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Expression, localization and functions in acrosome reaction and sperm motility of $Ca_V3.1$ and $Ca_V3.2$ channels in sperm cells: an evaluation from $Ca_V3.1$ and $Ca_V3.2$ deficient mice

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Number of figures : 9
Number of words: 7077
Run header: **$Ca_V3.1/3.2$ channel functions in sperm physiology**

Keywords **: Calcium channels; $Ca_V3.1$; $Ca_V3.2$; spermatogenic cell; acrosome reaction; sperm motility.**

SUMMARY

In sperm cell, voltage-dependent calcium channels (VDCC) have been involved in different cellular functions like acrosome reaction (AR) and sperm motility. Multiple types of VDCC are present and their relative contribution in these functions is still a matter of debate. Based mostly on pharmacological studies, Low-Voltage-Activated Calcium Channels (LVA-CC), responsible of the inward current in spermatogenic cells, were described as essential for AR in sperm cell. The development of $Ca_V3.1$ or $Ca_V3.2$ null mice provides the opportunity to evaluate the involvement of such LVA-CC in acrosome reaction and sperm motility, independently of pharmacological tools. The depolarization-induced current is fully abolished in spermatogenic cells from $Ca_V3.2$ deficient mice. The spermatogenic inward current is thus only due to $Ca_V3.2$ channels. We showed that $Ca_V3.2$ channels are maintained in mature sperm cells by Western blot and immunohistochemistry experiments. Calcium imaging experiments revealed that calcium influx in response to KCl is reduced but not abolished in $Ca_V3.2$ null mature sperm in comparison to control sperm cells, demonstrating that $Ca_V3.2$ channels are functional in mature sperm cell. On the other hand, no difference was noticed in calcium signaling induced by solubilized zona pellucida. Moreover, neither Western blot or immunohistochemistry, nor calcium imaging experiments, suggest the presence of $Ca_V3.1$ in sperm head. Despite the $Ca_V3.2$ channels contribution in KCl-induced calcium influx, the reproduction parameters (number of sperm per epididymis, pups number, delay between mating and delivery), and the cellular functions (AR and sperm motility) remain intact in $Ca_V3.2$ deficient mice. These data demonstrate that in sperm, besides $Ca_V3.2$ channels, other types of VDCC are activated during the voltage-dependent calcium influx of AR, these channels likely belonging to high-voltage activated Ca^{2+} channels family. The conclusion is that voltage-dependent calcium influx during acrosome reaction is due to the opening of redundant families of calcium channels.

INTRODUCTION

The acrosome reaction (AR), the first step of fertilization, is an exocytotic event, allowing sperm to cross the zona pellucida (ZP) and to become competent for fusion with the oocyte, by unmasking the izumo protein (Inoue et al, 2005). ZP3, one of the three glycoproteins present in the zona pellucida, represents the main physiological agonist of the AR in rodent. The fusion of the outer acrosomal membrane with the plasma membrane is dependent of a cytoplasmic calcium rise. The calcium signaling requires the successive opening of at least three different types of calcium channels. Voltage-dependent calcium channels (VDCC), localized in the plasma membrane, are the first to be activated. Subsequently, an Inositol-1,4,5-triphosphate (InsP3) receptor, localized in the outer membrane of the acrosome, is activated (Walensky and Snyder, 1995; O'Toole et al, 2000) and leads to the calcium depletion of the acrosome, an operational calcium store (De Blas et al, 2002; Herrick et al, 2004). The depletion of this store finally leads to the activation of store-operated calcium channels like TRPC2 (Jungnickel et al, 2001).

The successive activation of these channels determines two phases in the sperm acrosome calcium signaling: i) during a first phase, the opening of VDCCs elicits a very short transient calcium rise, of about 100 msec duration (Arnoult et al, 1999), ii) a second phase, corresponding to the activations of the InsP3 receptor and subsequently TRPC store-operated calcium channels, is characterized by a slow calcium increase (between few second to few minutes, dependent of reports), followed by a long lasting calcium entry. Different types of VDCC blockers like amiloride, PN200-110 (a dihydropyridine) or pimozide (a diphenylbutylpiperidine) inhibit both the calcium transient and the subsequent slow calcium increase and calcium plateau (Arnoult et al, 1996; Arnoult et al, 1999). In consequence, the calcium influx through VDCC is currently understood as the key triggering element for the second phase of calcium signaling in sperm cells.

Voltage-dependent calcium channels are subdivided in high-voltage (HVA) and low-voltage-activated (LVA) calcium channels, represented by L, N, P/Q, R and by T-type calcium channels, respectively. At the molecular level, 10 different genes encode for VDCC α_1 subunits, the pore subunit. The molecular characterization of VDCC present in sperm cells and their respective role in the sperm physiology is a hotly debated and studied question (Zhang et al, 2006; Arnoult et al, 1996; Santi et al, 1996; Goodwin et al, 1997; Goodwin et al, 1998; Arnoult et al, 1999; Westenbroek and Babcock, 1999; Serrano et al, 1999; Jagannathan et al, 2002; Stamboulian et al, 2004).

Owing to the high difficulties to record voltage-dependent calcium currents in mature sperm cells, many groups have worked on the molecular characterization of voltage-dependent channels in spermatogenic cells (Lievano et al, 1996; Arnoult et al, 1996; Espinosa et al, 1999; Sakata et al, 2001; Jagannathan et al, 2002; Stamboulian et al, 2004). In these cells, only T-type calcium currents are recorded during patch-clamp experiments. Although mRNA encoding for both $Ca_V3.1$ (Sakata et al, 2001) and $Ca_V3.2$

(Jagannathan et al, 2002) have been found in spermatogenic cells, results from $Ca_V3.1$ deficient mice suggested that $Ca_V3.2$ is the main calcium channel in spermatogenic cells (Stamboulian et al, 2004). On the other hand, several authors have claimed that part of the T-type current in spermatogenic cells could be due to activation of HVA channels, $Ca_V1.2$ (Goodwin et al, 1999) or $Ca_V2.2$ and $Ca_V2.3$ (Wennemuth et al, 2000). The development of transgenic mice deficient in $Ca_V3.2$ channels provides the opportunity to evaluate the actual contribution of $Ca_V3.2$ channels in the inward calcium current of spermatogenic cells.

In this manuscript, we compare for the first time the inward calcium current density from wild-type spermatogenic cells with those of spermatogenic cells isolated from $Ca_V3.1$ or $Ca_V3.2$ deficient mice. The inward current elicited by depolarization is fully abolished in spermatogenic cells obtained from $Ca_V3.2$ deficient mice. This result ends a long-term controversy over the molecular identity of the LVA inward current in spermatogenic cells: this current is due only to activation of $Ca_V3.2$ channels. In mature sperm cells, Western blotting experiments demonstrate for the first time the presence of $Ca_V3.2$ channel protein. By immunohistochemistry, we illustrate that $Ca_V3.2$ is localized in a specific patch of the plasma membrane located at the base of the acrosome, an area known to be the starting point of the calcium signaling during the acrosome reaction. Moreover, this area of the plasma membrane that contains the $Ca_V3.2$ channels overlays the external acrosomal membrane involved in exocytosis during acrosome reaction, since the $Ca_V3.2$ immunoreactivity is lost on acrosome reacted spermatozoa. Because of the presence of $Ca_V3.2$ channels in mature sperm cells, it was important to evaluate the contribution of $Ca_V3.2$ channels in sperm calcium signaling. First, we evaluated the contribution of $Ca_V3.2$ channels in voltage-dependent calcium influx, induced by high external K^+. The depolarization-induced calcium signaling is biphasic: a calcium rise followed by a calcium plateau. In $Ca_V3.2$ deficient mice, both calcium rise and calcium plateau are reduced, demonstrating the functional state of $Ca_V3.2$ channels in mature sperm cell. Second, we evaluated the importance of such channels in calcium signaling induced by solubilized ZP. In $Ca_V3.2^{-/-}$ sperm, the calcium signaling induced by ZP was identical to the one measured in wild type sperm.

Concerning $Ca_V3.1$ channels, we were unable to find any specific staining with an antibody designed specifically against $Ca_V3.1$ channels, in agreement with the electrophysiological data. Moreover, the calcium signaling induced by high K^+ is not affected in $Ca_V3.1$ deficient mice. These data, taken together, strongly suggest that $Ca_V3.1$ channels do not play a physiological role in sperm.

Despite the presence of $Ca_V3.2$ channels and their contribution in K^+-induced calcium influx, the reproduction parameters (sperm concentration per epididymis, pups number, delay between mating and delivery), and the cellular functions (AR and sperm motility) of $Ca_V3.2$ deficient mice remain intact. These data demonstrate that in sperm, besides $Ca_V3.2$ channels, other types of VDCC are activated during the voltage-dependent calcium influx of AR. The absence of reproduction phenotype at the animal or cellular levels, indicates that the expression of different types of VDCC are redundant.

Finally, we show in this paper that the motility parameters of sperm from $Ca_V3.1$ or $Ca_V3.2$ deficient animals are not statistically different to those obtained from sperm of wild type animals, suggesting that neither $Ca_V3.1$ nor $Ca_V3.2$ are involved in sperm motility.

MATERIALS AND METHODS

Biological preparations

Knock-out mice: $Ca_V3.1$ (Kim et al, 2001b) and $Ca_V3.2$ (Chen et al, 2003) deficient mice have been described previously. Control mice corresponded to C57BL/6 males if non specified (Charles River-France). The genetic backgrounds of both knockout mice are different. The genetic background of $Ca_V3.1$ deficient mice is a cross between C57Bl/6 strain and SVJ129 strain. On the other hand, the genetic background of $Ca_V3.2$ deficient mice is a C57Bl/6 strain.

Spermatogenic cells preparation: Seminiferous tubules were isolated from the testes of mice (8-16 weeks old) and incubated at 37°C for 30 minutes in 3 ml of a solution containing (mM): NaCl (150), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), NaH_2PO_4 (1), $NaHCO_3$ (12), D-glucose (11), pH 7.3 and collagenase type IA (1 mg/ml - Sigma). Tubules were rinsed twice in collagenase-free medium and cut into 2 mm sections. Spermatogenic cells were obtained by manual trituration and attached to culture dishes coated with Cell-Tak (Beckton Dickinson France). Pachytene spermatocytes and round spermatids are the prominent cell types obtained from the diploid meiotic and the haploid post-meiotic stages of spermatogenesis, respectively. These cells are readily distinguished based on cellular and nuclear morphology (Romrell et al, 1976). These stages were routinely used for electrophysiological recordings. However, similar results were obtained with both stages and data were pooled for presentation.

Sperm membrane preparation: Sperm cells, obtained by manual trituration of caudae epididymes from $Ca_V3.1$, $Ca_V3.2$ deficient mice or control C57Bl/6 mice (16 weeks old, Charles River), were pelleted (500 g , 10 minutes) and re-suspended in RIPA buffer containing in (mM) Tris (50), NaCl (150), 0,1 % SDS, 1% NP-40, 0,5 % DOC) complemented with a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche). Sperm cells were then sonicated (SONICATOR ULTRASONIC PROCESSOR XL, MISONIX INCORPORATED) for 1 sec on ice, repeated 30 times, with 7 sec intervals. Cell debris were pelleted (500 g, 15 minutes) and the supernatant used for Western blotting. To obtain a head-plasma membrane enriched fraction, sperm were capacitated in M16 (Sigma-Aldrich France) supplemented with 2% BSA (fraction V, Sigma-Aldrich France) pH 7.4 for 1 hour at 37°C. Sperm were pelleted (500 g, 10 minutes) and treated with 10 µM A23187 during 30 minutes at 37°C in the presence of a cocktail of protease inhibitors. Sperm were then centrifuged 5 minutes at 1000 g. The supernatant was collected and subsequently ultra-centrifuged at 100 000 g for 1 hour at 4°C. The pellet was re-suspended in RIPA buffer.

Cell culture and transfection: HEK-293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin and transiently transfected with a plasmid containing $Ca_V3.1$ cDNA (generous gift of Dr Anne Feltz) or $Ca_V3.2$ cDNA (generous gift of Dr Emmanuel Bourinet), using JetPEI from Qbiogene according to the

instructions of the manufacturer. Two days after transfection, transfected and control cells were collected and re-suspended in RIPA buffer complemented with a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche).

Electrophysiological recordings

Ca^{2+} currents were recorded in the whole-cell configuration of the patch-clamp technique. Pipettes were pulled from Corning #7052 glass (Gardner Glass Co., CA) and fire polished. Pipette resistance was 1-3 MΩ. Currents were obtained with an Axopatch 200B amplifier (Axon Instruments). All traces were corrected for leak currents, filtered at 2 kHz, and digitized at 20 kHz.

The pipette solution was designed to eliminate all K^+ currents and consisted of the following components (mM): Cs-glutamate (130), D-glucose (5), HEPES (10), $MgCl_2$ (2.5), Mg_2ATP (4), EGTA-Cs (10) pH 7.2 (adjusted with 1 N CsOH). For experiments, the bath solution was changed to a recording solution containing (mM): NaCl (100), KCl (5), $CaCl_2$ (10), $MgCl_2$ (1), TEA-Cl (26), Na-lactate (6), HEPES (10), D-glucose (3.3) and pH 7.4 (adjusted with 1 N NaOH). All experiments were done at room temperature (~25 °C).

Primary antibodies

For Western blotting, Antibodies against $Ca_V3.2$ (sc-16261) were from Santa Cruz Biotechnology, Inc. CA U.S.A. In the absence of specific commercial antibodies designed for immuno-histochemistry, we designed two antipeptide antibodies against the II-III loop of the $Ca_V3.2$ channels, a region presenting no homology with other VDCC. The peptides have been chosen in the II-III loop of the $alpha_1$ subunit and correspond to the amino acids 1025-1034 (Ab-1025) and 1169-1180 (Ab-1169) of the swissprot locus CAC1H_MOUSE, accession O88427. Antibodies against $Ca_V3.1$ were from Alomone Labs - Israel.

Western blot analysis

Proteins of sperm enriched plasma membrane preparation were separated on 10% polyacrylamide denaturing gels and elecrotransferred for 90 minutes at 350 mA to Immobilon P transfer membrane (Millipore). The membranes were then blocked 60 minutes with 4% non-fat dry milk (Biorad) in PBS Tween 0.1%. The primary antibody was added and incubated overnight at 4°C. After washing in PBS Tween 0.1%, the secondary antibody was added at 1:10,000 during 3 hours at room temperature. Brain and testis proteins were separated on 7% polyacrylamide denaturing gels and elecrotransferred over night at 22 V to Immobilon P transfer membrane (Millipore). The membranes were then blocked over night with 5% non-fat dry milk (Biorad) in PBS Tween 0.1%. The primary antibody was added and incubated for 2h00 at room temperature. After washing with 5% non-fat dry milk (Biorad) in PBS Tween 0.1%, the secondary antibody was added at 1:10,000 during 30 minutes at room temperature. The reactive proteins were detected using chemiluminescence assay followed by exposure to Biomax film (Kodak).

Immunohistochemistry and indirect immunofluorescence

Sperm cells were harvested from the caudae epididymes, washed in PBS and fixed in 4% PFA for 30 minutes on ice. Fixed spermmatozoa were allowed to air-dry on poly-L-Lysine coated slides. The slides were washed in PBS (3 x 5 minutes), 50 mM NH_4Cl (2 x 15 min), PBS (3 x 5 min), 0.1% triton X-100 in PBS (15 min) and PBS (3 x 5 min). Slides were blocked with 1% BSA and 2% normal goat serum during 60 min at room temperature. Antibodies against $Ca_V3.1$ or $Ca_V3.2$ alpha1 subunit, as described above were diluted in blocking solution at 1/100 and slides were incubated overnight at 4°C. Slides were then incubated 60 minutes with secondary alexa fluor546-conjugated antibodies (Molecular probes), diluted at 1/1000 and washed in PBS (3 x 5 minutes). For a double stain of the acrosomal status and $Ca_V3.2$ channels, lectin from Pisum sativum conjugated to fluorescein isothiocyanate (PSA-FITC, Sigma Aldrich, France) was used to label the acrosomal matrix, allowing to identify the PSA-FITC negative cells as acrosome reacted cells. For the double staining experiments, sperm cells were first treated with 10 µM A23187 for 30 minutes after a 60 minutes capacitation period in a solution (M2, Sigma-A.) containing 2% BSA, to obtain acrosome reacted sperm. Staining with PSA-FITC was used to evidence acrosome reacted sperm. Fixed sperm were washed in PBS for 3 x 5 minutes and incubated with PSA-FITC (10 µg/ml in PBS) for 15 minutes at room temperature, in the dark before starting the immunostaining protocol for calcium channels detection.

Slides were analyzed on a confocal laser scanning microscope (Leica TCS-SP2, Mannheim)

Calcium imaging

Sperm cells were obtained from C57Bl/6 mice (12 weeks old, Charles Rivers, France), $Ca_V3.1$ and $Ca_V3.2$ deficient mice from caudae epididymides by manual trituration. The fraction of motile sperm was determined by visual inspection within 10 minutes, and preparations with <75% motile cells were discarded. Sperm were loaded with 5 µM of the calcium indicator Oregon green BAPTA 1-AM (Molecular probes) by a 40 minutes incubation (37°C) in a solution A containing (in mM): NaCl (109), KCl (4.8), $MgCl_2$ (1.2), $CaCl_2$ (1.7), KH_2PO_4 (1.2), $NaHCO_3$ (10), HEPES (25), sodium lactate (25), sodium pyruvate (1), D-glucose (5.6) and BSA 0.1%.

Sperm were then centrifuged (5 minutes, 1200 rpm) and re-suspended in the solution A containing 2% BSA in order to promote *in vitro* capacitation for a period of 120 minutes incubation (37°C). Sperm were then centrifuged and re-suspended in the solution A without BSA and kept at 37°C until imaging experiment. 100 µl aliquots of capacitated sperm cells (6×10^6 /ml) were transferred to an imaging chamber, in which the lower surface was a glass coverslip treated with Cell-Tak (Beckton-Dickinson, France). The chamber was then connected to the perfusion apparatus and at least 10 ml of fresh medium was washed through. All experiments were carried out at 37° C in a continuous flow of medium. Solubilized zona pellucida, prepared as precisely described (Rockwell and Storey, 2000), was introduced in the recording medium by adding 10 µL of a solution containing 20 solubilized ZP by µL (final volume

100 µL). Thus, the final solubilized ZP concentration is 2 ZP/µL. Only sperm presenting a motile flagellum were studied. Cells were imaged on an inverted Nikon TE200U microscope, fitted with an X-cite 120 EXFO source. Images were acquired with an IXON ANDOR TE885KCS-VP camera and the frame acquisition frequencies are 4 Hz for KCl and 1 Hz for ZP.

Data were processed offline using ANDOR IQ 1.5 software. Fluorescence was measured in the whole head of the sperm.

Acrosome reaction assay

Sperm cells from the caudae epididymes were allowed to swim in M2 medium for 10 minutes. Then sperm cells were capacitated for 45 minutes at 37°C in M16 medium containing 20 mg/ml BSA. The AR was triggered by lacto-N-fucopentaose III-BSA (LNFP III-BSA). The compounds were injected in the capacitation medium and sperm were incubated for an additional period of 80 minutes. Sperm cells were then fixed in 4% PFA and stained with coomassie blue, as previously described (Arnoult et al., 1996).

M2 and M16 medium were purchased from Sigma and LNFP III-BSA from Dextra laboratories (Reading, UK). 1 mg of LNFP III-BSA was diluted in 500 µl of solution A, corresponding to a concentration of 23.6 µM. LNFP III-BSA was used at a final concentration of 5 µM.

Computer-assisted motility analysis

Five hundred µl of sperm suspension in M2 medium were mixed with the same volume of M2 medium containing 4% BSA (Fraction V Sigma) to initiate the capacitation. After incubation for 1 hour at 37°C , 10 µl of the sperm suspension was immediately placed onto analysis chamber (2X-CEL Slides , 80 µm depth, Leja Products B.V., Netherlands) kept to 37°C for microscopic quantitative study of sperm movement.

Sperm motility parameters were measured at 37°C using a sperm analyzer (Hamilton Thorn Research, Beverley , USA). The settings employed for analysis were as followed: acquisition rate: 60 Hz; number of frames: 30; minimum contrast: 30; minimum cell size: 4; low-size gate: 0.13; high-size gate: 2.43; low-intensity gate: 0.10; high-intensity gate: 1.52; minimum elongation gate: 5; maximum elongation gate: 100 ; magnification factor: 0.81.

The motility parameters measured were: straight line velocity (VSL); curvilinear velocity (VCL) ; averaged path velocity (VAP) ; amplitude of lateral head displacement (ALH) ; beat cross frequency (BCF); linearity (LIN); straightness (STR). A minimum of 100 motile spermatozoa by sample was analysed.

RESULTS

RECORDING OF INWARD CURRENT IN SPERMATOGENIC CELLS

In order to determine the molecular identity of the channels responsible for the inward calcium current in spermatogenic cells, still unresolved since their first recording in 1984 (Hagiwara and Kawa, 1984), we first performed patch-clamp experiments, in the whole cell configuration, on spermatogenic cells from $Ca_V3.x$ deficient mice. In Fig. 1A, we compared the current-density of inward current elicited by depolarization from a holding potential of -90 mV to a test potential of -20 mV of spermatogenic cells obtained from three different mouse strains: wild type-OF1 (control), $Ca_V3.1$ and $Ca_V3.2$ deficient mice. The current density was fully abolished in spermatogenic cells from $Ca_V3.2$ deficient mice. In comparison, no modification of inward current in $Ca_V3.1$ deficient pachytene spermatocytes was noticed, as already published (Stamboulian et al, 2004). The absence of $Ca_V3.2$ channels in testis was confirmed by Western Blot analysis. A specific antibody against $Ca_V3.2$ channels immunodecorates around 250 kD a band on brain and testis loaded proteins, obtained from wild type animals. In the other hand, no band is present at the same molecular weight of 250 kD on brain and testis loaded proteins, obtained from $Ca_V3.2^{-/-}$ animals (Fig. 1B). Fig. 1C shows representative traces of inward calcium current of pachytene spermatocytes from wild type and both deficient mice.

PRESENCE AND LOCALIZATION OF $CA_V3.2$ CHANNELS IN MATURE SPERM CELL

The molecular identification of channels responsible for the inward current in spermatogenic cells made us to wonder if the $Ca_V3.2$ channels are still present in the mature sperm cell.

By Western blot, two different antibodies, designed against $Ca_V3.2$ channels and named Ab-1025 and Ab-1169, immunodecorate a band at the same molecular weight (around 250 kD) on wild type head-plasma membrane enriched fraction (Fig 2A, lanes 2 and 4 respectively). To check that both antibodies immunodecorate the same band, both antibodies have been mixed: in these conditions, only one band is elicited around 250 kD (Fig 2A, lane 5). Moreover, these bands are absent on sperm microsomes from $Ca_V3.2^{-/-}$ sperm (Fig 2A, lanes 1 and 3 respectively). The $Ca_V3.2$ deficient mice guaranty the specificity against $Ca_V3.2$ channels of the immunostaining. The $Ca_V3.2$ channels are thus present in mature sperm cells.

By immuno-histochemistry, the antibody Ab-1169 evidenced a head staining, localized at the base of the acrosome, whereas no staining was observed in the sperm head of $Ca_V3.2$ deficient sperm, attesting again the specificity of this reactivity (Fig. 2B). The same antibody gave unspecific staining in the flagellum, making impossible to conclude whether the $Ca_V3.2$ channel was present in the tail (data not shown).

It is well known that membrane reorganization occurs during capacitation. We followed the $Ca_V3.2$ staining during capacitation and we observed that it was not modified during this step (Fig 3A).

From studies in neurons, we know that calcium channels involved in neurotransmission are localized in close vicinity to the secretion vesicles (Kim and Catterall, 1997). In order to further study the specific localization of $Ca_V3.2$ channels, we investigated if $Ca_V3.2$ channels are positioned in the pool of the plasma membrane merging with the outer acrosomal membrane during acrosome reaction. After capacitation, sperm cells were incubated 30 minutes in the presence of the Ca^{2+} ionophore A23187, and a double staining was performed using PSA-FITC to assess the status of the acrosome and Ab-1169 to localize the $Ca_V3.2$ channels. Acrosome reacted sperm were evidenced by the loss of the specific acrosomal matrix staining (negative PSA staining). Fig. 3B shows clearly that the $Ca_V3.2$ staining was strongly diminished or lost during acrosome reaction since $Ca_V3.2$ immunostaining was very strongly reduced in acrosome reacted spermatozoa. From the western blot of figure 2A and the immuno-histochemistry of figure 3 results, we can conclude that at least, part of the $Ca_V3.2$ channels are localized in the plasma membrane that merged with the acrosome membrane during acrosome reaction.

CALCIUM SIGNALING IN $Ca_V3.1$ AND $Ca_V3.2$ DEFICIENT MICE.

The localization of $Ca_V3.2$ channels at the base of the sperm acrosome, is of potential interest since it has been demonstrated that calcium signaling induced by agonists as progesterone or ZP3 starts precisely in the same subcellular area (Shirakawa and Miyazaki, 1999; Fukami et al, 2003; Kirkman-Brown et al, 2000). We therefore studied the voltage-dependent calcium signaling in sperm from $Ca_V3.2$ deficient mice, to further analyze the impact of the loss of $Ca_V3.2$ channels. We also studied for the first time the impact of the loss of $Ca_V3.1$ channels on voltage-dependent calcium signaling, using sperm from $Ca_V3.1$ mice. Control capacitated sperm cells, loaded with the calcium dye Oregon green, presented a large calcium increase in the head sperm, when the plasma membrane was suddenly depolarized with a high concentration of external potassium (Fig. 4). For $Ca_V3.1$ deficient sperm cells, the amplitude and kinetics of high-K^+-induced calcium signaling were similar to those recorded for control sperm cells. By contrast, for $Ca_V3.2$ deficient sperm cells, both amplitude and kinetics of high-K^+-induced calcium signaling were modified: the mean amplitude was decreased by 30 % and the calcium signal was more transient (Fig. 4). By comparing the integrated calcium signal from control and $Ca_V3.2$ deficient sperm cells, a decrease of 68 % was observed in the latter.

Because K^+-induced calcium increase was modified in $Ca_V3.2$ deficient sperm cells, we wondered if the calcium signaling induced by solubilized ZP, the physiological agonist, would be also altered. Indeed, voltage-dependent calcium channel antagonists were described to inhibit both the amplitude of the short calcium transient (Arnoult et al, 1999) and the rate of calcium increase which follows the short calcium transient (Arnoult et al, 1996). Solubilized ZP elicited a fast calcium increase, followed by a calcium plateau, returning slowly to zero, in both wild type and $Ca_V3.2$ deficient sperm cells (Fig. 5A). In order to characterize calcium signaling, we calculated for each sperm cell responding to ZP, the time to peak, the

normalized maximum amplitude and the time necessary to decrease the calcium peak by two ($t_{1/2}$). In our experimental conditions, peaks were reached in 14.5 ± 6.9 sec and in 11.4 ± 3.9 sec in wild type (n=9) and in $Ca_V3.2^{-/-}$ sperms (n=7), respectively (Fig. 5B), showing no statistical difference. These values fall in the range of previously described ZP-induced kinetics. Indeed, a wide range of ZP-induced calcium increase kinetics have been reported in the past and peaks were reached from few seconds (Rockwell and Storey, 2000; Shirakawa and Miyazaki, 1999; Fukami et al, 2003) to minutes (Arnoult et al, 1996; Fukami et al, 2003). This range is likely due to experimental differences between groups, like ZP concentration, capacitation time or medium composition. No difference was also noticed for normalized peak amplitude and $t_{1/2}$ (Fig. 5B). In our experimental conditions, normalized peak amplitudes were 1.45 ± 0.1 and in 1.47 ± 0.3 and the time necessary to decrease the calcium peak by two were 66 ± 35 sec and in 62 ± 52 sec, in wild type (n=9) and in $Ca_V3.2^{-/-}$ sperms (n=7), respectively.

The facts that we still recorded voltage-dependent calcium influx in $Ca_V3.2$ deficient sperm and that $Ca_V3.1$ deficient sperm did not present calcium signaling defect, strongly suggests that the remaining voltage-dependent calcium influx observed in $Ca_V3.2$ deficient sperm is due to the opening of different type of VDCC. High-voltage-activated calcium channels or $Ca_V3.3$ channels, present in sperm cells, could be responsible of the remaining calcium influx in $Ca_V3.2$ deficient spermatozoa.

REPRODUCTION PARAMETERS OF $Ca_V3.1$ AND $Ca_V3.2$ DEFICIENT MICE

We showed that $Ca_V3.2$ channels are present in mature sperm cell and that $Ca_V3.2$ deficient sperm cells present an altered K^+-induced calcium signaling profile, with lower amplitude and a more transient calcium signals. These changes in calcium signaling induced by the loss of $Ca_V3.2$ channels may decrease the fertility of $Ca_V3.2$ deficient males. We studied two macroscopic reproduction parameters, the interval between mating and birth and the litter size. No difference in these two parameters was noticed (Fig. 6A, B) when males of C57Bl/6, $Ca_V3.1$ and $Ca_V3.2$ deficient strains were interbred with OF1 females.

The absence of a specific macroscopic reproductive phenotype defect does not exclude i) an alteration of spermatogenesis leading to a reduction of sperm number or ii) a lowering of some sperm functions at the cellular level. Although membrane potential of spermatogenic cells does not allow T-type calcium activation, the fact that inward calcium current was totally absent in $Ca_V3.2$ deficient spermatogenic cells indeed raises the question of a role of $Ca_V3.2$ channels in sperm production. We measured the sperm concentration in cauda epididymis and no difference was noticed between wild type and in $Ca_V3.2^{-/-}$ sperms (Fig. 7), both having a standard sperm concentration of around 5 millions of sperm per milliliters. Because voltage-dependent calcium channels are supposed to play a role in both sperm acrosome reaction and sperm motility, we focused our studies on these two cellular functions. Again, the development of

transgenic mice deficient in $Ca_V3.1$ or $Ca_V3.2$ channels provided a unique opportunity to test the physiological importance of such channels in sperm functions. We compared the number of acrosome reacted sperm induced by the lacto-N-fucopentaose III-BSA (LNFP III-BSA), a synthetic molecule designed to replace the signaling sugars of ZP3 (Hanna et al, 2004), after a capacitation of 120 minutes in 2% BSA (Fig. 8A). In these peculiar conditions, we did not observe a lower rate of acrosome reacted sperm in $Ca_V3.2$ deficient mice, when compared to control mice, suggesting that the remaining voltage-dependent calcium influx in $Ca_V3.2$ deficient mice is sufficient to promote a complete acrosome reaction. From experiments using pharmacological tools, it has been suggested that LVA calcium channels could be involved in sperm motility (Trevino et al, 2004). Using a sperm movement analyzer, the different parameters characterizing sperm movement were studied for $Ca_V3.1$ and $Ca_V3.2$ null mice or control capacitated sperm (Fig. 8B). No statistical difference was observed between the mean values of the different parameters measured for the three populations of sperm.

PRESENCE AND IMMUNOLOCALIZATION OF $Ca_V3.1$ CHANNELS IN SPERM

The absence of inward calcium currents in spermatogenic cells from $Ca_V3.2$ deficient mice, the specific staining of $Ca_V3.2$ channels in sperm head and the decrease of depolarization-induced calcium influx in sperm from $Ca_V3.2$ deficient animals, clearly demonstrate that $Ca_V3.2$ channels are present and functional in sperm. On the other hand, the presence of $Ca_V3.1$ channels in sperm is still controversy since i) $Ca_V3.1$ deficient spermatogenic cells present no alteration of the inward current and ii) $Ca_V3.1$ deficient sperm exhibit a similar depolarization-induced calcium to control sperm cells. The presence of $Ca_V3.1$ channels in rodent sperm is only supported by RT-PCR experiments, which clearly shows specific products amplification (Trevino et al, 2004). In order to better understand this apparent discrepancy between functional and molecular data, we did important efforts to attempt to evidence $Ca_V3.1$ channels by immunohistochemistry and Western blotting. For immunohistochemistry experiments, we tested a commercial antibody (Alomone, anti $Ca_V3.1$). In our experimental conditions, this antibody did not evidence a specific staining by immunochemistry in sperm (Fig. 9A). The flagellum is stained by the antibody but the $Ca_V3.1$ deficient sperm present an identical staining. In Western blotting, the same antibody specifically recognized a band of approximately at 240 kD in HEK-293 cells transfected with $Ca_V3.1$ clone. This band corresponds to $Ca_V3.1$ channels since in non transfected HEK-293 cells, no band is evidenced in the same area of molecular weight. In the whole sperm plasma membrane preparations, no signal was detected by the Alomone antibody (Fig. 9B), even if, to compensate an eventual low level of $Ca_V3.1$ channels expression in sperm, 1.7 times more of sperm protein (25 µg) than control HEK cells extract (14 µg) was loaded in the gel.

DISCUSSION

IN SPERMATOGENIC CELLS, INWARD CURRENT IS ONLY DUE TO $Ca_V3.2$ CHANNELS

The molecular identity of voltage-dependent inward calcium current in spermatogenic cells has been a debated question. If the pharmacological and biophysical properties of this current are close to those expected for $Ca_V3.2$ channels, some doubts were still present because the spermatogenic calcium current present some specific biophysical (Stamboulian et al, 2004) and pharmacological (Arnoult et al, 1998; Wennemuth et al, 2000) properties in comparison to those obtained by re-expression of cloned channels. The fact that no inward current is present in $Ca_V3.2$ deficient spermatogenic cells, represent a conclusive proof that spermatogenic inward calcium current is only due to the opening of $Ca_V3.2$ channels. It would be interesting to clone spermatogenic $Ca_V3.2$ channels in order to further understand the biophysical and pharmacological specificities carried by this channel at the molecular level.

$Ca_V3.2$ CHANNELS ARE PRESENT IN MATURE SPERM HEAD AND PARTICIPATE TO THE VOLTAGE-DEPENDENT CALCIUM INFLUX DURING ACROSOME REACTION

In this manuscript, we showed that $Ca_V3.2$ channels are present from pachytene stages, by patch-clamp experiments, to mature sperm cells, by Western blot and immuno-histochemistry experiments. The $Ca_V3.2$ deficient mice channels provide the unique opportunity to guaranty the specificity of immuno-localization. Its localization, at the base of the acrosome is unique, and different to the localizations already described for other voltage-gated channels (Westenbroek and Babcock, 1999; Trevino et al, 2004) or store-operated channels (Jungnickel et al, 2001; Sutton et al, 2004; Castellano et al, 2003; Trevino et al, 2001). The localization of $Ca_V3.2$ channels is particularly interesting since its localization seems to coincide with the locus of calcium signaling initiation during progesterone or ZP3-activated acrosome reaction (Fukami et al, 2003; Shirakawa and Miyazaki, 1999; Kirkman-Brown et al, 2000). This result indicates that it may be one of the first calcium channel activated during the depolarization event that follows ZP3 receptor activation. Moreover, we demonstrated herein that the plasma membrane, which merges with the acrosomal membrane during acrosome reaction, contains $Ca_V3.2$ channels. This result provides essential information. First, although $Ca_V3.2$ channels are expressed early during spermatogenesis, $Ca_V3.2$ channels play a physiological role in a narrow temporal window between the end of the capacitation, when it becomes functional, and the end of the acrosome reaction, when it is lost in membrane vesicle secretion. Indeed, as a low-voltage-activated calcium channel, $Ca_V3.2$ is fully inactivated at potential above -60 mV. Spermatogenic cells and non-capacitated sperm cells have a membrane potential around -30 mV and only fully capacitated sperm present a hyperpolarized membrane potential (Arnoult et al, 1999). Among all voltage-dependent calcium channels present in sperm (Westenbroek and Babcock, 1999), only $Ca_V3.2$ channels present an early expression pattern during

spermatogenesis and the reasons of this specific expression pattern are still not understood. Secondly, the fact that immunostaining of $Ca_V3.2$ disappears in acrosome reacted sperm demonstrates that the surface of the plasma membrane merging with the outer acrosomal membrane is not restricted to the acrosomal crescent when AR is induced by A23187 but extents to the equatorial segment. The fact that $Ca_V3.2$ channels are present at a strategic localization for calcium signaling in sperm head made us wonder if calcium signaling is affected during acrosome reaction. In $Ca_V3.2$ deficient sperm, the peak calcium increase, elicited by KCl-induced depolarization, is diminished by 30% and the calcium signal is more transient. The integrated calcium signal is reduced overall by 68%. These results indicate that sperm $Ca_V3.2$ channels are functional and contribute significantly but partially to KCl-induced calcium signaling. This result differs from those obtained previously (Wennemuth et al, 2000), where KCl-induced calcium signaling has been attributed mainly to HVA calcium channels. This difference evidences the recurrent problem of the specificity of calcium channels blockers. The localization of $Ca_V3.2$ channels in sperm head and their functionality strongly suggest that they contribute significantly to the calcium signaling during acrosome reaction.

FUNCTIONAL $Ca_V3.1$ CHANNEL PROTEIN IS LIKELY ABSENT IN RODENT SPERM

The presence of $Ca_V3.1$ channels in mature sperm cell was previously suggested, based on RT-PCR experiments (Trevino et al, 2004), *in situ* hybridization (Jagannathan et al, 2002) and immuno-histochemistry experiments. From inhibition experiments, it was suggested that $Ca_V3.1$ channels may play a role in flagellum beat (Trevino et al, 2004). The $Ca_V3.1$ deficient mice gave us the opportunity to search for the $Ca_V3.1$ channels implications in two important sperm functions: acrosome reaction and sperm motility. Acrosome reaction is activated by an increase of intracellular calcium concentration. The initial rise is due to voltage-dependent calcium channels opening. If $Ca_V3.1$ channels were present and activated during this initial phase, calcium signaling elicited by KCl-induced depolarization should be affected in $Ca_V3.1$ deficient sperm in comparison to control sperm, in a similar way to $Ca_V3.2$ deficient sperm. No difference was observed in $Ca_V3.1$ deficient sperm in comparison to control sperm. This result clearly shows that $Ca_V3.1$ does not play a role in calcium signaling during acrosome reaction. In order to test a putative role of $Ca_V3.1$ in sperm motility, we compared with a sperm movement analyzer the different characteristics of sperm motility after capacitation of $Ca_V3.1$ sperm versus control sperm. No obvious difference was observed.

The lack of cellular phenotype of $Ca_V3.1$ deficient mice is in good accordance with biochemical or immunohistology results obtained. Indeed, we were unable to obtain a specific staining of $Ca_V3.1$ channels using the same antibody previously used (Trevino et al, 2004). We did obtain a signal in flagellum as well, but $Ca_V3.1$ deficient sperm presented identical staining. From our experiences, a lot of antibodies, known to give specific staining in several tissues, stain also sperm flagellum in a non specific

manner. In Western blot, the absence of signal in sperm extract does not necessary mean that $Ca_V3.1$ channels are absent, but this result is in good accordance with the absence of phenotype of $Ca_V3.1$ deficient mice.

Although the presence of $Ca_V3.1$ channels in flagellum sperm remains to be established, this result demonstrates that the contribution of $Ca_V3.1$ channels to sperm flagellum beat would be very minor.

$Ca_V3.2$ CHANNELS ARE NOT THE ONLY VDCC ACTIVATED DURING ACROSOME REACTION.

The facts that i) the K^+-induced calcium signaling was not abolished and ii) the second phase of ZP-induced calcium signaling was not altered in $Ca_V3.2$ deficient sperm, suggest that other voltage-dependent calcium channels are present and functional in sperm head, as well. Because calcium signaling is not affected in $Ca_V3.1$ deficient sperm, it is unlikely that the remaining voltage-dependent calcium increase in $Ca_V3.2$ deficient sperm is due to $Ca_V3.1$ channels opening.

Different publications pointed out the presence of several other VDCC in spermatogenic or mature sperm cells. High-voltage-activated (HVA) calcium channels, but also $Ca_V3.3$, the last member of the LVA calcium channel family, have been described to be present.

$Ca_V3.3$ RNA have been amplified in spermatogenic cells. In mature sperm cells, two groups have showed by immunohistochemistry realized with two different antibodies that the channels is restricted to the flagellum and is absent of the head (Trevino et al, 2004; Zhang et al, 2006) and then, $Ca_V3.3$ unlikely contributes to the remaining calcium influx in sperm head from $Ca_V3.2$ deficient mice.

HVA channels, $Ca_V1.2$, $Ca_V2.2$ and $Ca_V2.3$, are present in the sperm head (Goodwin et al, 1999; Westenbroek and Babcock, 1999; Wennemuth et al, 2000). These HVA channels are likely activated during the physiological depolarization induced by ZP3 and contribute to the transient calcium influx sensitive to different classes of calcium blockers (Arnoult et al, 1999).

EXPRESSION OF VDCC IN SPERM IS REDUNDANT.

Most of the studies pointing out the central role of LVA channel in acrosome reaction were based on pharmacological studies. The different calcium channel blockers used were potent inhibitors of the spermatogenic $Ca_V3.2$ channels (Arnoult et al, 1998) and were also potent inhibitors of acrosome reaction (Arnoult et al, 1996; Arnoult et al, 1999). From these results, we have contributed with others groups (Santi et al, 1996; Florman et al, 1998; Trevino et al, 2004) to popularize the fact that LVA channels were key channels in sperm physiology. However the specificity of voltage-dependent calcium channel blockers among the different types of VDCC is only relative, the specificity representing generally a 10-20 fold difference in affinity. An LVA calcium channel blocker which is used at a concentration that blocks most of the LVA channels, also blocks a small proportion of HVA channels. Then, during an inhibition experiment, when 100% of the LVA calcium channels are blocked, a small proportion of HVA

channels (around 20 %) are blocked, as well. Because $Ca_V3.2$ is responsible of 62 % of the integrated calcium entry, in the presence of a $Ca_V3.2$ channel blocker, an extra inhibition of 20% of the remaining calcium influx, via the inhibition of HVA channels, could diminish the voltage-dependent calcium influx in such way that acrosome reaction would be then blocked. Although the channel antagonists are important tools in the characterization of ionic channels involved in a specific physiological function, the response obtained is always dependent of the specificity these tools. In the other hand, the development of transgenic mice deficient in $Ca_V3.1$ or $Ca_V3.2$ channels provided a unique opportunity to evaluate the physiological importance of these channels in sperm physiology in general and in the acrosome reaction or sperm motility in particular, independently of pharmacological agents.

The reproduction parameters of $Ca_V3.2$ deficient mice studied at different levels are normal. We did not observe alteration in the number of pups by litter, the delay between mating and delivery, the rate of acrosome reaction induced by an agonist of ZP3 receptor, sperm motility and the ZP-induced calcium signaling. We can then conclude that there is no macroscopic fertility or cellular functions troubles in $Ca_V3.2$ deficient mice, although we demonstrated that $Ca_V3.2$ channels are present and functional in wild type sperm cells. These data suggest that $Ca_V3.2$ channels are redundant calcium channels. Similar results were obtained with $Ca_V2.2$ (Kim et al, 2001a) or $Ca_V2.3$ (Sakata et al, 2001) deficient mice, which do not present any fertility trouble, as well. The likely conclusion is that voltage-dependent calcium influx during acrosome reaction is due to the opening of redundant families of calcium channels. Finally, the redundancy of VDCC seems to be a general feature. Indeed, there is a large discrepancy between the high number of cellular types where VDCC are expressed and the low number of physiological anomalies generated by the lack of only one VDCC in the different knock-out mice (Kim et al, 2001a; Saegusa et al, 2000; Jun et al, 1999; Kim et al, 2001b; Chen et al, 2003).

Acknowledgments.

We are thankful to Florence Appaix (INSERM U836-Grenoble-F) and Emmanuel Bourinet (IGH/CNRS-Montpellier-F) for Westernblotting expertise and to Isabelle Marty for her skills in antibody design (INSRM U836-Grenoble-F).

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FIGURE LEGENDS

Figure 1. Inward calcium current in spermatogenic cells measured in the whole cell configuration of the patch-clamp technique.

A. Current densities measured at the peak current, corresponding to a depolarization of a holding potential of -90 mV to a test potential of -20 mV in spermatogenic cells from wild type, $Ca_V3.1$ and $Ca_V3.2$ deficient mice. Results are expressed as mean ± s.d. Above histrograms, n indicates the number of cells recorded.

B. Western blots of mouse testis membrane proteins obtained with anti-$Ca_V3.2$ Ab. Membrane proteins were loaded on polyacrylamide gel and transferred to nitrocellulose. The apparent molecular size markers are indicated on the left. Antibody is from Santa-Cruz, USA.

C. Representative superimposed traces of inward current recorded in spermatogenic cells from wild type, $Ca_V3.1$ or $Ca_V3.2$ deficient mice. Holding potential -90 mV, test potentials from -60 mV to +30 mV, 10 mV increments.

Figure 2. **Immunodetection and localization of $Ca_V3.2$ channels in mature sperm cells.**

A. Western blot showing the presence of $Ca_V3.2$ channels in mature sperm cells. The microsomes recovered by centrifugation after acrosome reaction of sperm from $Ca_V3.2$ deficient animals (lanes 1 and 3) or wild-type OF1 animals (lanes 2, 4 and 5) were loaded on polyacrylamide gel and transferred to nitrocellulose. Lanes 1 and 2, antibody Ab-1169; lanes 3 and 4, antibody Ab-1025, lane 5 both antibodies.

B. Specific antibody against $Ca_V3.2$ channel evidences a specific localization of $Ca_V3.2$ channels, at the base of the acrosome (red staining in control sperm). The staining is absent in sperm from $Ca_V3.2$ deficient mice. The fluorescence and phase contrast pictures were superimposed to precisely localize the staining in relation to structures. The staining obtained in flagellum is unspecific since identical staining was observed in flagellum of wild type or $Ca_V3.2^{-/-}$ sperm.

Figure 3. $Ca_V3.2$ channels localization after capacitation and acrosome reaction.

Double-staining with anti-$Ca_V3.2$ antibody and with lectins from Pisum sativum conjugated to FITC (green), localized $Ca_V3.2$ channels and the acrosome matrix, respectively.

A. Capacitation does not modify $Ca_V3.2$ staining (images of the right column): capacitated sperm cells displayed a staining at the base of the acrosome, identical to that observed on non capacitated sperm. B. Acrosome reacted sperm loose the $Ca_V3.2$ channel staining (images of the two left columns). AR is evidenced by the absence of PSA staining

Figure 4. Comparison of high-K^+-induced calcium signaling in sperm from wild type, $Ca_V3.1$ or $Ca_V3.2$ deficient animals.

KCl-induced depolarization elicits a large calcium signaling in control sperm (●, n=7) and a similar calcium signaling in sperm from $Ca_V3.1$ deficient animals (○, n=10). On the other hand, calcium signaling is reduced in sperm from $Ca_V3.2$ deficient animals (□, n=13). Results are expressed as normalized mean intensities ± s.d.

Figure 5. Comparison of calcium signaling induced by solubilized ZP in sperm from wild type or $Ca_V3.2$ deficient animals.

A. Solubilized ZP (2 ZP/μL) elicits a fast calcium increase, followed by a calcium plateau in both control sperm (●) and sperm from $Ca_V3.2$ deficient animals (○). Acquisition rate, 1 Hz.

B. Characteristics of calcium signaling induced by solubilized ZP. Histograms showing the time to peak, the normalized maximum amplitude, and the time necessary to decrease the calcium peak by two ($t_{1/2}$) for wild type (black bars, n=9) and for $Ca_V3.2^{-/-}$ sperms (white bars, n=7). In our experimental conditions, peaks were reached in 14.5 ± 6.9 sec and in 11.4 ± 3.9 sec, normalized peak amplitudes were 1.45 ± 0.1 and in 1.47 ± 0.3 and the time necessary to decrease the calcium peak by two were 66 ± 35 sec and in 62 ± 52 sec in wild type and in $Ca_V3.2^{-/-}$ sperms, respectively. Results are expressed as mean ± s.d.

Figure 6. Macroscopic reproduction parameters of $Ca_V3.1$ and $Ca_V3.2$ deficient animals.

A. Interval between mating and pups delivery in function of the different strains of mice.

B. Average litter size in function of the different strains of mice. Results are expressed as mean ± s.d. Above histrograms, n indicates the number of litter studied.

Figure 7. **Testis weight and sperm count of $Ca_V3.2$ deficient animals.**

A t-test indicates that neither testis weight nor sperm count are different between wild type animals (n=6) and $Ca_V3.2^{-/-}$ animals (n=6).

Figure 8. Cellular reproduction parameters of $Ca_V3.1$ and $Ca_V3.2$ deficient animals.

A. Acrosome reaction induced by 5 μM LNFP III-BSA of $Ca_V3.2$ deficient mice and wild type mice.

The two left columns correspond to sperm incubated during 125 minutes in the capacitation medium, the two right columns correspond to sperm incubated first during 45 minutes in the capacitation medium, and subsequently in the presence of 5 μM of LNFP III-BSA for an extra period of 80 minutes in the

capacitation medium. Mean ± s.d. of 3 independent experiments. In each experiment, more than 150 sperm cells have been analyzed.

B. Sperm motility parameters of $Ca_V3.1$ and $Ca_V3.2$ deficient animals in comparison to wild type sperm. The motility parameters measured were: averaged path velocity (VAP); straight line velocity (VSL); curvilinear velocity (VCL); amplitude of lateral head displacement (ALH); beat cross frequency (BCF); straightness (STR); linearity (LIN). Black columns: control sperm cells, light grey columns: sperm cells from $Ca_V3.1$ null mice and dark grey columns: sperm cells from $Ca_V3.2$ null mice. Results are expressed as mean ± s.d.

Figure 9. No evidence for the presence of $Ca_V3.1$ channel in mouse sperm.

A. Immunostaining with anti-$Ca_V3.1$ antibody from Alomone on two types of mature sperm: control sperm (OF1) and $Ca_V3.1$ deficient sperm.

B. Western Blot with anti-$Ca_V3.1$ Alomone antibody (upper panel). The specificity of the antibody was checked on HEK-293 cells transiently transfected with plasmids containing $Ca_V3.1$ versus non transfected cell. A polyacrilamide gel, loaded with the identical protein amounts as used for the Western Blot, was stained with coomassie blue, to indicate the protein loading (lower panel).

FIGURE 1

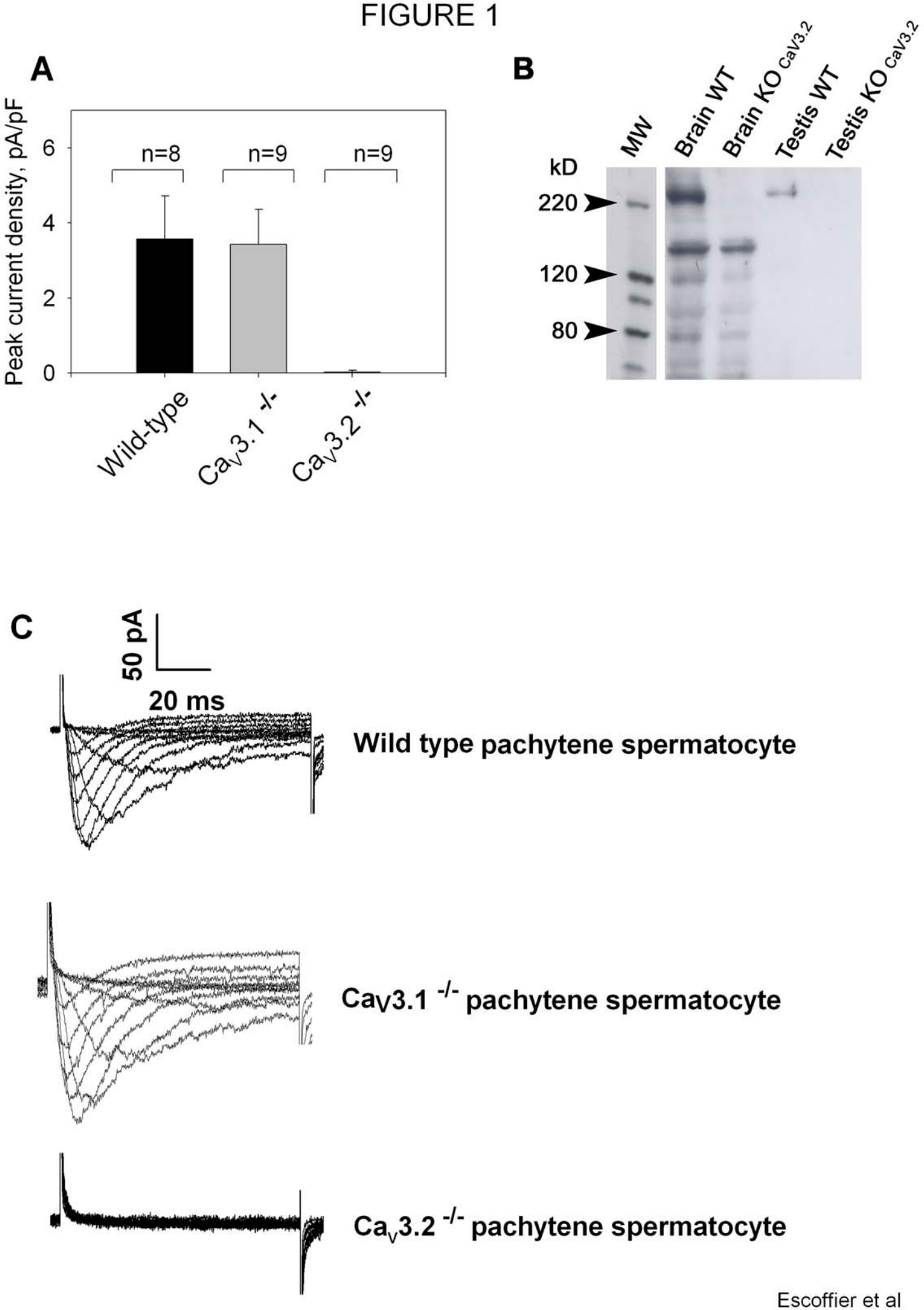

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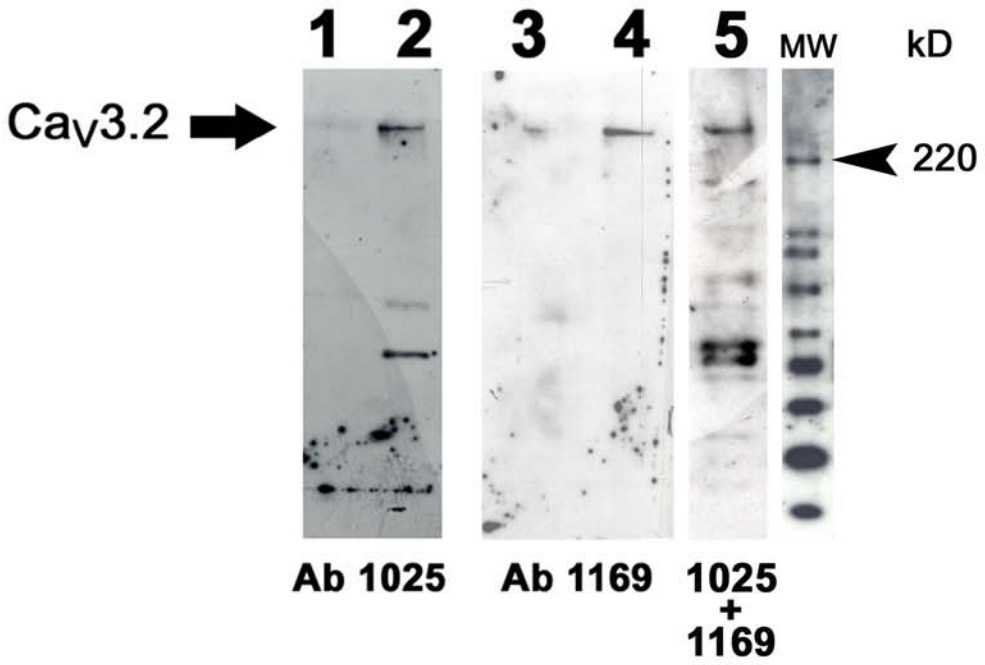
Figure 2

A



B

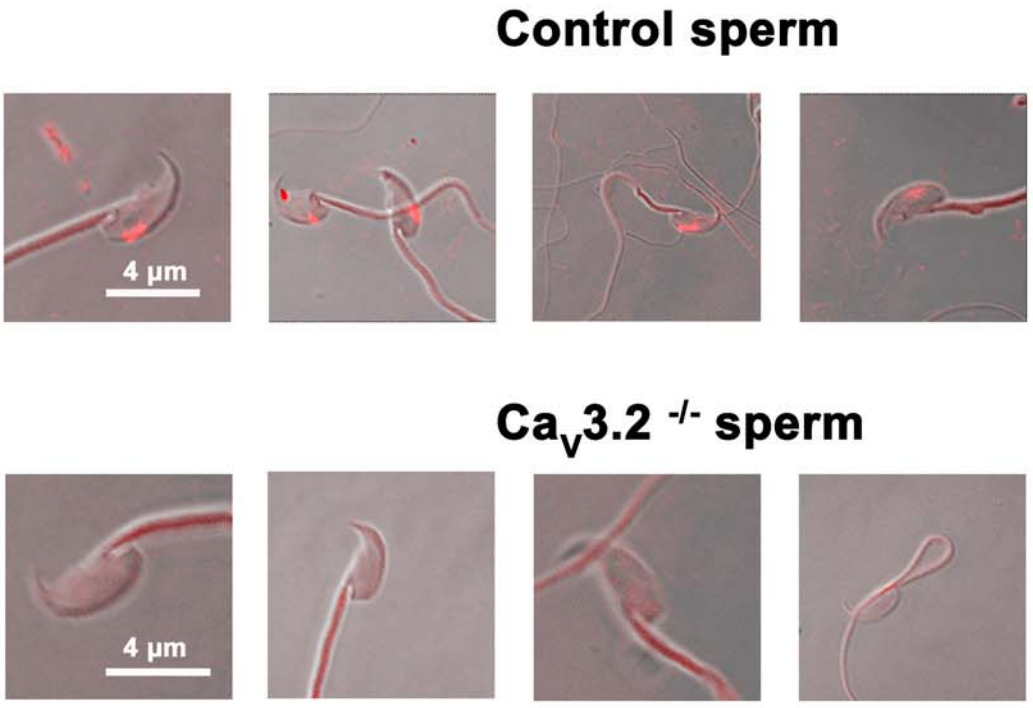


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FIGURE 3

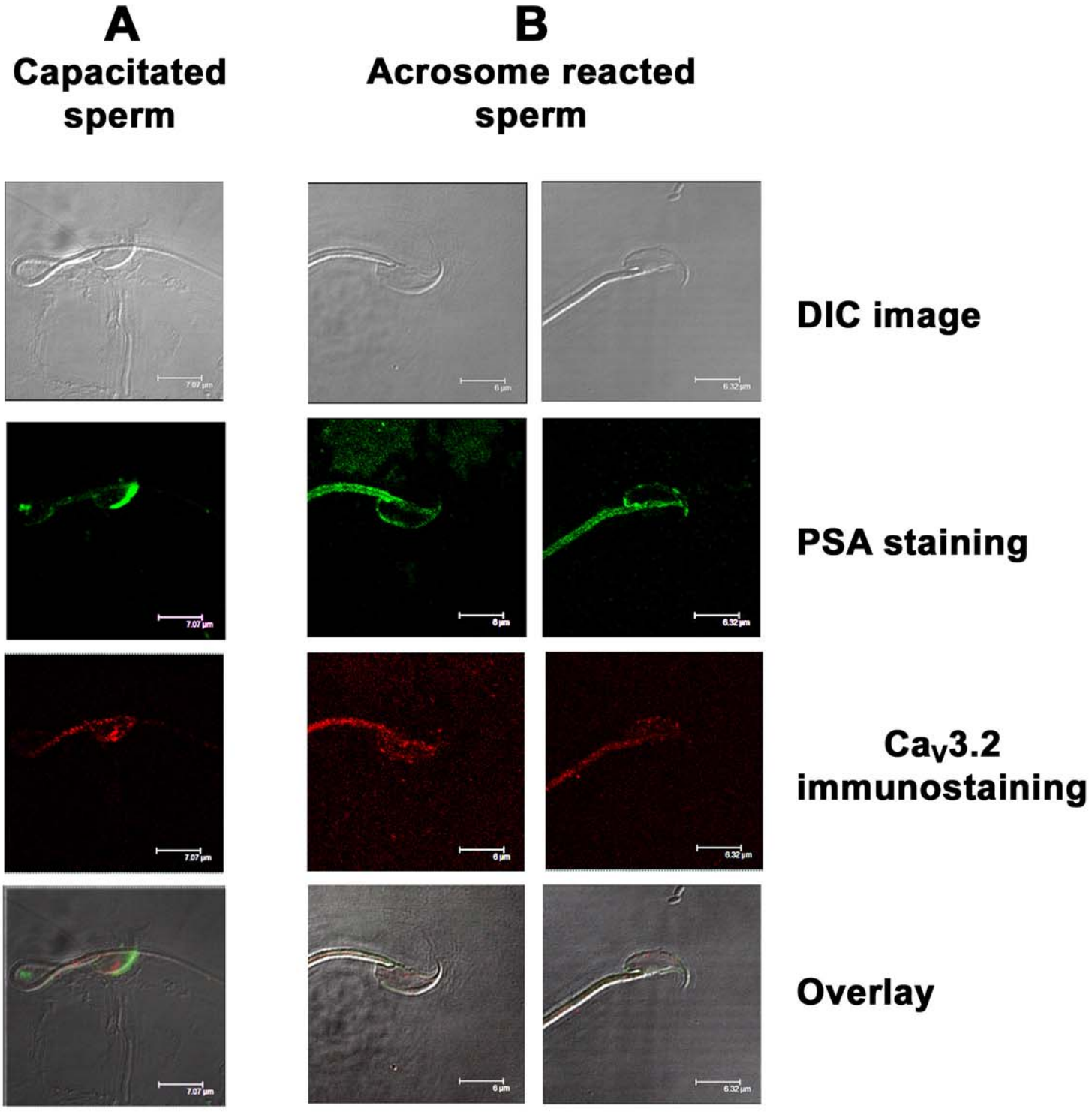

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FIGURE 4

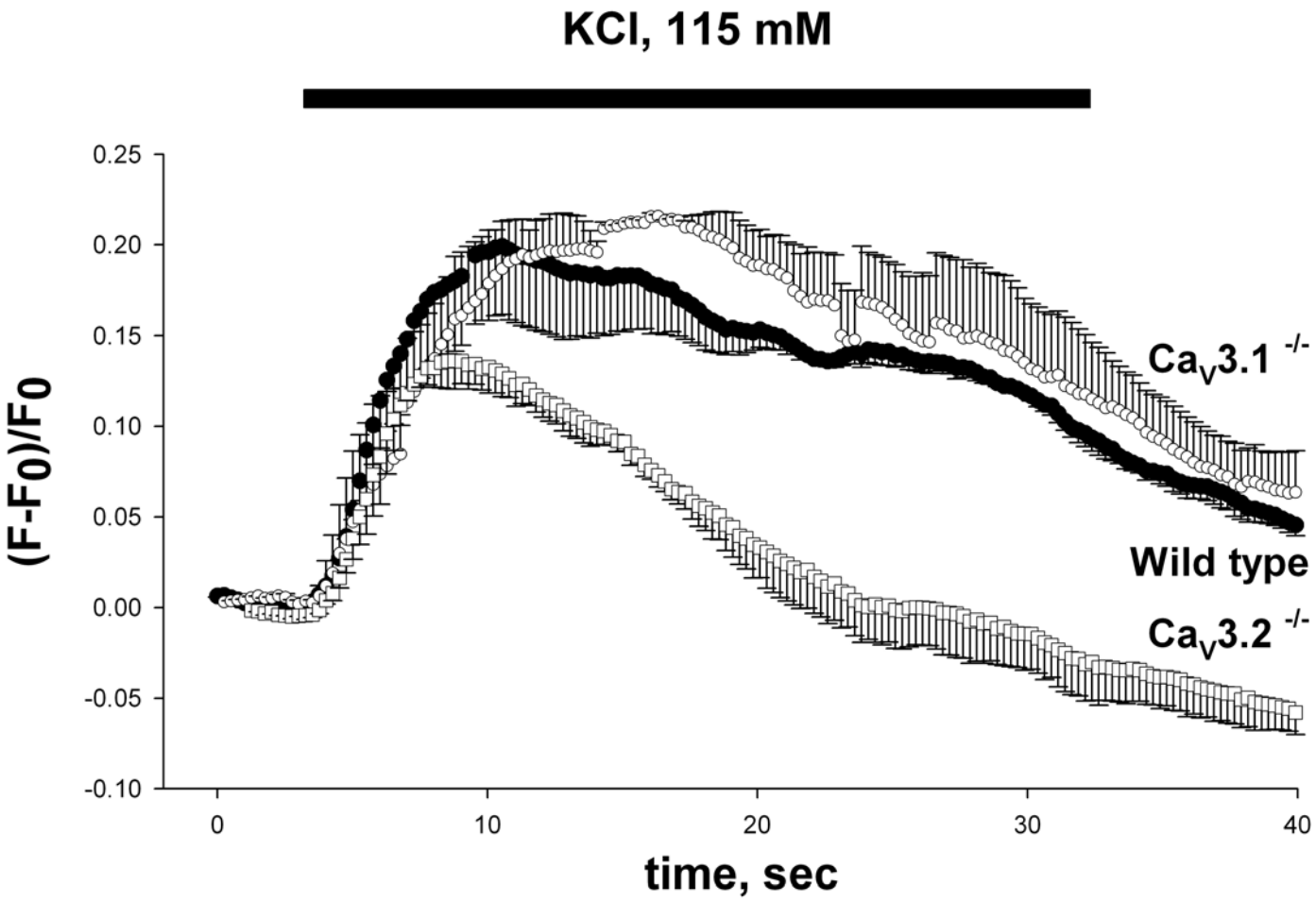

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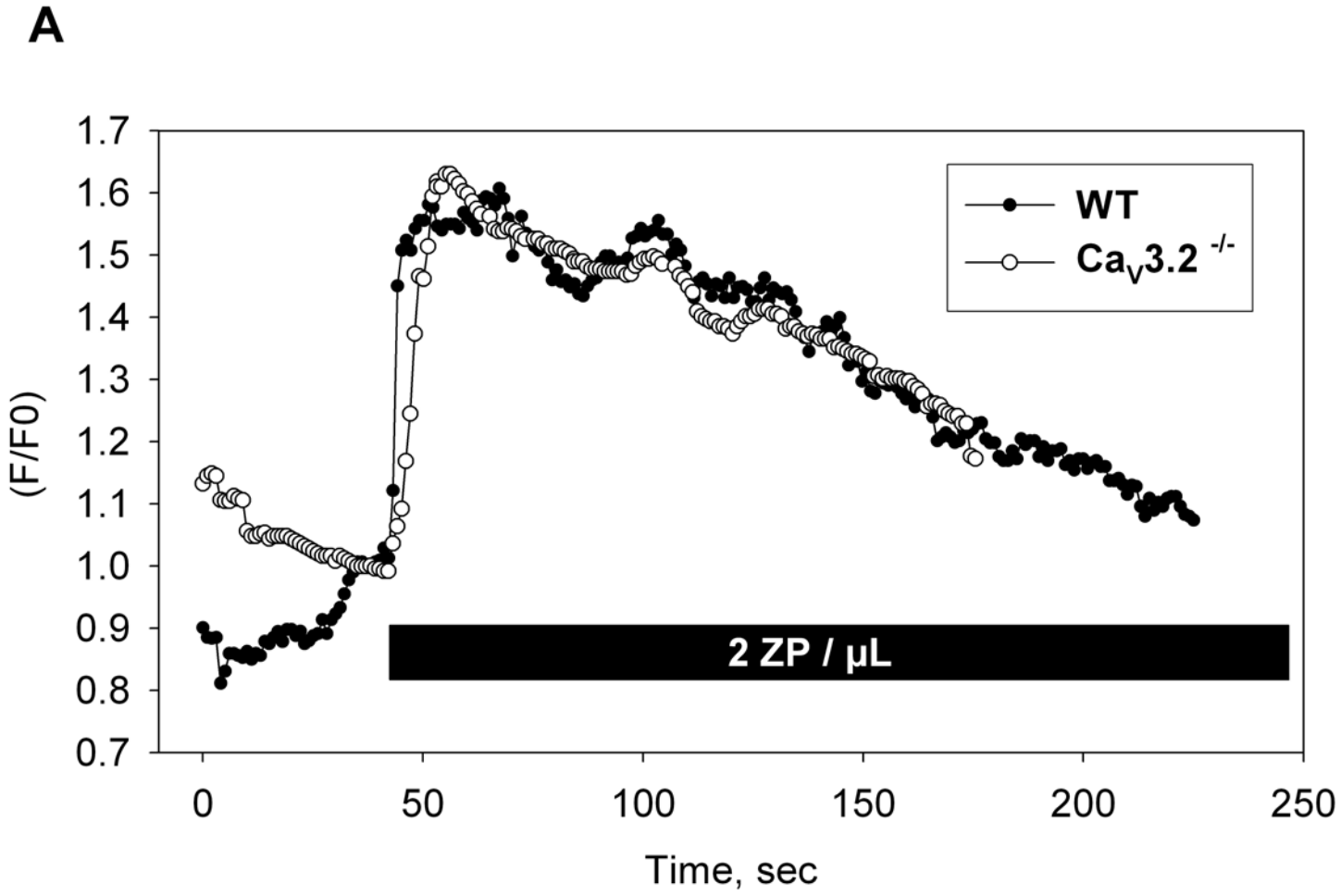
FIGURE 5



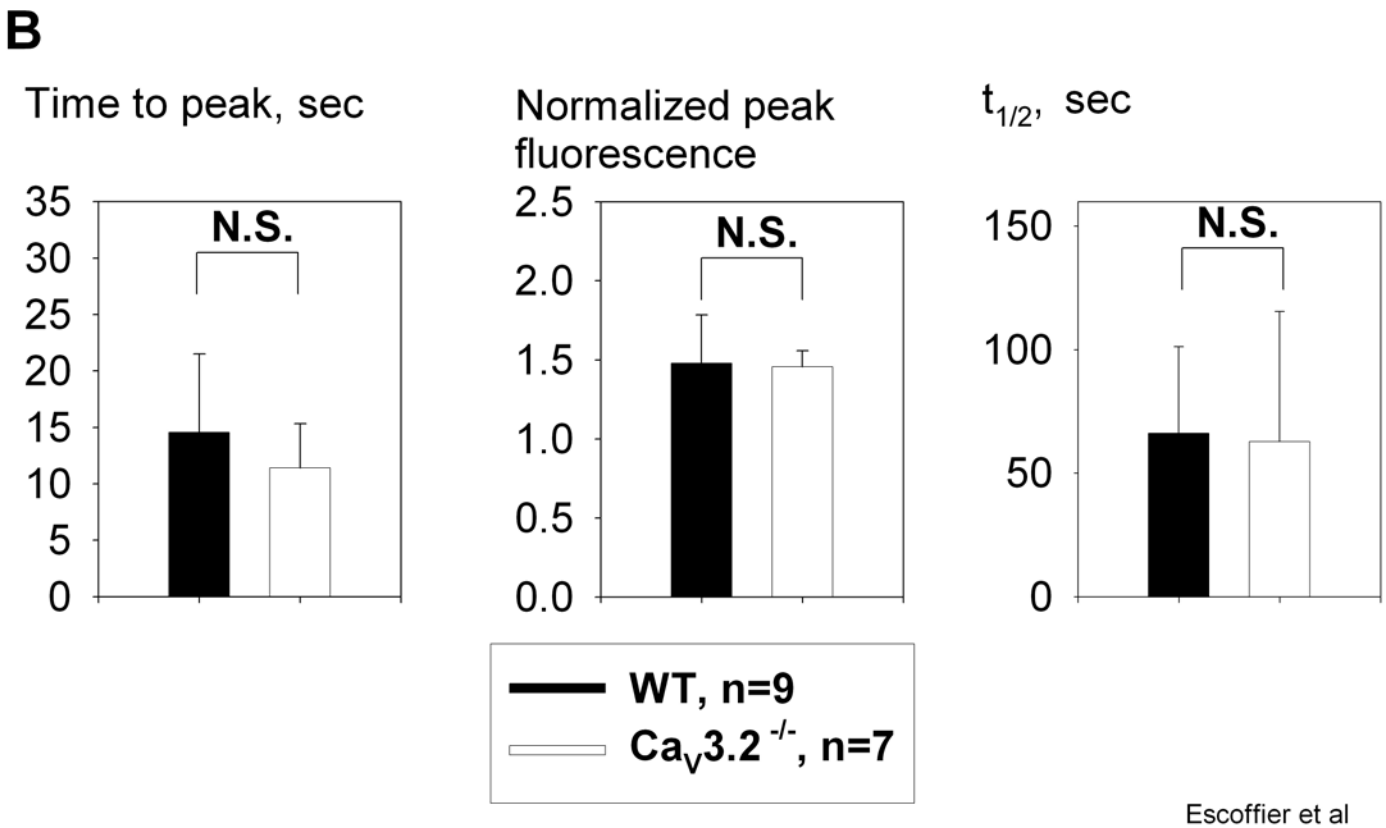


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FIGURE 6

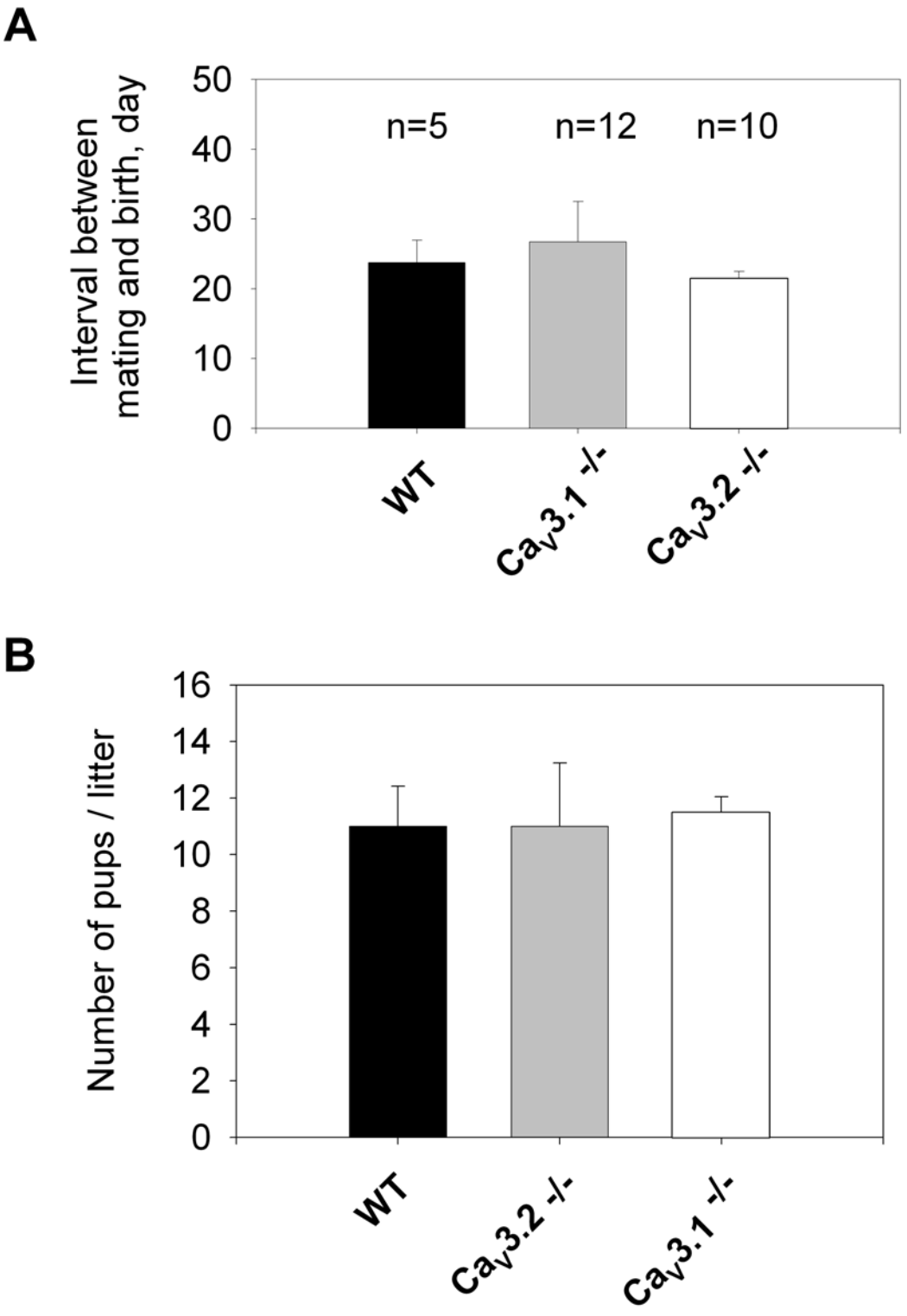
A
Interval between mating and birth, day
50
40
30
20
10
0
n=5
n=12
n=10
WT
Cav3.1 -/-
Cav3.2 -/-
B
Number of pups / litter
16
14
12
10
8
6
4
2
0
WT
Cav3.2 -/-
Cav3.1 -/-

FIGURE 7

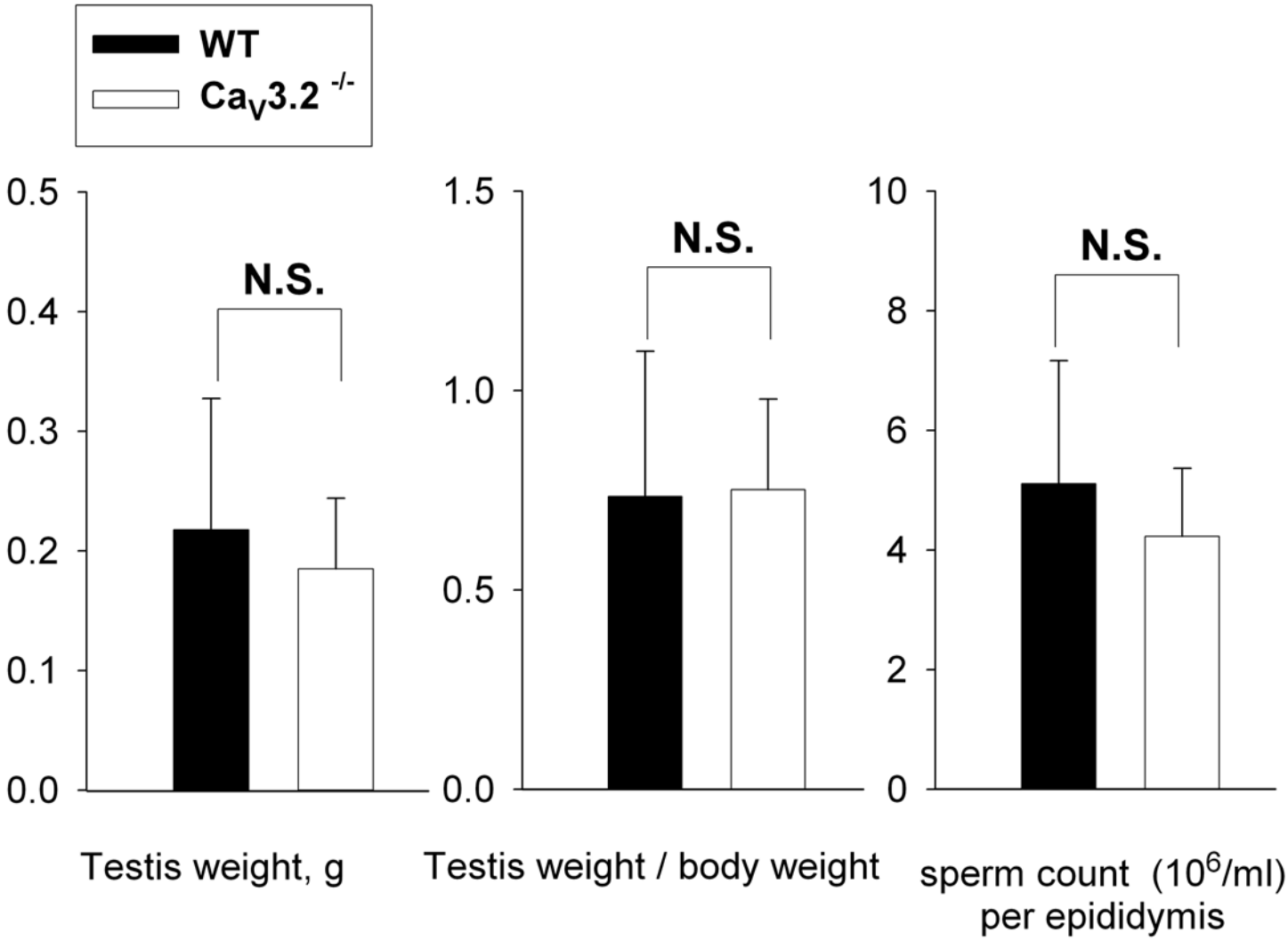
WT
Ca$_V$3.2 $^{-/-}$
0.5
0.4
0.3
0.2
0.1
0.0
N.S.
Testis weight, g
1.5
1.0
0.5
0.0
N.S.
Testis weight / body weight
10
8
6
4
2
0
N.S.
sperm count (10^6/ml)
per epididymis

FIGURE 8

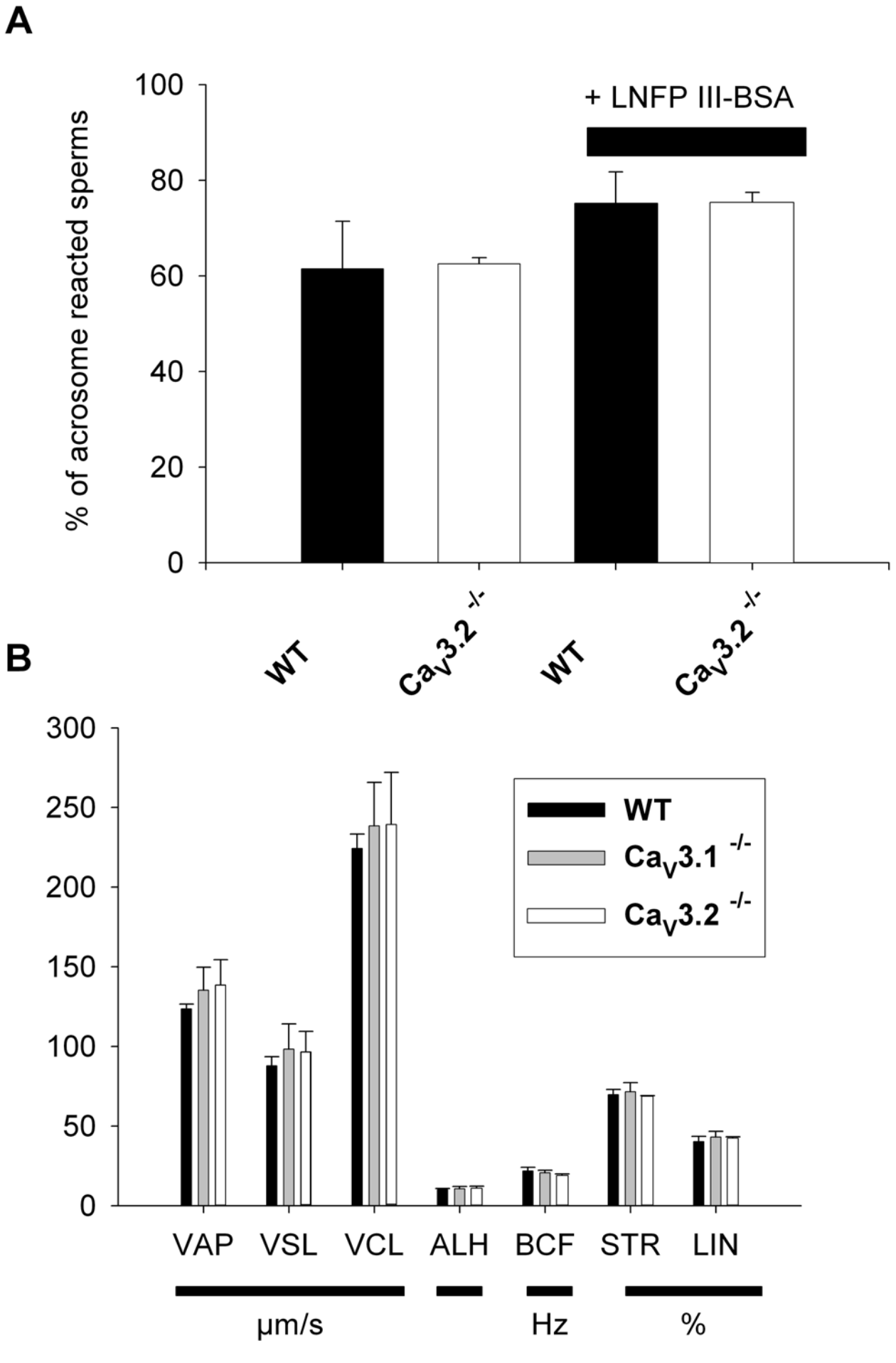

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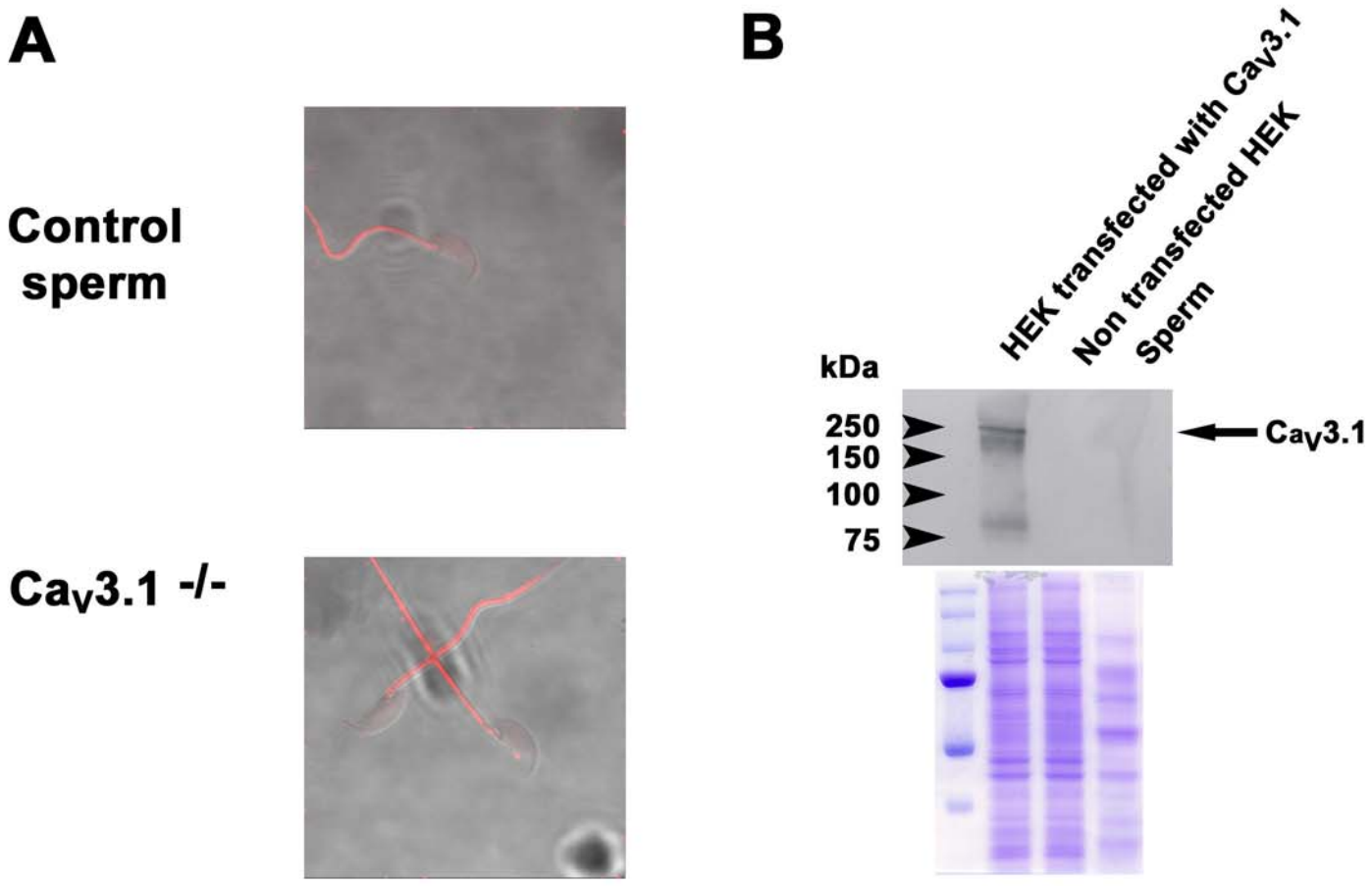
FIGURE 9



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