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Expression of Serotonin Receptors in Mouse Oocytes and Preimplantation Embryos

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Summary

Serotonin receptors have been found in several reproductive organs as well as in the central nervous system. Serotonin-binding sites have been demonstrated in duck ovarian follicles and the testis, hamster ovaries, human granulosa cells and mouse placenta. Local production of serotonin by the rat ovary, oviduct, uterus and testis has also been reported. We analyzed the expression of three types of serotonin receptors: 5-HT1B, 5-HT2C and 5-HT1D by reverse transcription-polymerase chain reaction in mouse unfertilized oocytes and preimplantation embryos from zygotes to the blastocyst stage *in vivo*. Transcripts for 5-HT1B and 5-HT2C serotonin receptors were detected neither in unfertilized oocytes nor at any stages of *in vivo* developing preimplantation embryos. Serotonin 5-HT1D receptor mRNA was present in unfertilized oocytes, zygotes, 2-cell embryos, compacted morulae and *in vivo* produced expanded blatocysts. The expression of the mRNA 5-HT1D serotonin receptor was also detected in blastocysts cultured *in vitro*. When added to the culture medium, specific serotonin 5-HT1D agonist sumatriptan (1 μM) significantly inhibited the development of mouse embryos cultured *in vitro*. Demonstration of the expression of 5-HT1D serotonin receptor in mouse oocytes and preimplantation embryos supports the idea of a functional serotonin (5-HT1D) receptor in early mammalian development.

Key words

Expression • Oocyte • Preimplantation • Serotonin • Sumatriptan

Introduction

Serotonin and melatonin regulate reproductive processes not only through the hypothalamo-hypophysial system but also by direct effects on reproductive organs. Their importance is supported by the presence of indoleamine and indoleamine receptors in the reproductive organs, by the actions of indoleamines on the production of hormones and growth factors, by their effects on oocyte maturation and on the secretory functions of the testis and oviduct (Sirotkin and Schaeffer 1997). High levels of serotonin are present in human ovarian follicular fluid, fluctuating in association with the ovulatory cycle (Bodis *et al*. 1993). It was shown that serotonin has a positive effect on progesterone and estradiol secretion by cultured rat preovulatory follicles (Tanaka *et al*. 1993) and human granulosa cells (Bodis *et al*. 1992). Furthermore, serotonin treatment stimulated oxytocin and cGMP and inhibited vasopressin and cAMP output by porcine granulosa cells (Sirotkin 1995). A decrease in oxytocin and an increase in insulin-like growth factor-I output after serotonin addition was also found in these cells (Sirotkin and Schaeffer 1997). Stricker and Smythe (2001) demonstrated that serotonin

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(5-hydroxytryptamine, 5-HT) can cause immature oocytes to undergo an increase in cAMP, which stimulates rather than inhibits meiotic maturation in marine memertean worms. A cAMP-mediated stimulation of germinal vesicle breakdown (GVBD) during 5-HT-triggered maturation is further supported by the fact that protein kinase A (PAK) inhibitors effectively block 5-HT-induced GVBD.

While the role of serotonin in the regulation of oocyte maturation is widely documented, a direct serotonin effect on preimplantation embryo development is not well established. There are very few data in the literature concerning a direct serotonin effect during the preimplantation period of embryo development. According to Khozhai *et al*. (1995), decreased levels of endogenous serotonin during the early periods of pregnancy lead to the absence of cytokinesis at the zygote stage. Formation of blastocysts is impaired, although cavitation may take place at the same time as in the control group. Markova *et al.* (1990) tested the effect of serotonin and adrenaline antagonists on preimplantation mouse embryos. All the substances tested produced an arrest or inhibition of cleavage division and produced the appearance of anomalies. Serotonin added to the incubation medium was effective against some serotoninolytics. However, more information is available concerning the role of serotonin during postimplantation embryo development. Differential expression of serotonin 5-HT2 receptors in the head during rat embryogenesis ranging from embryonic day (ED) 9 to 21 has been reported (Wu *et al*. 1999). 5-HT2A receptor levels gradually increased from embryonic day 11 to 21. The expression of 5-HT2B receptor decreased from ED 9 to ED 11 and then remained relatively constant till ED 21.

To our knowledge, however, no 5-HT serotonin receptors have yet been described in the oocytes and embryos of higher vertebrates. To investigate the possible role of serotonin in early preimplantation development and to fill the information gap regarding the existence of serotonin receptors in mammalian preimplantation embryo stages, we analyzed the mRNA expression of three representative types of serotonin receptors in mouse oocytes and preimplantation embryos.

Methods

Animals

ICR females, 4-5 weeks old, were superovulated by an injection of 10 IU pregnant mare serum gonadotropin (PMSG), followed by 10 IU human chorionic gonadotropin (hCG) 46-47 h later, and were mated with males of the same strain. Detection of vaginal plug following overnight mating was designated as Day 1 of pregnancy. The unfertilized oocytes and preimplantation embryos (zygotes, late 2-cell embryos, compacted morulae and expanded blastocysts) were isolated by flushing from the oviduct and uterus, respectively. Oocytes and embryos were washed with several drops of flushing-holding medium (FHM) with 1 % bovine serum albumin (BSA) and pooled according to their morphology. Cumulus cells were recovered with 0.1 % hyaluronidase (Sevac, Prague, Czech Republic). Two-cell embryos isolated 57 h after hCG treatment were cultured in KSOM medium (Lawitts *et al*. 1992) and collected at the expanded blastocyst stage on Day 5.

Serotonin 5-HT1D agonist sumatriptan

Two-cell embryos were also cultured in KSOM medium with addition of specific serotonin 5-HT1D agonist sumatriptan (Glaxo Wellcome, Verona, Italy) at 1 μM concentration. *In vitro* cultured embryos were morphologically evaluated after 67 h and stained with propidium iodide (PI) and Hoechst 33342 (Liu *et al*. 1999). To eliminate experimental bias, at least two independent series were performed in each group and the results were pooled.

RNA preparation and RT-PCR products

Unfertilized oocytes and zygotes were collected 24 h after hCG, 2-cell embryos 57 h after hCG, compacted morulae 71 h after hCG and expanded blastocysts 112 h after hCG treatment. Carrier rRNA (20 μg) was added to each batch of oocytes or preimplantation embryos before RNA preparation. For RT-PCR a modified procedure (Pampfer *et al*. 1992) was used. Total RNA was extracted from batches of 200 unfertilized mouse oocytes at metaphase II, and from 100 preimplantation embryos using a micro-adaptation method of the guanidium isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987) using RNA blue (Top-bio, Prague, Czech Republic).

Reverse transcription was done in 20 μl of RT buffer (Perkin-Elmer Gene Amp RNA PCR Core Kit, Part No. N808-0143) with Oligo d(T) 16. The final volume of each PCR tube was 50 μl. Amplification was performed for 35 cycles in Progene thermocycler (Techne, UK). Each cycle included target denaturation for 1 min, at 94 ºC, primer annealing for 2 min, at 60 ºC and extension for 1 min at 72 ºC. Positive control

included reverse transcribed RNA from mouse brain. Each RT-PCR was repeated 3 times.

The serotonin 5´ and 3´ 5-HT1B primer AGACAGGGGTACCTCTCACCAACC and ATGAGC GCCAACAAAGCAACCAGC (cDNA fragment 115bp), 5´ and 3´ 5-HT2C primer TAATGGTGAACCTGGGCA CTGCGG and TAAAAGTGTCAGTTACTATAGCTGC (cDNA fragment 116 bp), 5´ and 3´ 5-HT1D primer AAACCAGTCCCTAGAAGGCCTTCC and GCCAGTG TGATGACGGACAGCAC (cDNA fragment 132 bp) were used.

Detection of ß-actin transcript (539 bp fragment) using ß-actin primer pair (Temeles *et al.* 1994) served as an internal positive control in each experiment. Reverse transcriptase negative controls were carried out in parallel, using the same RNA samples without reverse transcriptase in the reaction buffer.

PCR products were separated in 2 % agarose gels containing 0.1 % ethidium bromide (Sigma) and photographed. For the verification of RT-PCR products, DNA bands were digested with diagnostic restriction enzyme PstI. Digestion of the 132 bp PCR product with PstI produced expected fragments of 49 and 83 bp.

Statistical analysis

Results are given as means ± S.E.M. The chi-square (χ^2) test was used to detect differences in the distribution of preimplantation embryos. Statistical analysis of the total cell number in blastocysts was done using Student´s t-test. $P<0.05$ was considered as significant.

Table 1. Effect of 5-HT1D agonist on preimplantation embryo development – morphological analysis at the end of *in vitro* culture

	Control		Sumatriptan 1 μM
No. of cultured embryos (%)	79 (100 %)		77 (100 %)
No. of blastocysts (%)	73 (92.4 %)		59 (76.6 %)
No. of morulae (%)	6 (7.6 %)		14 (18.2 %)
No. of degenerated (%)	0 (0 %)		4 (5.2 %)
χ^2 test Sumatriptan versus control		**p<0.001**	
Cell number in blastocysts	59.9±1.7		51.4±1.3
-test Sumatriptan versus control		**p<0.001**	

Results

Total RNA extracted from 200 unfertilized ovulated oocytes and from 100 preimplantation embryos collected between Day 1 and Day 5 were analyzed by RT-PCR for the expression of serotonin receptors: 5-HT1B, 5-HT2C and 5-HT1D. RNA from mouse brain was used as a positive control and ß-actin primers were used as internal control. DNA contamination was eliminated using RNA samples without enzyme reverse trancriptase in the RT.

As shown in Figure 1, serotonin 5-HT1D receptor mRNA was present in unfertilized oocytes, zygotes, 2-cell embryos, compacted morulae and in the *in vivo* developed expanded blastocysts. Comparable expression of mRNA 5-HT1D serotonin receptor was also detected in the *in vitro* cultured blastocysts. Using RT-PCR we were not able to detect transcripts for 5-HT1B and 5-HT2C serotonin receptors in the used developmental design. However all three types of serotonin receptors (5-HT1B, 5-HT2C, 5-HT1D) were detected in the mouse brain (data not shown).

Preimplantation embryos at each developmental stage expressed mRNA of β-actin, thus confirming the integrity of the RNA and the RT-PCR process.

Further evidence about the expression of functional serotonin 5-HT1D receptor mRNA in preimplantation embryos was tested by the addition of specific 5-HT1D agonist sumatriptan to the culture medium. Sumatriptan (1 μM) after its addition to the culture medium significantly inhibited *in vitro* development of mouse embryos (Table 1). We observed a decrease in blastocyst numbers and increased embryo degeneration (χ^2 test $p<0.001$). After sumatriptan treatment the cell number in blastocysts was significantly decreased in the experimental group (t-test $p<0.001$).

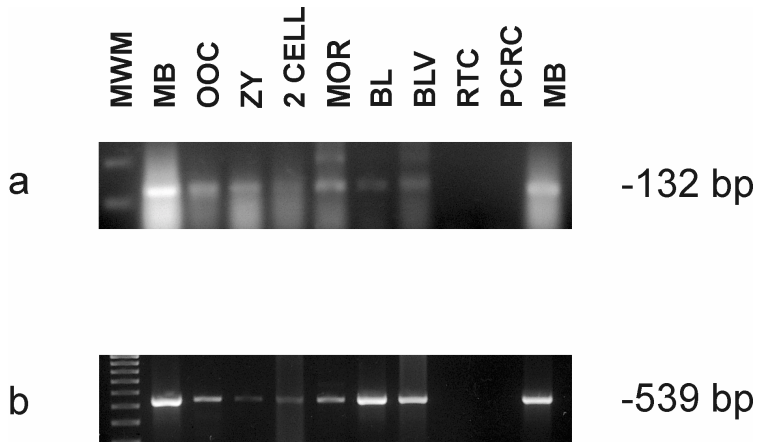

Fig. 1. *RT-PCR analysis of serotonin (5-HT1D) receptor mRNA expression in oocytes and preimplantation embryos: ethidium bromide staining of PCR-amplified serotonin (5-HT1D) cDNA product separated by electrophoresis ethidium bromide staining of PCR-amplified ß-actin cDNA product synthesized in the same cDNA preparations used in (a). MRK – molecular weight markers (100bp), MB – mouse brain, OOC – unfertilized oocytes, ZY – zygotes, 2CE – 2-cell embryos, MOR – morulae, BL – blastocysts, BLV – blastocysts cultured in vitro, RTC – reverse transcriptase negative control, PCRC – control for PCR contamination. The sizes of amplified serotonin (5-HT1D) cDNA (amplicon size 132 bp) and ß-actin cDNA (amplicon size 539 bp) are indicated to the right of panels (a) and (b), respectively.*

Discussion

Our results show that serotonin 5-HT1D receptor mRNA was detectable in mouse unfertilized oocytes in the metaphase II and/or after fertilization at all developmental stages of preimplantation embryos. The mRNA expression was also documented in the *in vitro* developed expanded blastocysts. It is known that several neurotransmitters, in particular serotonin (5-HT), could influence early embryo development. Transcripts for 5-HT were observed in the mouse oocyte, where it appeared to function as a regulator of cell cleavage (Moiseiwitsch 2000). *In vitro* studies have shown that the treatment of follicle-enclosed immature oocytes of *Fundulus heteroclitus* with 5-HT leads to increased levels of cAMP and inhibition of steroid 17,20 ßP-induced oocyte maturation. On the other hand, the treatment of oocytes with 17,20 ßP alone reduced the total cAMP levels and triggered meiosis reinitiation (Cerda *et al*. 1998). Despite the existing differences in oocyte maturation of the teleost compared with mammals, these findings support our evidence of the presence of mRNA oocyte-associated serotonin (5-HT1D) receptors in mice, which may be involved in the regulation of oocyte maturation.

Neurotransmitter serotonin (5-HT) plays an important role throughout the various stages of embryo development. In the postimplantation stage, developmental regulation in the expression of 5-HT2 receptors was confirmed in rat fetuses from Day 9 to 21 (Wu *et al*. 1999). Using RT-PCR, we analyzed the expression of three types of serotonin (5-HT1B, 5-HT2C, 5-HT1D) receptor mRNA in mouse preimplantation embryo stages. Only the serotonin 5-HT1D receptor mRNA was detected in zygotes, 2-cell embryos, compacted morulae and expanded blastocysts. These data suggest that serotonin (5-HT) could play a role in early mammalian development through the expressed receptors.

For confirmation of serotonin (5-HT1D) receptor function, we cultured 2-cell mouse embryos to the blastocyst stage in a medium with addition of specific serotonin 5-HT1D agonist sumatriptan (Peroutka and McCarthy 1989). 5–HT1D agonist sumatriptan in the concentration 1 µM significantly inhibited the development of blastocysts cultured *in vitro*. Existing literature data regarding the direct effect of serotonin on preimplantation embryo development are very sparse.

A positive effect of serotonin on blastocyst development has been reported which was contradictory to our results and was probably due to a different experimental design. The administration of p-chlorophenylalanine to mouse females leading to a decreased level of endogenous serotonin resulted in impaired formation of blastocysts. The blastocysts consisted of a small number of large blastomeres and the separation into trophoblast and ICM was characteristically indistinct (Khozhai *et al*. 1995). The positive effect of pretreatment with serotonin (5-HT, 5

μM) on the development of early mouse embryos after cryopreservation has also been observed (Sakharova *et al*. 1997). In the teleost *Fundulus heteroclitus*, serotonin (5-HT) inhibited 17,20 ßP-induced resumption of meiosis both in the follicle-enclosed and denuded oocytes, which indicates the presence of specific oocyte-associated 5-HT sensitive sites. The response of oocytes to 5-HT was characterized pharmacologically using serotonergic agonists and antagonists which mimicked or blocked the 5-HT inhibition of steroid-induced oocyte maturation (Cerda *et al*. 1997).

The interrelationships between central and peripheral indoleamine systems remain to be studied. Whereas hypothalamic serotonin acts as an inhibitor, peripheral serotonin seems to be a stimulator or inhibitor of reproductive processes, dependent upon ovarian cyclicity.

Our results indicate that specific serotonin agonist sumatriptan inhibited *in vitro* development in preimplantation embryos. *In vitro* cultures with 1 μM of sumatriptan significantly increased the percentage of degenerated embryos and we observed lower cell numbers in experimental mouse blastocysts in comparison with the control group.

In conclusion, serotonin (5-HT1D) receptor mRNAs are present both as maternal and embryonic transcripts since they are continuously expressed during oocyte maturation and after fertilization. The results of our study showed an expression pattern of the serotonin receptor mRNA at the earliest stages of mouse embryo development. The addition of specific serotonin agonist sumatriptan to the culture medium may influence mouse preimplantation embryo development, thus indicating the existence of the expression of a functional serotonin (5-HT1D) receptor.

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