Plasma membrane and vesicular monoamine transporters in normal endometrium and early pregnancy decidua

Barbara Bottalico, Radovan Pilka, Irene Larsson, Bertil Casslen, Karel Marsal and Stefan R. Hansson

Department of Obstetrics and Gynecology, Lund University Hospital, 221 85 Lund, Sweden

1To whom correspondence should be addressed. E-mail: stefan.hansson@mphy.lu.se

The uterus is innervated by adrenergic sympathetic fibres, and the endometrium has a capability for endogenous monoamine synthesis. Extracellular monoamine levels are regulated primarily through re-uptake by specific membrane-bound transporter proteins dopamine transporter (DAT), norepinephrine transporter (NET) and serotonin transporter (SERT). Intracellular storage of monoamines involves vesicular transporter proteins (VMAT1 and VMAT2). This study explored gene expression of the monoamine transporters in normal endometrium throughout the menstrual cycle and early decidua. In-situ hybridization histochemistry revealed three general classes of expression patterns: (i) epithelial expression of NET mRNA; (ii) increasing stromal expression of VMAT2 mRNA in the proliferative phase; and (iii) increasing epithelial expression of VMAT2 mRNA during the secretory phase. Real time PCR showed low expression levels of NET in all phases of the endometrial cycle and a higher expression of VMAT2 mRNA in the mid-secretory phase. Our results suggest that several monoamine transporters may have menstrual cycle phase-specific functions in endometrial biology by maintaining adequate levels of monoamines. Re-uptake and regulated release of monoamines may also modulate several steps of the reproductive processes such as embryo implantation and decidua formation.

Key words: dopamine transporter/in-situ hybridization histochemistry/norepinephrine transporter/real time PCR serotonin transporter

Introduction

Monoamines such as norepinephrine (NE), serotonin (5-HT), dopamine (DA) and histamine (HI) are all signalling molecules in the central nervous system (CNS). In addition, NE is the principal neurotransmitter in the sympathetic nervous system. These signal molecules take part in a wide variety of processes such as motor functions, arousal, attention, mood and anxiety. Moreover, NE together with epinephrine are potent stress hormones released by the adrenal medulla during the ‘fight and flight’ response. Neuroendocrine cells of the gastrointestinal and respiratory tracts also contain monoamines, which play important paracrine roles in these organs.

Since monoamines have potent physiological effects throughout the body, their extracellular concentrations are tightly regulated. Specific membrane-bound transporter proteins mediate re-uptake of monoamines from the synaptic cleft and the extracellular fluid (Amara and Kuhar, 1993; Brownstein and Hoffman, 1994). In the last decade, several genes coding for transporter proteins have been cloned and characterized (Erickson and Eiden, 1993; Borowsky and Hoffman, 1995; Chang et al., 1996). These include the transporters for 5-HT (SERT) (Blakely et al., 1991; Hoffman, 1994), NE (NET) (Amara and Kuhar, 1993) and DA (DAT) (Kilty et al., 1991; Usdin et al., 1991).

Monoamine transporters have been of particular interest because of the central role of NE and 5-HT re-uptake inhibition (serotonin specific re-uptake inhibition: SSRI) in modern treatment of depression and anxiety (Pacholczyk et al., 1991; Tatsumi et al., 1997), and because of their involvement in mechanisms for drug addiction (Schuldiner et al., 1993). Cocaine and amphetamine block monoamine transporters in a way similar to the effect of SSRI, leading to increased levels of extracellular monoamines.

Monoamine transporters are located in the cell membrane of monoaminergic neurons as well as in several non-neural cells (Hoffman et al., 1998). For example, both SERT (Balkovetz et al., 1989) and NET (Ramamoorthy et al., 1993b) are expressed in the placenta. Chemical signalling by presynaptic neurons is terminated by re-uptake of the monoamine from the synaptic cleft by the signalling cell. The intracellular fate of monoamines is either degradation by catechol-O-methyltransferase (COMT) or monoamine oxidase (MAO), or repacking in vesicles in order to be available for release again. Accumulation of monoamines from the cytoplasm into vesicles requires yet another set of transporter proteins, i.e. the vesicular monoamine transporters (VMAT). Two isoforms of the VMAT have been cloned, VMAT1 and VMAT2 (Peter et al., 1995). They show different anatomical distribution; VMAT2 is mainly expressed in the CNS (Hoffman et al., 1991; Usdin et al., 1991) and the adrenal medulla (Erickson et al., 1992; 1996), whereas VMAT1 is mainly found in the adrenal medulla and neuroendocrine cells of the gastrointestinal tract (Weih et al., 1994). Thus monoaminergic cells are capable of not only releasing monoamines, but also of controlled re-uptake and storage.

The uterus has an extensive innervation of sympathetic neurons, and adrenergic nerve signalling is involved in uterine contractility. Enzymes responsible for monoamine synthesis have been demonstrated in normal endometrium as well as in early pregnancy deciduas, and locally synthesized monoamines have physiological functions through paracrine signalling (Manyonda et al., 1998).

Since the transporter proteins critically regulate extracellular monoamine concentrations, knowledge of their distribution and cyclic variation in normal endometrial tissue is of great importance to further
understand the contribution of monoaminergic mechanisms in the reproductive process. Thus, we used in-situ hybridization and real time PCR quantification to study tissue distribution and cyclic variation of monoamine transporter mRNA in endometrium and early decidua.

Materials and methods

**Patients and tissue sampling**

Endometrial tissue was collected at the Department of Obstetrics and Gynecology, Lund University Hospital. Sampling after informed consent was approved by the Research Ethical Committee Review Board for studies in human subjects. Only patients without oral and intrauterine contraceptives and with regular menstrual cycles were included in the study. Endometrial tissue was collected from healthy women, 34–50 years, undergoing hysterectomy or with regular menstrual cycles were included in the study. Endometrial tissue was collected according to an ideal 28 day cycle prior to hybridization. Fresh frozen tissue, rather than fixative-treated tissue, was used in order to maximize mRNA detection. Tissue sections (12 μm) were cut on a cryostat, thaw-mounted onto silanized slides, and stored at −80°C prior to hybridization. Fresh frozen tissue, rather than fixative-treated tissue, was used in order to maximize mRNA detection. Thawing of tissue did not occur prior to sectioning in order to ensure best possible tissue integrity.

**RNA probes**

For the human 5HTT mRNA, a probe was used corresponding to 475 nucleotides (nt) (1845–2320), GenBank accession no. NM001045 (Ramamoorthy et al., 1993a). For the human NET mRNA, a probe was used corresponding to 561 nt (316–877, exons 1–5), GenBank accession no. NM001043 (Pacholczyk et al., 1991). For the human DAT mRNA, a probe was used corresponding to 467 nt (1088–1555), GenBank accession no. S44626 (Uhl and Kiyatama, 1993). For the vesicular monoamine transporters (VMAT1), a probe was used corresponding to 436 nt (1741–2177), GenBank accession no. NM_003053.1 (Erickson and Eiden, 1993). For the vesicular monoamine transporters (VMAT2), a probe was used corresponding to different exons of the investigated gene in order to avoid possibly generate sense and antisense probes respectively.

**Hybridization**

**In-situ hybridization histochemistry**

**Tissue preparation**

Tissue sections (12 μm) were cut on a cryostat, thaw-mounted onto silanized slides, and stored at −80°C prior to hybridization. Fresh frozen tissue, rather than fixative-treated tissue, was used in order to maximize mRNA detection. Thawing of tissue did not occur prior to sectioning in order to ensure best possible tissue integrity.

**DNA probes**

For the human 5HTT mRNA, a probe was used corresponding to 475 nucleotides (nt) (1845–2320), GenBank accession no. NM001045 (Ramamoorthy et al., 1993a). For the human NET mRNA, a probe was used corresponding to 561 nt (316–877, exons 1–5), GenBank accession no. NM001043 (Pacholczyk et al., 1991). For the human DAT mRNA, a probe was used corresponding to 467 nt (1088–1555), GenBank accession no. S44626 (Uhl and Kiyatama, 1993). For the vesicular monoamine transporters (VMAT1), a probe was used corresponding to 436 nt (1741–2177), GenBank accession no. NM_003053.1 (Erickson et al., 1993) and (VMAT2) corresponding to 486 nt (1300–1786, exons 12–14), GenBank accession no. L23205 (Erickson and Eiden, 1993).

**Table 1. Data on primers and probes used for real time PCR amplification**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Accession no.</th>
<th>Size (nt)</th>
<th>Primers, forward (F) and reverse (R)/ assay on demand number</th>
<th>Probe/part of ampiclon sequence</th>
<th>Reporter dye</th>
<th>Quencher dye</th>
<th>Exons</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-NET</td>
<td>NM_001043</td>
<td>75 F: CTTCCCCTACCTCTGTCATACAGAA R: CCCGGCAGTATAGGAAACAG</td>
<td>CGCGGGTGCCCTTCGTGATCC</td>
<td>Fam</td>
<td>Tamra</td>
<td>1–2</td>
<td></td>
</tr>
<tr>
<td>h-NET</td>
<td>NM_001043</td>
<td>150 F: GAAACCAGGCTACAACTGAGGAT R: GCCCTGGATACATGGAAGAAGT</td>
<td>CACAGAGGAGGTG</td>
<td>Fam</td>
<td>None</td>
<td>7–8</td>
<td></td>
</tr>
<tr>
<td>h-VMAT2</td>
<td>L23205</td>
<td>150 H000161858_m1 433762F</td>
<td>GTGGATCCGTCAATGATGCGCTATCA</td>
<td>Fam</td>
<td>None</td>
<td>11–12</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_001101</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

nt = nucleotides; Fam = fluorescent dye, 6-carboxyfluorescein; Tamra = 6-carboxytetramethylrhodamine.
genomic DNA contamination (Table I). Oligonucleotide probes labelled with fluorogenic dye, 6-carboxyfluorescein (Fam) and quenched with 6-carboxytetramethylrhodamine (Tamra) (Table I). PCR reactions were carried out in a 25 μl final volume containing final concentrations: 1× Universal PCR Master Mix (Applied Biosystems), 0.5 μmol/l TaqMan probe, 0.9 μmol/l of forward and reverse primers respectively, and 1 μl of 10 ng/ml of a DNA aliquot. For transcripts analysed with pre-manufactured probes, the reactions were carried out in a 25 μl final volume containing final concentrations: 1× Universal PCR Master Mix (Applied Biosystems), 1× Assaymix (Applied Biosystems), 0.25 μmol/l probe, 0.9 μmol/l of forward and reverse primers respectively, and 1 μl of 10 ng/ml of a DNA aliquot. The thermal cycling conditions were initiated by UNG activation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s, annealing at 60°C for 1 min. Two negative controls, without template, were included in every amplification. RNA samples were tested for genomic DNA contamination prior to further investigation. For each reaction, triplicate or duplicate assays were carried out. Transcript of β-actin mRNA, as a housekeeping gene, was quantified to normalize each sample. Quantification was achieved through a calibration curve obtained by serial 10-fold dilutions of the template DNA (0.08–80 ng). Results are expressed as relative values.

**Microphotograph and figure preparation**

Microphotographs were prepared using an Axiohot microscope (Olympus) equipped for darkfield and brightfield microscopy with a digital camera (Olympus DP50-CU). Captured images were assembled electronically using Adobe Photoshop 5.0. Figures were printed onto matt-finished paper by a Fuji Pictrography 3000 (Fuji) printer at 400 dpi resolution.

**Statistics**

Results are presented as box plots. The Mann–Whitney U-test was used to evaluate the significant differences between the proliferative and secretory groups. P < 0.05 was considered significant.

**Results**

The different in-situ hybridization histochemistry probes used in the study were hybridized on control tissue to verify their specificity (data not shown). None of the sense probes showed specific hybridization (data not shown). Hybridizations were repeated at least twice with consistently reproducible results.

**Plasma membrane transporters**

Endometrial expression of NET mRNA was found consistently in the late proliferative phase (Figure 1, Table II), and in two samples of the early proliferative phase. The signal was clear and exclusively detected in glandular epithelial cells. NET was not detected in secretory phase endometrium, or in early decidual tissue. In contrast, real time PCR revealed low expression levels of NET in all phases. The peak observed in the late proliferative phase with in-situ hybridization histochemistry could not be confirmed with real time PCR. The results were reproduced using two non-overlapping TaqMan probes (Table I). No significant difference was found between the examined phases of the menstrual cycle, but a trend towards lower expression in the secretory than in the proliferative phase (P = 0.09) was observed (Figure 2). ISSH showed sporadic expression of the other two plasma membrane transporters (data not shown). DAT mRNA was not detected in any endometrial tissue from non-pregnant women obtained at all stages of the menstrual cycle, but was found in epithelial cells in one case of early pregnancy decidua. SERT mRNA was found in occasional stromal cells of two secretory phase endometria, and in sporadic epithelial cells of early decidua.

**Vesicular transporters**

VMAT1 mRNA was not detected in the tissues examined (data not shown). Endometrial expression of VMAT2 mRNA showed a dynamic pattern over the menstrual cycle (Figure 3, Table II). Stromal expression of VMAT2 mRNA was weak in the early proliferative phase, increased to a maximum in the late proliferative phase, then decreased and disappeared in the late secretory phase. On the other hand, epithelial expression, which was negative in the early proliferative phase, increased gradually from early secretory, to reach a maximum in the late secretory phase. Glandular epithelial cells in general showed a strong signal, and some of these cells expressed a

<table>
<thead>
<tr>
<th>mRNA</th>
<th>EP</th>
<th>MP</th>
<th>LP</th>
<th>ES</th>
<th>MS</th>
<th>LS</th>
<th>ME</th>
<th>Decidua</th>
</tr>
</thead>
<tbody>
<tr>
<td>NET/stroma</td>
<td>–</td>
<td>–</td>
<td>--</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NET/epithelium</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VMAT2/stroma</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VMAT2/epithelium</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Expression was graded from background (–), more than background (+), less than half maximal (++), more than half maximal (+++) and maximal (++++) signal intensity.

EP = early proliferative; MP = mid-proliferative; LP = late proliferative; ES = early secretory; MS = mid-secretory; LS = late secretory phase.

Figure 1. In-situ hybridization showing norepinephrine transporter (NET) mRNA in normal endometrial tissue. Brightfield (A) and darkfield (B) views of NET mRNA in glandular (G) epithelial cells (arrow) in the late proliferative phase (only). No signal was observed in the stroma (S). Scale bar in A = 100 μm.
more robust hybridization signal (Figure 3E, inset). No VMAT2 mRNA was detected in the menstrual endometria or in early decidua. Real time PCR quantification confirmed higher expression of VAT2 mRNA in the secretory than in the proliferative phase ($P = 0.0004$) (Figure 4). However, levels peaked in the mid-secretory instead of the late secretory as shown with in-situ hybridization histochemistry.

**Discussion**

In-situ hybridization revealed endometrial expression of monoamine transporter genes. Three distinguishable patterns were observed over the menstrual cycle: (i) epithelial expression of NET; (ii) stromal expression of VMAT2 with maximal signal intensity in the late secretory phase; and (iii) expression of DAT, which was weak or absent in the endometrium but intense in the glandular epithelium in the late secretory phase.

**Figure 2.** Real time PCR quantification of NET mRNA. The amount of NET mRNA was normalized to β-actin mRNA and the relative values are presented in a box plot diagram. No significant changes were observed between the proliferative and secretory phases. One extreme value was excluded from the EP and is not shown in the figure. EP = early proliferative; MP = mid-proliferative; LP = late proliferative; ES = early secretory; MS = mid-secretory; LS = late secretory phase.

**Figure 3.** In-situ hybridization showing VMAT2 mRNA in normal endometrial tissue. Early proliferative phase in brightfield (A) and darkfield (B) images reveal VMAT2 mRNA positive cells (arrows) in the stroma (S). No signal was detected in the glandular (G) epithelial cells. In the late proliferative phase, brightfield (C) and darkfield (D) images, VMAT2 mRNA positive cells (arrows) in the stroma showed a more intense signal and glandular epithelial cells began to express a weak signal (arrow heads). From late secretory phase, brightfield (E) and darkfield (F) images, VMAT2 mRNA expression gradually increased throughout the secretory phase and peaked in the late secretory phase. Some cells of the glandular epithelium (arrows) were distinguishable by an even stronger signal, as shown in the magnified area in E. Note the absence of signal in the stroma. Scale bars in A, C, E = 100 μm.

**Figure 4.** Real time PCR quantification of VMAT2 mRNA. The amount of VMAT2 mRNA normalized to the amount of β-actin mRNA and the relative values are presented in a box plot diagram. Significant changes were obtained between the proliferative and the secretory phases, $P = 0.0004$. EP = early proliferative; MP = mid-proliferative; LP = late proliferative; ES = early secretory; MS = mid-secretory; LS = late secretory phase.
proliferative phase; and (iii) increasing epithelial expression of VMAT2 with a maximum in the late secretory phase. These patterns of NET and VMAT2 expression are likely to reflect distinct and transient functional roles that may relate to endometrial biology and the reproductive process.

The pattern of NET expression suggests a role for uptake of NE in the proliferative phase. However, the lack of overlapping VMAT2 expression in the epithelial cells indicates that regulated release and storage does not take place.

It has been previously shown that uptake of norepinephrine (NE) by glandular epithelial cells is cocaine-sensitive and enhanced by estradiol in animal experiments, supporting our results on the presence of NET in human endometrium and its distribution over the menstrual cycle (Alm et al., 1975; Declercq de Perez Bedes and Garcia Bienere, 1975; Kennedy and de la Lande, 1986). In-vitro uptake of NE has also been shown in human endometrium as well as myometrium (Pedroza-Garcia et al., 1975). DAT mRNA was absent in endometrium, but dopamine (DA) cannot be ruled out as a potential transporter since it is more efficiently transported by NET than NE (Pacholczyk et al., 1991; Eisenhofer, 2001).

We found VMAT2 mRNA in stromal cells with peak expression in the late proliferative phase, suggesting transiently increased storage capacity and a potentially regulated release of monoamines. Interestingly, none of the examined plasma membrane transporters showed an overlapping expression pattern with VMAT2 mRNA, implying that the monoamines, stored in stromal cell vesicles, either are synthesized locally or taken up by an as yet unidentified membrane transporter. VMAT2 mRNA positive cells in the stroma could be mast cells or macrophages, since these cell types are known to contain serotonin and histamine. However, since SERT and VMAT2 are normally co-expressed in both these cell types, lack of SERT expression makes them less likely candidates. Neuroendocrine argyrophil cells have been described in the stroma of normal and neoplastic endometrium (Sivridis et al., 1984; Ueda et al., 1989). These cells have been shown to have capacity for monoamine uptake (Inoue et al., 1982) and are therefore possible candidates.

Neural as well as extraneuronal monoamines have been shown to play a role in the process of decidualization of endometrial tissue. The mechanisms of action are, however, not known. Histamine has been extensively studied in this respect (Dey et al., 1979; Dey, 1981) (Barkai and Kraicer, 1996). A gradual increase of VMAT2 mRNA throughout the secretory phase suggests a gradual accumulation of monoamines in the glandular epithelium. VMAT2 transports all monoamines with highest affinity for histamine (Erickson et al., 1992). Histamine reportedly plays a role in deciduization (Hatanaka et al., 1982), implantation (Dey, 1981), immune modulation (Cocchiara et al., 1986) and blood flow regulation (Barkai and Kraicer, 1996). De-novo synthesis by endometrial cells as well as release from mast cells are possible sources of histamine (Hatanaka et al., 1982). Endogenous synthesis by endometrial cells is however most likely, since decidua formation has been shown to be intact in genetically mast cell-deficient mice (Hatanaka et al., 1982). Accumulation of histamine in intracellular vesicles during the secretory phase presumably represents a pool which, when released, can stimulate initial decidual formation. After implantation, the embryo is a source of histamine needed for further decidua formation (Dey and Johnson, 1980). This might then explain the lack of VMAT2 mRNA expression seen in early decidua. Among the plasma membrane transporters, only SERT was detected in epithelial cells of early decidua. 5-HT has been shown to inhibit decidualization (Mitchell et al., 1983; Maekawa and Yamanouchi, 1996). Expression of the SERT gene might possibly represent a protective mechanism, which maintains low levels of 5-HT thereby assuring decidualization.

Monoamines in general are potent vasoactive agents. Regulation of uterine blood flow is important in cycling endometrium and during menstruation, but most of all during pregnancy when blood flow increases markedly. Depressed levels of NE correlate with endometrial hyperaemia seen during decidua formation in guinea-pigs (Garris and Dar, 1985). Cocaine and hydrocortisone have been shown to inhibit neuronal and extraneuronal NE uptake respectively. By inhibiting NE uptake, changes of the contractile response of the porcine uterine artery have been demonstrated in early pregnancy (Laporte and DeRoth, 1997). NET may have a role as a protective mechanism, preventing sympathetic hyperactivity from reducing uterine blood flow. In non-fertile cycles the secretory endometrium eventually disintegrates and menstruation is initiated. Monoamines accumulated into vesicles by VMAT2 during the secretory phase could be released en mass and play a role in vasocostriction of the uterine spiral arteries and thereby diminish excessive bleeding.

Peak expression of NET in the late proliferative phase could not be verified with quantitative PCR. Instead, real time PCR showed low expression levels throughout the cycle which, however, tended to be higher in the proliferative phase. In order to rule out hybridization artefacts to a specific part of the gene, the real time probes were designed as one overlapping with the in-situ probe and one non-overlapping. Both probes showed identical patterns, thus ruling out artefacts as an explanation. The overall dynamics of VMAT2 mRNA expression in the endometrium were confirmed with real time PCR. The methods used can detect low levels of RNA in tissue but only ISSH allows identification of low intensity expression in single cells. A possible risk when analysing low expression transcripts, as NET appears to be, from heterogeneous tissue, is that cyclic variations can be masked as the tissue composition changes. Endometrial morphology changes significantly from the proliferative to the secretory phase when the glands increase in size and the stroma becomes denser. Such changes may help to explain discrepancies between results obtained with in-situ hybridization histochemistry and real time PCR.

In summary, our results show that monoamine transporters are expressed in endometrial tissue in a cycle-dependent way, which suggests involvement in the reproductive process by regulating re-uptake and release of monoamines. However, it should be emphasized that demonstration of NET and VMAT2 mRNA in endometrium and decidua does not necessarily prove the presence of functional transporter protein activities. Future studies applying other methods will address this question.

Acknowledgements

The author is grateful to Drs. Susan Amara and Jeff Erickson for providing NET and VMAT cDNA respectively. The Swedish Research Council 14358, 14187 and The Tegger Foundation supported this study.

References


Bradley, D.J., Towle H.C. and Young, W.S. III (1992) Spatial and temporal expression of a- and b-thyroid hormone receptor mRNAs, including the b2-subtype, in the developing mammalian nervous system. *J. Neurosci.*, 12, 2288–2302.

Submitted on February 17, 2003; accepted on March 21, 2003