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Intracellular cAMP and calcium signaling by serotonin in mouse cumulus-oocyte complexes

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List of abbreviations : 5-HT: serotonin, RT-PCR: reverse transcription polymerase chain reaction, BAPTA-AM: Glycine, N,N'-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-[(acetyloxy) methoxy]-2-oxoethyl]]-, bis[(acetyloxy)methyl] ester, GVBD : germinal vesicle breakdown, GPCR : G-protein-coupled receptors, SAGE : serial analysis of gene expression, IBMX : 3-isobutyl-1-methylxanthine, hCG : human chorionic gonadotropin, COC : cumulus-oocyte complex, 5-CT : 5-carboxamidotryptamine maleate, 8-OH DPAT : 8-hydroxy-2-(di-n-propylamino) tetralin, PPB : 2-[1-(4-piperonyl) piperazinyl] benzothiazole, DMSO : dimethyl sulfoxide, kDa : kilo Dalton

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Abstract

Cyclic AMP (cAMP) and intracellular Ca^{2+} are important second messengers involved in mammalian follicular growth and oocyte meiotic maturation. We investigated the capacity of the neurohormone serotonin (or 5-hydroxytryptamine, 5-HT) to regulate intracellular cAMP and Ca^{2+} in mouse oocytes and surrounding cumulus cells. Based on a RT-PCR study, 5-HT_7 receptor mRNA is expressed in cumulus cells, oocytes and embryos up to the 4-cell stage and 5-HT_{2A} and 5-HT_{2B} receptor mRNAs in cumulus cells only, while 5-HT_{2C}, 5-HT_4 and 5-HT_6 receptors are expressed neither in oocytes nor cumulus cells. The addition of 5-HT (10 nM-10 μM) to isolated metaphase II oocytes had no effect on their internal cAMP or Ca^{2+} levels whereas it caused dose-dependent cAMP and Ca^{2+} increases in cumulus cells. This cAMP increase in cumulus cells could be mimicked by 5-HT agonists with the following order of potency: 5-HT > 8-OH DPAT = α-methyl-5-HT = 5-CT, thereby supporting a preferential involvement of 5-HT_7 receptors. As measured with cumulus cells pre-loaded with fura-2-AM, the addition of 5-HT also caused dose-dependent Ca^{2+} rises, likely linked to detected 5-HT_{2A} and 5-HT_{2B} receptors. Adding the Ca^{2+} ionophore ionomycin to cumulus cells resulted in both Ca^{2+} and cAMP elevations whereas preincubation of cells with the Ca^{2+} chelator BAPTA-AM abolished the 5-HT-induced Ca^{2+} increase and reduced the cAMP rise, indicating cross-talk between the 5-HT-sensitive Ca^{2+} and cAMP pathways. Our results show that 5-HT may be a local regulator in mouse cumulus-oocyte complexes through its actions on cAMP and Ca^{2+} signaling, as mediated by 5-HT_{2A}, 5-HT_{2B} and 5-HT_7 receptors.

Introduction

Cyclic AMP (cAMP) and intracellular Ca^{2+} are involved in several important reproductive functions in mammals. One role of cAMP, in the mammalian ovary, is the maintenance of the oocytes arrested in prophase I until ovulation. The high internal cAMP required for the maintenance of oocyte meiotic arrest appears to be provided, in part, by granulosa cells tightly connected to oocytes through gap junctions, allowing free transfer of both cAMP and Ca^{2+} ions (Conti *et al.*, 2002; Webb *et al.*, 2002a,b). In the whole follicle, cAMP, along with intracellular Ca^{2+}, is also involved in FSH-induced expressions of P450scc, aromatase, and LH receptor in granulosa cells (Gore-Langton and Armstrong, 1988; Leung and Steele, 1992). LH also promotes cAMP and Ca^{2+} increases in theca cells, leading to CYP11A and CYP17 gene expressions (Gore-Langton and Armstrong, 1988; Leung and Steele, 1992). At fertilization, the oocyte undergoes an initial Ca^{2+} increase, followed by periodic oscillations that are essential for meiosis reinitiation, cortical granule exocytosis and proper embryo development (Kline and Kline, 1992; Lawrence *et al.*, 1998). Thus, cAMP and Ca^{2+} signaling is required not only for various functions within both oocytes and their surrounding granulosa cells, but also for their intimate coordination one with the other. Despite this central role of Ca^{2+} and cAMP, little is known about their upstream regulation by potential ligands and receptors, to be identified, that might influence their cellular levels.

One such potential ligand is serotonin (5-hydroxytryptamine, 5-HT), whose action is well-known as a regulator of spawning and oocyte maturation in several invertebrates (Colas and Dubé, 1998; Stricker and Smythe, 2000) and also of follicular growth in fishes (Cerda *et al.*, 1998), but whose potential functions in mammalian reproductive tissues, through Ca^{2+} and

cAMP signaling, are poorly documented. Among the indications that 5-HT might be such a local regulator is its detection in female rodent genital tracts (Amenta *et al.*, 1992) and in human follicular fluid (Bodis *et al.*, 1993). Moreover, 5-HT has also been recently reported in isolated mouse oocytes and embryos (Il'kova *et al*., 2004; Amireault and Dubé, 2005) as well as in surrounding cumulus cells that also possess the rate-limiting enzyme tryptophan hydroxylase (TPH1) for 5-HT production, thus making these cells a potential immediate direct source of 5-HT (Amireault and Dubé, 2005). Also, *in vitro*, 5-HT promotes estradiol secretion by rat (Tanaka *et al.*, 1993) and hamster (Terranova *et al.*, 1990) pre-ovulatory follicles, and progesterone secretion by cultured bovine luteal cells (Battista *et al*., 1987). Finally, it was also shown that an antidepressant-sensitive specific 5-HT transporter was active in mouse oocytes and embryos to accumulate external 5-HT (Amireault and Dubé, 2005). All these observations suggest the existence of a local and functional serotonergic network in reproductive tissues in general, and in mouse cumulus-oocyte complexes, in particular. A proper identification of the specific 5-HT receptors involved in the regulation of this serotonergic network remains however to be established.

Mammalian 5-HT receptors are divided into seven subfamilies (5-HT_{1-7}) sharing common sequences, pharmacological properties and signaling pathways, and most of them are G-protein-coupled receptors (GPCR) regulating cAMP or intracellular Ca^{2+}. For example, 5-HT_1 receptors are coupled preferentially to $G_{i/o}$ to inhibit cAMP formation (Barnes and Sharp, 1999) while 5-HT_4, 5-HT_6 and 5-HT_7 receptors are coupled to G_s and, hence, positively regulate adenylate cyclase, causing cAMP increases when activated (Hamblin *et al*., 1998). 5-HT_2 receptors are coupled to G_q and linked to phospholipase C, thus mobilizing intracellular Ca^{2+} (Roth *et al.*, 1998). Only a few of these 5-HT receptor subtypes have been reported in mammalian

reproductive tissues and cells. First, the 5-HT_7 receptor was detected in cultured human granulosa-lutein cells (Graveleau *et al.*, 2000) in which 5-HT promotes the expected cAMP elevation. Also, in isolated metaphase II hamster oocytes, 5-HT induces intracellular Ca^{2+} oscillations sensitive to 5-HT_2 antagonists (Miyazaki *et al.*, 1990), suggesting the presence of this receptor type in oocytes. Moreover, a polymerase chain reaction-serial analysis of gene expression (PCR-SAGE) study reported the expression of 5-HT_{2A} receptor mRNA in human oocytes (Neilson *et al.*, 2000) and an RT-PCR analysis suggested the expression of 5-HT_{1D} receptor mRNA in mouse oocytes and embryos (Vesela *et al*., 2003).

All these findings support an involvement of 5-HT and some of its receptors in various key processes in oocytes and surrounding cells, likely to involve cAMP and/or Ca^{2+} signaling. We, therefore, decided to clarify the effects of 5-HT on cAMP and Ca^{2+} levels in mouse oocytes and cumulus cells, as well as to identify the 5-HT receptors regulating these effects, with special attention given to those two G_q and G_s subtypes, 5-HT_2 and 5-HT_7, previously detected in other mammalian reproductive tissues or cells.

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Material and methods

Oocyte and embryo collection

Fully-grown germinal vesicle stage oocytes, ovulated metaphase II oocytes and pre-implantation embryos at various stages were obtained from 3-4-week-old female B6C3F1 mice (Charles River) after standard gonadotropin injection. For germinal vesicle stage oocytes, the mice were primed with 5 IU of pregnant mare's serum (Sigma), and cumulus-enclosed, fully-grown oocytes were collected 46-48 h later, by puncturing of the antral follicles with a 30-gauge needle under a dissecting microscope, in M2 medium containing 100 μM 3-isobutyl-1-methylxanthine (IBMX). When needed, the cumulus cells were removed by repeated pipetting with a small bore pipette. For metaphase II arrested eggs, the mice were primed with 5 IU of pregnant mare's serum, followed (44-48 h later) by a 5 IU human chorionic gonadotropin (pregnyl-hCG, Organon Canada) injection, and cumulus-enclosed eggs (COC-metaphase II) were collected from the oviduct 18-20 h later in M2 medium. When needed, the cumulus cells were dispersed in M2 medium containing 10 mg/ml bovine testis hyaluronidase (Sigma), and the eggs were washed and collected in M2 medium. For embryos, female mice were submitted to the gonadotropin protocol and were allowed to mate with a male the night after the second injection. Embryos were collected by flushing, with M2 medium, the oviducts or uteri with a 30-gauge needle mounted on a syringe. The timing of embryo collection was as follows: 1-cell, 19 h post-hCG; 2-cell, 43 h; 4-cell, 50 h; 8-cell, 67 h; morula, 74 h; early blastocysts, 91 h.

mRNA isolation and reverse transcription (RT)-PCR

Collected cells were incubated in acidic Tyrode's solution (Sigma) to remove the zona pellucida of germinal vesicle oocytes, metaphase II eggs, and 1-cell embryos. The cells were

kept in a minimum of M2 medium at -80°C until mRNA isolation. mRNAs of 10 oocytes, 10 embryos or cumulus cells from 30-50 COC-metaphase II were isolated according to the micro-scale protocol with the Dynabeads mRNA Direct kit (Dynal). The mRNAs were reverse transcribed using Superscript II enzyme (Gibco BRL) in a 20 µL reaction at 42°C, for 45 min, to construct a cDNA library immobilized on beads, following the manufacturer's specifications. The first PCR run (50 µL) was performed on cDNA beads in suspension. The PCR program, of 26 cycles with a hot start, consisted of denaturation of 90 sec at 95°C, primer annealing of 90 sec at 65°C (5-HT_7 and 5-HT_{2A}) or 60°C (5-HT_{2B}, 5-HT_{2C}, 5-HT_4 and 5-HT_6), and primer extension of 90 sec at 72°C (last primer extension of 15 min). The second PCR run was performed with 1/10 of a µL of products from the first amplification and the same PCR program (28 cycles). For 5-HT_7, 2 pairs of primers in a nested PCR strategy produced final amplicons of 174 bp (5-HT_{7a}), 179 bp (5-HT_{7b}) or 272 bp (5-HT_{7c}). The primers were: 1st forward 5'-cagccaaacacaagttctcag-3', 1st reverse 5'-cccctgttctgcattacttctt-3', 2nd forward 5'-tccagtgccagtaccggaatatcaac-3' and 2nd reverse 5'-tacttcttctccagggttccgctct-3'. For 5-HT_{2A}, 2 pairs of primers were used in a nested PCR strategy to produce amplicons of 627 bp (forward 5'-tcttctccacggcatccatcatgcac-3' and reverse 5'-caaacacattgagcagggctccaatgac-3') and 419 bp (forward 5'-accatagccgcttcaactccagaacc-3' and reverse 5'-tgctttttgctcattgctgatggactgc-3'). For 5-HT_{2B}, 2 pairs of primers were used in a nested PCR strategy to produce amplicons of 678 bp (forward 5'-tgtctgaacaaagcacaacttctgagc-3' and reverse 5'-ccatgatggtgagaggtacgaagaaag-3') and 451 bp (forward 5'-actcagtagcagaggaaatgaagcaga-3' and reverse 5'-gcgatgcctattgaaattaaccatacc-3'). For 5-HT_{2C}, 2 pairs of primers were used in a nested PCR strategy to produce amplicons of 658 bp (forward 5'-gcagtacgtaatcctattgagcatagcc-3' and reverse 5'-ttttgttgaagagagtgtacaccagagg-3') and 411 bp (forward 5'-ttcttcatcccgttgacaattatgg-3' and reverse

5'-cacatagccaatccaaacaaacaca-3'). For 5-HT_4, 2 pairs of primers were used in a nested PCR strategy to produce amplicons of 649 bp (forward 5'-ctaatgtgagttccaacgagggtttc-3' and reverse 5'-tgctccttagcagtgacatagattcg-3') and 512 bp (forward 5'-gttccttgcagtggttatcctgatg-3' and reverse 5'-tgatagcatagggcttgttgaccat-3'). For 5-HT_6, 2 pairs of primers were used in a nested PCR strategy to produce amplicons of 790 bp (forward 5'-caacacgtctaacttcttcctggtgt-3' and reverse 5'-gatgatagggttcatggtgctattacag-3') and 454 bp (forward 5'-ctaacttcttcctggtgtcgctcttc-3' and reverse 5'-aagatcctgcagtaggtgaagcagat-3'). Primers for β-actin as positive controls yielded 540-bp (forward 5'-gtgggccgctctaggcaccaa-3' and reverse 5'-ctctttgatgtcacgcacgatttc-3') and 277-bp (forward 5'-tgtgatggtgggaatgggtcagaaggac-3' and reverse 5'-tacgtacatggctggggtgttgaagg-3') amplicons. 25 μL of each reaction was loaded on agarose gel stained with ethidium bromide. Each amplification was executed at least 3 times, yielding similar results. All PCR products obtained were cloned in pCRII (Invitrogen) and sequenced on both strands, using the Université Laval sequencing service to confirm the sequence.

Indirect immunofluorescence confocal microscopy for 5-HT_7 and 5-HT_{2A} detection

Oocytes and embryos were collected and treated as described above, then fixed in fresh paraformaldehyde 4% for 30 min at room temperature. They were washed 3 times for 5 min in Dulbecco's phosphate-buffered saline (D-PBS), before a 1-h blocking step in D-PBS/milk 5%/Triton 0.5%/normal goat serum (NGS) 5%. They were next incubated overnight at 4°C with a primary antibody in D-PBS/milk 1%/Triton 0.1%/NGS 1%. After 3 washes in D-PBS, they were incubated for 1 h at room temperature in a Cy-3-conjugated goat anti-rabbit antibody (1/2,000, Jackson Immunoresearch), and washed 3 times in D-PBS. Finally, metaphase II arrested eggs, COC-metaphase II, cumulus cells and 4-cell embryos were mounted with

Fluoromount (Electron Microscopy System), and blastocysts were mounted with 50% glycerol in D-PBS. Images were collected with a 63X/1.4 oil DIC plan-apochromat objective and a Zeiss Axiovert 100M microscope coupled with the LSM510 system.

For 5-HT_7, a rabbit anti-rat antibody directed against amino acids 8-23 (Oncogene) and diluted 1/200 served as primary antibody. For 5-HT_{2A}, a rabbit anti-rat antibody directed against amino acids 22-41 (Oncogene) was diluted 1/150. Controls without the first antibody were also included for each cell type tested.

Western blotting

For each detection of 5-HT_{2A} and 5-HT_7, approximately 300 and 500 oocytes, respectively, and corresponding surrounding cumulus cells were lysed in 30 µL of 0.5% sodium dodecyl sulphate (SDS) and kept at -80ºC until electrophoresis. The frozen samples were diluted in 4X sample buffer, loaded on 7.5% SDS-polyacrylamide gel, run at 200 V for 45 min and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% milk and 0.1% Tween 20 in D-PBS for 1 h at room temperature before overnight incubation at 4°C, in fresh blocking solution containing the appropriate diluted first antibody. The membrane was then washed 3 times in 0.1% Tween 20 in D-PBS and incubated in fresh blocking solution containing the appropriate diluted second antibody. Finally, the membrane was washed several times in 0.1% Tween 20 in D-PBS before the detection protocol, using the enhanced chemiluminescence plus assay kit (Amersham). The same 5-HT_7 (1/500) and 5-HT_{2A} (1/500) antibodies as for indirect immunofluorescence detection were deployed for Western blotting with a goat anti-rabbit-horse radish peroxydase secondary antibody (1/20,000, Bio-Rad).

cAMP extraction and measurement after cell treatments

Groups of 10 COC-metaphase II or 50 metaphase II oocytes were collected, as described above, and treated for 5 min in small Petri dishes containing a drop of M16 medium supplemented with 200 μM IBMX, under paraffin oil, in a humidified chamber at 37°C and 5% CO_2. Stock solutions of 10 mM 5-carboxamidotryptamine maleate (5-CT, Tocris), 5-hydroxytryptamine creatinine sulfate complex (5-HT, Sigma), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH DPAT, Tocris) or α-methyl 5-HT maleate (α-methyl-5-HT, Tocris) were prepared at 10 mM in H_20 and kept in aliquots at -20°C. Stock solutions of 2-[1-(4-piperonyl) piperazinyl] benzothiazole (PPB, Tocris) at 10 mM in DMSO, forskolin at 10 mM in DMSO, BAPTA AM (Molecular Probes) at 20 mM in DMSO and ionomycin (Sigma) at 1 mM in ethanol were also prepared and kept the same way. One 10 mM aliquot of the appropriate drug was thawed and diluted before each experiment. In the BAPTA-containing conditions, groups of cells were incubated in M16 medium containing 50 μM BAPTA AM and 0.02% Pluronic F-127 (Molecular Probes) for 30 min, under paraffin oil, in a humidified chamber at 37°C and 5% CO_2 before the 5-min treatment. Treatments were stopped by transfer of the cells, with a minimum of medium, to a new tube and its immersion in liquid nitrogen. The cells were kept at -80°C until cAMP extraction. Frozen cells were thawed and frozen in liquid nitrogen 2 more times to ensure complete lysis of the cells. Then, 100 μL of cold 95% ethanol 0.1% trichloroacetic acid was added to each tube, and the cells were centifuged at 3,000 rpm for 10 min at 4°C. The supernatant was evaporated, and the remaining pellet was resuspended in 50 μL of assay buffer (cAMP Biotrak enzyme immunoassay system kit, Amersham Biosciences).

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cAMP was measured according to the acetylation procedure described in the manufacturer's booklet.

Intracellular Ca^{2+} measurement in oocytes and cumulus cells

For cumulus cell Ca^{2+} measurements, COC-metaphase II were collected, as described above, and incubated in M2 medium containing 5 μM fura-2-AM and 0.02% Pluronic F-127 for 25 min at 37°C. After 3 washes in M2 medium, the COCs were transferred into a 100-μl plastic chamber, containing M2 medium supplemented with 10 mg/ml bovine testis hyaluronidase, on a poly-lysine coverslip installed on the stage of an inverted microscope (Diaphot, Nikon). After a few minutes to allow dispersion of the cumulus cells and their adherence to the coverslip, the chamber was perfused (5 ml/ min) with M2 medium until a stable baseline signal was obtained. M2 medium or M2 medium containing 10 nM, 100 nM, 1 μM 5-HT or 200 μM ATP, maintained at 37°C was perfused at a rate of 5ml-min throughout each experiment. For metaphase II oocyte measurements, oocytes without zona pellucida were prepared like cumulus cells but for the hyaluronidase-containing step. Fluorescence signals were obtained from a fluorescence lamp coupled to a high speed filter changer (Lambda DG-4 , Sutter Instrument Company) and a refrigerated CCD Camera (Photometrics Cool SNAP HQ, Roper Scientific). Excitation wavelenghts were 340 and 380 nm, and fluorescence emission was measured at 510 nm. The collected data were then analysed by Metafluor program 6.1 (Universal Imaging Corporation).

Statistical analysis

The results of cAMP measurement are expressed as means ± SEM. Each experiment was performed at least 3 times in duplicate. Statistically significant differences between group means and the control mean were analysed by unpaired Student's t test.

Results

Expression of 5-HT_{2A} and 5-HT_7 receptor mRNAs in COCs and embryos

Adopting a nested RT-PCR strategy with oligonucleotides flanking intron sequences and specific for mouse 5-HT_7 mRNA, we detected a band of the proper size in preparations from the ovary, cumulus cells, germinal vesicle stage oocytes, metaphase II oocytes, 1-cell embryos, 2-cell embryos and 4-cell embryos, but not in 8-cell embryos, morula and blastocysts (Fig. 1A, upper panel). Taking a similar RT-PCR approach for 5-HT_{2A} mRNA, an amplified band of the expected size (confirmed by sequencing), was detected in mRNA preparations of ovary and cumulus cells, but not in oocytes or pre-implantation embryos of any developmental stage (Fig. 1A, middle panel).

In addition to the known mouse 5-HT_7 sequence, to which our band (called a) was identical (as verified by sequencing), we detected 2 additional isoforms (herein called isoforms b and c, sequences deposited in GenBank #AY450670 and AY450671) that had not been described previously for the mouse, but are homologous to rat isoforms b and c, known to result from alternative splicing (Heidmann *et al.*, 1997). Mouse isoform a mRNA disappears by the 8-cell stage, whereas isoforms b and c disappear sooner, by the 4-cell and 2-cell stages respectively (Fig. 1A, upper panel).

Figure 1B compares the deduced C-terminal amino acid sequence of mouse, rat and human 5-HT_7 receptor isoforms. As already described, mouse and rat isoforms a are identical in their C-terminal region while human isoform a differs for 5 amino acids (Fig. 1B, underlined). The isoform b that we identified in the mouse is identical to rat isoform b and differs only by 1 amino acid from the human sequence. For the last isoform, the C-terminal tail following the

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alternative splicing site differs between rats (isoform c) and humans (isoform d) and yields 2 different C-terminal sequences with no homology. The sequencing of this last isoform in the mouse showed that the mouse expresses an isoform c which differs only for 6 amino acids compared to the rat. As with rat isoform c, mouse isoform c has no homology with human isoform d (Fig. 1B).

Expression of 5-HT_{2B}, 5-HT_{2C}, 5-HT_4 and 5-HT_6 receptor mRNAs in metaphase II oocytes and cumulus cells

A similar nested RT-PCR strategy was also conducted to verify the mRNA expression of the other G_q-coupled receptor subtypes, 5-HT_{2B} and 5-HT_{2C}, and G_s-coupled receptor subtypes 5-HT_4 and 5-HT_6. None of these receptors could be detected in metaphase II oocytes (Fig. 2, lane 1). In cumulus cell preparations, the 5-HT_{2B} receptor mRNA could be detected but not the 5-HT_{2C}, 5-HT_4 or 5-HT_6 receptor mRNAs (Fig. 2, lane 2). Appropriate positive and negative controls were also conducted with, respectively, brain preparations (Fig. 2, lane 3) and preparations without cDNAs for each receptor (Fig. 2, lane 4).

Expression of 5-HT_{2A} and 5-HT_7 receptor proteins in COCs and embryos

In addition to 5-HT_7 and 5-HT_{2A} mRNAs, we verified the presence of corresponding proteins by Western blotting and indirect immunofluorescence microscopy, utilizing specific antibodies for each receptor. Figure 3 depicts the positive detection of both receptors, at expected size ranges, with a common band (53 kDa) for 5-HT_7 in cumulus cells and isolated oocytes (Fig. 3A), and a doublet of bands (51-63 kDa) for 5-HT_{2A} in cumulus cells but not in oocytes (Fig. 3B). With the same antibodies under indirect immunofluorescence microscopy, COCs exhibited

strong 5-HT_{2A}-associated peripheral immunoreactivity in cumulus cells (Fig. 4A-A'), but no staining of enclosed or isolated oocytes (Fig. 4A-A' and 4B-B'), 4-cell embryos (Fig. 4C-C') or blastocysts (data not presented). No cell labeling was found when the anti-5-HT_{2A} antibody had been previously depleted by pre-incubation with a 5-HT_{2A} blocking peptide or when only a secondary antibody was used (data not shown). In contrast, 5-HT_7-associated immunoreactivity in COCs revealed strong oocyte labeling compared to weaker labeling at the periphery of cumulus cells (Fig. 5A-A'). Isolated oocytes also showed strong immunoreactive 5-HT_7-labeling (Fig. 5B-B'). In 4-cell embryos, in which 5-HT_7 mRNA was at the limit of detection, we could still identify strong immunoreactivity associated with each blastomere (Fig. 5C-C'), whereas blastocysts, in agreement with our RT-PCR detecting no 5-HT_7 mRNA, did not display any labeling with the anti-5-HT_7 antibody (data not presented). In controls, no labeling of any cell type was seen when the first antibody was omitted (data not reported).

Serotonin- and agonist-induced cAMP elevation in COCs

Since the 5-HT_7 receptor is known to be coupled to a rise of cAMP through G_s (Shen *et al.*, 1993), we measured cAMP levels of COCs and metaphase II oocytes after 5-HT treatments. COCs were treated for 5 min with different concentrations of 5-HT in M2 medium containing IBMX to inhibit endogenous phosphodiesterases. Treatments of COCs with 10 nM or 100 nM 5-HT produced, respectively, 19% and 29% rises in their cAMP content, but these differences were not statistically significant (Fig. 6A). Higher doses of 1 μM and 10 μM 5-HT resulted in significant increases of 67% ($p<0.01$) and 79% ($p<0.001$). Incubating isolated oocytes in M2 medium containing 1 μM or 10 μM 5-HT did not affect their cAMP content while incubation in 10 μM forskolin resulted in a robust elevation of nearly 300% (Fig. 6B). Since 5-HT failed to

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raise the cAMP content of isolated oocytes, the cAMP increase observed in COCs is likely attributable to cumulus cells through activation of one or multiple 5-HT receptors.

We thus decided to test the effect of different agonists on the cAMP content of COCs, targeting G_s-coupled 5-HT_4, 5-HT_6 and 5-HT_7 receptors. We first used 5-CT, which has mixed 5-HT_6 and 5-HT_7 affinities (Kohen *et al.*, 1996; Shen *et al.*, 1993). Exposure to 1 μM or 10 μM 5-CT increased the cAMP content of COCs by 12% and 30%, respectively, with only the 10-μM dose yielding a significant increment ($p<0.01$, Fig. 7A). With 1 or 10 μM 8-OH DPAT, a 5-HT_{1A} and 5-HT_7 agonist (Stam *et al.*, 1992), the cAMP content of COCs rose by 18% and 36% ($p<0.05$), respectively (Fig. 7B). Next, 1 μM or 10 μM PPB, a 5-HT_4 agonist (Ramirez *et al.*, 1997), did not significantly elevate the cAMP content of COCs (Fig. 7C). Finally, since cumulus cells also express 5-HT_{2A} and 5-HT_{2B} receptors, the 5-HT_2 agonist α-methyl-5-HT (Baxter *et al.*, 1995) was tested and induced increases in cAMP of 13% (1 μM) and 34% (10 μM, $p<0.01$) (Fig. 7D). The estimated order of potency was thus 5-HT > 8-OH DPAT = α-methyl-5-HT = 5-CT > PPB, which excludes the possibility of a 5-HT_4 receptor and strongly suggests the involvement of the 5-HT_7 receptor, since 8-OH DPAT was able to evoke a cAMP rise, in agreement with our RT-PCR study in which the only G_s-coupled receptor mRNA detected was the 5-HT_7 receptor (Figs. 1-2).

Serotonin-induced Ca^{2+} rise in COCs

Expression of the 5-HT_{2A} and 5-HT_{2B} receptor in cumulus cells led us to investigate the effect of 5-HT on the Ca^{2+} level of cumulus cells, since these receptors are known to be coupled to an increase in intracellular Ca^{2+} through G_q in other cell types. Dispersed cumulus cells were

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constantly perfused for the duration of the recording and, when adding 1 μM 5-HT, an increment of intracellular Ca^{2+} was detected in 56% of the cells (Fig. 8A, Table 1). At the end of each experiment, the cells were perfused with 200 μM ATP, and a strong Ca^{2+} increase was observed in 92% of them. This provided a positive control, since it is known that cumulus cells express a P2Y2 receptor whose activation results in Ca^{2+} increases (Webb *et al.*, 2002a). Intracellular Ca^{2+} chelation with 50 μM BAPTA-AM before the 5-HT perfusion completely blocked the Ca^{2+} rise (Fig. 8D). Table 1 summarizes the characteristics of the 5-HT responses observed including the dose-response effect of 5-HT on the amplitude of the Ca^{2+} elevation (positive correlation: R^2=0,081 and P<0,0001), the percentage of reacting cells, and the effect of a BAPTA pre-incubation. The time delay of this Ca^{2+} increase was relatively short, occurring always within the first 12 sec of perfusion with the 5-HT-containing solution. When a sharp spike was observed, it lasted for 20-25 sec, and a long recovery time of approximately 60 sec was needed to return to the original Ca^{2+} level. Experiments carried out with 100 nM or 10 nM 5-HT resulted in lower Ca^{2+} rises, but the time delay of the response, the duration of the peak and recovery time were similar to the 1 μM-dose response (Fig. 8B and 8C respectively). 5-HT at 1 nM was also tested but clear Ca^{2+} rises were not detectable over the background (data not shown) and the 10 nM dose was considered the critical minimum concentration. It has been reported previously that, in hamster oocytes, 5-HT triggers Ca^{2+} increases that are sensitive to 5-HT_2 antagonists (Miyazaki *et al.*, 1990). Even though we did not detect any 5-HT_2 receptor in mouse oocytes, we decided to investigate the effect of 5-HT on the Ca^{2+} level of mouse oocytes. Perfusion with 10 μM 5-HT failed to elicit any Ca^{2+} upsurge (0/7, Table 1) even though these oocytes could respond to a 100 μM carbachol dose as a positive control (7/7).

Cross-talk between Ca^{2+} and cAMP in COCs

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Finally, since 5-HT regulates the cAMP and calcium levels of cumulus cells, we decided to investigate possible cross-talk between these 2 signaling pathways. When COCs were exposed to 5 μM ionomycin for 5 min to raise intracellular Ca^{2+}, a 280% increase in their cAMP content was observed, indicating that solely augmenting intracellular Ca^{2+} somehow activated endogenous adenylate cyclase, resulting in elevated cAMP (Fig. 9A). This cAMP increment could be prevented by pre-incubation in the presence of BAPTA, confirming the Ca^{2+}-specificity of this ionomycin-induced cAMP rise. We further evaluated whether ionomycin could increase the cAMP level of isolated oocytes. Figure 9B shows that the oocyte cAMP level is not affected by ionomycin, suggesting that the cAMP elevation in COCs is, in this condition, again attributable only to cumulus cells. When COCs were incubated in the presence of BAPTA before the 5-HT addition, the cAMP increment was limited to 36% ($p<0.01$ vs control) rather than 60% ($p<0.001$ vs control), but the difference between the 2 conditions was not statistically significant (Fig. 9A). Taken altogether, these results suggest that when COCs were incubated in the presence of BAPTA, the cAMP rise induced by 5-HT was somewhat lower but still significant over untreated control cells (Fig. 9A). Part of the cAMP increase induced by 5-HT in cumulus cells could thus be due to an augmentation of intracellular Ca^{2+}. However, intracellular Ca^{2+} chelation does not preclude part of the 5-HT-induced rise in cAMP which, expectedly, is largely Ca^{2+}-independent.

Discussion

The present work extends our previous demonstration of a local serotonergic network in mouse cumulus-oocyte complexes and early embryos, including the presence of 5-HT itself, of the 5-HT synthesizing enzyme TPH1 in cumulus cells, and a 5-HT-specific uptake driven by a classical antidepressant-sensitive transporter within oocytes and embryos (Amireault and Dubé, 2005). We further show here that 5-HT might exert its local effect through 5-HT_{2A}, 5-HT_{2B} and 5-HT_7 receptors in cumulus cells, oocytes and embryos, and that 5-HT affects intracellular Ca^{2+} and cAMP in cumulus cells as expected from the activation of these identified receptors. Our work therefore completes the panel of required components for a local functional serotonergic network and confirms or extends scattered reports involving 5-HT in reproductive tissues or cells.

We have thus shown that 5-HT induces a dose-dependent increase of cAMP in mouse cumulus cells most likely through a 5-HT_7 receptor. This supports the previous demonstration that 5-HT could elevate the cAMP content of human granulosa-lutein cells in culture, and their progesterone secretion, through activation of a 5-HT_7 receptor (Graveleau *et al.*, 2000). The expression of a 5-HT_7 receptor in these closely-related cell types, from two species, suggests that it might be universally expressed in mammalian follicles. Cyclic AMP in granulosa cells is already known to transduce the effects of FSH and LH and, thus, turns on multiple distinct pathways, depending on the maturational stage of the follicle (Conti, 2002). Our present work adds 5-HT as a new potential intermediate in these processes turned on by cAMP. Our pharmacological and molecular studies further confirm this assumption, since 5-CT and 8-OH

DPAT, both 5-HT_7 agonists, could increase the cAMP content of cumulus cells while 5-HT_4 and 5-HT_6 receptor mRNAs could not be detected in these cells.

We detected both mRNA and protein of the 5-HT_7 receptor from germinal vesicle stage oocytes to 4-cell embryos. However, after adding 5-HT to isolated metaphase II oocytes, in contrast to cumulus cells, none of the expected cAMP increment was detectable, suggesting little if any activity of the 5-HT_7 receptor at this specific stage which also shows internal rather than peripheral receptor immunostaining, a condition already reported for inactive and internalized 5-HT_7 receptor in rat brain (Muneoka and Takigawa, 2003). Still, it remains possible that an oocyte 5-HT_7 receptor might be active at earlier maturational stages when a tighter communication network with surrounding cells is most necessary to further oocyte progression. In this respect, recent evidence indicates that active maintenance of oocytes in prophase I, before ovulation, requires high cAMP, a tight communication with somatic cells, and constant G_s protein activity in mouse oocytes (Mehlmann *et al.*, 2002; Kalinowski *et al.*, 2004). This constant G_s protein activity was shown to rely on the orphan GPR3 receptor since most oocytes (~90%) from *Gpr3* knockout mice resume meiosis prematurely within antral follicles (Mehlmann *et al.*, 2004). If an additional oocyte G_s-linked receptor was participating in the maintenance of meiotic arrest, as suggested by these authors (Mehlmann *et al.*, 2004), at similar or more likely earlier follicular stages, then the G_s-linked 5-HT_7 receptor reported here would be a candidate fulfilling some of the expected attributes with its ligand, 5-HT, being produced by neighbouring somatic cells (Amireault and Dubé, 2005). Alternatively, whether the 5-HT_7 receptor becomes functional at later stages, e.g. in cleavage-stage embryos, remains to be established. In this respect, it is noteworthy that 5-HT antagonists were reported to block or inhibit the progression of early cleavage divisions while 5-HT prevents this effect (Buznikov *et*

al., 1996) whereas other studies reported a negative effect on later blastocyst formation following exposure to 5-HT (Ill'kova *et al.*, 2004) or to the agonist sumatriptan (Vesela *et al.*, 2003). Along this line, interestingly, we detected three distinct 5-HT_7 receptor isoforms in mouse oocytes and embryos that appear to be homologous to known rat (a,b,c) or human (a,b) isoforms (Heidmann *et al.*, 1997). While these three isoforms were not found to differ significantly one with another in their pharmacological properties or functions (Heidmann *et al.*, 1998), their respective abundance observed in various tissues in the rat (most abundant a, then b, then c isoform) seems conserved for mouse oocytes and embryos, with the sequential disappearance of the isoforms (c, b, then a), in our RT-PCR study, possibly reflecting an earlier decline, below a detectable threshold level, of the least expressed isoforms in 2-cell and 4-cell embryos. Therefore, the reported serotonergic effects at the blastocyst stage are unlikely to be linked to the 5-HT_7 receptor whose mRNA has long disappeared by that time but could be due, as suggested, to a 5-HT_{1D} receptor whose effective expression would however require additional confirmation (Vesela *et al.*, 2003).

One surprising finding in cumulus cells was that the 5-HT_2 agonist α-methyl-5-HT could also increase their cAMP content. However, this α-methyl-5-HT-induced cAMP rise might involve the observed cross-talk between Ca^{2+} and cAMP signaling in these cells. Indeed, the large increment of cAMP seen after ionomycin treatment of cumulus cells in COCs, and blocked by BAPTA, reveals a Ca^{2+}-sensitive effect on cAMP levels. An elevation of intracellular Ca^{2+} could lead to such a cAMP increase through activation of calmodulin-sensitive adenylate cyclase isoforms I and VIII (Taussig and Zimmermann, 1998). These adenylate cyclases have never been reported in mouse cumulus cells, but they are expressed in human granulosa cells (Asboth *et al.*, 2001), and could link an α-methyl-5-HT-induced Ca^{2+} rise, through a 5-HT_2

receptor, to increased cAMP. Also, part of the 5-HT-evoked cAMP elevation in cumulus cells could be mediated by this Ca^{2+} increment since BAPTA-pretreated cells showed a smaller rise in cAMP after 5-HT addition.

We investigated the presence of 5-HT_{2A-B-C} receptors in oocytes, embryos and cumulus cells because of the known capacity of 5-HT to cause Ca^{2+} increases in hamster oocytes (Miyazaki *et al.*, 1990; Fujiwara *et al.*, 1993). Our various data clearly establish that none of the 5-HT_2 receptors is expressed in oocytes in agreement with the fact that their Ca^{2+} level is 5-HT-insensitive in the mouse, in contrast to the hamster (our data not shown, and S. Miyazaki, personal communication). Therefore, a species difference exists between the mouse and hamster that presumably reflects a differential expression of the 5-HT_2 and/or 5-HT_7 receptors in mammalian oocytes. Indeed, we have detected, by RT-PCR, 5-HT_{2A} mRNA, but not 5-HT_7 mRNA, in hamster metaphase II oocytes whereas hamster cumulus cells express both subtypes as in the mouse (golden hamster 5-HT_{2A} and 5-HT_7 receptor cDNAs were cloned and sequenced, see GenBank, accession numbers DQ015678 and DQ015679, Amireault and Dubé, unpublished results). This explains the observed Ca^{2+}-mobilizing effect of 5-HT in hamster but not mouse oocytes and, additionally, underscores the possibility of species differences in the type(s) of 5-HT receptors expressed in oocytes from diverse mammalian species. This also lends support to the possibility that human oocytes might indeed express a 5-HT_{2A} receptor as suggested by the detection of a 5-HT_{2A}-specific expressed sequence tag (EST, Neilson *et al.*, 2000).

On the other hand, the expression of the 5-HT_{2A} and 5-HT_{2B} receptor in cumulus cells reveals that these receptors could be involved in follicle growth and steroidogenesis. Indeed, 5-HT has been demonstrated to stimulate estradiol secretion in rat pre-ovulatory follicles, and this could be inhibited by ketanserin, a preferential 5-HT_{2A} antagonist (Tanaka *et al.*, 1993). Also, 5-HT_{2A}

receptor densities increase in the rat forebrain at the time of the spontaneous estrogen-induced LH surge, compared to diestrous females (Sumner and Fink, 1997), while ovariectomy reduces 5-HT_{2A} receptor mRNA and protein in the rat frontal cortex (Bethea *et al.*, 1998). Thus, 5-HT_{2A} receptors expressed in cumulus cells could promote steroidogenesis and could be regulated by steroids in a feedback loop, leading to coordinated follicle maturation. It seems likely that the Ca^{2+} responses of cumulus cells to 5-HT are largely mediated by a 5-HT_2 receptor. Preliminary experiments showing that α-methyl-5-HT, an agonist for 5-HT_{2A-B-C} receptors (Baxter *et al.*, 1995) can induce calcium responses in cumulus cells further confirm this assumption (data not shown). Our RT-PCR analysis, showing the expression of both 5-HT_{2A} and 5-HT_{2B} receptor, but not of the 5-HT_{2C} receptor, makes these two receptors likely candidates to generate the observed Ca^{2+} responses in cumulus cells. However, 5-HT can also evoke Ca^{2+} rises in HEK 293 cells transfected with the 5-HT_7 receptor (Baker *et al*., 1998). Hence, the activation of all three 5-HT receptors expressed by cumulus cells could generate, at least in part, the observed Ca^{2+} responses after 5-HT addition, even though this is not supported by the lack of effect of 8-OH DPAT on their Ca^{2+} levels (data not shown). The expression of 5-HT_{2A}, 5-HT_{2B} and 5-HT_7 receptors that we report here does not exclude the potential expression of other 5-HT receptor subtypes in both oocytes and cumulus cells, other than the 5-HT_{2C}, 5-HT_4 and 5-HT_6 receptors not detected here, with possible species differences, as mentioned earlier. Further investigations on this local ovarian serotonergic network should therefore include a more thorough survey of other potential 5-HT receptors that might be expressed, along with pharmacological analyses that are however hindered by the heterogeneity of cell populations expressing multiple 5-HT receptors and exhibiting interconnected signaling pathways such as that linking cAMP and Ca^{2+} increases.

In the *in vivo* context, the serotonergic network displayed in mouse COCs, could be implicated in the autocrine and paracrine regulation of coordinated follicular growth, known to involve bidirectional communication between the oocyte and the somatic compartment, but through communicating channels still largely unresolved (Picton *et al.*, 1998; Eppig, 2001). This local ovarian serotonergic network might regulate the cAMP and Ca^{2+} levels not only of cumulus cells, but also of the oocytes themselves, either directly through their expressed 5-HT_7 receptor, or indirectly through the physical bridging with cumulus cells by gap junctions.

In conclusion, our work demonstrates that 5-HT can regulate the Ca^{2+} and cAMP levels of mouse cumulus cells most likely through expressed 5-HT_{2A}, 5-HT_{2B} and 5-HT_7 receptors. Also, oocytes and embryos up to the 4-cell stage express the 5-HT_7 receptor, but further work is needed to determine at which stage(s) this receptor is functional. Such uncovering of an ovarian local serotonergic network opens new avenues for understanding the intricate processes underlying follicle maturation, meiotic maturation and, eventually, early embryonic development.

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Footnotes

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Figure legends

Figure 1. Expressions of 5-HT_7 and 5-HT_{2A} receptors mRNAs in cumulus cells, oocytes and embryos and C-terminal amino acid sequences of mouse, rat and human 5-HT_7 receptor isoforms. (A) RT-PCR-amplified bands for 5-HT_7 (isoforms a, b and c), 5-HT_{2A} receptors and actin control obtained with mRNA extracted respectively from (lanes 1 to 11) total ovaries (1), isolated cumulus cells (2), germinal vesicle stage oocytes (3), metaphase II oocytes (4), 1-cell (5), 2-cell (6), 4-cell (7), and 8-cell embryos (8), morulas (9), blastocysts (10) and a representative negative control sample without cDNA (11). Depicted bands, at their expected sizes as described in Materials and Methods, were the only ones detected in three to four separate determinations. (B) Comparison of the deduced C-terminal amino acid of the mouse with its rat and human homologues. Amino acid differences are underlined.

Figure 2. Expressions of 5-HT_{2B}, 5-HT_{2C}, 5-HT_4 and 5-HT_6 receptor mRNAs in oocytes and cumulus cells. RT-PCR-amplified bands for 5-HT_{2B}, 5-HT_{2C}, 5-HT_4 and 5-HT_6 receptors and actin control obtained with mRNA extracted respectively from (lanes 1 to 4) metaphase II oocytes (1), isolated cumulus cells (2), brain (3) and a representative negative control sample without cDNA (4). Depicted bands, at their expected sizes as described in Materials and Methods, were the only ones detected in three to four separate determinations.

Figure 3. Expressions of 5-HT_7 and 5-HT_{2A} proteins in cumulus cells and metaphase II oocytes. Western blots against samples from isolated oocytes (1) or cumulus cells (2) showing a

common band at 53 kDa with an anti-5HT_7 antibody (A) and doublet bands only in cumulus cells with an anti-5-HT_{2A} antibody (B).

Figure 4. Expression of 5-HT_{2A} receptor protein in COCs, isolated metaphase II oocytes and early embryos. Immunofluorescence of cells prepared with an anti-5-HT_{2A} antibody (A'-C') and observed by confocal microscopy. Phase contrast (A-C) and corresponding fluorescence (A'-C') images of a mouse COC (A-A'), isolated metaphase II oocyte (B-B') and 4-cell stage embryo (C-C'). Scale bars, 10 μm.

Figure 5. Expression of 5-HT_7 receptor protein in COCs, isolated metaphase II oocytes and early embryos. Immunofluorescence of cells prepared with an anti-5-HT_7 antibody (A'-C') and observed by confocal microscopy. Phase contrast (A-C) and corresponding fluorescence (A'-C') images of a mouse COC (A-A'), isolated metaphase II oocyte (B-B'), and 4-cell stage embryo (C-C'). Scale bars, 10 μm.

Figure 6. Effect of 5-HT on the cAMP content of mouse COCs and isolated metaphase oocytes. COCs (A) and metaphase II oocytes (B) were submitted to different concentrations of 5-HT for 5 min in M2 medium containing 200 μM IBMX. A 10-μM forskolin treatment was included in the oocyte experiments. Mean results (± S.E.M.) of at least 3 duplicate experiments. **= $p<0.01$, ***= $p<0.001$ compared to the control.

Figure 7. Effect of 5-HT agonists on the cAMP content of mouse COCs. COCs were treated with 5- CT (A), 8-OH DPAT (B), PPB (C) or α-methyl-5-HT (D), for 5 min in M2 medium containing 200 μM IBMX. Mean results (± S.E.M.) of at least 3 duplicate experiments. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ compared to control.

Figure 8. Effect of 5-HT on resting Ca^{2+} concentration in mouse cumulus cells. A typical trace of a cumulus cell response is shown when perfused with M2 medium containing 1 μM (A), 100 nM (B) or 10 nM 5-HT (C). Pre-treatment of the cumulus cells with 50 μM BAPTA AM before the addition of 1 μM 5-HT blocks the Ca^{2+} increase (D). 200 μM ATP was added at the end of each experiment as a positive control. Agonist perfusion time is indicated by the arrow and its duration, by the bar.

Figure 9. Cross-talk between intracellular calcium concentration and cAMP in mouse COCs and metaphase II oocytes. Groups of cells (A: COCs; B: metaphase II oocytes) were treated with 1 μM 5-HT, 5 μM ionomycin or 10 μM forskolin for 5 min in M2 medium containing 200 μM IBMX. Under BAPTA-containing conditions, the cells were pre-treated for 30 min in 50 μM BAPTA-AM before the 5-min treatment. Mean results (± S.E.M.) of at least 3 duplicate experiments. **= $p<0.01$, ***= $p<0.001$ compared to the control.

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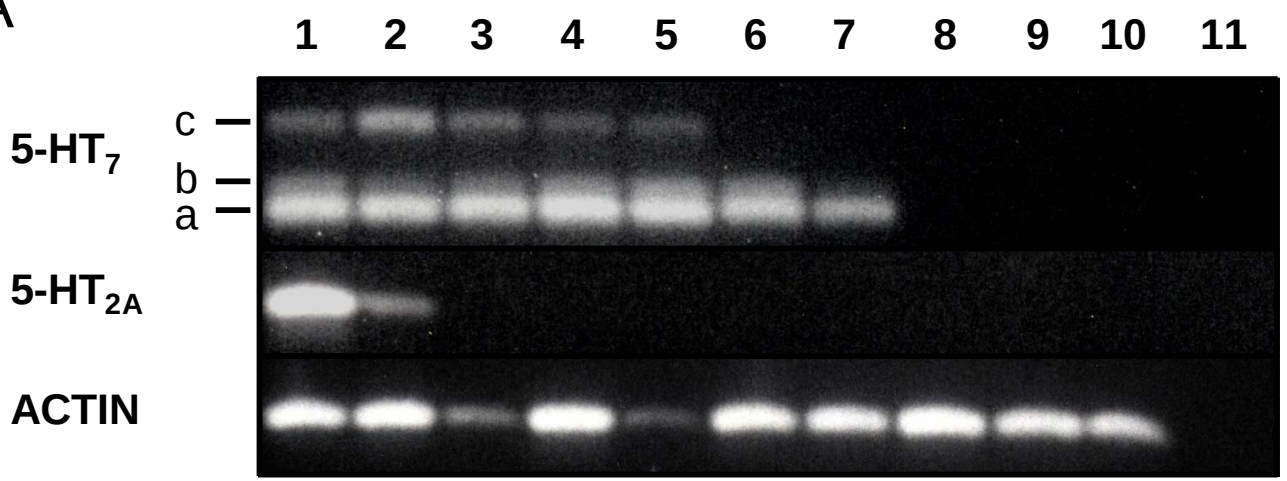
Table 1 : Ca^{2+} measurement, following 5-HT perfusion, in mouse cumulus cells and oocytes.

Agonist	Cell Type	Number of experiments	Number of cells	Number of reacting cells	% of reacting cells	Amplitude Mean response (ratio 340/380) +/- SEM
5-HT 1 µM	Cumulus	7	336	189	56.3	0.34 +/- 0,02
5-HT 100 nM	Cumulus	5	272	128	47.1	0.21 +/- 0,02
5-HT 10 nM	Cumulus	3	115	36	34.5	0.14 +/- 0,01
5-HT 1 µM + BAPTA	Cumulus	3	211	8	3.8	0.09 +/- 0,01
5-HT 10 µM	MII oocyte	3	7	0	0	-

A



B

Alternative splicing site

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5-HT7a mouse   MHEALKLAERPERSEFVL QNSDHCGKKGHDT
5-HT7a rat     MHEALKLAERPERSEFVL QNSDHCGKKGHDT
5-HT7a human   MHEALKLAERPERPEFVL QNADYCRKKGHDS

5-HT7b mouse   MHEALKLAERPERSEFVL
5-HT7b rat     MHEALKLAERPERSEFVL
5-HT7b human   MHEALKLAERPERPEFVL

5-HT7c mouse   MHEALKLAERPERSEFVL MTGASGVQKALENLPWGNGVNTGIKAVNSVALTKL
5-HT7c rat     MHEALKLAERPERSEFVL MTRASGVQQALENFPWGNGMKAGIKAVNSVALTKL
5-HT7d human   MHEALKLAERPERPEFVL RACTRRVLLRPEKRPPVSVWVLQSPDHHNWLADKMLTTVEKKVMIHD
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Figure 1

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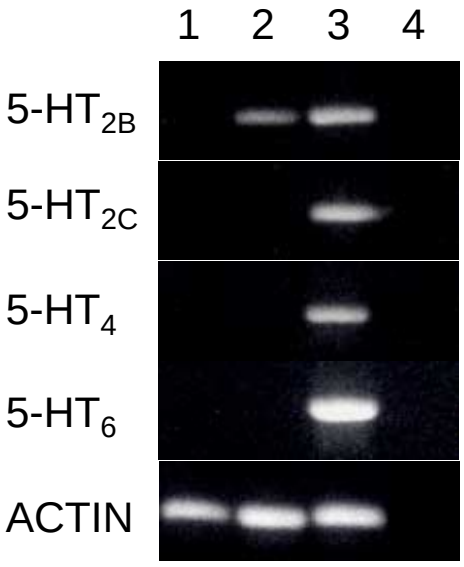

Figure 2

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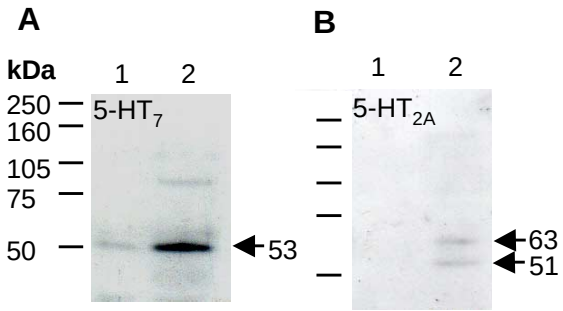

Figure 3

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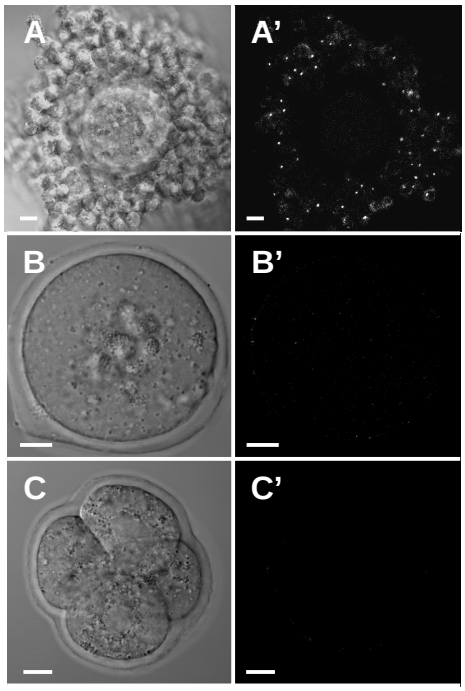

Figure 4

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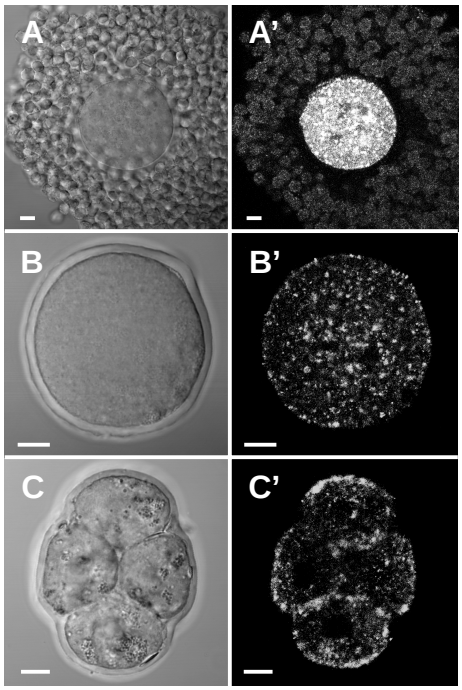

Figure 5

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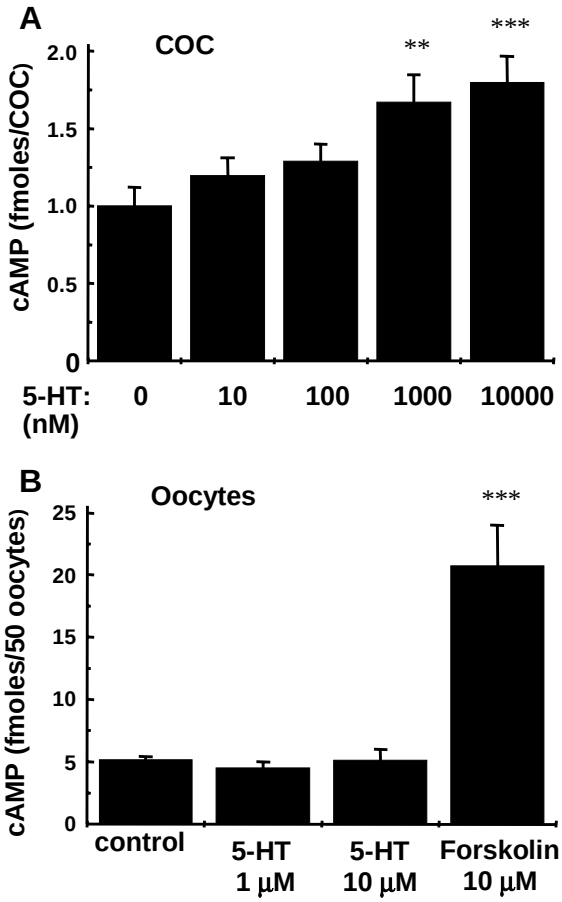

Figure 6

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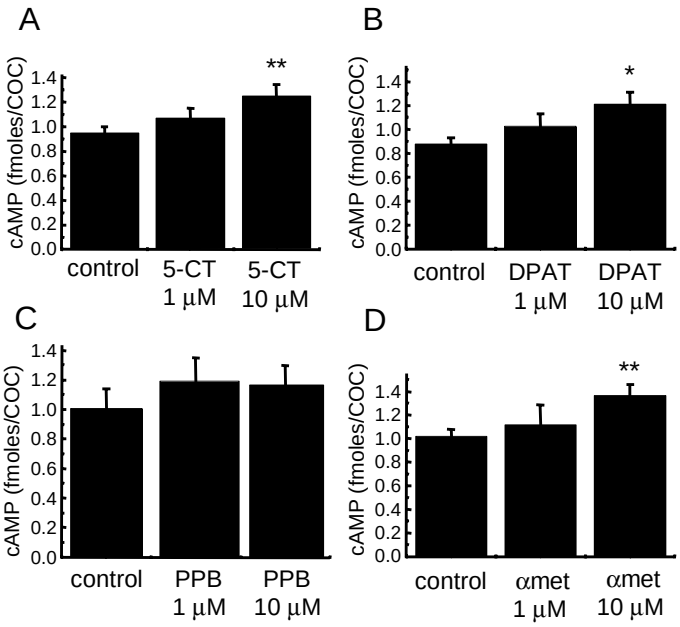

Figure 7

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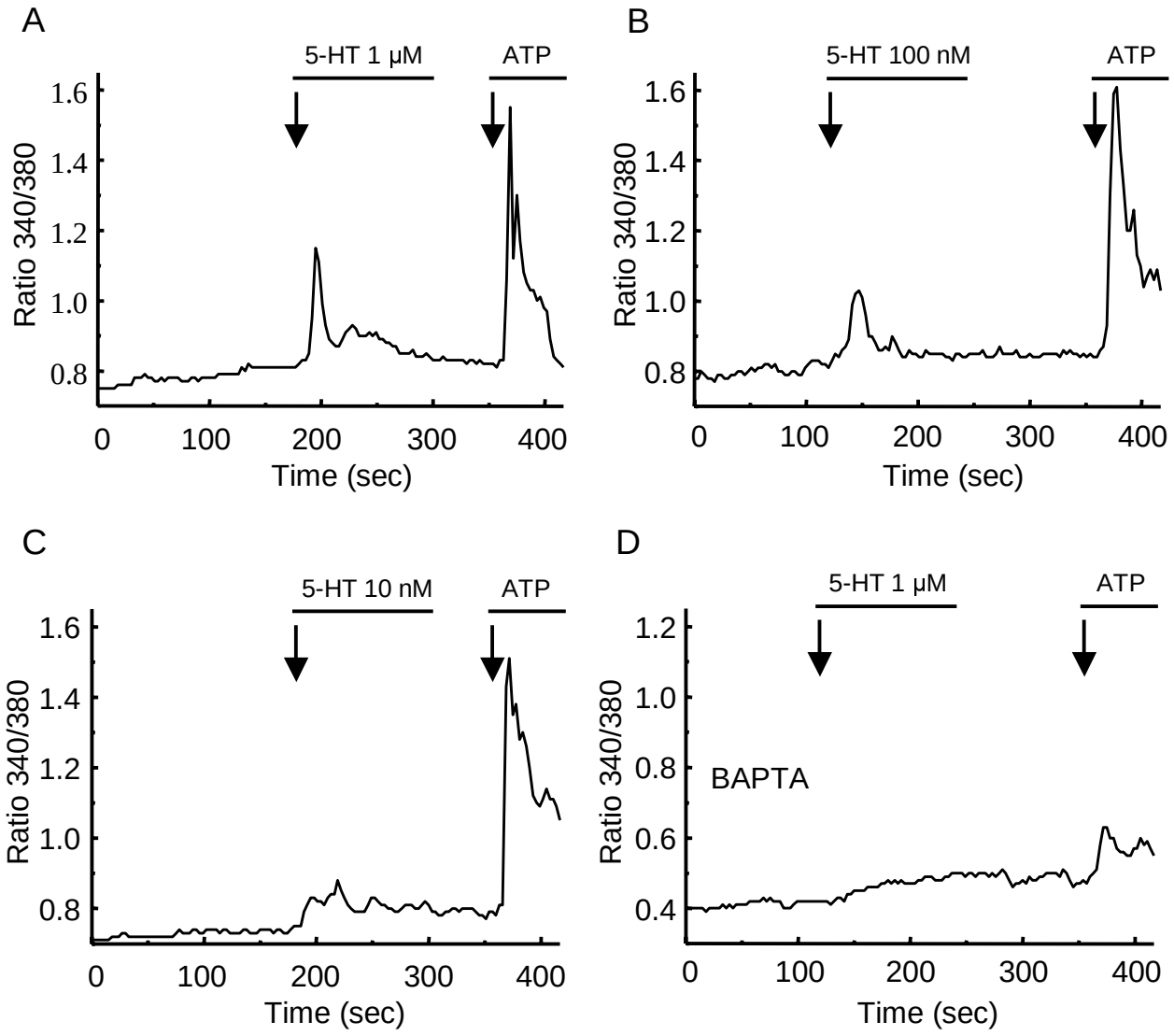

Figure 8

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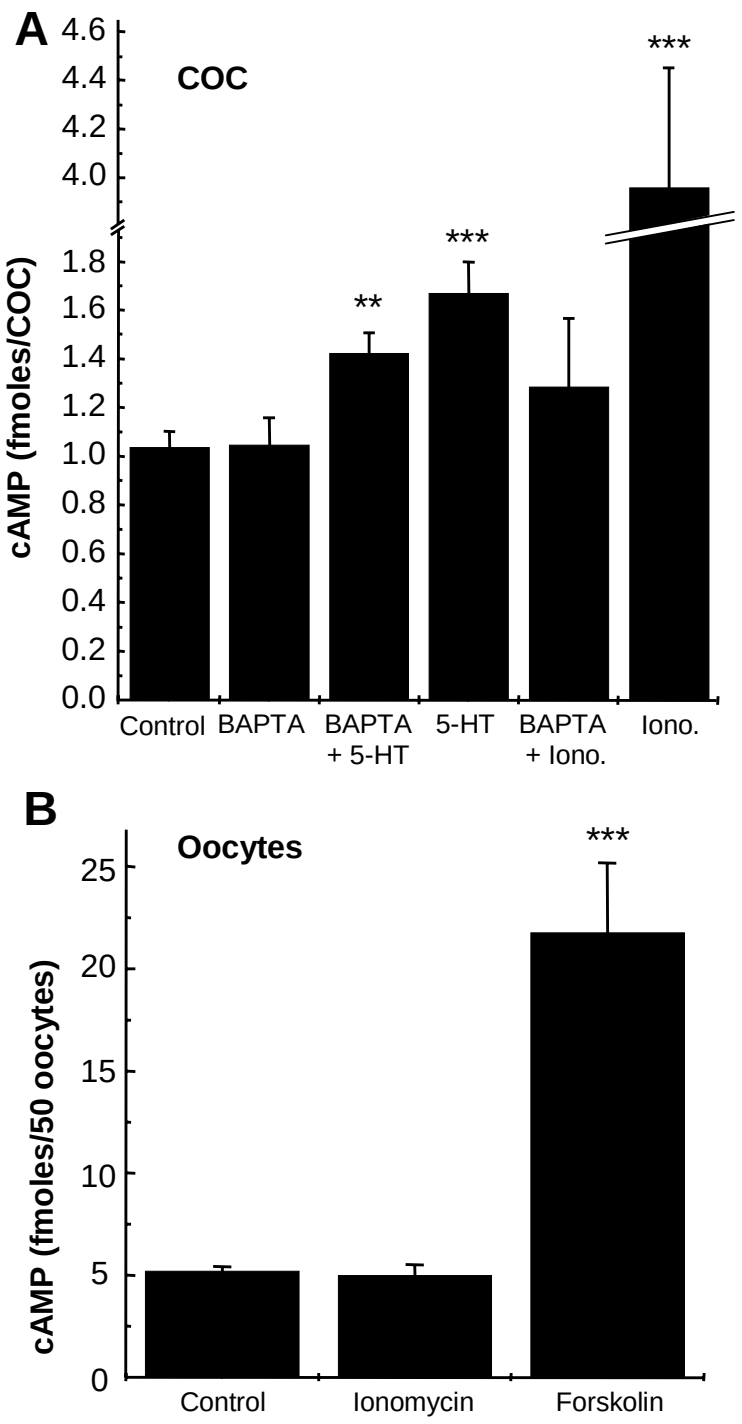

Figure 9