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

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ABSTRACT
cAMP and intracellular Ca²⁺ 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 Ca²⁺ 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, whereas 5-HT₂A, 5-HT₄, and 5-HT₆ receptors are expressed in neither oocytes nor cumulus cells. The addition of 5-HT (10 nM to 10⁻⁶ M) to isolated metaphase II oocytes had no effect on their internal cAMP or Ca²⁺ levels, whereas it caused dose-dependent cAMP and Ca²⁺ increases in cumulus cells. This cAMP increase in cumulus cells could be mimicked by 5-HT agonists with the following order of potency: 5-HT₇ > 5-HT₂A > 5-HT₂B, and 5-HT₇ receptor mRNA is expressed in cumulus cells. On the basis of a reverse transcription-polymerase chain reaction study, 5-HT₇ receptor mRNA is expressed in cumulus cells. The addition of 5-HT (10 nM to 10⁻⁶ M) to isolated metaphase II oocytes had no effect on their internal cAMP or Ca²⁺ levels, whereas it caused dose-dependent cAMP and Ca²⁺ increases in cumulus cells. This cAMP increase in cumulus cells could be mimicked by 5-HT agonists with the following order of potency: 5-HT₇ > 5-HT₂A > 5-HT₂B, and 5-HT₇ receptor mRNA is expressed in cumulus cells. On the basis of a reverse transcription-polymerase chain reaction study, 5-HT₇ receptor mRNA is expressed in cumulus cells...
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 (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 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-HT\(_1\)–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-HT\(_1\) receptors are coupled preferentially to G\(_{\alpha}\) to inhibit cAMP formation (Barnes and Sharp, 1999), whereas 5-HT\(_4\), 5-HT\(_6\), and 5-HT\(_7\) receptors are coupled to G\(_i\) and, hence, positively regulate adenylate cyclase, causing cAMP increases when activated (Hamblin et al., 1998). 5-HT\(_2\) receptors are coupled to G\(_i\) 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-HT\(_2\) receptor was detected in cultured human granulosa-lutein cells (Gravelleau 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-HT\(_2\) 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\(_{\alpha}\) and G\(_i\) subtypes, 5-HT\(_2\) and 5-HT\(_7\), detected previously in other mammalian reproductive tissues or cells.
shire, UK). The same 5-HT7 (1/500) and 5-HT2A (1/500) antibodies as the detection protocol using the Enhanced Chemiluminescence Plus assay kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). 5-HTr, two pairs of primers were used in a nested PCR strategy to produce amplicons of 790 bp (forward, 5′-cacaacgctacttctctggg-3′), and reverse, 5′-gtagatgctagttggtatcaagn-3′) and 454 bp (forward, 5′-ccacaacgctacttctctggg-3′, and reverse, 5′-gtagatgctagttggtatcaagn-3′) amplifications. 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-HT2A 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 the detection protocol using the Enhanced Chemiluminescence Plus assay kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). For 5-HT7, a rabbit anti-rat antibody directed against amino acids 22 to 41 (Calbiochem) was diluted 1/150. Control experiments included pre-adsorption of the primary antibody with its corresponding antigen.

**Statistical Analysis.** The results of cAMP measurement are expressed as means ± 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-HT2A and 5-HT7 Receptor mRNAs in COCs and Embryos.** Oocytes and embryos were collected and treated as described above. Groups of 10 COC-metaphase II or 50 metaphase II oocytes were deployed for Western blotting. For 5-HT7, 5′-gtgctgacctttgacctttg-3′ and 5′-ttttgcttctttgcttctttg-3′ were used in a nested PCR strategy to produce amplicons of 790 bp and 200 bp. The 790-bp amplicon was used to test the expression of 5-HT7 mRNA, whereas the 200-bp amplicon was used to test the expression of 5-HT2A mRNA. COCs were collected as described above and incubated in M2 medium containing 5 μM fura-2/acetoxymethyl ester and 0.02% Pluronic F-127 at 37°C for 25 min. After three 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 polylysine 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 μM 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).

**Expression of 5-HT2A 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-HT, 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-HT2A 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 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, 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-HT2B, 5-HT2C, 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 Gq-coupled receptor subtypes, 5-HT2B and 5-HT2C, and Gs-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-HT2B receptor mRNA could be detected but not the 5-HT2C, 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-HT2A and 5-HT7 Receptor Proteins in COCs and Embryos. In addition to 5-HT7 and 5-HT2A 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-HT2A in cumulus cells but not in oocytes (Fig. 3B). With the same antibodies under indirect immunofluorescence microscopy, COCs exhibited strong 5-HT2A-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-HT- and 5-HT receptors mRNAs in cumulus cells, oocytes, and embryos, and C-terminal amino acid sequences of mouse, rat, and human 5-HT receptor isoforms. A, RT-PCR-amplified bands for 5-HT (isoforms a, b, and c), 5-HT, 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.](https://molpharm.aspetjournals.org/doi/fig/10.1124/jpet.2b.5-HT-2a,-5-HT-7,-5-HT-2b,-5-HT-2c,-5-HT-4,-5-HT-6-receptor-mRNAs-in-metaphase-II-oocytes-and-cumulus-cells)
or blastocysts (data not presented). No cell labeling was found when the anti-5-HT2A antibody had been depleted previously by preincubation with a 5-HT2A-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 Gs (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 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 or 10 μM 5-HT did not affect their cAMP content, whereas incubation in 10 μM forskolin resulted in a robust elevation of nearly 300% (Fig. 6B).

Fig. 2. Expressions of 5-HT2B, 5-HT2C, 5-HT4, and 5-HT6 receptor mRNAs in oocytes and cumulus cells. RT-PCR-amplified bands for 5-HT2B, 5-HT2C, 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-HT2A 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-5HT7 antibody (A) and doublet bands only in cumulus cells with an anti-5-HT2A antibody (B).

Fig. 4. Expression of 5-HT2A receptor protein in COCs, isolated metaphase II oocytes, and early embryos. Immunofluorescence of cells prepared with an anti-5-HT2A 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 μm.

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 μm.
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<sub>a</sub>-coupled 5-HT<sub>4</sub>, 5-HT<sub>α</sub>, and 5-HT<sub>7</sub> receptors. We first used 5-CT, which has mixed 5-HT<sub>α</sub> and 5-HT<sub>7</sub> affinities (Shen et al., 1993; Kohen et al., 1996). Exposure to 1 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<sub>1A</sub> and 5-HT<sub>7</sub> 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 μM PPB, a 5-HT<sub>4</sub> agonist (Ramirez et al., 1997), did not significantly elevate the cAMP content of COCs (Fig. 7C). Finally, because cumulus cells also express 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptors, the 5-HT<sub>2</sub> 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<sub>4</sub> receptor and strongly suggests the involvement of the 5-HT<sub>7</sub> receptor, because 8-OH DPAT was able to evoke a cAMP increase, in agreement with our RT-PCR study in which the only G<sub>a</sub>-coupled receptor mRNA detected was the 5-HT<sub>7</sub> receptor (Figs. 1 and 2).

Serotonin-Induced Ca<sup>2+</sup> Increase in COCs. Expression of the 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptor in cumulus cells led us to investigate the effect of 5-HT on the Ca<sup>2+</sup> level of cumulus cells, because these receptors are known to be coupled to an increase in intracellular Ca<sup>2+</sup> through G<sub>a</sub> in other cell types. Dispersed cumulus cells were constantly perfused for the duration of the recording, and when adding 1 μM 5-HT, an increment of intracellular Ca<sup>2+</sup> was detected in 56% of the cells (Fig. 8A and Table 1). At the end of each experiment, the cells were perfused with 200 μM ATP, and a strong Ca<sup>2+</sup> 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<sup>2+</sup> increases (Webb et al., 2002a). Intracellular Ca<sup>2+</sup> chelation with 50 μM BAPTA-AM before the 5-HT perfusion completely blocked the Ca<sup>2+</sup> 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<sup>2+</sup> elevation (positive correlation, R<sup>2</sup> = 0.81 and p < 0.0001), the percentage of reacting cells, and the effect of a BAPTA preincubation. The time delay of this Ca<sup>2+</sup> 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<sup>2+</sup> level. Experiments carried out with 100 or 10 nM 5-HT resulted in lower Ca<sup>2+</sup> increases, but the time delay of the response, the duration of the peak, and recovery time were similar to the 1 μM dose response (Fig. 8, B and C, respectively). 5-HT at 1 nM was also tested, but clear Ca<sup>2+</sup> 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<sup>2+</sup> increases that are sensitive to 5-HT<sub>2</sub> antagonists (Miyazaki et al., 1990). Even though we did not detect any 5-HT<sub>2</sub> receptor in mouse oocytes, we decided to investigate the effect of 5-HT on the Ca<sup>2+</sup> level of mouse oocytes. Perfusion with 10 μM 5-HT failed to elicit any Ca<sup>2+</sup> surge (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<sup>2+</sup> 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 μM ionomycin for 5 min to increase intracellular Ca<sup>2+</sup>, a 280% increase in their cAMP content was observed, indicating that solely augmenting intracellular Ca<sup>2+</sup> 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<sup>2+</sup> 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...
and early embryos, including the presence of 5-HT itself, of the 5-HT synthesizing enzyme tryptophan hydroxylase in cumulus cells, and a 5-HT-specific uptake driven by a classic 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-HT2A, 5-HT2B, and 5-HT7 receptors in cumulus cells, oocytes, and embryos and that 5-HT affects intracellular Ca2+ 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-HT7 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-HT7 receptor (Graveleau et al., 2000). The expression of a 5-HT7 receptor in these closely related cell types from two species suggests that it might be universally expressed in mammalian follicles. cAMP in granulosa cells is already known to transduce the effects of follicle-stimulating hormone 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, because 5-CT and 8-OH DPAT, both 5-HT7 agonists, could increase the cAMP content of cumulus cells, whereas 5-HT2A and 5-HT6 receptor mRNAs could not be detected in these cells.

We detected both mRNA and protein of the 5-HT7 receptor from germinall 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 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 Gs protein activity in mouse oocytes (Kalinowski et al., 2004; Mehlmann et al., 2004). This constant Gs protein activity was shown to rely on the orphan GPR3 receptor, because most oocytes (~90%) from Gpr3 knockout mice resume meiosis prematurely within antral follicles (Mehlmann et al., 2004). If an additional oocyte Gs-linked receptor were 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 Gs-linked 5-HT7 receptor reported here would be a candidate fulfilling some of the expected attributes with its ligand, 5-HT, being produced by neighboring somatic cells (Amireault and Dubé, 2005). On the other hand, whether the 5-HT7 receptor becomes functional at later stages (e.g., in cleavage-stage embryos) remains to be established. In this

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 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 pretreated for 30 min in 50 μM BAPTA-AM before the 5-min treatment. Mean results (± S.E.M.) of at least three duplicate experiments are shown. **, p < 0.01; *** p < 0.001 compared with the control.

### Table 1

Ca2+ measurement after 5-HT perfusion in mouse cumulus cells and oocytes

<table>
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<tr>
<th>Agonist</th>
<th>Cell Type</th>
<th>No. of Experiments</th>
<th>No. of Cells</th>
<th>No. of Reacting Cells</th>
<th>Reacting Cells</th>
<th>Amplitude Mean Response (Ratio 340/380)</th>
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<td>Cumulus</td>
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<td>336</td>
<td>189</td>
<td>56.3</td>
<td>0.34 ± 0.02</td>
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<td>Cumulus</td>
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<td>36</td>
<td>34.5</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>5-HT 1 μM + BAPTA</td>
<td>Cumulus</td>
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<td>211</td>
<td>8</td>
<td>3.8</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>5-HT 10 μM</td>
<td>Metaphase II oocyte</td>
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<td>7</td>
<td>0</td>
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Amplitude mean response presented as mean ± S.E.M.
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-HT2 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-HT2 receptor whose mRNA has long disappeared by that time but could be caused, as suggested, by a 5-HT1D 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 (Tausig 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-HT2A-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-HT2A 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-HT2A 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-HT2A receptor, as suggested by the detection of a 5-HT2A-specific expressed sequence tag (Neilson et al., 2000).

On the other hand, the expression of the 5-HT2A and 5-HT7 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-HT2 antagonist (Tanaka et al., 1993). In addition, 5-HT7 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-HT2A receptor mRNA and protein in the rat frontal cortex (Bethea et al., 1998). Thus, 5-HT7A 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-HT2A receptor. Preliminary experiments showing that α-methyl-5-HT, an agonist for 5-HT2A-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-HT2A and 5-HT2B receptors but not of the 5-HT2C 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-HT2A, 5-HT2B, 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-HT2A, 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 conclusion, our work demonstrates that 5-HT can regulate the Ca2+ and cAMP levels of mouse cumulus cells, most likely through expressed 5-HT2A, 5-HT2B, 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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Baker LP, Nielsen MD, Impey S, Chan G, Obrietan K, Metcalf MA, Poser SW, and Sharp T (1999) A review of central 5-HT receptors and their subtypes. 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.

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