Complexin-I-deficient sperm are subfertile due to a defect in zona pellucida penetration

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Abstract

Upon adhesion to the zona pellucida, sperm undergo regulated exocytosis of the acrosome. Although it is necessary for sperm to penetrate the zona pellucida and fertilize an egg, the acrosomal membrane fusion process is poorly understood. Complexins I and II are small, cytosolic proteins that bind to a complex of proteins termed the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor complex to regulate synaptic vesicle exocytosis. Complexin-II-deficient mice are fertile but the fertility of sperm from complexin-I-deficient male mice is unclear because the mice have ataxia and cannot mate. Here, we show that the genes encoding complexins I and II are expressed in primary spermatocytes and spermatids. Complexin proteins were found in/near the developing acrosome in spermatids and in or around the acrosome of mature sperm. Cell fractionation demonstrated that complexins I and II were predominantly found in the cytosolic fraction. Furthermore, sperm from complexin-I-deficient mice had normal morphology, number, and only small differences in motility, as assessed by computer-assisted semen analysis. Complexin-I-deficient sperm capacitated normally and bound to the zona pellucida. But when sperm from complexin-I-deficient mice were inseminated into females, a defect in fertility was observed, in concordance with previous data showing that *in vitro* fertilization rate was also reduced. If the zona pellucida was removed prior to *in vitro* fertilization, fertility was normal, demonstrating that zona pellucida penetration was defective, a step requiring acrosomal exocytosis. Therefore, complexin-I-deficient sperm are subfertile due to faulty zona pellucida penetration.

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Introduction

The sperm acrosome reaction is a required step for fertilization, but how membrane fusion is regulated in this large vesicle is not clear (Yanagimachi 1994). The sperm acrosome is located between the nucleus and the plasma membrane, and in mouse sperm envelops two-thirds of the nucleus, extending 3–4 μm along each side of the head; it is much larger than a typical secretory vesicle (Flaherty & Olson 1991, Yanagimachi 1994, Nolan & Hammerstedt 1997). Zona pellucida protein binding to the sperm plasma membrane induces an increase in intracellular calcium leading to the acrosome reaction (Florman *et al.* 1998, Jungnickel *et al.* 2001). During the acrosome reaction, the outer acrosomal membrane fuses with the adjacent sperm plasma membrane at hundreds of points simultaneously, first forming fenestrae. As the fenestrae enlarge and fusion continues, hybrid membrane vesicles form around the small amount of sperm cytosol and the acrosomal contents are released, eventually exposing the inner acrosomal membrane on the sperm head (Barros *et al.* 1967, Russell *et al.* 1979,

Yudin *et al.* 1988). In contrast to the more heavily studied neuronal exocytosis, acrosomal exocytosis is a much slower process and there is no vesicle recycling after exocytosis. Although the acrosome reaction is a distinct event, it shares some similarities in membrane fusion with synaptic vesicle exocytosis. Similar to the requirement of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) for synaptic vesicle membrane fusion (Sollner 2003), there is growing evidence that acrosomal exocytosis is regulated by SNAREs (Katafuchi *et al.* 2000, Ramalho-Santos *et al.* 2000, Tomes *et al.* 2002).

The core neuronal SNARE complex is made up of three proteins: two proteins on the plasma membrane, syntaxin and synaptosome-associated protein 25 (SNAP25), and one on the vesicle, vesicle-associated membrane protein-2 (VAMP2; also called synaptobrevin) (Sollner 2003, Jahn & Scheller 2006). Although these three proteins are sufficient for membrane fusion *in vitro*, there are numerous proteins that bind to the SNARE complex to modulate its function *in vivo*. Among these SNARE-regulating proteins are the complexins

(also called synaphins). Complexins are composed of four isoforms in mammals (McMahon *et al.* 1995, Reim *et al.* 2005). Complexins I and II are 15–16 kDa cytosolic proteins that are expressed highly in the brain but are much less abundant in other tissues (McMahon *et al.* 1995). Complexins I and II are 84% identical at the amino acid level and functionally redundant in neurons (McMahon *et al.* 1995, Reim *et al.* 2001). They bind tightly to the assembled SNARE complex and stabilize the complex to facilitate membrane fusion during synaptic vesicle exocytosis (Pabst *et al.* 2000, 2002, Tokumaru *et al.* 2001, Chen *et al.* 2002, Hu *et al.* 2002). Very recent studies suggest that complexins I and II have two distinguishable functions: they promote full assembly of SNARE complexes into a ‘super-primed’ metastable state but then block completion of the fusion reaction in these complexes until a calcium signal is provided (Giraudo *et al.* 2006, Schaub *et al.* 2006, Tang *et al.* 2006). Formation of a ‘super-primed’ metastable state is proposed to be necessary for synchronized exocytosis when intracellular calcium is elevated (Tang *et al.* 2006). The domains within complexin I, which facilitate membrane fusion, are distinct from an accessory α -helical domain that inhibits fast synaptic exocytosis (Xue *et al.* 2007).

Complexins III and IV were recently discovered and are expressed predominantly in the retina (Reim *et al.* 2005). Although complexins III and IV are highly homologous to each other, their amino acids residues are only 24–28% identical to complexins I and II (Reim *et al.* 2005).

In mice, deletion of both complexins I and II impairs the fast synchronous calcium-triggered exocytosis but does not affect the slower calcium-triggered asynchronous exocytosis that also depends on SNAREs (Reim *et al.* 2005). Mice deficient in both complexins I and II have severely reduced synaptic transmission and die within a few hours after birth (Reim *et al.* 2001).

Although complexins I and II appear to have similar intracellular functions, in the brain, they are expressed by distinct subsets of neurons (Freeman & Morton 2004). Therefore, mice deficient in each complexin have different behavioral phenotypes (Reim *et al.* 2001, Glynn *et al.* 2003, 2005, 2007). Mice deficient in complexin II have normal reproductive ability (Takahashi *et al.* 1999, Reim *et al.* 2001). Mice lacking complexin I, on the other hand, are unable to reproduce, but it is unclear whether this is due only to their profound ataxia or also to an additional specific defect in the reproductive axis (Reim *et al.* 2001, Glynn *et al.* 2005).

Our very recent data showed that sperm lacking complexin I did not undergo the acrosome reaction induced by soluble zona pellucida proteins, and this resulted in a 50% decrease in *in vitro* fertilization rates (Zhao *et al.* 2007). Studies in this report have two goals: (1) to investigate complexin I and II gene expression in germ cells and protein localization in sperm and

(2) to identify the fertility defect in complexin-I-deficient sperm, both of which are necessary to clarify the role for complexin I in sperm fertility.

Results

Complexin I and II genes are expressed in spermatocytes and spermatids

As we have shown previously, complexins I and II are expressed in mouse testis (Zhao *et al.* 2007). To determine which germ cells expressed complexins I and II, we obtained RNA from rat primary spermatocytes round spermatids (provided by Dr David Bunick, University of Illinois) and RNA from testes of 15-day-old mice, in which the majority of germ cells are spermatocytes. Primers specific to complexin I or II were used to amplify reverse-transcribed cDNA. The 509 bp complexin I product was detected in the cDNA from rat pachytene spermatocytes and round spermatids (Fig. 1, lanes 7 and 9). Similarly, the complexin I product was found in testes cDNA from 15-day-old mice, in which the majority of germ cells are spermatocytes (Fig. 1, lane 1). Likewise, the 489 bp complexin II product was detected in testis cDNA from 15-day-old mice (Fig. 1, lane 4). Thus, genes encoding both complexins I and II are expressed by male germ cells

Figure 1 Complexin (*Cplx*) RT-PCR of testis from 15-day-old mice (majority of germ cells are spermatocytes) and isolated rat pachytene spermatocytes and round spermatids demonstrates all express complexins I and II. Primers were designed to amplify the entire complexin I and II cDNA. RT-PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Products were loaded in lanes as follows: 100 bp DNA Ladder (M), oligo-dT-primed testis cDNA from 15-day-old mice (lanes 1 and 4), testis RNA from 15-day-old mice (lanes 2 and 5; no reverse transcriptase was used), water rather than cDNA (lanes 3 and 6), oligo-dT-primed rat pachytene spermatocyte cDNA (lane 7), rat pachytene spermatocyte RNA (lane 8; no reverse transcriptase was used), oligo-dT-primed rat round spermatid cDNA (lane 9), rat round spermatid RNA (lane 10). Complexin I primers were used in lanes 1, 2, 3, 7, 8, 9, and 10, and complexin II primers were used in lanes 4–6. Complexin I primers amplified a 509 bp product from the testis of 15-day-old mice, rat pachytene spermatocytes, and rat round spermatids. Complexin II primers amplified a 483 bp product of testis from 15-day-old mice. Controls using RNA (no reverse transcriptase) or water did not result in a product.

from two rodent species. Controls lacking reverse transcriptase or using water rather than cDNA did not generate a product (Fig. 1, lanes 2, 3, 5, 6, 8, and 10).

Complexin I and II proteins are localized around the developing acrosome of spermatids

To determine the localization of complexin I and II proteins in germ cells, immunofluorescence was performed using antibodies that recognize both complexins I and II (antibody B) on mouse testicular sections. Although this antibody does not distinguish each complexin, previous reports using western blots demonstrated that both complexins were present in the testes (McMahon *et al.* 1995, Redecker *et al.* 2003). The antibodies detected complexin I and II proteins in or around the acrosomal vesicle of round spermatids (Fig. 2A and C) and the acrosomal region of elongated spermatids (Fig. 2A and D). No other cells in the testis showed detectable complexin protein. These results were in agreement with the previous reports using a different complexin antibody (Redecker *et al.* 2003). Our experiments using immunohistochemistry with a peroxidase-labeled secondary antibody confirmed that complexins I and II were found around the acrosomal region of spermatids (data not shown). Complexins I and II were first detected around the proacrosomal vesicle of round spermatids and continued to be present around the acrosomal region of the elongated spermatids (data not shown). These data demonstrate that complexin I and II proteins are localized around or in the developing acrosome of spermatids.

Figure 2 Immunofluorescence demonstrates complexin I and II proteins are found in the developing acrosome of round and elongated spermatids. Anti-complexin I and II IgG (antibody B) detected with a fluorescein-labeled secondary antibody detects complexin in the acrosomal region of round and elongated spermatids (A, C, and D). (B) Control IgG does not bind to any testicular cell types. Cell nuclei are counterstained with propidium iodide. Images were captured at 200 $\times$ (A and B) or 400 $\times$ (C and D) at different stages of spermatogenesis. Scale bar, 50 μ m.

Complexins I and II are localized to the acrosomal region of mature sperm and behave as cytosolic proteins

To determine the localization of complexin I and II proteins in mature sperm, immunofluorescence experiments were performed using two different antibodies that recognized both complexins I and II (antibodies A and B, described in Materials and Methods). Both antibodies detected complexins I and II only in the acrosomal region of sperm (Fig. 3A and C). When the complexin antibody was replaced with normal IgG in the experiment, no fluorescence was observed (Fig. 3B). When antibody B was pre-incubated with the blocking peptide, there was also no fluorescence (Fig. 3D). These data indicate that complexins I and II are around or within the acrosome. Fluorescence was not observed in other regions of sperm, demonstrating that complexins I and II are confined to the acrosomal region.

To compare more precisely the location of complexins I and II with the acrosome, immunofluorescence was performed on cauda epididymal sperm using fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin (FITC-PSA) to detect the acrosome and Texas Red-labeled complexin antibody to detect the complexins. Antibody A, which recognizes both complexins I and II (Reim *et al.* 2001, Redecker *et al.* 2003), detected complexins in the acrosomal region of sperm (Fig. 3E). FITC-PSA identified the crescent-shaped acrosome (Fig. 3F). The signals for the acrosome and complexin largely overlapped (Fig. 3G), indicating that complexins I and II are in the correct region to regulate acrosomal exocytosis.

To identify the subcellular localization of complexins I and II in sperm, membrane and cytosolic fractions of mouse sperm were prepared and probed on western blots. Most (83%) of the complexin I or II proteins were found in the cytosolic fraction and little was detectable in the detergent-soluble fraction (Fig. 4). Antibody against protein kinase C α (PKC α), which is a cytosolic protein in bovine sperm (Lax *et al.* 1997), did not detect PKC α in the membrane fraction (Fig. 4), signifying that the detergent-soluble fraction was not contaminated with cytosolic proteins. These results suggest that complexins I and II are primarily cytosolic proteins in germ cells as they appear to be in secretory cells (McMahon *et al.* 1995).

Phenotype of complexin-I-deficient mice

To understand the function of complexin I, we examined the effect of targeted deletion of complexin I. Complexin I knockout mice (lacking the first exon of the complexin I gene) were generated previously (Reim *et al.* 2001). Mice deficient in complexin I develop strong ataxia, suffer from sporadic seizures, and males do not reproduce (Reim *et al.* 2001, Glynn *et al.* 2005). Males lacking complexin I do not mate due to ataxia; we have never observed vaginal plugs in female mice that have

been paired with complexin-I-deficient males for several months (data not shown). Testes from complexin-I-deficient mice lack complexin I but produce complexin II more abundantly (Zhao *et al.* 2007).

Because we did not observe a different phenotype between wild-type and complexin I heterozygote mice, we pooled data from these mice. Male complexin-I-deficient mice were much smaller than wild-type mice in the same litter. The body weight of adult male complexin-I-deficient mice was 21.5 ± 0.9 g, compared with 30.7 ± 0.7 g for wild-type animals at the same age (Table 1). Their smaller size was probably due to their difficulty consuming food, caused by their ataxia. There were no gross anomalies that could be observed along the reproductive tract of complexin-I-deficient male

mice. Testes and accessory glands from complexin-I-deficient mice had a normal shape. The mean weight of testes of complexin-I-deficient mice was 187.5 ± 5.7 mg compared with 213.2 ± 8.6 mg for wild-type animals (Table 1). However, the ratios of testicular to body weight of wild-type and complexin-I-deficient mice were 0.00694 and 0.00872 respectively (Table 1), demonstrating that testicular weight of complexin-I-deficient mice as a fraction of body size was not reduced but, in fact, increased. In addition, hematoxylin and eosin and periodic acid Schiff staining of mouse testicular sections showed that seminiferous tubules from complexin-I-deficient mice were morphologically normal (data not shown). All spermatogenic stages could be observed in complexin-I-deficient testes with a normal proportion of the different germ cell stages. Also, the total number and morphology of cauda epididymal sperm from complexin-I-deficient mice were normal as observed by light microscopy (data not shown). Together, these data indicate that complexin I is not required for spermatogenesis and sperm acrosome formation.

Analysis of motility in complexin-I-deficient sperm

To determine whether complexin I is required for normal sperm motility, individual cauda epididymal sperm from wild-type and complexin-I-deficient littermates were analyzed in real-time using computer-assisted semen assay (CASA). After 30 min capacitation, the motility percentage, progressive motility, and amplitude of lateral head displacement, an indicator of hyperactivated motility (Zhang & Baker 1997) of complexin-I-deficient sperm were reduced slightly, compared with sperm from wild-type mice (Table 2). However, after 60 min capacitation, these parameters were not different between wild-type and complexin-I-deficient sperm. Two other indicators of hyperactivation (Zhang &

Figure 3 Complexins I and II are localized around the acrosomal region of mature sperm. Antibodies that detect both complexins I and II were used to localize complexins in fixed permeabilized cauda epididymal mouse sperm. (A) Purified complexin IgG (antibody A) binds to the acrosomal region of mouse sperm. (B) Control IgG does not bind to sperm. (C) The same results were obtained when a different antibody (antibody B) was used. (D) No signal was observed when antibody B was pre-incubated with blocking peptide. Sperm nuclei were counterstained with propidium iodide. These results are representative of more than five experiments. (E–G) Complexins co-localize with acrosomal markers. Sperm were stained with antibody A (rhodamine-labeled secondary) and with FITC-PSA to identify the acrosomal region of mouse sperm. (E) Fluorescein signal showing FITC-PSA localization of acrosome. (F) Rhodamine-labeled secondary antibody detection of complexin–antibody localization of complexins. (G) Overlay of E and F showing co-localization of complexins and PSA detection of acrosome. (H) Capacitated sperm treated with A23187 that were 90% acrosome reacted were stained with complexin antibody A (fluorescein-labeled secondary) and co-stained with propidium iodide. All but the sperm on the right edge showed no complexin staining. Scale bars correspond to 5 μ m.

Figure 4 Complexins (CPLX) I and II fractionated as cytosolic proteins from sperm. An equal amount of protein (26 µg) from the cytosol or membrane fraction was loaded in each lane and detected by anti-complexin (antibody A) and anti-PKCα antibodies. Protein kinase Cα (PKCα) is a cytosolic protein in sperm and was used as an indicator of efficiency of cell fractionation. Complexin antibody detects complexins I and II mainly in the cytosol of mouse sperm.

Baker 1997), curvilinear velocity and linearity as well as other parameters including path velocity (Table 2), straight line velocity, beat frequency, and straightness (data not shown), were not different between complexin-I-deficient and wild-type animals during capacitation. Together, these results indicate that sperm lacking complexin I had only a small difference in normal motility and hyperactivation parameters and this small motility difference was only apparent before 60 min of capacitation.

Complexin-I-deficient sperm are subfertile when used in artificial insemination

Because of the ataxia, complexin-I-deficient male mice cannot mate and do not reproduce. To determine whether deletion of complexin I affects sperm fertility, sperm from wild-type and complexin-I-deficient mice were inseminated into superovulated C57BL female mice. Embryos were collected from the oviducts 32–36 h after insemination. The fertilization rate of wild-type sperm was $48.4 \pm 4.9\%$, whereas the fertilization rate of complexin-I-deficient sperm was reduced to $20.3 \pm 2.8\%$ (Fig. 5). This diminution suggests that deletion of complexin I results in a considerable loss in fertility.

Complexin-I-deficient sperm capacitate normally

Because there was a defect in fertility when complexin-I-deficient sperm were used in artificial insemination, our next goal was to identify the defective step(s).

Table 1 Body weight, total testicular weight, and testis/body weight ratio of wild-type or complexin I heterozygous or homozygous null mice.

Genotype	Number of mice	Body weight (g)	Testicular weight (mg)	Testicular/body weight (g/g)
+/+ or +/-	9	30.7 ± 0.7	213.2 ± 8.6	0.00694
-/-	9	$21.5 \pm 0.9^*$	$187.5 \pm 5.7^*$	0.00872*

*Differences between wild-type and complexin-I-deficient mice ($P < 0.05$). Values represent the mean $\pm$ S.E.M.

Fertilization is an ordered multi-step process during which sperm must first undergo capacitation to complete maturation and obtain the ability to fertilize oocytes. To evaluate the capacitation ability of complexin-I-deficient sperm, the chlortetracycline (CTC) fluorescence assay was performed (Ward & Storey 1984, Lee *et al.* 1987). Uncapacitated sperm show fluorescence over the entire head (Fig. 6A, left), whereas capacitated sperm have bright fluorescence only on the anterior part of the head (Fig. 6A, middle), and acrosome-reacted sperm lose fluorescence on the entire head (Fig. 6A, right). The CTC fluorescence assay has been used successfully to determine the time course of capacitation, as assessed by a decrease in pattern F and increase in pattern B (Ward & Storey 1984, Lee *et al.* 1987).

Wild-type and complexin-I-deficient mouse sperm were incubated for various time periods under conditions that promote capacitation and stained with CTC. Patterns F, B, and AR were evaluated at different times of incubation. The abundance of pattern F in wild-type sperm decreased with time (0 min, $69.3 \pm 1.7\%$ to 120 min $16 \pm 3.4\%$). At the same time, the percentage of sperm in pattern B increased with time (0 min, $27.5 \pm 0.7\%$ to 120 min $67 \pm 3\%$) (Fig. 6B). Similarly, the abundance of pattern F in complexin-I-deficient sperm decreased during capacitation (0 min, $69.5 \pm 2.2\%$ to 120 min $17.8 \pm 3.0\%$) and the percentage in pattern B increased (0 min, $25.9 \pm 1.1\%$ to 120 min $65.9 \pm 4.2\%$) in a time-dependent manner (Fig. 6C). The percentage of pattern F, B, and AR at each time point was not different between wild-type and complexin-I-deficient sperm. These data demonstrated that capacitation of sperm lacking complexin I proceeds at the same rate as wild-type sperm and they have a similar spontaneous acrosome reaction rate.

Complexin-I-deficient sperm have normal zona pellucida-binding ability

After capacitation, mouse sperm pass through the cumulus matrix and bind to the zona pellucida. To determine whether deletion of complexin I disturbed sperm binding with zona pellucida, a zona binding assay was performed. Capacitated sperm from wild-type and complexin-I-deficient mice were incubated with cumulus-free oocytes for 30 min at 37 °C. After carefully washing to remove loosely adherent sperm, the sperm–oocyte complexes were fixed and stained with propidium iodide to detect nuclei of bound sperm (Fig. 7A and B). Sperm nuclei were counted under light and fluorescence microscopies. Results showed 26.8 ± 2.5 complexin-I-deficient sperm bound to each oocyte, the same number as wild-type sperm (32.0 ± 1.6 sperm/oocyte; Fig. 7C). Therefore, sperm from complexin-I-deficient mice have normal ability to bind to the zona pellucida.

Table 2 Computer-assisted motility analysis wild-type and complexin-I-deficient mice.

Genotype	Capacitation time (min)	Motile (%)	Progressive (%)	Path velocity (μm/s)	Curvilinear velocity (μm/s)	Lateral displacement (μm)	Linearity (%)
+/+	0	77.7±8.5	48.0±3.0*	110.7±1.9	214.0±4.7	12.8±0.9	37.7±1.5
+/+	30	75.3±3.2*	41.7±4.6*	115.4±6.4	226.7±3.4	13.2±0.5*	35.3±2.3
+/+	60	66.0±15.7	32.0±10.0	98.7±11.8	191.5±11.0	12.5±0.9	34.3±4.7
-/-	0	62.0±7.8	33.3±7.8*	94.3±11.2	193.0±15.9	12.0±0.7	36.0±1.0
-/-	30	52.3±5.0*	27.0±7.0*	104.5±8.2	212.4±14.6	11.8±0.3*	34.3±5.1
-/-	60	52.7±2.1	22.7±1.5	90.8±3.0	185.4±5.5	11.5±0.9	32.7±1.5

Cauda epididymal sperm from three pairs of wild-type and complexin-I-deficient mice were incubated under capacitating conditions. At each time point, a sample of sperm was added to a pre-warmed chamber and motility parameters were assessed by computer-assisted semen analysis (CASA). *Differences between sperm from wild-type and complexin-I-deficient mice ($P<0.05$). Values represent the mean $\pm$ S.E.M.

Zona removal rescues the fertilizing ability of complexin-I-deficient sperm

After they bind to the zona pellucida, sperm undergo the acrosome reaction, which is necessary for the sperm to penetrate the zona pellucida and fertilize the oocyte (Yanagimachi 1994). Previously, we found that sperm lacking complexin I could not undergo the acrosome reaction in response to soluble zona pellucida proteins *in vitro* (Zhao *et al.* 2007). Complexin-I-deficient sperm, when used for *in vitro* fertilization of cumulus-enclosed oocytes, produced markedly lower fertilization rates compared with wild-type sperm (Zhao *et al.* 2007). To determine whether removal of the zona pellucida from oocytes could allow complexin-I-deficient sperm that had spontaneously undergone the acrosome reaction to fertilize oocytes, *in vitro* fertilization with zona-free oocytes was used. If faulty zona-induced acrosomal exocytosis were the only defect in complexin-I-deficient sperm, one would expect that removing the zona pellucida would alleviate the reduced fertility. After 60 min of capacitation, sperm from wild-type or complexin-I-deficient mice were incubated with zona-free oocytes. After a 6-h co-incubation, $71\pm13\%$ and $70\pm6\%$ of oocytes were fertilized by complexin-I-deficient sperm and wild-type sperm respectively, as assessed by the formation of the second polar body (Fig. 8). Similarly, $59\pm21\%$ and $68\pm12\%$ of oocytes

Figure 5 Complexin-I-deficient sperm are defective in fertilization following artificial insemination (AI). The sperm suspension (3×10^6 sperm in 50 μ l) from wild-type ($n=6$) or complexin-I-deficient mice ($n=4$) was inseminated into mouse cervix via the vagina. About 32–36 h post-AI, oocytes were flushed from the oviducts and examined. The presence of two normal sized blastomeres was taken as evidence of fertilization. Around 48.4% of oocytes (81/174 oocytes) were fertilized by wild-type sperm, while 20.3% of oocytes (29/142 oocytes) were fertilized by complexin-I-deficient sperm. WT, wild-type; KO, knockout.

Figure 6 Chlortetracycline fluorescence assays of sperm from wild-type and complexin-I-deficient mice. (A) Fluorescence pattern observed on the sperm head stained by chlortetracycline. Pattern F (F, left) shows uniform fluorescence over the head. Pattern B (B, middle) shows fluorescence on the anterior portion of head and a dark post-acrosomal region. Pattern AR (AR, right) is observed in sperm that have undergone the acrosome reaction, showing barely detectable fluorescence over the whole surface of the head. (B and C) Comparison of capacitation states between complexin-I-deficient mouse sperm and wild-type mouse sperm. Sperm from (B) wild-type and (C) complexin-I-deficient mice were incubated in dmKRBT medium and stained with chlortetracycline before (0 min) and after incubation for 30, 60, 90, or 120 min. WT, wild-type; KO, knockout.

Figure 7 An equal number of wild-type and complexin-I-deficient sperm bound to the zona pellucida of oocytes. (A and B) Ovulated oocytes from CD-1 mice were incubated with 1×10^6 capacitated sperm from (A) wild-type and (B) complexin-I-deficient mice for 30 min at 37 °C. After washing to remove non-adherent sperm, oocyte–sperm complexes were fixed with 2% paraformaldehyde, stained with 10 µg/ml of propidium iodide, and observed using fluorescence microscopy. (Inserts) Two-cell mouse embryos that do not bind sperm with high affinity were used as negative controls. (C) Similar numbers of sperm from wild-type mice (32.0 ± 1.6 S.E.M./oocyte) or complexin-I-deficient mice (26.8 ± 2.5 S.E.M./oocyte) bound to each oocyte. Data are overall means of five experiments using 20 oocytes per droplet in triplicate droplets. WT, wild-type; KO, knockout.

incubated with complexin-I-deficient sperm and wild-type sperm respectively were fertilized and developed to the two-cell stage (Fig. 8). The fertilization rate of zona-free oocytes was not different between wild-type and complexin-I-deficient sperm. These results indicate that the fertilizing ability of complexin-I-deficient sperm is normal if the zona pellucida is removed.

Complexin II expression is upregulated in complexin-I-deficient testis and the protein is in the acrosomal region of complexin-I-deficient sperm

Complexins I and II are 84% identical at the amino acid level (McMahon *et al.* 1995) and appear to be functionally redundant in most cells, although many neurons only express one complexin (Eastwood *et al.* 2000, Freeman & Morton 2004). The different phenotype of each knockout appears to be due to the withdrawal of complexin I or II from cells that only express one complexin (Reim *et al.* 2001, Glynn *et al.* 2003, 2005, 2007). In the brain, if the complexin that is normally expressed is deficient, the other is not upregulated to fulfill the function; there appears to be no compensatory

Figure 8 The fertility of complexin-I-deficient sperm is normal when allowed to fertilize zona-free oocytes. Mouse zona-free oocytes were co-incubated with 1×10^5 /ml of capacitated sperm from complexin-I-deficient mice or wild-type mice in HTF medium at 37 °C. After 6- or 28-h co-incubation, embryos and oocytes were observed under light microscopy to determine the fertilization rate. The presence of the second polar body (6 h) or two-cell embryo (28 h) is considered as fertilization. Black bars refer to wild-type or heterozygote complexin I sperm. Gray bars refer to complexin-I-deficient sperm. Data are overall means of five experiments using 10–20 oocytes per droplet in triplicate droplets. WT, wild-type; KO, knockout.

upregulation in the brain. On the contrary, deletion of some sperm proteins has been shown to affect the abundance of other gene products in sperm (Nishimura *et al.* 2001, 2007). Previously, we found that complexin II abundance was, in fact, increased in the testes deficient in complexin I (Zhao *et al.* 2007).

To confirm that the localization of complexin II was normal in sperm from complexin-I-deficient mice, sperm lacking complexin I were stained with antibody A that detects both complexins I and II. The antibody labeled the acrosomal region of complexin-I-deficient sperm (Fig. 9A), demonstrating that the localization of complexin II was unaffected in sperm from mice lacking complexin I.

Based on these results, we conclude that sperm from complexin-I-deficient mice are subfertile due to a defect in the penetration of zona pellucida. Complexin II, which is highly upregulated in complexin-I-deficient testes and is targeted properly in complexin-I-deficient sperm, does not compensate for the loss of complexin I during the sperm acrosome reaction.

Figure 9 Complexin II is present in acrosomes of sperm lacking complexin I. The acrosomal region of sperm from mice lacking complexin I was stained by the (A) complexin antibody and (B) control rabbit IgG does not bind to sperm. Sperm nuclei are counterstained with propidium iodide.

Discussion

Our previous data showed that sperm deficient in complexin I are faulty in acrosomal exocytosis and fertilization *in vitro* (Zhao *et al.* 2007). Herein, we identified the germ cells that express complexins I and II and determined the cellular location of complexin I/II proteins as a basis for a detailed analysis of the biological effects of complexin I deficiency in sperm. Using RT-PCR, we found that complexin I and II transcripts are both expressed in spermatocytes and spermatids. Their proteins were localized in or around the developing acrosome of spermatids and the acrosomal region of cauda epididymal sperm. This is consistent with a recent study localizing complexins I and II in human sperm, which are, in contrast to mouse sperm, paddle-shaped (Roggero *et al.* 2007). Furthermore, we demonstrated that sperm complexins I and II fractionate as mainly cytosolic proteins. Because complexins I and II are lost during the acrosome reaction, we speculate that complexins I and II are present in the cytosol between the outer acrosomal membrane and the sperm plasma membrane. Due to the very small amount of cytosol in this region and difficulty in distinguishing cytosolic and acrosomal components, we could not draw a definite conclusion about the precise location of complexins I and II.

Complexin-I-deficient male mice are totally infertile due to defective muscular coordination that prevents the mice from mating. This prevents routine assessment of sperm fertility; however, when fertility was assessed by *in vivo* fertilization (artificial insemination), we found that sperm fertility was diminished markedly (Fig. 8). The curtailed fertility appears to be due to a specific defect in zona pellucida penetration. Other problems were not observed in sperm from complexin-I-deficient mice. Seminiferous tubules from complexin-I-deficient mice were morphologically normal, as determined by light microscopy. Complexin-I-deficient sperm were normal in number, morphology, and only altered slightly in motility. During fertilization, complexin-I-deficient sperm capacitated normally and bound in typical number to the zona pellucida. However, previous results showed that complexin-I-deficient sperm are defective in the zona pellucida-induced acrosome reaction and have conspicuously lower fertilization rates of cumulus-enclosed oocytes, *in vitro* (Zhao *et al.* 2007). The observation that the lower fertility by *in vitro* fertilization parallels the reduction in fertility following artificial insemination indicates that the cause of reduced fertility is probably not an inability of sperm to be stored in the isthmus, transported to the site of fertilization, or to penetrate the cumulus mass. Furthermore, removal of the barrier that requires the acrosome reaction, the zona pellucida, allowed complexin-I-deficient sperm to fertilize oocytes at wild-type frequencies indicating that deletion of complexin I did not affect the ability of sperm to fuse with and activate oocytes. Because the

small temporary decrease in motility was not sufficient to affect sperm binding to the zona pellucida and to zona-free oocytes and motility was not different at 60 min of capacitation, when sperm were added to oocytes, it is unlikely that the decrease in fertility was due to a change in motility. Based on these results and our previous work (Zhao *et al.* 2007), we conclude that complexin I is necessary for the zona-induced acrosome reaction, zona penetration, and normal fertility.

The dramatic outcome of complexin I deficiency in cells that express both complexins I and II is novel. In the brain, complexins I and II are expressed by distinct subsets of neurons (Eastwood *et al.* 2000, Freeman & Morton 2004). They are encoded by two different genes and differently regulated (Abderrahmani *et al.* 2004); however, they have a similar function, to regulate SNARE-mediated exocytosis (McMahon *et al.* 1995). A recent study using permeabilized sperm suggested that either complexin I or II is necessary to promote acrosomal exocytosis (Roggero *et al.* 2007). By contrast, the observation that sperm contain both complexins I and II but that only complexin I deficiency affects fertility suggests that complexin I has a unique function in acrosomal exocytosis; complexin II cannot compensate for the absence of complexin I during this process, even though complexin II is in the acrosomal region of complexin-I-deficient sperm (Fig. 9) and its expression is highly upregulated in complexin-I-deficient testis (Zhao *et al.* 2007). Because it was reported that complexins behave as clamps on membrane fusion (Giraudo *et al.* 2006, Schaub *et al.* 2006, Huntwork & Littleton 2007), we cannot exclude the possibility that the abundant complexin II in the complexin-I-deficient mice acts to clamp fusion and cause subfertility. This appears to be unlikely, however, because the opposite does not occur. Complexin I does not act as a clamp in complexin-II-deficient male mice and they have no reproductive defects as assessed by *in vivo* fertility (Takahashi *et al.* 1999, Reim *et al.* 2001, Glynn *et al.* 2003). Therefore, our data suggest that complexins I and II play a different role in cells, in which they are expressed at the same level and in the same cellular location. In contrast to sperm, neurons lacking complexin I undergo normal secretion if complexin II is expressed and even the more sequence-divergent complexins III and IV can substitute for the function of complexin I (Reim *et al.* 2005).

An explanation for the specific requirement for complexin I in sperm is not obvious. One possibility is that, in germ cells, complexin I may interact with different SNAREs than complexin II. In the synaptic SNARE complex, the affinities of complexins I and II for the core SNARE complex including syntaxin 1A, VAMP2, and SNAP25 are similar (Pabst *et al.* 2002). However, replacement of syntaxin 1 with syntaxin 2 in the core complex reduces the affinity of complexin I for the complex, but the affinity of complexin II is unchanged. And if syntaxin 1 is replaced by syntaxin 4,

both complexins I and II lose the binding ability with the SNARE complex (Pabst *et al.* 2000). Similarly, a more recent report demonstrated that complexins and synaptotagmin have a difference in affinity for SNARE complexes composed of varying syntaxins (Bajohrs *et al.* 2005). Based on these studies with neuronal SNAREs, we speculate that complexin I in sperm interacts with novel germ cell SNAREs that have a specific affinity for it and the interaction is necessary for acrosomal exocytosis. However, early experiments have not detected any difference in proteins bound to complexins I and II (Zhao *et al.* 2007).

Based on these data, we infer a working model of complexin I during the acrosome reaction. Complexin binds to SNARE complexes and binding to SNAREs may be regulated by complexin phosphorylation (Shata *et al.* 2007). Complexin binding to SNARE complexes allows them to move to a 'super-primed' state and then to hold SNARE complexes at each fusion site in a release-competent state until calcium concentrations are increased (Tang *et al.* 2006). 'Super-priming' and then stabilization appear to be accomplished by separable domains in complexins (Xue *et al.* 2007). This model implies a sequential positive role of complexins for stability and a subsequent negative role to prevent premature fusion. Because complexin-I-deficient sperm do not undergo acrosomal exocytosis, it supports a role for complexin I in the formation of a 'super-primed' complex. Because this complex is not formed in complexin-I-deficient sperm, we could not assess a blocking role for complexin I in sperm. But it is clear that complexin I acts as more than a simple clamp for a second reason; complexin-I-deficient sperm do not spontaneously acrosome react at higher rates than wild-type sperm, as would be predicted if the only role of complexin I is to block fusion (Fig. 6 in the present paper and Zhao *et al.* (2007). Furthermore, antibodies to complexins I and II, when added to permeabilized human sperm, block the acrosome reaction (Roggero *et al.* 2007). Additional data from permeabilized human sperm suggest that the calcium sensor synaptotagmin VI can displace complexins from the SNARE complex, allowing acrosomal exocytosis (Roggero *et al.* 2007). This displacement may be regulated by synaptotagmin phosphorylation (Roggero *et al.* 2007). Our results are consistent with this putative function for complexin I but not complexin II. Understanding the interplay between calcium/synaptotagmin and complexin I that regulates SNARE function and sperm membrane fusion will help us understand the unique exocytosis carried out by sperm.

Materials and Methods

RT-PCR of testis/germ cell RNA

Fifteen-day-old mice were killed by CO₂ asphyxiation and testis were removed and placed immediately in liquid nitrogen. Total RNA was prepared from frozen testis using TRIzol

Reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using the RETROscript kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions using Moloney murine leukemia virus reverse transcriptase.

Primers were designed to amplify the entire coding region of complexin I (up: 5'-CCTGAGGAACCAAGCCATCA-3' and down: 5'-CTGCTCCCAACATCCCTGT-3') or complexin II (up: 5'-CCAGCCAGGAGTGCTGAAT-3' and down: 5'-CGGAGGATGGGTACTT-3') based on published murine complexin sequences (GenBank accession numbers: NM_007756 and NM_009946 respectively; Takahashi *et al.* 1995). Five microliters of reverse-transcribed cDNA or RNA were amplified in a 50 µl reaction containing 10× PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 250 µM dNTPs, 10 pmol of each primer, and 1 U *Taq* DNA polymerase (Invitrogen Life Technologies). RNA (not reverse transcribed) or water was used as a template instead of cDNA for controls. PCR amplifications were performed in a RoboCycler (Stratagene, La Jolla, CA, USA) with an initial denaturation step of 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. A final extension step was carried out at 72 °C for 10 min and the reactions were incubated at 6 °C until the products were analyzed in 1.5% agarose gel.

Immunofluorescence

To collect testes, mature male CD-1 mice were killed by CO₂ asphyxiation. Animals were transcardially perfused with pre-wash and fixative solution (4% paraformaldehyde in PBS) as detailed (Redecker & Bargsten 1993). Testes were immersed in fixative overnight at 4 °C. Fixed testicular samples were embedded in OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in liquid nitrogen. Cryostat sections were cut at a thickness of 10 µm.

To prepare mouse sperm, cauda epididymides were placed in 2 ml PBS. Cauda epididymides were macerated with a 30 gauge needle and then placed at room temperature for 10 min to release sperm. Twenty microliters of sperm solution were smeared on X-tra-coated slides (Surgipath, Richmond, IL, USA) and allowed to air-dry at room temperature. In some experiments, sperm were collected into dmKRBT and incubated *in vitro* at 37 °C for 1 h and then for another 1 h in 10 µM calcium ionophore A23187 (Sigma) to induce the acrosome reaction. At each time point (0, 60, and 120 min after collection), an aliquot of sperm was dried on the slides and fixed with 95% ethanol and 5% glacial acetic acid at -20 °C for 10 min. After fixing, the slides were air dried.

Tissue sections or sperm on the slides were washed twice with solution A (127 mM NaCl, 5.3 mM KCl, 18.2 mM sodium HEPES, and 0.02% sodium azide) containing 3% BSA for 5 min and blocked and permeabilized for 45 min with solution A containing 3% BSA and 0.5% Triton X-100.

Two antibodies were used to detect complexin proteins. Both recognize complexins I and II. Some samples were incubated with 1 µg/ml antibody A made against a peptide corresponding to amino acid residues 41–91 of complexin II (affinity purified; Synaptic Systems, Gottingen, Germany) or control rabbit IgG

(Sigma), diluted in solution A. Other samples were incubated with 1 µg/ml complexin antibody made against a peptide mapping to the carboxy terminus of human complexin I (antibody B; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or antibody pre-incubated with fivefold excess of blocking peptide for 1 h. The slides were washed, incubated with biotinylated goat anti-rabbit or rabbit anti-goat secondary antibody for 1 h, washed, and detected with FITC-avidin (Vector Laboratories, Burlingame, CA, USA) diluted 1:100 in solution A with 3% BSA for 45 min. Cell nuclei were counterstained with 100 µg/ml (tissue) or 1 µg/ml (sperm) propidium iodide in PBS. The slides were mounted with a fade reductant (DakoCytomation, Carpinteria, CA, USA). Cells were examined using a Zeiss Axioskop at 400× and 630× and images obtained with a Zeiss AxioCam and AxioVision software (Carl Zeiss, Inc., Thornwood, NY, USA). At least 200 sperm were examined.

Double immunofluorescence

Ethanol-fixed sperm on the slides were washed with solution A containing 3% BSA twice for 5 min each and blocked and permeabilized for 45 min with solution A containing 3% BSA and 0.2% Triton X-100. The sperm were incubated with affinity-purified antibody A or control rabbit IgG (Sigma), diluted to 20 µg/ml in solution A for 1 h. The sperm were washed thrice and Texas Red-labeled goat anti-rabbit secondary antibody (Vector Laboratories) diluted 1:50 in solution A containing 3% BSA was added. The slides were incubated for 1 h, washed, and stained with 15 µg/ml FITC-PSA in PBS for 10 min. The slides were rinsed briefly with solution A, mounted with a fade reductant (DakoCytomation), and analyzed by fluorescence microscopy.

Sperm fractionation

Sperm fractionation was performed as described (NagDas *et al.* 2002). Briefly, sperm were washed twice with PBS by centrifugation, suspended in TNI (150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 2 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.05% sodium azide), and sonicated for four 10-s intervals with a Branson Sonifier at a medium power setting. The sonicated suspensions were centrifuged at 50 000 *g* for 60 min. The supernatant was removed and will be referred to as the cytosolic fraction. The detergent-soluble fraction of sperm was prepared by extraction of the pellet with 0.1% Triton X-100 in TNI for 1 h at 4 °C followed by centrifugation at 12 000 *g* for 10 min.

Western blots

Proteins were separated electrophoretically in 10% acrylamide SDS-PAGE gels and transferred to nitrocellulose. The membranes were blocked in TBST (100 mM Tris (pH 7.4), 0.9% NaCl, and 0.1% Tween 20) containing 3% nonfat dry milk and 1% BSA at 4 °C overnight. Affinity-purified rabbit anti-complexin IgG (antibody A) was diluted 1:2000 in TBST and added to the blocked membranes for 1 h at room temperature. Blots were washed and incubated with a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (BD Transduction Laboratories, San Jose, CA, USA)

diluted 1:20 000 in TBST. The blots were then washed, incubated with SuperSignal Pico chemiluminescent substrate (Pierce, Rockford, IL, USA), and exposed to autoradiography film. Benchmark pre-stained standards were used to estimate molecular sizes (Invitrogen). As a fractionation control, the membranes were washed with Restore western blot stripping buffer (Pierce Biotechnology, Inc.) to remove the antibodies and incubated with anti-PKCα antibodies (Santa Cruz Biotechnology, Inc.) diluted 1:200 in TBST. Quantitation of band intensity was performed using Adobe Photoshop.

Sperm motility analysis

CASA was performed to determine the sperm motility characteristics. Sperm from three pairs of mature wild-type and complexin-I-deficient mice of the same age were incubated in dmKRBT (120 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 10 mM NaHCO₃, 1.2 mM MgSO₄, 0.36 mM NaH₂PO₄, 5.6 mM glucose, 1.1 mM pyruvic acid, 25 mM TAPSO, 18.5 mM sucrose, 0.6% BSA, 10 units/ml penicillin, and 10 µg/ml streptomycin (pH 7.3)) at 37 °C for 1 h. At 0, 30, and 60 min, aliquots of prepared sperm samples were added to a 37 °C sperm analysis chamber and motility parameters were assessed using the HTM-IVOS Vs 12 Integrated Visual Optical System (Hamilton-Thorne Research Danvers, MA, USA). The analysis parameter settings were as follows: frames acquired 30; frame rate, 60 Hz; magnification, 0.82×; fields acquired, 6; video source, 60 Hz, dark field; illumination intensity, 4095; and temperature set, 37.0 °C.

The motility parameters measured were as follows: percentage of motile sperm (motile); percentage of progressively motile sperm (progressive); average cell path velocity; straight line velocity; curvilinear velocity; amplitude of lateral head displacement; beat cross frequency; straightness; and linearity.

Artificial insemination

C57BL/6 female mice were injected with pregnant mare serum gonadotropin (7.5 IU i.p.) and 48 h later were injected with human chorionic gonadotropin (hCG; 7.5 IU i.p.) (Miller *et al.* 1993). Wild-type and complexin-I-deficient mice (littermates) were killed by CO₂ asphyxiation. Cauda epididymides were placed in 400–500 µl dmKRBT covered by mineral oil. Cauda epididymides were macerated with a 30 gauge needle and placed at 37 °C for 5 min to release the sperm. Cauda epididymides were removed from the medium and the sperm were allowed to capacitate at 37 °C for 30–60 min. The sperm concentration was adjusted to 6 × 10⁷ sperm/ml.

The method of artificial insemination is described as follows (Olds-Clarke & Wivell 1992). Briefly, 11–12 h after hCG injection, female mice were anesthetized using a small cone containing cotton soaked with isoflurane as an inhalant. A speculum was inserted into the vagina and advanced to the cervix. A 50 µl volume of the sperm suspension (3 × 10⁶ sperm) was inseminated into the cervix transvaginally with a bent, blunted 23 gauge needle. Approximately 32–36 h post-AI, mice were killed and oocytes were flushed from the oviducts. The presence of two normal sized blastomeres was considered as fertilization.

CTC fluorescence assay

Sperm were collected and incubated in dmKRBT medium. The final concentration of sperm was adjusted to 1×10^6 sperm/ml. The protocol was modified from a previously published version (Ward & Storey 1984). The CTC solution was made by dissolving 750 mM CTC-HCl (Sigma) in a chilled buffer containing 20 mM Tris-HCl, 130 mM NaCl, and 5 mM cysteine-HCl (pH 7.0). The tubes were wrapped with a foil to prevent exposure to light and stored at 4 °C. Fresh solutions were made for daily use. At 0, 30, 60, 90, and 120 min of incubation, 10 µl of sperm suspension were added to a warmed (37 °C) slide immediately after which an equal column of CTC solution was added with mixing. The CTC-sperm suspension was incubated for 1 min at 37 °C. The slides were coverslipped and immediately observed by fluorescence microscopy (Carl Zeiss, Inc.). Staining patterns were distinguished as described (Ward & Storey 1984).

Sperm-oocyte binding assay

Six-week-old female CD-1 mice were superovulated as described above. Oviducts were removed from females at 13-h post-hCG, and the oocyte-cumulus complexes were collected. The oocytes were treated with 0.1% hyaluronidase for 10 min to remove their cumulus cells and washed with dmKRBT. Groups of 20 oocytes were added in 50 µl drops of dmKRBT under paraffin oil. Three to five two-cell embryos flushed from the oviducts of superovulated females who mated with a male were included in every drop as negative controls. Fifty microliters of capacitated sperm solution (10 000 cells) from wild-type or complexin-I-deficient mice were added to the oocyte-containing drops and binding was allowed to proceed for 30 min at 37 °C and 5% CO₂. Sperm-oocyte complexes were washed free of unbound or loosely adherent sperm. Washing was stopped when less than three sperm remained bound to two-cell embryos. Sperm-oocyte complexes were fixed with 2% paraformaldehyde for 10 min and stained with 10 µg/ml propidium iodide in PBS for 15 min. They were carefully transferred to the slides with 2 µl fluorescent mounting medium (DakoCytomation) and coverslipped. The number of sperm bound to the oocyte zona pellucida was determined by fluorescence microscopy.

In vitro fertilization with zona pellucida-free oocytes

Oocytes were recovered from superovulated mice 13–14 h after hCG injection and placed in HTF medium (Irvine Scientific, Santa Ana, CA, USA). Preparation of ZP-free oocytes is described as follows (Yamagata *et al.* 2002). Oocytes were treated with 0.1% hyaluronidase for 10 min at room temperature and washed with HTF medium. Cumulus-free oocytes were transferred into 50 µl drops of acidic Tyrode's solution (Sigma) and pipetted several times until the ZP were dissolved under a stereoscopic microscope (~30 s). The oocytes were then washed twice with HTF medium and incubated in HTF medium for more than 1 h to allow surface proteins to recover (Evans *et al.* 1997).

For *in vitro* fertilization, the sperm were collected from the cauda epididymis of wild-type or complexin-I-deficient mice

and capacitated in HTF medium at 37 °C for 1 h. Capacitated sperm at a concentration of 10^5 sperm/ml were co-incubated with 10–20 zona-free oocytes in a 100 µl drop of HTF covered by embryo-tested mineral oil (Sigma) at 37 °C and 5% CO₂. After 6- or 28-h co-incubation, the embryos were observed under light microscopy. The presence of a second polar body (after 6-h incubation) or development to the two-cell stage (after 24-h incubation) was considered as fertilization.

Statistical analysis

Data were evaluated using one-way ANOVA. Student's *t*-test was used for pairwise comparisons. $P < 0.05$ was considered a significant difference.

Declaration of interest

Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the National Institutes of Health. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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