

Intracellular cAMP and Calcium Signaling by Serotonin in Mouse Cumulus-Oocyte Complexes

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ABSTRACT

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 (5-hydroxytryptamine, 5-HT) to regulate intracellular cAMP and Ca^{2+} in mouse oocytes and surrounding cumulus cells. On the basis of a reverse transcription-polymerase chain reaction study, 5-HT₇ receptor mRNA is expressed in cumulus cells, oocytes, and embryos up to the four-cell stage, and 5-HT_{2A} and 5-HT_{2B} receptor mRNAs are expressed in cumulus cells only, whereas 5-HT_{2C}, 5-HT₄, and 5-HT₆ receptors are expressed in neither oocytes nor cumulus cells. The addition of 5-HT (10 nM to 10 μ M) 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-hydroxy-2-(di-*n*-propylamino) tetralin = α -methyl-5-HT =

5-carboxamidotryptamine maleate > 2-[1-(4-piperonyl)piperazinyl]benzo-triazole, thereby supporting a preferential involvement of 5-HT₇ receptors. As measured with cumulus cells preloaded with fura-2/acetoxymethyl ester (AM), the addition of 5-HT also caused dose-dependent Ca^{2+} increases, which were probably 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 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)-AM abolished the 5-HT-induced Ca^{2+} increase and reduced the cAMP increase, 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-HT₇ receptors.

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 seems to be provided, in part, by granulosa cells tightly connected to oocytes through gap junctions, allowing the 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 follicle-stimulating hormone-induced expressions of cytochrome P450 side-

chain cleavage enzyme, 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-hydroxytrypta-

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ABBREVIATIONS: LH, luteinizing hormone; 5-HT, 5-hydroxytryptamine; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; AM, acetoxymethyl ester; IBMX, 3-isobutyl-1-methylxanthine; 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; α -methyl-5-HT, α -methyl-5-hydroxytryptamine maleate; D-PBS, Dulbecco's phosphate-buffered saline; bp, base pair(s).

mine, 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 of follicular growth in fishes (Cerdeira 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 reported recently in isolated mouse oocytes and embryos (Il'kova et al., 2004; Amireault and Dubé, 2005) and in surrounding cumulus cells that also possess the rate-limiting enzyme tryptophan hydroxylase for 5-HT production, thus making these cells a potential immediate direct source of 5-HT (Amireault and Dubé, 2005). In addition, in vitro, 5-HT promotes estradiol secretion by rat (Tanaka et al., 1993) and hamster (Terranova et al., 1990) preovulatory 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 of 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 to be established.

Mammalian 5-HT receptors are divided into seven subfamilies (5-HT₁₋₇) sharing common sequences, pharmacological properties, and signaling pathways, and most of them are G-protein-coupled receptors regulating cAMP or intracellular Ca^{2+} . For example, 5-HT₁ receptors are coupled preferentially to G_{i/o} to inhibit cAMP formation (Barnes and Sharp, 1999), whereas 5-HT₄, 5-HT₆, and 5-HT₇ receptors are coupled to G_s and, hence, positively regulate adenylate cyclase, causing cAMP increases when activated (Hamblin et al., 1998). 5-HT₂ receptors are coupled to G_q and are 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-HT₇ receptor was detected in cultured human granulosa-lutein cells (Graveleau et al., 2000), in which 5-HT promotes the expected cAMP elevation. In addition, in isolated metaphase II hamster oocytes, 5-HT induces intracellular Ca^{2+} oscillations sensitive to 5-HT₂ antagonists (Miyazaki et al., 1990), suggesting the presence of this receptor type in oocytes. Moreover, a polymerase chain reaction (PCR)-serial analysis of gene expression study reported the expression of 5-HT_{2A} receptor mRNA in human oocytes (Neilson et al., 2000), and a reverse transcription (RT)-PCR analysis suggested the expression of 5-HT_{1D} receptor mRNA in mouse oocytes and embryos (Vesela et al., 2003).

All of these findings support an involvement of 5-HT and some of its receptors in various key processes in oocytes and surrounding cells, probably involving 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 and to identify the 5-HT receptors regulating these effects, with special attention given to those two G_q and G_s subtypes, 5-HT₂ and 5-HT₇, detected previously in other mammalian reproductive tissues or cells.

Materials and Methods

Oocyte and Embryo Collection. Fully grown germinal vesicle stage oocytes, ovulated metaphase II oocytes, and preimplantation embryos at various stages were obtained from 3- to 4-week-old female B6C3F1 mice (Charles River, Margate, Kent, UK) after standard gonadotropin injection. For germinal vesicle-stage oocytes, the mice were primed with 5 IU of pregnant mare's serum (Sigma-Aldrich, St. Louis, MO), and cumulus-enclosed, fully grown oocytes were collected 46 to 48 h later by puncturing 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 5 IU of human chorionic gonadotropin (pregnynl-human chorionic gonadotropin; Organon Canada Ltd., Scarborough, ON, Canada) injection, and cumulus-enclosed eggs (COC-metaphase II) were collected from the oviduct 18 to 20 h later in M2 medium. When needed, the cumulus cells were dispersed in M2 medium containing 10 mg/ml bovine testis hyaluronidase (Sigma-Aldrich), 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 the oviducts or uteri with M2 medium by using a 30-gauge needle mounted on a syringe. The timing of embryo collection was as follows: one-cell embryo, 19 h after human chorionic gonadotropin; two-cell embryo, 43 h; four-cell embryo, 50 h; eight-cell embryo, 67 h; morula, 74 h; and early blastocysts, 91 h.

mRNA Isolation and RT-PCR. Collected cells were incubated in acidic Tyrode's solution (Sigma-Aldrich) to remove the zona pellucida of germinal vesicle oocytes, metaphase II eggs, and one-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 to 50 COC-metaphase II were isolated according to the microscale protocol with the Dynabeads mRNA Direct kit (DynaL Biotech, Lake Success, NY). The mRNAs were reverse-transcribed using Superscript II enzyme (Invitrogen, Carlsbad, CA) 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 s at 95°C , primer annealing of 90 s at 65°C (5-HT₇ and 5-HT_{2A}), or 60°C (5-HT_{2B}, 5-HT_{2C}, 5-HT₄, and 5-HT₆), and primer extension of 90 s at 72°C (last primer extension of 15 min). The second PCR run was performed with .10 μl of products from the first amplification and the same PCR program (28 cycles). For 5-HT₇, two 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 used were the following: first forward, 5'-cagccaacacaagttctcag-3'; first reverse, 5'-ccctgttctgcattacttctt-3'; second forward, 5'-tccagtccagtcaggaatatacaac-3'; and second reverse, 5'-tacttcttccagggtccgctct-3'. For 5-HT_{2A}, two pairs of primers were used in a nested PCR strategy to produce amplicons of 627 bp (forward, 5'-tcttctccacggcatcatcatgacac-3', and reverse, 5'-caaacacattgagcagggtccaatgac-3') and 419 bp (forward, 5'-accatagccgcttcaactccagaacc-3', and reverse, 5'-tgcttttgcctattgctgatgagctgc-3'). For 5-HT_{2B}, two pairs of primers were used in a nested PCR strategy to produce amplicons of 678 bp (forward, 5'-tgtctgaacaagaacacaaacttctgagc-3', and reverse 5'-catgatgtgtgagaggtacgaagaag-3') and 451 bp (forward, 5'-actcagtagcagaggaatgaagcaga-3', and reverse, 5'-gcgatgcctattgaaattaaccatacc-3'). For 5-HT_{2C}, two pairs of primers were used in a nested PCR strategy to produce amplicons of 658 bp (forward, 5'-gcagtagcgaatcattgagcatagacc-3', and reverse, 5'-ttttgttgagagaggtgacacagagg-3') and 411 bp (forward, 5'-ttcttcattccggtgacaaattatgg-3', and reverse, 5'-cacatagccaatcaaacacacaca-3'). For 5-HT₄, two pairs of primers were used in a nested PCR strategy to produce amplicons of 649 bp (forward, 5'-ctaattgtgagttccaacagggttttc-3', and reverse, 5'-tgctccttagcagtgacata-

gattcg-3') and 512 bp (forward, 5'-gttctctgcagtggttatctctgatg-3', and reverse, 5'-tgatagcatagggctgttgaccat-3'). For 5-HT₆, two pairs of primers were used in a nested PCR strategy to produce amplicons of 790 bp (forward, 5'-caacacgtctaactcttctctgtgt-3', and reverse, 5'-gatgataggggttcatgggtctattacag-3') and 454 bp (forward, 5'-ctaactcttctctgtgtcgtcttc-3', and reverse, 5'-aagatctgcagtaggtgaagcagat-3'). Primers for β -actin as positive controls yielded 540-bp (forward, 5'-gtggcgctctctaggcaccac-3', and reverse, 5'-ctcttctgatgtcagcagcagatttc-3') and 277-bp (forward, 5'-tgtgatgggtggaatgggtcagaaggac-3', and reverse, 5'-tacgtacatggctgggtgtgaagg-3') amplicons. Twenty-five microliters of each reaction was loaded on agarose gel stained with ethidium bromide. Each amplification was executed at least three times, yielding similar results. All PCR products obtained were cloned in pCRII (Invitrogen) and were sequenced on both strands using the Université Laval sequencing service to confirm the sequence.

Indirect Immunofluorescence Confocal Microscopy for 5-HT₇ and 5-HT_{2A} Detection. Oocytes and embryos were collected and treated as described above and then fixed in fresh paraformaldehyde 4% for 30 min at room temperature. They were washed three 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 5%. They were next incubated overnight at 4°C with a primary antibody in D-PBS/milk 1%/Triton 0.1%/normal goat serum 1%. After three washes in D-PBS, the oocytes and embryos were incubated for 1 h at room temperature in a Cy-3-conjugated goat anti-rabbit antibody (1/2000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and washed three times in D-PBS. Finally, metaphase II-arrested eggs, COC-metaphase II, cumulus cells, and four-cell embryos were mounted with Fluoromount (Electron Microscopy System), and blastocysts were mounted with 50% glycerol in D-PBS. Images were collected with a 63 \times /1.4 oil differential interference contrast plan-apochromat objective and a Zeiss Axiovert 100M microscope coupled with the LSM510 system (Carl Zeiss Inc., Thornwood, NY).

For 5-HT₇, a rabbit anti-rat antibody directed against amino acids 8 to 23 (Calbiochem, San Diego, CA) and diluted 1/200 served as primary antibody. For 5-HT_{2A}, a rabbit anti-rat antibody directed against amino acids 22 to 41 (Calbiochem) 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-HT₇, approximately 300 and 500 oocytes, respectively, and corresponding surrounding cumulus cells were lysed in 30 μ l of 0.5% SDS and kept at -80°C until electrophoresis. The frozen samples were diluted in 4 \times sample buffer, loaded onto 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 three 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 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The same 5-HT₇ (1/500) and 5-HT_{2A} (1/500) antibodies as for indirect immunofluorescence detection were deployed for Western blotting with a goat anti-rabbit-horseradish peroxidase secondary antibody (1/20,000; Bio-Rad, Hercules, CA).

cAMP Extraction and Measurement after Cell Treatments. Groups of 10 COC-metaphase II or 50 metaphase II oocytes were collected, as described above, and were treated for 5 min in small Petri dishes containing a drop of M16 medium supplemented with 200 μ M IBMX, under paraffin oil, in a humidified chamber at 37°C and 5% CO₂. Stock solutions of 10 mM 5-carboxamidotryptamine maleate (5-CT; Tocris Cookson Inc., Ellisville, MO), 5-hydroxytryptamine creatinine sulfate complex (5-HT; Sigma-Aldrich), 8-hydroxy-

2-(di-*n*-propylamino) tetralin (8-OH DPAT; Tocris Cookson), or α -methyl 5-HT maleate (α -methyl-5-HT; Tocris Cookson) were prepared at 10 mM concentration in H₂O and kept in aliquots at -20°C. Stock solutions of 2-[1-(4-piperonyl) piperazinyl]benzothiazole (PPB; Tocris Cookson) at 10 mM in DMSO, forskolin at 10 mM in DMSO, BAPTA-AM (Invitrogen) at 20 mM in DMSO, and ionomycin (Sigma-Aldrich) 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 μ M BAPTA-AM and 0.02% Pluronic F-127 (Invitrogen) for 30 min under paraffin oil in a humidified chamber at 37°C and 5% CO₂ 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 two more times to ensure complete lysis of the cells. Then, 100 μ l of cold 95% ethanol/0.1% trichloroacetic acid was added to each tube, and the cells were centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was evaporated, and the remaining pellet was resuspended in 50 μ l of assay buffer (cAMP Biotrak enzyme immunoassay system kit; GE Healthcare). cAMP was measured according to the acetylation procedure described by the manufacturer.

Intracellular Ca²⁺ Measurement in Oocytes and Cumulus Cells. For cumulus cell Ca²⁺ measurements, COC-metaphase II were collected as described above and incubated in M2 medium containing 5 μ M fura-2/acetoxymethyl ester and 0.02% Pluronic F-127 for 25 min at 37°C. After three washes in M2 medium, the COCs were transferred into a 100- μ l 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, Tokyo, Japan). 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, or 1 μ M 5-HT, or 200 μ M ATP maintained at 37°C was perfused at a rate of 5 ml/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, Novato, CA) and a refrigerated charge-coupled device camera (Photometrics Cool SNAP HQ; Roper Scientific, Trenton, NJ). Excitation wavelengths were 340 and 380 nm, and fluorescence emission was measured at 510 nm. The collected data were then analyzed by Metafluor program 6.1 (Universal Imaging Corporation, Downingtown, PA).

Statistical Analysis. The results of cAMP measurement are expressed as means \pm S.E.M. Each experiment was performed at least three times in duplicate. Statistically significant differences between group means and the control mean were analyzed by unpaired Student's *t* test.

Results

Expression of 5-HT_{2A} and 5-HT₇ Receptor mRNAs in COCs and Embryos. Adopting a nested RT-PCR strategy with oligonucleotides flanking intron sequences and specific for mouse 5-HT₇ mRNA, we detected a band of the proper size in preparations from the ovary, cumulus cells, germinal vesicle stage oocytes, metaphase II oocytes, one-cell embryos, two-cell embryos, and four-cell embryos, but not in eight-cell embryos, morula, and blastocysts (Fig. 1A, top). 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 preimplantation embryos of any developmental stage (Fig. 1A, middle).

In addition to the known mouse 5-HT₇ sequence, with which our band (called a) was identical (as verified by sequencing), we detected two additional isoforms (herein called 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, which are known to result from alternative splicing (Heidmann et al., 1997). Mouse isoform a mRNA disappears by the eight-cell stage, whereas isoforms b and c disappear sooner, by the four- and two-cell stages, respectively (Fig. 1A, top).

Figure 1B compares the deduced C-terminal amino acid sequence of mouse, rat, and human 5-HT₇ receptor isoforms. As described already, mouse and rat isoforms a are identical in their C-terminal region, whereas human isoform a differs for five amino acids (Fig. 1B, underlined). The isoform b that we identified in the mouse is identical with rat isoform b and differs only by one amino acid from the human sequence. For the last isoform, the C-terminal tail after the alternative splicing site differs between rats (isoform c) and humans (isoform d) and yields two different C-terminal sequences with no homology. The sequencing of this last isoform in the mouse showed expression of an isoform c, which differs only in six amino acids compared with 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-HT₄, and 5-HT₆ 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-HT₄ and 5-HT₆. 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-HT₄, or 5-HT₆ receptor mRNAs (Fig. 2, lane 2). Appropriate positive and negative controls were also conducted with brain preparations (Fig. 2, lane 3) and preparations without cDNAs for each receptor (Fig. 2, lane 4), respectively.

Expression of 5-HT_{2A} and 5-HT₇ Receptor Proteins in COCs and Embryos. In addition to 5-HT₇ and 5-HT_{2A} mRNAs, we verified the presence of corresponding proteins by Western blotting and indirect immunofluorescence microscopy using 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-HT₇ 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. 4, A-A' and B-B'), four-cell embryos (Fig. 4C-C'),

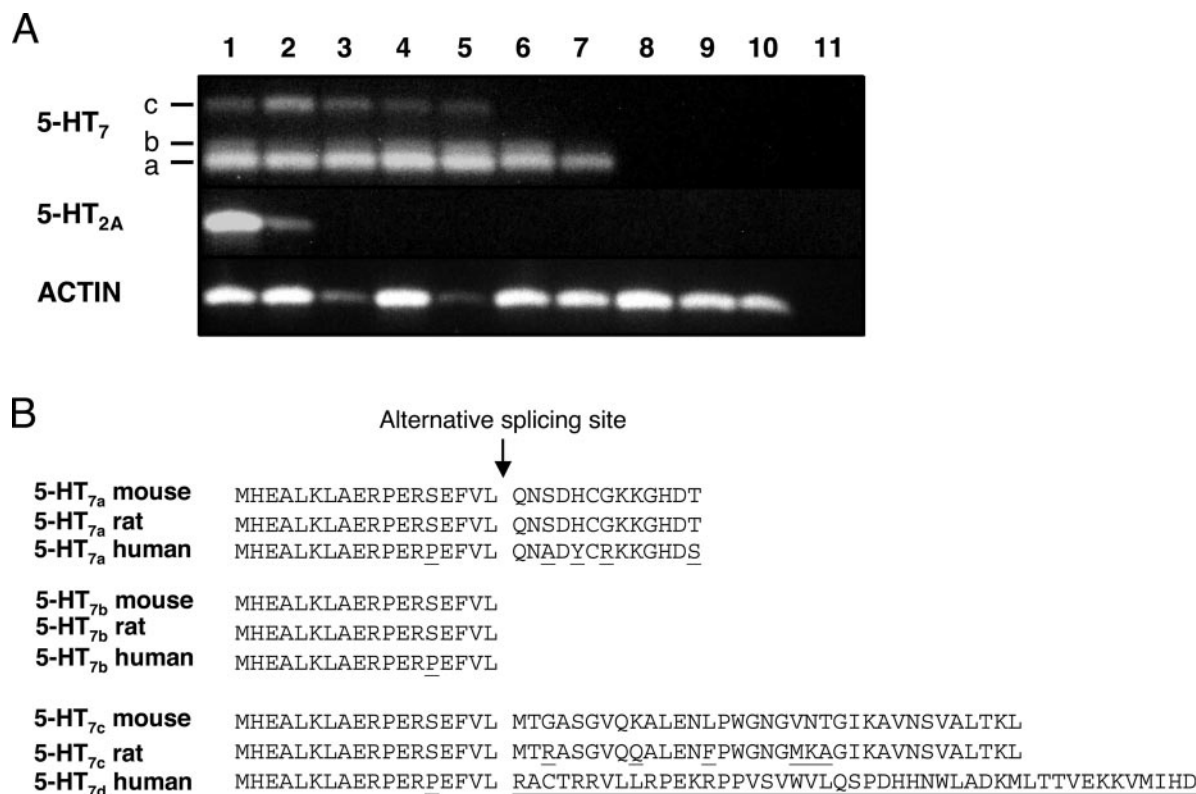


Fig. 1. Expressions of 5-HT₇ and 5-HT_{2A} receptors mRNAs in cumulus cells, oocytes, and embryos, and C-terminal amino acid sequences of mouse, rat, and human 5-HT₇ receptor isoforms. A, RT-PCR-amplified bands for 5-HT₇ (isoforms a, b, and c), 5-HT_{2A} receptors, and actin control obtained with mRNA extracted, respectively, from total ovaries (lane 1), isolated cumulus cells (lane 2), germinal vesicle stage oocytes (lane 3), metaphase II oocytes (lane 4), one-cell (lane 5), two-cell (lane 6), four-cell (lane 7), and eight-cell embryos (lane 8), morulae (lane 9), blastocysts (lane 10), and a representative negative control sample without cDNA (lane 11). Depicted bands, at their expected sizes as described under *Materials and Methods*, were the only ones detected in three to four separate determinations. B, comparison of the deduced C-terminal amino acids of the mouse isoforms with their rat and human homologs. Amino acid differences are underscored.

or blastocysts (data not presented). No cell labeling was found when the anti-5-HT_{2A} antibody had been depleted previously by preincubation with a 5-HT_{2A}-blocking peptide or when only a secondary antibody was used (data not shown). In contrast, 5-HT₇-associated immunoreactivity in COCs revealed strong oocyte labeling compared with weaker labeling at the periphery of cumulus cells (Fig. 5A-A'). Isolated oocytes also showed strong immunoreactive 5-HT₇-labeling (Fig. 5B-B'). In four-cell embryos, in which 5-HT₇ 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-HT₇ mRNA, did not display any labeling with the anti-5-HT₇ 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. Because the 5-HT₇ receptor is known to be coupled to an increase 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 or 100 nM 5-HT produced, respectively, 19 and 29% increases in their cAMP content, but these differences were not statistically significant (Fig. 6A). Higher doses of 1 and 10 μ M 5-HT resulted in significant increases of

67 ($p < 0.01$) and 79% ($p < 0.001$). Incubating isolated oocytes in M2 medium containing 1 or 10 μ M 5-HT did not affect their cAMP content, whereas incubation in 10 μ M forskolin resulted in a robust elevation of nearly 300% (Fig.

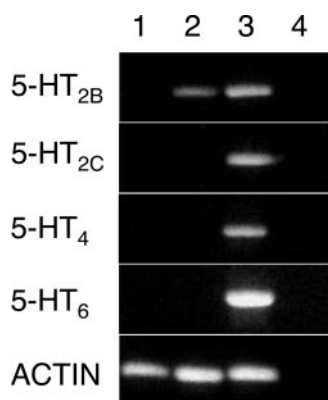


Fig. 2. Expressions of 5-HT_{2B}, 5-HT_{2C}, 5-HT₄, and 5-HT₆ receptor mRNAs in oocytes and cumulus cells. RT-PCR-amplified bands for 5-HT_{2B}, 5-HT_{2C}, 5-HT₄, and 5-HT₆ receptors and actin control obtained with mRNA extracted, respectively, from metaphase II oocytes (lane 1), isolated cumulus cells (lane 2), brain (lane 3), and a representative negative control sample without cDNA (lane 4). Depicted bands, at their expected sizes as described under *Materials and Methods*, were the only ones detected in three to four separate determinations.

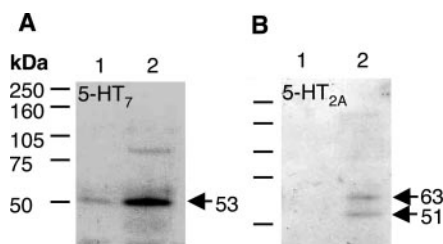


Fig. 3. Expressions of 5-HT₇ and 5-HT_{2A} proteins in cumulus cells and metaphase II oocytes. Western blots against samples from isolated oocytes (lane 1) or cumulus cells (lane 2) showing a common band at 53 kDa with an anti-5-HT₇ antibody (A) and doublet bands only in cumulus cells with an anti-5-HT_{2A} antibody (B).

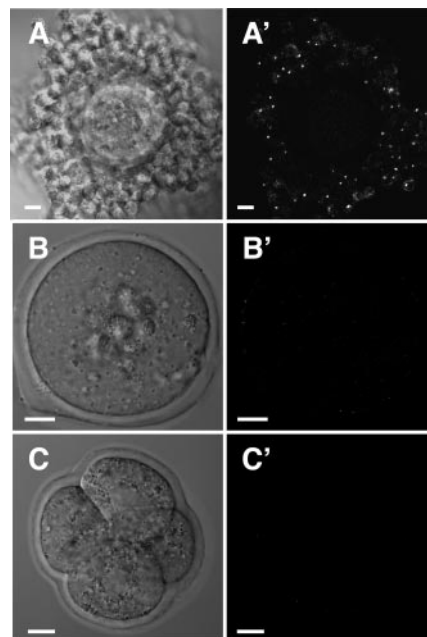


Fig. 4. Expression of 5-HT_{2A} receptor protein in COCs, isolated metaphase II oocytes, and early embryos. Immunofluorescence of cells prepared with an anti-5-HT_{2A} antibody (A'-C') and observed by confocal microscopy. Phase contrast (A-C) and corresponding fluorescence (A'-C') images of a mouse COC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

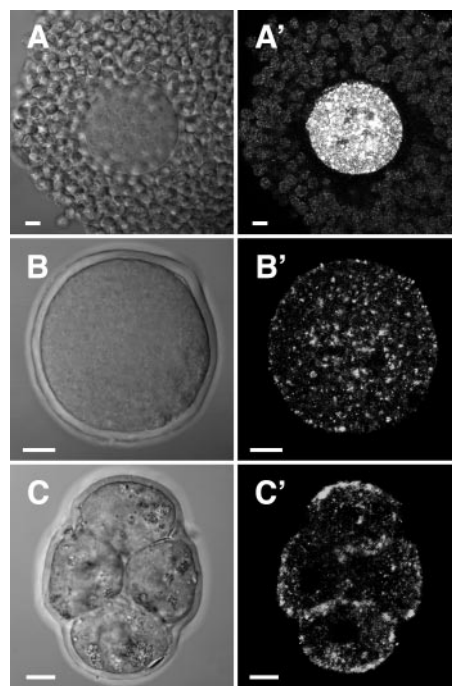


Fig. 5. Expression of 5-HT₇ receptor protein in COCs, isolated metaphase II oocytes, and early embryos. Immunofluorescence of cells prepared with an anti-5-HT₇ antibody (A'-C') and observed by confocal microscopy. Phase contrast (A-C) and corresponding fluorescence (A'-C') images of a mouse COC (A-A'), isolated metaphase II oocyte (B-B'), and four-cell stage embryo (C-C'). Scale bars, 10 μ m.

6B). Because 5-HT failed to increase the cAMP content of isolated oocytes, the cAMP increase observed in COCs is probably 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-HT₄, 5-HT₆, and 5-HT₇ receptors. We first used 5-CT, which has mixed 5-HT₆ and 5-HT₇ affinities (Shen et al., 1993; Kohen et al., 1996). Exposure to 1 or 10 μ M 5-CT increased the cAMP content of COCs by 12 and 30%, respectively, with only the 10- μ M dose yielding a significant increment ($p < 0.01$, Fig. 7A). With 1 or 10 μ M 8-OH DPAT, a 5-HT_{1A} and 5-HT₇ agonist (Stam et al., 1992), the cAMP content of COCs increased by 18 and 36% ($p < 0.05$), respectively (Fig. 7B). Next, 1 or 10 μ M PPB, a 5-HT₄ agonist (Ramirez et al., 1997), did not significantly elevate the cAMP content of COCs (Fig. 7C). Finally, because cumulus cells also express 5-HT_{2A} and 5-HT_{2B} receptors, the 5-HT₂ agonist α -methyl-5-HT (Baxter et al., 1995) was tested and induced increases in cAMP of 13 (1 μ M) and 34% (10 μ M, $p < 0.01$) (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-HT₄ receptor and strongly suggests the involvement of the 5-HT₇ receptor, because 8-OH DPAT was able to evoke a cAMP increase, in agreement with our RT-PCR study in which the only G_s -coupled receptor mRNA detected was the 5-HT₇ receptor (Figs. 1 and 2).

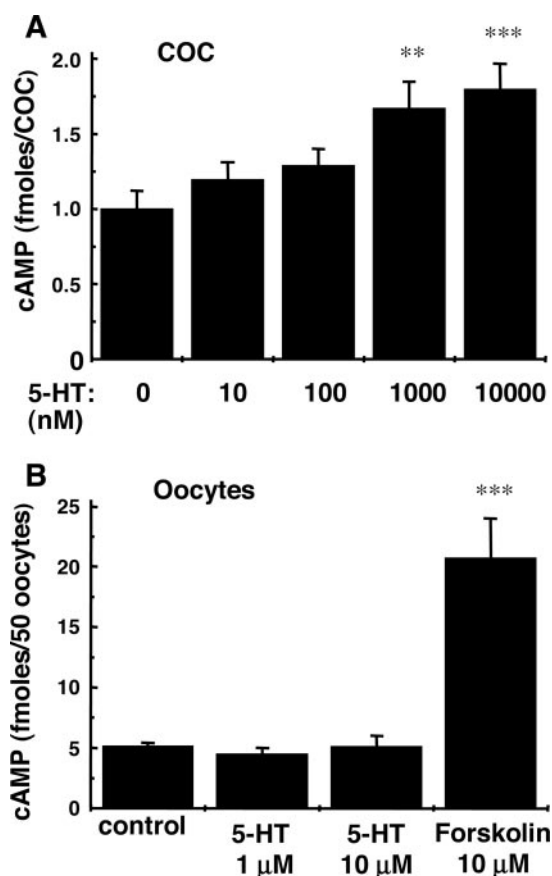


Fig. 6. Effect of 5-HT on the cAMP content of mouse COCs and isolated metaphase oocytes. COCs (A) and metaphase II oocytes (B) were submitted to different concentrations of 5-HT for 5 min in M2 medium containing 200 μ M IBMX. A 10 μ M forskolin treatment was included in the oocyte experiments. Mean results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

Serotonin-Induced Ca^{2+} Increase 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, because 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 constantly perfused for the duration of the recording, and when adding 1 μ M 5-HT, an increment of intracellular Ca^{2+} was detected in 56% of the cells (Fig. 8A and Table 1). At the end of each experiment, the cells were perfused with 200 μ M ATP, and a strong Ca^{2+} increase was observed in 92% of them. This provided a positive control, because 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 μ M BAPTA-AM before the 5-HT perfusion completely blocked the Ca^{2+} increase (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 preincubation. The time delay of this Ca^{2+} increase was relatively short, occurring always within the first 12 s of perfusion with the 5-HT-containing solution. When a sharp spike was observed, it lasted for 20 to 25 s, and a long recovery time of approximately 60 s was needed to return to the original Ca^{2+} level. Experiments carried out with 100 or 10 nM 5-HT resulted in lower Ca^{2+} increases, but the time delay of the response, the duration of the peak, and recovery time were similar to the 1 μ M dose response (Fig. 8, B and C, respectively). 5-HT at 1 nM was also tested, but clear Ca^{2+} increases 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-HT₂ antagonists (Miyazaki et al., 1990). Even though we did not detect any 5-HT₂ receptor in mouse oocytes, we decided to investigate the effect of 5-HT on the Ca^{2+} level of mouse oocytes. Perfusion with 10 μ M 5-HT failed to elicit any Ca^{2+} upsurge (0/7, Table 1), even though these oocytes could respond to a 100 μ M carbachol dose as a positive control (7/7).

Cross-Talk between Ca^{2+} and cAMP in COCs. Finally, because 5-HT regulates the cAMP and calcium levels of cumulus cells, we decided to investigate possible cross-talk between these two signaling pathways. When COCs were exposed to 5 μ M ionomycin for 5 min to increase 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 preincubation in the presence of BAPTA, confirming the Ca^{2+} specificity of this ionomycin-induced cAMP increase. 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% ($p < 0.01$ versus control) rather than 60% ($p < 0.001$ versus control), but the difference between the two conditions was not statistically significant (Fig. 9A). Taken altogether, these results

suggest that when COCs were incubated in the presence of BAPTA, the cAMP increase induced by 5-HT was somewhat lower but was still significant over untreated control cells (Fig. 9A). Part of the cAMP increase induced by 5-HT in cumulus cells could thus be caused by an augmentation of intracellular Ca^{2+} . However, intracellular Ca^{2+} chelation

does not preclude part of the 5-HT-induced increase in cAMP which, as expected, is largely Ca^{2+} -independent.

Discussion

This work extends our previous demonstration of a local serotonergic network in mouse cumulus-oocyte complexes

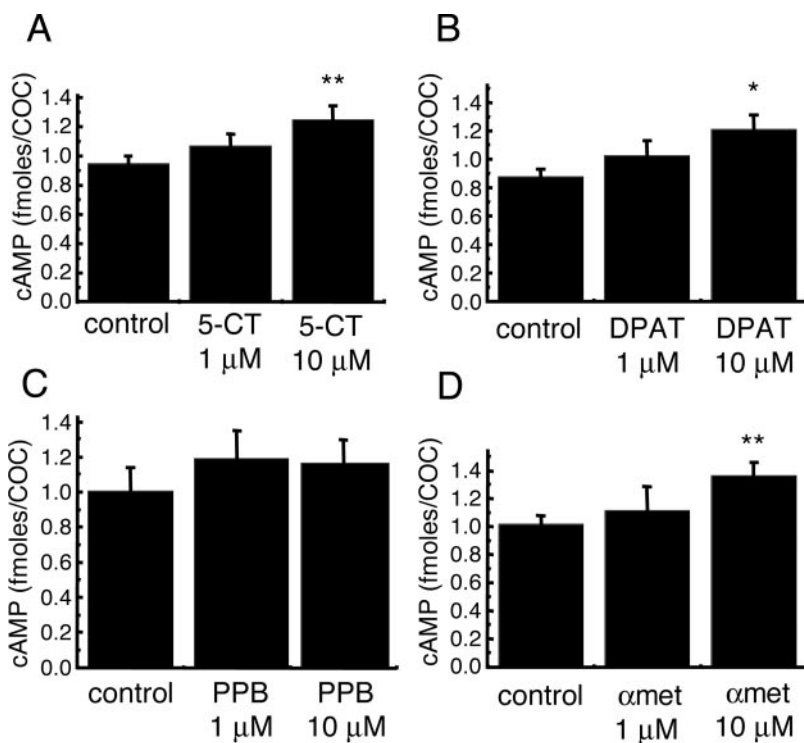


Fig. 7. Effect of 5-HT agonists on the cAMP content of mouse COCs. COCs were treated with 5-CT (A), 8-OH DPAT (B), PPB (C), or α -methyl-5-HT (D) for 5 min in M2 medium containing 200 μM IBMX. Mean results (\pm S.E.M.) of at least three duplicate experiments are shown. *, $p < 0.05$; **, $p < 0.01$;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

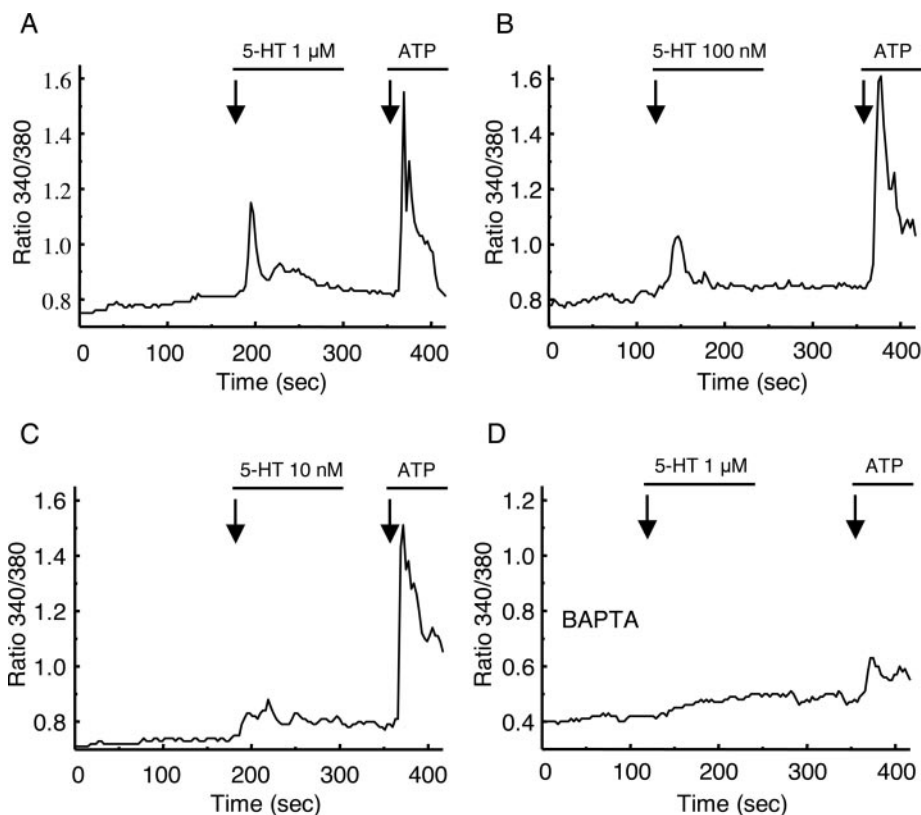


Fig. 8. Effect of 5-HT on resting Ca^{2+} concentration in mouse cumulus cells. A typical trace of a cumulus cell response is shown when perfused with M2 medium containing 1 μM (A), 100 nM (B), or 10 nM 5-HT (C). Pretreatment of cumulus cells with 50 μM BAPTA-AM before the addition of 1 μM 5-HT blocks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

We detected both mRNA and protein of the 5-HT₇ receptor from germinal vesicle stage oocytes to four-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-HT₇ receptor at this specific stage, which also shows internal rather than peripheral receptor immunostaining, a condition already reported for inactive and internalized 5-HT₇ receptor in rat brain (Muneoka and Takigawa, 2003). Still, it remains possible that an oocyte 5-HT₇ 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 (Kalinowski et al., 2004; Mehlmann et al., 2004). This constant G_s protein activity was shown to rely on the orphan

Fig. 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 μ M 5-HT, 5 μ M ionomycin, or 10 μ M forskolin for 5 min in M2 medium containing 200 μ M IBMX. Under BAPTA-containing conditions, the cells were pretreated for 30 min in 50 μ M BAPTA-AM before the 5-min treatment. Mean results (\pm S.E.M.) of at least three duplicate experiments are shown. **, $p < 0.01$; ***, $p < 0.001$ compared with the control.

Agonist	Cell Type	No. of Experiments	No. of Cells	No. of Reacting Cells	Reacting Cells	Amplitude Mean Response (Ratio 340/380)
					%	
5-HT 1 μ M	Cumulus	7	336	189	56.3	0.34 \pm 0.02
5-HT 100 nM	Cumulus	5	272	128	47.1	0.21 \pm 0.02
5-HT 10 nM	Cumulus	3	115	36	34.5	0.14 \pm 0.01
5-HT 1 μ M + BAPTA	Cumulus	3	211	8	3.8	0.09 \pm 0.01
5-HT 10 μ M	Metaphase II oocyte	3	7	0	0	

respect, it is noteworthy that 5-HT antagonists were reported to block or inhibit the progression of early cleavage divisions, whereas 5-HT prevents this effect (Buznikov et al., 1996), although other studies reported a negative effect on later blastocyst formation after exposure to 5-HT (Il'kova et al., 2004) or to the agonist sumatriptan (Vesela et al., 2003). Along this line, we detected three distinct 5-HT₇ receptor isoforms in mouse oocytes and embryos that seem to be homologous to known rat (a, b, and c) or human (a and b) isoforms (Heidmann et al., 1997). Although 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 decrease below a detectable threshold level of the least expressed isoforms in two- and four-cell embryos. Therefore, the reported serotonergic effects at the blastocyst stage are unlikely to be linked to the 5-HT₇ receptor whose mRNA has long disappeared by that time but could be caused, as suggested, by a 5-HT_{1D} receptor, whose effective expression would require additional confirmation (Vesela et al., 2003).

One surprising finding in cumulus cells was that the 5-HT₂ agonist α -methyl-5-HT could also increase their cAMP content. However, this α -methyl-5-HT-induced cAMP increase might involve the observed cross-talk between Ca²⁺ 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 Ca²⁺-sensitive effect on cAMP levels. An elevation of intracellular Ca²⁺ could lead to such a cAMP increase through the 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 Ca²⁺ increase through a 5-HT₂ receptor to increased cAMP. In addition, part of the 5-HT-evoked cAMP elevation in cumulus cells could be mediated by this Ca²⁺ increment because BAPTA-pretreated cells showed a smaller increase 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 Ca²⁺ increases in hamster oocytes (Miyazaki et al., 1990; Fujiwara et al., 1993). Our various data clearly establish that none of the 5-HT₂ receptors is expressed in oocytes, which is in agreement with the fact that their Ca²⁺ level is 5-HT-insensitive in the mouse, in contrast to the hamster (data not shown; S. Miyazaki, personal communication). Therefore, a species difference exists between the mouse and hamster that presumably reflects a differential expression of the 5-HT₂ and/or 5-HT₇ receptors in mammalian oocytes. Indeed, we have detected, by RT-PCR, 5-HT_{2A} mRNA but not 5-HT₇ mRNA in hamster metaphase II oocytes, whereas hamster cumulus cells express both subtypes as in the mouse (golden hamster 5-HT_{2A} and 5-HT₇ receptor cDNAs were cloned and sequenced; see GenBank accession numbers DQ015678 and DQ015679; P. Amireault and F. Dubé, unpublished data). This explains the observed Ca²⁺-mobilizing effect of 5-HT in hamster but not mouse oocytes and 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 (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 preovulatory follicles, and this could be inhibited by ketanserin, a preferential 5-HT_{2A} antagonist (Tanaka et al., 1993). In addition, 5-HT_{2A} receptor densities increase in the rat forebrain at the time of the spontaneous estrogen-induced LH surge, compared with diestrous female rats (Sumner and Fink, 1997), whereas 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 Ca²⁺ responses of cumulus cells to 5-HT are largely mediated by a 5-HT₂ 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 5-HT_{2A} and 5-HT_{2B} receptors but not of the 5-HT_{2C} receptor, makes these two receptors likely candidates to generate the observed Ca²⁺ responses in cumulus cells. However, 5-HT can also evoke Ca²⁺ increases in human embryonic kidney 293 cells transfected with the 5-HT₇ 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 Ca²⁺ responses after 5-HT addition, even though this is not supported by the lack of effect of 8-OH DPAT on their Ca²⁺ levels (data not shown). The expression of 5-HT_{2A}, 5-HT_{2B}, and 5-HT₇ 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-HT₄, and 5-HT₆ 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 hindered by the heterogeneity of cell populations expressing multiple 5-HT receptors and exhibiting interconnected signaling pathways, such as that linking cAMP and Ca²⁺ 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 Ca²⁺ levels not only of cumulus cells but also of the oocytes themselves, either directly through their expressed 5-HT₇ receptor or indirectly through the physical bridging with cumulus cells by gap junctions.

In conclusion, our work demonstrates that 5-HT can regulate the Ca²⁺ and cAMP levels of mouse cumulus cells, most likely through expressed 5-HT_{2A}, 5-HT_{2B}, and 5-HT₇ recep-

tors. In addition, oocytes and embryos up to the four-cell stage express the 5-HT₇ 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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