (yellow box; shown by yellow shading on sperm flagellum) are activated by diverse stimuli  
820 including intracellular pH ( $pH_i$ ), membrane potential ( $E_m$ ), progesterone, prostaglandins and other  
821 organic molecules.  $Ca^{2+}$  from the flagellum diffuses forward, raising  $[Ca^{2+}]_i$  at the sperm neck and can  
822 mobilise stored  $Ca^{2+}$  by  $Ca^{2+}$ -induced  $Ca^{2+}$  release (CICR). Susceptibility of the store to CICR is  
823 potentially regulated/sensitised by processes occurring during capacitation including cAMP  
824 signalling, oxidative stress, S-nitrosylation as well as  $Ca^{2+}$  store filling and effects of agonists on  $Ca^{2+}$ -  
825 store release channels.

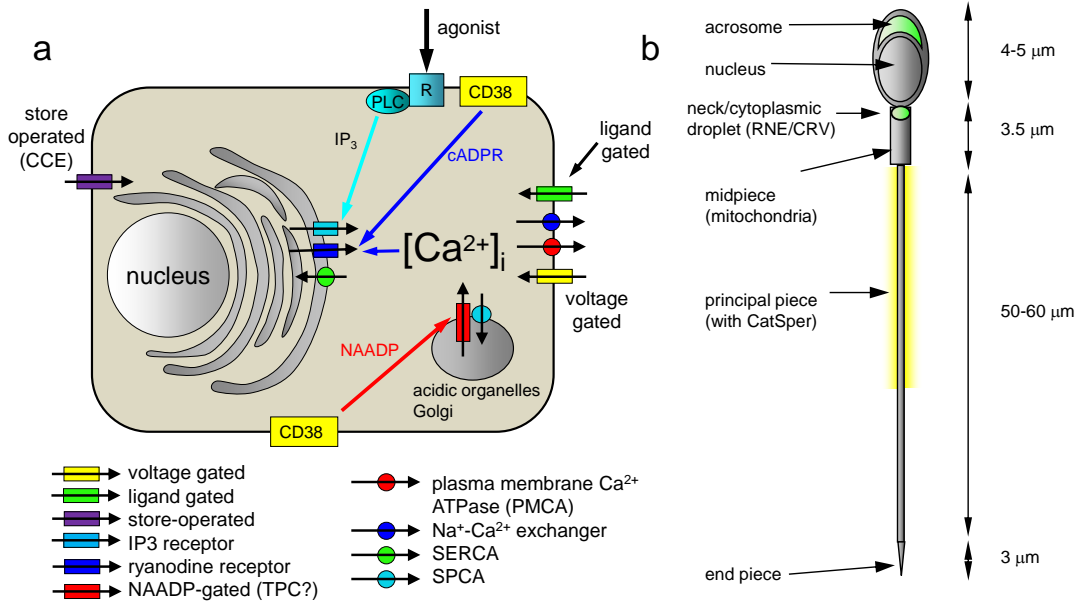


Figure 1

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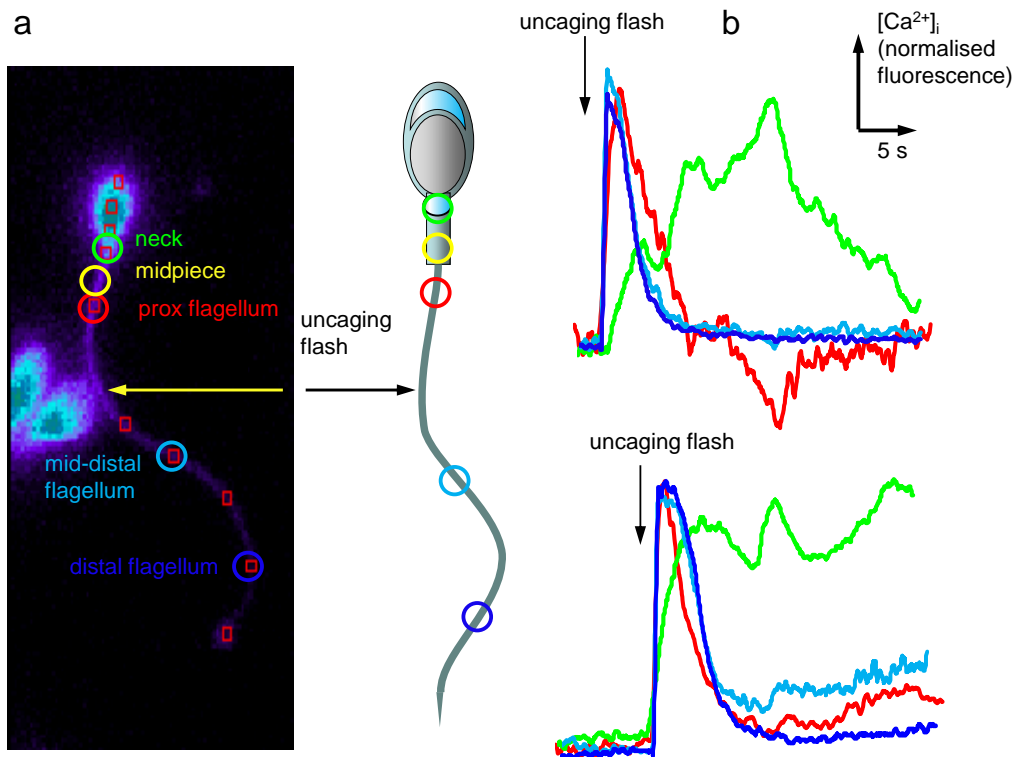


Figure 2

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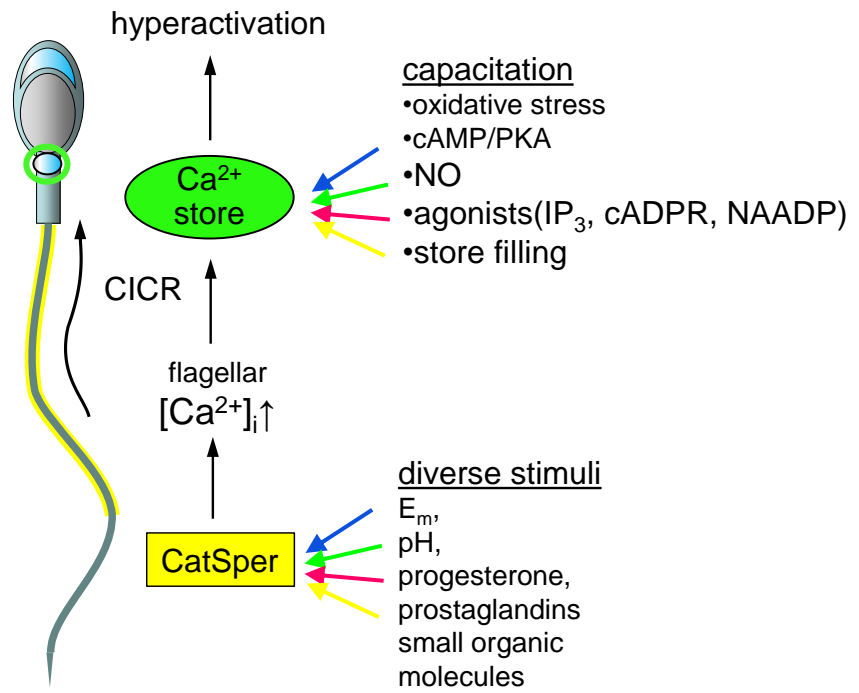


Figure 3