REPRODUCTION
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Transcriptional status of mouse oocytes corresponds with their ability to generate Ca^{2+} release

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Abstract

In fully grown ovarian follicles both transcriptionally active (NSN) and inactive (SN) oocytes are present. NSN oocytes have been shown to display lower developmental potential. It is possible that oocytes that have not completed transcription before meiosis resumption accumulate less RNA and proteins required for their further development, including those responsible for regulation of Ca^{2+} homeostasis. Oscillations of the cytoplasmic concentration of free Ca^{2+} ions ($[Ca^{2+}]_i$) are triggered in oocytes by a fertilizing spermatozoon and are crucial for inducing and regulating further embryonic development. We showed that NSN-derived oocytes express less inositol 1,4,5-triphosphate receptor type 1 (IP3R1), store less Ca^{2+} ions and generate weaker spontaneous $[Ca^{2+}]_i$ oscillations during maturation than SN oocytes. Consequently, NSN oocytes display aberrant $[Ca^{2+}]_i$ oscillations at fertilization. We speculate that this defective regulation of Ca^{2+} homeostasis might be one of the factors responsible for the lower developmental potential of NSN oocytes.

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Introduction

In all animal species, including mammals, oocytes arrested in prophase of the 1st meiotic division (hereinafter called germinal vesicle (GV) oocytes) undergo an intense growth and accumulate organelles, proteins and mRNAs required for their further functioning (reviewed in Masui 1985, Gilchrist *et al.* 2008). In mice, pre-antral ovarian follicles contain mainly transcriptionally active oocytes (Mattson & Albertini 1990, Debey *et al.* 1993, Zuccotti *et al.* 1995), whereas oocytes that ceased transcription are present only in antral follicles (Wickramasinghe *et al.* 1991). However, in such fully grown follicles transcriptionally active oocytes can be observed as well (Liu & Aoki 2002).

Nuclei of oocytes that completed their growth-related transcription are characterized by a tightly condensed chromatin forming a ring around the nucleolus (so called surrounded nucleolus (SN) oocytes). Oocytes that still transcribe can be distinguished by an amorphous, decondensed chromatin; these are called non-surrounded nucleolus (NSN) oocytes (Bouniol-Baly *et al.* 1999; reviewed in Tan *et al.* 2009). Greater chromatin condensation in SN than in NSN oocytes, however, does not correlate with a decreased histone acetylation. On the contrary, H4K5 and H4K12 acetylation level is higher in SN compared to NSN oocytes. Albeit, the methylation level of histones (H3K9) and DNA (5-MeC) is also higher in SN oocytes than in NSN oocytes (Kageyama *et al.* 2007).

Transcriptionally active NSN oocytes (NSN-GV) can undergo *in vitro* meiotic maturation without terminating transcription first (Debey *et al.* 1993); however, GV oocytes with distinct chromatin configurations (SN-GV and NSN-GV) differ in their ability to undergo the first meiotic division, respond to fertilization or undergo further embryonic development. NSN-GV oocytes resume meiosis during *in vitro* culture less often than SN-GV oocytes. If they do, their meiotic maturation progresses slower and less frequently results in the first meiotic division (Wickramasinghe *et al.* 1991, Debey *et al.* 1993, Liu & Aoki 2002). Moreover, when fertilized, oocytes in metaphase of the 2nd meiotic division (MII) derived from NSN-GVs (NSN-MII oocytes) have lower developmental potential as compared to MII oocytes derived from SN-GVs (SN-MII oocytes). When cultured *in vitro*, embryos originating from NSN oocytes arrest usually at the 2-cell stage, while SN-derived embryos continue to the blastocyst stage (Zuccotti *et al.* 2002, Inoue *et al.* 2008). We hypothesize that hindered Ca^{2+} homeostasis caused by prematurely terminated transcription and, in consequence, incomplete accumulation of mRNAs encoding proteins key for this process, might be one of the reasons for the lower developmental capabilities of NSN oocytes.

Proper regulation of Ca^{2+} homeostasis is crucial for embryo development. In mammals, including mice, fertilization induces in oocyte oscillations in cytoplasmic concentration of free Ca^{2+} ions ($[Ca^{2+}]_i$;

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Parrington *et al.* 1996, Saunders *et al.* 2002; reviewed in Swann & Lai 2013), which trigger a series of processes essential for the initiation of the embryonic development, such as establishment of the block to polyspermy and completion of the 2nd meiotic division, and regulate mitochondrial functionality, recruitment of maternal mRNAs and embryonic gene expression (Dumollard *et al.* 2004, Ozil *et al.* 2005, Campbell & Swann 2006, Madgwick *et al.* 2006, Ozil *et al.* 2006, Shoji *et al.* 2006, Tóth *et al.* 2006, Burkart *et al.* 2012; reviewed in Ducibella *et al.* 2006). $[Ca^{2+}]_i$ oscillations are triggered by a sperm-specific phospholipase C zeta (PLCz) (Saunders *et al.* 2002). PLCz hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which binds to its type 1 receptor, IP3R1, localized in the endoplasmic reticulum (ER). This stimulates Ca^{2+} channels to open and Ca^{2+} ions are released from the ER into the cytoplasm. When $[Ca^{2+}]_i$ rises above a certain threshold, Ca^{2+} channels close and Ca^{2+} ions are pumped back to the ER by the ATP-dependent pump SERCA. This in turn decreases $[Ca^{2+}]_i$ and IP3R1 channels can open again (Corbett *et al.* 1999, Li & Camacho 2004; reviewed in Berridge *et al.* 2003, Ajduk *et al.* 2008). The oscillations last until the pronuclei are formed (Marangos *et al.* 2003, Larman *et al.* 2004, Yoda *et al.* 2004). $[Ca^{2+}]_i$ oscillations are also observed during meiotic maturation of mouse oocytes, when they are a response to IP_3 produced during the metabolism of phosphatidylinositol (Carroll & Swann 1992, Carroll *et al.* 1994).

In the present paper, we show that NSN oocytes display defective Ca^{2+} homeostasis, that is, store less Ca^{2+} ions, express lower amount of IP3R1 protein and display weaker spontaneous $[Ca^{2+}]_i$ oscillations during *in vitro* maturation than SN oocytes. In consequence, fertilized NSN-derived MII oocytes generate aberrant $[Ca^{2+}]_i$ oscillations consisting of few $[Ca^{2+}]_i$ transients of low amplitude and lasting only for approx. 20 min. As $[Ca^{2+}]_i$ oscillations are important for initiating the embryonic development, inadequate Ca^{2+} homeostasis may be one of the factors causing lower developmental potential of NSN oocytes.

Materials and methods

Animals and reagents

GV oocytes were obtained from 1.5- to 3.5-month-old unprimed F1 (C57Bl6/Tar × CBA/Tar) mice. Sperm was obtained from 3- to 10-month-old males of the same breed. Animals were maintained in the animal facility of the Faculty of Biology, University of Warsaw at 14:10 light/darkness cycle and provided with food and water *ad libitum*. Animals were killed by a cervical dislocation. All experiments were performed in compliance with the national regulations: as animals used in the experiments were not injected with hormones before culling, a specific permission from the local ethical committee was not required. If not stated otherwise, reagents were purchased from Sigma-Aldrich Poland.

Isolation of GV oocytes and in vitro *maturation*

Fully grown GV oocytes were released from ovarian antral follicles by puncturing with a needle into M2 medium (M16 medium buffered with HEPES) (Fulton & Whittingham 1978) with 3′,5′-cyclic adenosine monophosphate (dbcAMP, 150 µg/mL). Oocytes were separated from the cumulus cells by gentle pipetting. In order to distinguish SN and NSN oocytes, their DNA was stained with Hoechst 33342 (100 ng/mL in M2 supplemented with dbcAMP) for 30 min in 37.5°C and the chromatin configuration was assessed with fluorescence microscopy (Zeiss AxioObserver Z1). Oocytes with dispersed chromatin were identified as NSN oocytes, whereas those with chromatin condensed in a ring around the nucleolus – as SN oocytes (Fig. 1A). Oocytes with a transitional chromatin morphology, with features of both SN and NSN oocytes, were excluded from further experiments. *In vitro* maturation, if required, was performed for 15–16 h in M16 medium in 37.5°C and in 5% CO_2 in the air.

Time lapse-imaging of $[Ca^{2+}]_i$ *in oocytes*

Oocytes were incubated for 30 min in M2 medium with 5 µM fluorescent Ca^{2+} indicator Oregon Green 488 BAPTA-1AM (Molecular Probes, Thermo Fisher Scientific) in 37.5°C. For imaging, oocytes were transferred to a glass-bottom dish (MatTek Corporations) on the time-lapse imaging system (Zeiss Axiovert microscope equipped with AxioCam HRm camera and an environmental chamber) and then either *in vitro* matured (in M16 medium), fertilized (in M2 medium without bovine serum albumin (BSA)) or treated with thapsigargin (10 µM in M2 without Ca^{2+} and Mg^{2+}) or A23187 ionophore (1 µM in M2 without Ca^{2+} and Mg^{2+}). Single-plane images were taken every 10 s. Oocytes were illuminated with 488 nm light, and the emitted light was collected with eGFP filter. Changes in $[Ca^{2+}]_i$ were assessed by measuring mean intensity of Oregon Green 488 BAPTA fluorescence in time. In order to avoid any additional variability between experiments caused by a different extent of dye loading, the initial (pre-fertilization or pre-treatment) mean intensity of fluorescence was calculated for each oocyte and then used to standardize the measurements in this oocyte. Therefore, the values we present are ratios: measured fluorescence intensity/initial fluorescence intensity.

In vitro *fertilization*

Sperm was extracted from epididymes into 500 µL of fertilization medium (Fraser 1982) with BSA (5 mg/mL) and incubated 1.5–2 h in 37.5°C and 5% CO_2 in the air in order to undergo capacitation. MII oocytes loaded with Oregon Green 488 BAPTA-1AM were subjected to acidic Tyrode's solution (pH 2.5; Fulton & Whittingham 1978) in order to remove *zonae pellucidae*. Denuded oocytes were transferred to M2 without BSA in a glass-bottom dish (MatTek Corporations) and

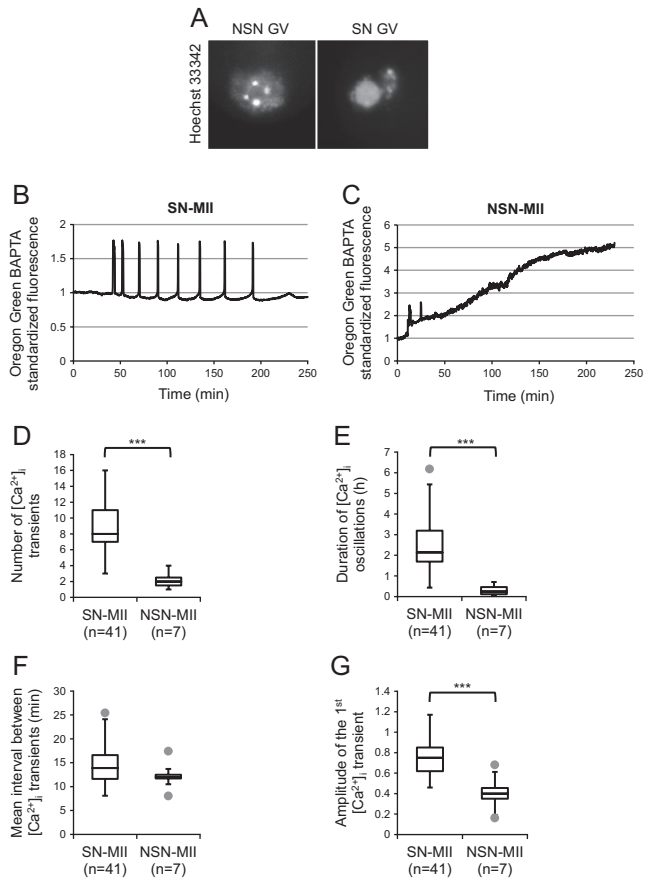

Figure 1 $[Ca^{2+}]_i$ oscillations in fertilized SN- and NSN-derived MII oocytes. (A) A representative Hoechst 33342 staining of NSN- and SN-GV nuclei. (B and C) Representative patterns of fertilization-induced $[Ca^{2+}]_i$ oscillations in SN- (B) and NSN-derived MII oocytes (C). (D) Number of $[Ca^{2+}]_i$ transients, (E) duration of $[Ca^{2+}]_i$ oscillations, (F) mean interval between $[Ca^{2+}]_i$ transients in the first hour of $[Ca^{2+}]_i$ oscillation and (G) amplitude of the first $[Ca^{2+}]_i$ transient in fertilized SN- and NSN-derived MII oocytes. Graphs (D, E, F and G) present median and the first and the third quartile values. The ends of the whisker are set at 1.5 × IQR above the third quartile and 1.5 × IQR below the first quartile. Dots show the minimum and maximum values if they are outside the range (outliers). ***$P < 0.001$.

allowed to stick to the glass bottom. 1–2 µL of capacitated sperm suspension was added to the oocytes. Time-lapse imaging was performed as described above for 4–5 h.

Real time RT-PCR

MII oocytes were transferred in groups of five into 10 µL of Lysis/Binding Buffer (Dynabeads mRNA DIRECT Micro Kit, Thermo Fisher Scientific) and stored in −80°C until further analysis. mRNA was isolated from pooled samples of 15–20 oocytes using the Dynabeads mRNA DIRECT Micro Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. In short, thawed samples were rotated with 10 µL of paramagnetic oligo-$(dT)_{25}$ bead suspension for 30 min at room temperature. mRNA was eluted from the beads by adding 10 µL of DEPC-treated water and heated for 10 min at 70°C with 0.5 µg oligo$(dT)_{25}$. The reverse transcription was performed in total volume of 20 µL using 200 U of Superscript II Reverse Transcriptase, 0.5 mM dNTPs and 40 U RNase inhibitor (Invitrogen, Thermo Fisher Scientific) at 42°C for 50 min. Synthesized cDNA was diluted twice with nuclease-free water (Thermo Fisher Scientific) and subjected to real-time PCR using TaqMan Gene Expression MasterMix and TaqMan Gene Expression Assays probes (*Itpr1*/IP3R1: cat. no. Mm00439907_m1; *Atp2a2*/SERCA2: Mm01275320_m1; *Actb*/Actin B: Mm01205647_g1) (Applied Biosystems, Thermo Fisher Scientific) in StepOne Real-Time PCR System thermocycler (Applied Biosystems, Thermo Fisher Scientific; 50°C/2 min; 60°C/10 min; 50 cycles: 95°C/15 s, 60°C/1 min). Relative level of expression was evaluated using $2^{-\Delta Ct}$ method (Livak & Schmittgen 2001), where actin B was used for normalization.

Western blot

The level of IP3R1 protein was examined in samples of 50 oocytes. Cell lysates were mixed with 4× NuPage LDS sample Buffer and 10× NuPage Sample Reducing Agent (Invitrogen, Thermo Fisher Scientific) and were heated for 10 min in 70°C. The samples were subjected to electrophoresis in NuPage Novex 3–8% Tris-Acetate gels (Invitrogen, Thermo Fisher Scientific) and separated proteins were transferred onto PVDF membranes (Hyperbond-P, Amersham Biosciences), which were probed with a rabbit polyclonal antibody (Rbt03) raised against a 15 amino acid peptide sequence of the C-terminal end of the IP3R1 (Parys *et al.* 1995) and mouse monoclonal antibody against HSP90 (Origene) diluted 1:500 and 1:1000 respectively in 5% non-fat milk in TTBS. Blotted proteins were incubated with primary antibodies for 1 h. A goat anti-rabbit (Pierce, Thermo Fisher Scientific or Bio-Rad) and goat anti-mouse (Bio-Rad) antibodies conjugated with horseradish peroxidase diluted 1:7000 and 1:10,000, respectively, were used as secondary antibodies in 1-h incubation. Detection was performed by the enhanced chemiluminescence technique using SuperSignal West Dura Extended Duration Substrate reagents (Pierce, Thermo Fisher Scientific) according to manufacturer's instruction.

Statistical analysis

Statistical analysis involved χ^2 test, the non-parametric Mann–Whitney test (*U*-test) and the Kruskal–Wallis ANOVA test. The differences between groups were considered statistically significant for $P < 0.05$. Values in the text show means ± standard deviation, but graphs display medians and quartiles.

Results

Among the GV oocytes collected from unprimed females 59.5% (263/442) comprised the SN oocytes and 40.5% (179/442) – NSN oocytes ($P < 0.01$). During *in vitro* maturation, 92.0% (242/263) of SN oocytes resumed meiosis (i.e. underwent germinal vesicle breakdown, GVBD) and 82.1% (216/263) reached

MII stage. Among the NSN oocytes 69.3% (124/179) underwent GVBD and 42.5% (76/179) reached MII stage. To summarize, SN oocytes more frequently went through GVBD and reached MII stage compared to NSN oocytes ($P<<0.0001$), which accords with the previous data (Wickramasinghe *et al.* 1991, Debey *et al.* 1993, Zuccotti *et al.* 1995, Liu & Aoki 2002).

As in some experiments oocytes were filmed during *in vitro* maturation, we were able to calculate mean timings of the main maturation events. GVBD occurred on average after 2.06 ± 2.01 h in SN oocytes ($n=76$) and after 5.22 ± 3.74 h in NSN oocytes ($n=47$; $P<<0.0001$). 1st meiotic division could be observed after 11.79 ± 1.25 h in SN ($n=48$) and 13.79 ± 1.58 h in NSN oocytes ($n=10$; $P<0.01$). Therefore, transcriptionally active GV oocytes resumed meiosis and divided later compared to those transcriptionally inactive, which is consistent with the literature data (Wickramasinghe *et al.* 1991, Debey *et al.* 1993, Liu & Aoki 2002).

Fertilization-induced $[Ca^{2+}]_i$ oscillations differ between oocytes with NSN or SN origin

In order to assess whether oocytes' transcriptional status at the beginning of meiotic maturation alters their ability to maintain Ca^{2+} homeostasis, we examined $[Ca^{2+}]_i$ oscillations generated in MII oocytes of the NSN or SN origin in response to fertilization. To this end, SN-GV (transcriptionally inactive) and NSN-GV (transcriptionally active) oocytes were matured *in vitro* to MII stage (SN-MII and NSN-MII respectively), loaded with Oregon Green 488 BAPTA-1AM dye, fertilized and filmed using a time-lapse technique.

Sperm-induced $[Ca^{2+}]_i$ oscillations in SN-MII oocytes ($n=41$; Fig. 1B) lasted for 2.52 ± 1.33 h and consisted of 8.6 ± 3.0 $[Ca^{2+}]_i$ transients. $[Ca^{2+}]_i$ transients occurred every 14.6 ± 3.8 min in the first hour. (As frequency of $[Ca^{2+}]_i$ oscillations changes with time, which may affect the analysis result, we calculated the mean inter-transient interval only for the first hour of $[Ca^{2+}]_i$ oscillations.) Amplitude of the first $[Ca^{2+}]_i$ transient was 0.74 ± 0.17 (Fig. 1D, E, F and G).

On the other hand, $[Ca^{2+}]_i$ oscillations in NSN-MII oocytes ($n=7$) differed vastly from those observed in SN-MII oocytes (Fig. 1C): they lasted for a much shorter time (18.4 ± 15.9 min, $P<0.0001$) with just 2.1 ± 1.1 $[Ca^{2+}]_i$ transients ($P<0.0001$). $[Ca^{2+}]_i$ transients occurred with frequency similar to this measured in SN-MII oocytes (every 12.3 ± 3.4 min, $P>0.05$), but had lower amplitude (for the first $[Ca^{2+}]_i$ transient 0.42 ± 0.17, $P<0.001$) (Fig. 1D, E, F and G).

In conclusion, $[Ca^{2+}]_i$ oscillations at fertilization were severely impaired in MII oocytes derived from transcriptionally active GV oocytes (NSN-MII), suggesting that the mechanism of Ca^{2+} homeostasis in those oocytes is hindered.

Oocytes of NSN origin contain less Ca^{2+} than SN-derived oocytes

To investigate whether the transcriptional status of an oocyte at the time of meiosis resumption affects its ability to accumulate Ca^{2+} ions, SN-MII and NSN-MII oocytes were treated with either thapsigargin or A23187 ionophore. Thapsigargin is a SERCA inhibitor and allows to assess an amount of Ca^{2+} stored in ER, whereas A23187 ionophore stimulates pore formation and therefore can be used to estimate amount of Ca^{2+} in all cellular compartments.

In SN-MII oocytes ($n=68$) thapsigargin triggered a multi-peaked $[Ca^{2+}]_i$ transient that lasted for 9.5 ± 2.6 min and rose up to 0.51 ± 0.15, while in NSN-MII oocytes ($n=15$), the average $[Ca^{2+}]_i$ transient was longer (12.8 ± 2.4 min, $P<0.0001$) but lower (0.31 ± 0.13, $P<0.0001$) (Fig. 2A, B and C). In case of the ionophore treatment, SN-MII oocytes ($n=51$) generated $[Ca^{2+}]_i$ transient lasting for 4.9 ± 0.9 min and reaching amplitude of 1.3 ± 0.18, while in NSN-MII oocytes ($n=17$), the $[Ca^{2+}]_i$ transient was shorter (3.5 ± 0.7 min, $P<<0.0001$) and had lower amplitude (0.84 ± 0.21, $P<<0.0001$) (Fig. 2D and E). Taken together, these results show that MII oocytes with the

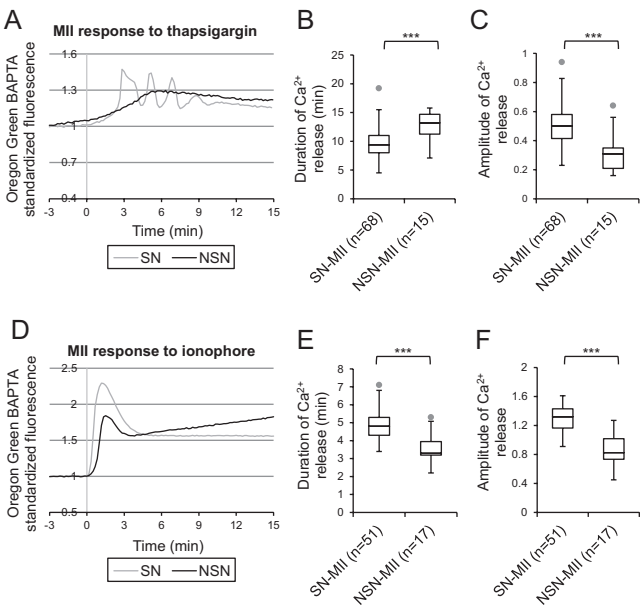

Figure 2 MII oocytes' response to thapsigargin and ionophore. (A) A representative Ca^{2+} response of SN- and NSN-derived MII oocytes to thapsigargin. Duration (B) and amplitude (C) of Ca^{2+} release in SN- and NSN-derived MII oocytes in response to thapsigargin. (D) A representative Ca^{2+} response of SN- and NSN-derived MII oocytes to A23187 ionophore. Duration (E) and amplitude (F) of Ca^{2+} release in SN- and NSN-derived MII oocytes in response to ionophore. Graphs (B and C, E and F) present median and the first and the third quartile values. The ends of the whisker are set at 1.5 × IQR above the third quartile and 1.5 × IQR below the first quartile. Dots show the minimum and maximum values if they are outside the range (outliers). ***$P<0.001$.

NSN origin (i.e. with unfinished transcription at the meiosis resumption) contain less Ca^{2+} ions than oocytes that completed transcription before the maturation onset (SN-MII oocytes).

As oocytes tend to accumulate Ca^{2+} during maturation (Tombes *et al.* 1992, Mehlmann & Kline 1994, Jones *et al.* 1995), we next wished to examine whether the difference in Ca^{2+} content is due to a decreased ability of NSN oocytes to store Ca^{2+} during this period or whether it is present already in the GV stage. To this end, we analyzed Ca^{2+} content in GV stage oocytes. In both the thapsigargin and the ionophore treatments $[Ca^{2+}]_i$ increase displayed by GV oocytes had similar amplitude as in MII oocytes, but lasted for shorter time, confirming that indeed oocytes accumulate Ca^{2+} ions during maturation. In SN-GV oocytes ($n=21$) thapsigargin-induced $[Ca^{2+}]_i$ transient with a mean amplitude of 0.55 ± 0.14 lasted for 3.77 ± 1.17 min. In NSN-GV oocytes ($n=32$) $[Ca^{2+}]_i$ transient was shorter, lasting for 1.87 ± 0.72 min and rose up only to 0.29 ± 0.14 (Fig. 3A, B and C; $P<<0.0001$). Ionophore-treated SN-GV oocytes ($n=38$) responded with a $[Ca^{2+}]_i$ transient with an average amplitude of 1.67 ± 0.31 and duration of 1.99 ± 0.23 min. NSN-GV ($n=38$) oocytes generated a $[Ca^{2+}]_i$ transient of a lower amplitude of 0.62 ± 0.37 and lasting for only 1.64 ± 0.3 min (Fig. 3D and E; $P<<0.0001$). These results indicate, that the difference in Ca^{2+} content between SN- and NSN-derived oocytes is already visible at GV stage, however, due to constraints of our analysis we cannot unequivocally determine whether it is additionally enhanced by a defective Ca^{2+} accumulation during *in vitro* maturation.

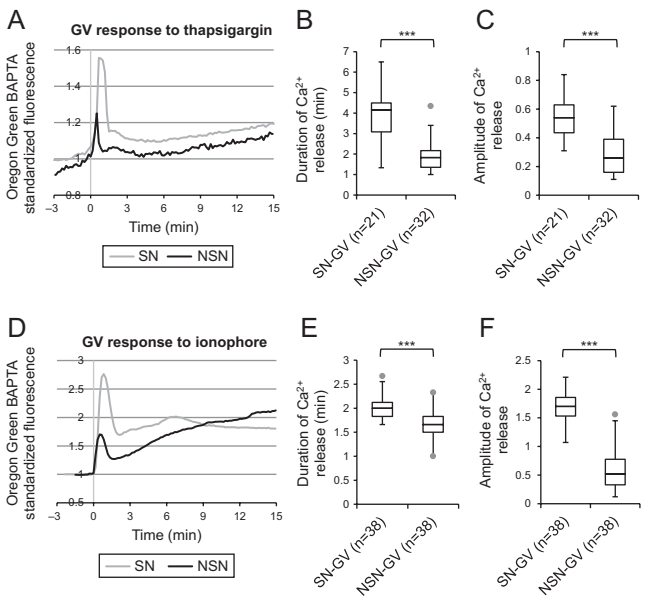

Figure 3 GV oocytes' response to thapsigargin and ionophore. (A) A representative Ca^{2+} response of SN and NSN GV oocytes to thapsigargin. Duration (B) and amplitude (C) of Ca^{2+} release in SN and NSN GV oocytes in response to thapsigargin. (D) A representative Ca^{2+} response of SN and NSN GV oocytes to A23187 ionophore (D). Duration (E) and amplitude (F) of Ca^{2+} release in SN and NSN GV oocytes in response to ionophore. Graphs (B and C, E and F) present median and the first and the third quartile values. The ends of the whisker are set at 1.5 × IQR above the third quartile and 1.5 × IQR below the first quartile. Dots show the minimum and maximum values if they are outside the range (outliers). ***$P<0.001$

Expression of IP3R1 is lower in NSN-derived oocytes

To investigate whether the differences in Ca^{2+} homeostasis between oocytes with the NSN and SN origin are related to altered expression of genes involved in this process, we analyzed mRNA and protein levels of two main regulators of $[Ca^{2+}]_i$ oscillations – IP3R1 and SERCA2. The real-time RT-PCR showed that there are no significant differences in the amount of mRNA for these two proteins between SN and NSN oocytes either in GV or in MII stage (Fig. 4A and B). However, when we examined the protein level of IP3R1 (in case of SERCA2 we were not able to detect it on a Western blot), we noticed that it is significantly less abundant in NSN-GV than in SN-GV oocytes. The difference was maintained after 16 h, when oocytes achieved MI or MII stage (Fig. 4C). In summary, although NSN-derived oocytes contain the same amount of mRNA for IP3R1 and SERCA as SN oocytes, the IP3R1 expression is decreased on the protein level, suggesting that either the protein synthesis is less effective or the protein itself is less stable.

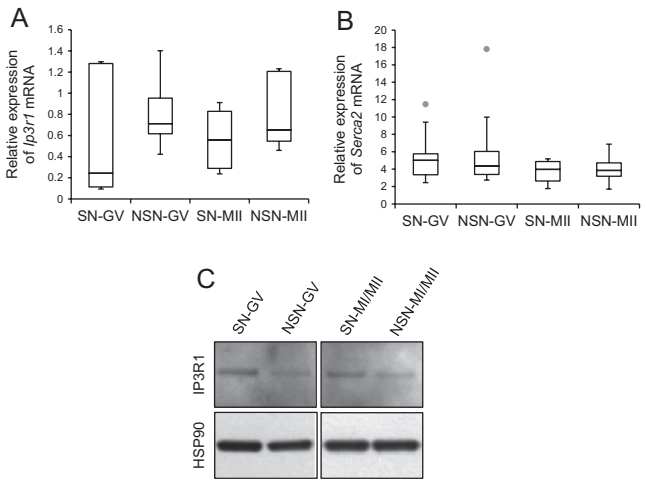

Figure 4 Expression of IP3R1 and SERCA2. Relative expression of *Ip3r1* (A) and *Serca2* (B) mRNA in SN- and NSN-derived oocytes. Graphs present median and the first and the third quartile values. The ends of the whisker are set at 1.5 × IQR above the third quartile and 1.5 × IQR below the first quartile. Dots show the minimum and maximum values if they are outside the range (outliers). (C) Western-blot of IP3R1 protein expressed in SN- and NSN GV oocytes and SN and NSN-derived oocytes that achieved MI or MII stage (MI/MII). HSP90 was used as a reference housekeeping protein.

SN and NSN oocytes differ in Ca^{2+} homeostasis regulation at maturation

$[Ca^{2+}]_i$ oscillations can also take place during GV stage and meiotic maturation (Carroll & Swann 1992, Carroll *et al.* 1994, Wakai & Fissore 2019), therefore, we decided to look into this process. We observed it in 72.4% (55/76) of transcriptionally inactive oocytes (SN) and 63.8% (30/47) of transcriptionally active oocytes (NSN) that resumed meiosis and in all (2/2) SN and 79.2% (19/24) NSN oocytes that remain arrested in the GV stage. We distinguished three pronounced patterns of Ca^{2+} release during maturation. Type 1 was characterized by at least 15 $[Ca^{2+}]_i$ transients of variable amplitude lasting for at least 100 min, type 2 had less than 15 $[Ca^{2+}]_i$ transients over less than 100 min, while in type 3 we did not observe any Ca^{2+} release (Fig. 5A, B and C). Although frequency of the type 2 and 3 responses in SN- and NSN-derived maturing oocytes was similar (64.5% (49/76) and 27.6% (21/76) vs 63.8% (30/47) and 36.2% (17/47) respectively), the type 1 pattern occurred only in SN-derived maturing oocytes (7.9%, (6/76)). On the other hand, all SN oocytes that remained arrested in the GV stage displayed the type 1 pattern, whereas none of the NSN-GV oocytes did so. 79.2% (19/24) of NSN oocytes arrested at the GV stage exhibited type 2 pattern, and 20.8% (5/24) – type 3 (Fig. 5D).

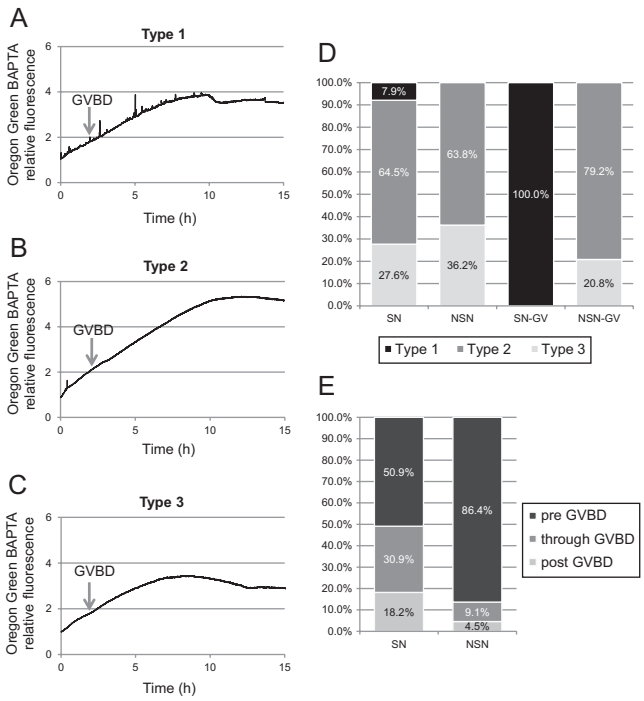

Figure 5 Spontaneous $[Ca^{2+}]_i$ oscillations during maturation of SN and NSN oocytes. (A, B and C) Three types of Ca^{2+} release observed in maturing oocytes. The arrowheads indicate the moment of GVBD. A detailed description of the types in the main text. (D) Frequency of the Ca^{2+} release patterns in SN and NSN oocytes. (E) Time of initiation and termination of $[Ca^{2+}]_i$ oscillations in relation to GVBD: 'pre GVBD' – $[Ca^{2+}]_i$ transients started and ended before GVBD; 'through GVBD' – $[Ca^{2+}]_i$ oscillations were initiated before GVBD, but ceased after GVBD; 'post GVBD' – $[Ca^{2+}]_i$ transients started and ended after GVBD.

In case of oocytes that resumed meiosis, we also examined when, in relation to the GVBD, $[Ca^{2+}]_i$ oscillations were initiated and when they ceased. In the vast majority (86.4% (19/22) of NSN oocytes $[Ca^{2+}]_i$ oscillations were initiated and ceased before GVBD ('pre GVBD'). SN oocytes were a more diversified group with only 50.9% (28/55) of oocytes having 'pre GVBD' $[Ca^{2+}]_i$ oscillations. 30.9% (17/55) of SN oocytes displayed $[Ca^{2+}]_i$ oscillations starting before and lasting through GVBD ('through GVBD'; vs only 9.1% in NSN) and 18.2% (10/55) – $[Ca^{2+}]_i$ oscillations initiated after GVBD ('post GVBD'; vs only 4.5% in NSN) ($P<0.05$) (Fig. 5E).

$[Ca^{2+}]_i$ oscillations in NSN-derived maturing oocytes lasted on average only for 30.6 ± 29.3 min with 2.33 ± 1.56 Ca^{2+} transients, while SN-derived maturing oocytes oscillated for 3.24 ± 4.37 h ($P<0.05$) with 10.71 ± 16.44 Ca^{2+} transients ($P<0.05$). Average transient amplitude equaled 0.21 ± 0.14 for maturing oocytes of the SN origin and 0.12 ± 0.08 ($P<0.001$) for those of the NSN origin (Fig. 6A, B and C).

Interestingly, oocytes of both SN- and NSN-origins that were not able to resume meiosis and remained in the GV stage, showed longer $[Ca^{2+}]_i$ oscillations with more numerous $[Ca^{2+}]_i$ transients than their maturing counterparts. SN GV-arrested oocytes ($n=2$) had $[Ca^{2+}]_i$ oscillations lasting for 3.8 ± 0.12 h with over 230, dynamically repeating $[Ca^{2+}]_i$ transients of 0.33 ± 0.04 amplitude. NSN GV-arrested oocytes (19/24) oscillated for 3.01 ± 3.9 h ($P<0.05$ in comparison to oocytes that resumed meiosis) with 4.47 ± 3.94 $[Ca^{2+}]_i$ transients ($P>0.05$) of 0.15 ± 0.17 amplitude ($P<0.05$) (Fig. 6D, E and F).

To conclude, $[Ca^{2+}]_i$ oscillations in NSN-derived oocytes are usually less numerous and shorter than in SN-derived oocytes. However, GV-arrested oocytes of both these origins tend to generate more intensive $[Ca^{2+}]_i$ oscillations than those that resumed meiosis.

Discussion

In this paper we investigated whether transcriptional status of mouse oocytes at the onset of meiotic maturation affects Ca^{2+} homeostasis in these cells. Taking into account the pertinence of Ca^{2+} signaling in oocytes, especially fertilization-induced $[Ca^{2+}]_i$ oscillations that trigger meiosis completion and initiate and regulate the embryonic development (Ozil *et al.* 2005, 2006, Campbell & Swann 2006, Madgwick *et al.* 2006, Shoji *et al.* 2006, Tóth *et al.* 2006, Burkart *et al.* 2012; reviewed in Ducibella *et al.* 2006), we hypothesized that if $[Ca^{2+}]_i$ oscillations were disrupted in NSN-derived oocytes, it could explain, at least partially, their low developmental capabilities (Zuccotti *et al.* 2002, Inoue *et al.* 2008; reviewed in Tan *et al.* 2009).

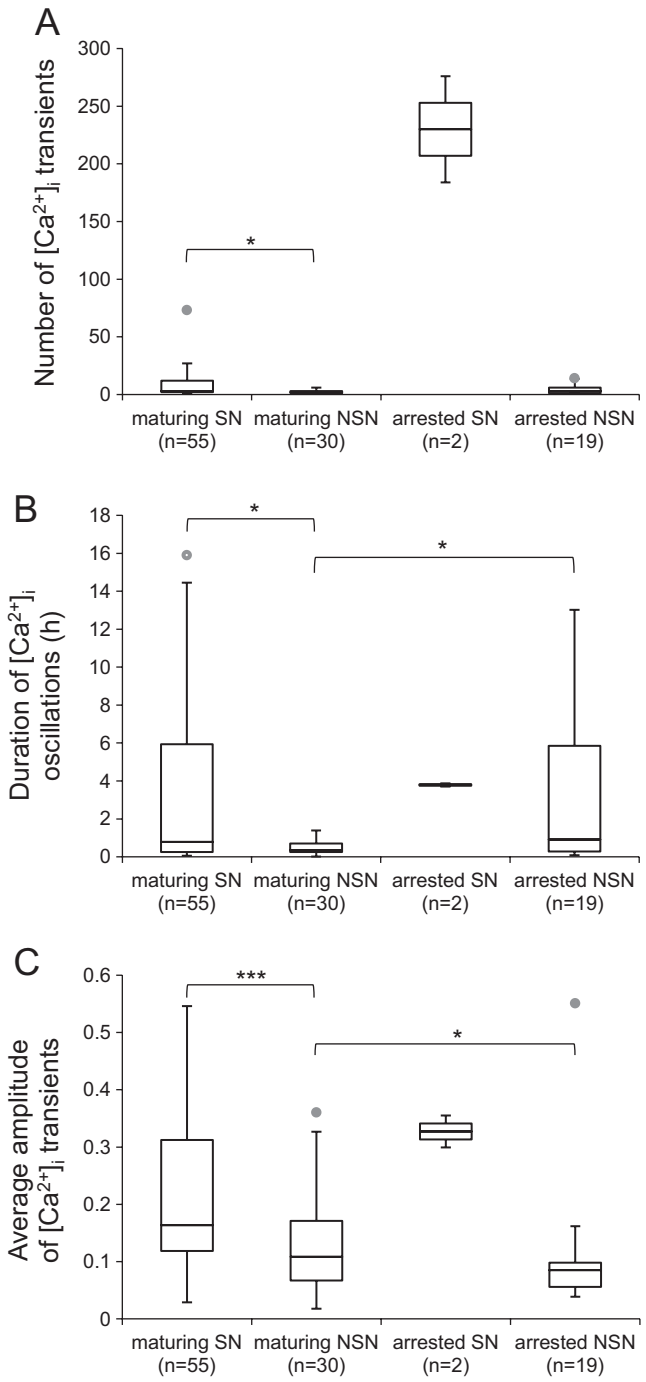

Figure 6 Quantitative analysis of the spontaneous Ca^{2+} release in maturing SN and NSN oocytes. (A) Number of $[Ca^{2+}]_i$ transients, (B) duration of $[Ca^{2+}]_i$ oscillations, (C) amplitude of the first $[Ca^{2+}]_i$ transient registered for the spontaneous Ca^{2+} release in maturing SN and NSN oocytes. Graphs (A, B and C) present median and the first and the third quartile values. The ends of the whisker are set at 1.5 × IQR above the third quartile and 1.5 × IQR below the first quartile. Dots show the minimum and maximum values if they are outside the range (outliers). $*P<0.05$, $***P<0.001$.

Indeed, we revealed that MII oocytes derived from transcriptionally active NSN-GV oocytes exhibit an abnormal pattern of sperm-triggered $[Ca^{2+}]_i$ oscillations compared to oocytes derived from transcriptionally inactive SN-GV oocytes. $[Ca^{2+}]_i$ oscillations in NSN-derived oocytes display fewer $[Ca^{2+}]_i$ transients of a lower amplitude and those $[Ca^{2+}]_i$ oscillations last for shorter time than in SN-derived oocytes. This result accords with our other observation that NSN-derived oocytes contain less Ca^{2+} and express less IP3R1 protein. It has been shown that decreased amount of Ca^{2+} stored in oocytes leads to aberrant pattern of $[Ca^{2+}]_i$ oscillations at fertilization: the whole event is shorter, $[Ca^{2+}]_i$ transients are less frequent and have lower amplitudes (Miao *et al.* 2012, Wakai *et al.* 2013). Ca^{2+} stores are similarly depleted in postovulatory aged oocytes (Takahashi *et al.* 2000, Lord & Aitken 2013), and $[Ca^{2+}]_i$ oscillations at fertilization in these oocytes are also anomalous, with the $[Ca^{2+}]_i$ transients being lower in amplitude but more frequent than in fresh oocytes (Igarashi *et al.* 1997, Takahashi *et al.* 2003). Importantly, we have also shown that difference in the Ca^{2+} content between SN- and NSN-derived oocytes is already visible at the GV stage. This could be due to the fact that in ovaries transport of ions and low molecular weight molecules, such as secondary messengers, amino acids and nucleotides, through the gap junctions between the cumulus cells and NSN oocytes may be still active, as it has been shown for bovine oocytes (Lodde *et al.* 2007). It is then possible, that NSN oocytes do not accumulate enough of these factors before their maturation *in vitro* is induced, which, in turn, negatively impacts their ability to maintain ionic homeostasis (Kumar & Gilula 1996), transcription (De La Fuente & Eppig 2001), or translation and protein phosphorylation (Colonna & Mangia 1983, Colonna *et al.* 1989, Haghighat & Van Winkle 1990).

Similarly, it has been indicated that correct pattern of $[Ca^{2+}]_i$ oscillations in fertilized oocytes depends on the amount of IP3R1 expressed (Parrington *et al.* 1998, Jellerette *et al.* 2000, 2004, Jędrusik *et al.* 2007, Lee *et al.* 2010). Interestingly, we showed that IP3R1 protein content is reduced in NSN as compared to SN oocytes, whereas mRNA for this protein is expressed at a similar level in both examined groups. This suggests that IP3R1 protein expression is deregulated translationally or post-translationally. Recent studies have suggested that ability of NSN oocytes to store mRNA may be reduced due to a lower number of cytoplasmic lattices (CPL), fibrillar matrices composed of protein and RNA, which seem to contain most of oocyte's ribosomes (Monti *et al.* 2013). CPL shortage may also deregulate translation (Yurttas *et al.* 2008). We cannot also exclude that IP3R1 undergoes a faster degradation in NSN oocytes due to for example amino acid deprivation (Dever 2002) that may be caused by potential premature closure of the

junctional transport between oocytes and cumulus cells (see the previous paragraph; Lodde *et al.* 2007).

It is also possible that in MII oocytes of NSN origin, metabolism of phosphatidylinositol (PI) is disturbed, which results in deficiency of indigenous IP_3. This hypothesis is in line with the literature reporting that in NSN oocytes expression of enzymes producing PI, PI(4)P (phosphatidylinositol 4-phosphate) and PI(4,5)P2 (phosphatidylinositol (4,5)-bisphosphate) is reduced (Ma *et al.* 2013). It is also possible that IP3R1 receptors in NSN-derived MII oocytes have lower affinity to IP_3. Previous studies have shown that the sensitivity of IP3R1 to IP_3 increases during oocyte maturation due to phosphorylation (Lee *et al.* 2006, Sun *et al.* 2009, Wakai *et al.* 2012, Zhang *et al.* 2015) and it is possible that this event is impaired in NSN oocytes. It has been shown that expression of MAP kinase, the main enzyme responsible for IP3R1 phosphorylation (Lee *et al.* 2006), is downregulated in mouse NSN oocytes (Ma *et al.* 2013).

Finally, we showed that NSN oocytes display altered Ca^{2+} signaling already during meiotic maturation. $[Ca^{2+}]_i$ oscillations in NSN oocytes are shorter and with fewer $[Ca^{2+}]_i$ transients. This corresponds with observations of Carroll *et al.* (1994) who reported that ability to generate $[Ca^{2+}]_i$ oscillations during maturation reflects developmental capabilities of oocytes: small, meiotically incompetent oocytes were less likely to generate $[Ca^{2+}]_i$ oscillations compared to competent oocytes. However, Carroll *et al.* (1994) showed also that oocytes arrested in GV stage did not exhibit $[Ca^{2+}]_i$ oscillations at all, whereas in our conditions such oocytes manifested more $[Ca^{2+}]_i$ transients and overall longer $[Ca^{2+}]_i$ oscillations than those that resumed meiosis. It is likely that impairment of $[Ca^{2+}]_i$ oscillations recorded in NSN-derived maturing oocytes, as compared to SN-derived ones, is caused by the same factors that negatively affect fertilization-induced $[Ca^{2+}]_i$ oscillations in NSN-MII oocytes, that is by the lower amounts of IP3R1 and Ca^{2+} ions stored in the cell. It is also possible that hindered PI metabolism and decreased functionality of IP3R1 are involved.

Although it has been suggested that Ca^{2+} participates in the signaling pathway linking a decrease in cAMP concentration in oocytes and their meiotic resumption (De Felici *et al.* 1991; reviewed in Tosti 2006), GVBD is Ca^{2+} independent (Carroll & Swann 1992, Tombes *et al.* 1992). Therefore, the lower rate of meiotic resumption in NSN oocytes probably is not caused by the hindered Ca^{2+} release during meiotic maturation. However, the altered pattern of $[Ca^{2+}]_i$ oscillations at maturation may influence the processes of cytoplasmic maturation. Recently, it has been reported that $[Ca^{2+}]_i$ oscillations in immature oocytes stimulate their mitochondrial metabolism (Wakai & Fissore 2019). Moreover, it has been shown that Ca^{2+} signaling has a role in cell membrane trafficking in somatic cells (reviewed in Li *et al.* 2013, van der Kant & Neefjes 2014) and a local rise in $[Ca^{2+}]_i$ may trigger closure of gap-junction channels (Rose & Loewenstein 1975). It is therefore possible that impaired Ca^{2+} homeostasis in oocytes affects their cellular trafficking and junctional communication with cumulus cells.

In summary, our results indicate NSN-derived oocytes display altered Ca^{2+} signaling, when compared to SN-derived counterparts. It may be, at least partially, caused by the unfinished transcription in NSN oocytes; however, it is likely that other properties of NSN oocytes, for example, potentially incomplete trans-junctional import from the surrounding cumulus cells, also play a role here. We postulate that defective Ca^{2+} homeostasis is one of the reasons for the low developmental capabilities of NSN oocytes. Importantly, better understanding of phenotypical differences between SN and NSN oocytes, as well as their molecular background, is crucial to optimize procedures of *in vitro* oocyte maturation. As this approach has been applied in assisted reproduction of farm animals and more and more intensely explored in infertility clinics (reviewed in Sauerbrun-Cutler *et al.* 2015), we definitely need to examine its mechanisms more closely.

Declaration of interest

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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Author contribution statement

M F conducted the experiments, analyzed the data and drafted the manuscript. K S consulted the PCR experiments. T I conducted some of the Western blot experiments. A A planned the experiments, conducted some of them and revised the manuscript.

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