The Effects of SSRI Treatment on Human Placenta and Embryo

HELENA KAIHOLA
Abstract


During pregnancy, 4 - 7% of women suffer from major depressive disorder. When antidepressive treatment is needed, selective serotonin reuptake inhibitors (SSRIs) are the most commonly used. Although severe complications from SSRI treatment are rare, association with a number of adverse pregnancy and fetal outcomes has been found. Also, antenatal depression per se has been shown to affect pregnancy outcomes. The overall aim of this thesis was to examine the effects of SSRIs on human placenta and embryo.

In the first study, gene expression was investigated in placenta from depressed, SSRI-treated and healthy pregnant women, using microarray analysis. Antenatal depression and SSRI treatment induced alterations in gene expression, but only 20 genes in common were noted. Validation with qRT-PCR showed that six out of seven selected genes were altered in SSRI-treated women compared with controls, and two genes were altered between depressed women and controls.

In study two, the protein levels in placenta from depressed, SSRI-treated and healthy pregnant women were investigated, focusing on the NGF signaling pathway. NGF, phosphorylated Raf-1, ROCK2 and phosphorylated ROCK2, were altered in both SSRI-treated and depressed women, although the proteins were regulated differently in the two groups.

In the third study, human embryos were treated with fluoxetine. Embryo development and protein expression were studied. Fluoxetine had some effect on the timing of embryo developmental stages. Also, several proteins were uniquely found in fluoxetine-treated embryos compared with untreated embryos. Fluoxetine also altered the levels of proteins secreted from the embryo.

In the fourth study, the human neuroblastoma cell line SH-SY5Y/TrkA was treated with TPA and NGF. The activation of Raf-1 was investigated and the involvement of Ras and PKC was studied. Both NGF and TPA activated Raf-1, but to a different extent and via different pathways. The NGF-induced activation of Raf-1 was mediated via Ras, while TPA induced signaling via PKC.

In conclusion, SSRI treatment and antenatal depression influence placental gene and protein expression. These findings may affect placental development and function, which in turn could affect fetal development. Also, direct exposure of embryos to fluoxetine has some effects on embryo development and protein expression, which may affect the development of the fetus.

Keywords: antenatal depression, embryo, embryo development, fluoxetine, gene expression, NGF, placenta, protein expression, Raf-1, ROCK, signaling pathways, SSRI

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To my family
List of Papers

This thesis is based on the following studies, which are referred to in the text by their Roman numerals. To be noted: my maiden name was Söderholm.


Reprints were made with permission from the respective publishers.
Methodological considerations.................................................................54
Choice of control group .......................................................................54
Sample size ..........................................................................................56
Human embryos ...................................................................................57
Experimental considerations ...............................................................57
The effects of SSRI and antenatal depression on placenta .......................58
ROCK1 and ROCK2 ...........................................................................59
Trophoblasts, placenta and pregnancy complications .........................60
Developmental programming ..............................................................62
Additive or separate effects? ...............................................................63
Fluoxetine affects embryo development ...........................................63
NGF signaling pathways ..........................................................................64
Conclusions and remarks .................................................................66

Summary of conclusions...............................................................................67
Sammanfattning på svenska (Summary in Swedish)...............................68
Acknowledgements .......................................................................................70
References.....................................................................................................74
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACOG</td>
<td>The American college of obstetricians and gynecologists</td>
</tr>
<tr>
<td>BASIC</td>
<td>Biology, affect, stress, imaging and cognition in pregnancy and puerperium</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid (DNA)</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EPDS</td>
<td>Edinburgh postnatal depression scale</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FLX</td>
<td>Fluoxetine</td>
</tr>
<tr>
<td>GST-MEK</td>
<td>Glutathione S-transferase-MEK</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamus-pituitary-adrenal</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity pathway analysis</td>
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<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MARCKS</td>
<td>Myristoylated alanine-rich C-kinase substrate</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen/extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NICE</td>
<td>The national institute of health and care excellence</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated, coiled-coil-containing protein kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecy1 sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tropomyosin receptor kinase A</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
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<tr>
<td>WB</td>
<td>Western blot</td>
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Introduction

In 2014, 10.4% of Swedish women of childbearing age (20 – 39 years) purchased prescribed antidepressant at least once. While the majority of these women are not pregnant when treatment is initiated, some will find themselves in a situation where they need to consider whether or not to continue treatment during a planned or unplanned pregnancy. The decision to continue treatment must be individualized, and be made together with the prescribing physician, and also be based on the best available evidence and risk-benefit assessments. While we have seen an increasing number of epidemiological reports on the side-effects of selective serotonin reuptake inhibitors (SSRI) during the past 15 years, little is known about the biological effects of this treatment during pregnancy. Thus, the major aim of this thesis is to expand our knowledge about the effects of antidepressant treatment on placental function and embryo development.

Antenatal depression and anxiety

Worldwide, approximately 20% of pregnant women experience depressive symptoms and 4 - 7% suffer from major depressive disorder [1-4]. Among women with postpartum depression, almost 40% already had depressive symptoms during pregnancy [5]. Besides the maternal and fetal outcomes associated with depression, discussed further below, the most severe consequence of depression is suicide. Indeed, in the United Kingdom, the Eighth Report of the Confidential Enquiries into Maternal Deaths [6] has consistently found mental health problems to be one of the leading causes of maternal death, with over half of these deaths being caused by suicide. In Sweden a similar pattern is noted, although it should be emphasized that maternal suicides predominantly occur in the postpartum period [7]. Out of 103 maternal suicides reported between 1980 and 2007 in Sweden, only three occurred during pregnancy (Esscher et al., in press).

However, major depressive disorder is not the only reason for women to continue SSRI treatment during pregnancy, as these drugs are often used for treatment of anxiety disorders as well. In addition, co-morbid depressive and anxiety disorders are common during pregnancy [8], ultimately affecting the choice of comparison group for this thesis, and the possibility of separating the effects of anxiety and depression on fetal outcomes.
Unfortunately, anxiety disorders are far less studied than depression in pregnancy, which is also one of the major reasons why this thesis focuses on cases of depression. The prevalence of most anxiety disorders in pregnancy appear similar to what is found at other times in women’s lives [8]; a population-based U.S. study reported a 13% one-year prevalence of any anxiety disorder in pregnancy, which was comparable to non-pregnant women [9]. By comparison, the point prevalence of any anxiety disorder in a population-based sample of Swedish pregnant women was 6.6% [2]. As to the prevalence rates of specific anxiety disorders, a recent systematic review reported on wide variations across studies. For instance, generalized anxiety disorder (GAD) was found in 0 – 10.5%, panic disorder in 0.2 – 5.7%, specific phobia in 3.2 – 19.9%, social phobia in 0.4 – 6.4% and posttraumatic stress disorder (PTSD) in 0 – 7.9% of pregnant women [8]. Generally, European and U.S. studies contributed with prevalence rates in the lower range.

The gold standard for depression and anxiety diagnoses is the structured psychiatric interview, with the Structured Clinical Interview of Diagnostics and Statistical Manual of Mental (DSM) disorders (SCID) being the most commonly used in research studies. However, for screening purposes during pregnancy and in the postpartum period, most researchers and clinicians use the Edinburgh Postnatal Depression Scale (EPDS) [10]. EPDS is a self-reporting questionnaire with 10 items [11], which has been translated into Swedish and validated for use in the postpartum period [12] and during pregnancy [13]. While the sensitivity of the instrument is not impressive, typically ranging between 0.47 – 0.71 depending on cutoff, the specificity as regards depressive disorders is excellent (pooled specificity 0.94 – 0.98) [14]. The EPDS is implemented in clinical routine in Sweden and is typically administrated at the first postnatal follow-up.

Untreated depression is strongly associated with poor quality of life, for patients and their relatives, and increased mortality [15-18]. The World Health Organisation predicts that major depressive disorder will be the most serious disease worldwide in this century [19]. Depressive disorders are associated with heavy social and financial burdens [20, 21] and results in a substantial public health disease burden [22]. Therefore, it is important to identify the symptoms of depression, to be able to treat the disease and increase quality of life for the patient.

Typical symptoms of depression are fatigue, trouble sleeping, reduced appetite, pessimistic thinking, anhedonia, reduced concentration and attention, as well as low self-esteem [23]. Important symptoms to notice are thoughts of self-harm or life not being worth living, since these thoughts increase the risk of suicide. In patients with more severe depression, psychomotor changes are noted. Both slowed psychomotor functioning, as well as agitated psychomotor activity, can be seen [23].

“Biological” symptoms can sometimes be seen in depressed patients. These symptoms, sometimes called melancholic, are e.g. weight loss, consti-
pation, loss of appetite, early morning waking, and diurnal mood variations, with the worst feelings in the morning and feeling better later in the evening [24]. Melancholic depression is a validated subgroup of depression; however, melancholic symptoms can be found in most depressed patients [25].

Causes of antenatal depression

Several risk factors for antenatal depression have been identified: prior history of depression or other mental health problems, low socioeconomic status, being a single mother or a teenage mother, domestic violence, smoking, and obesity are among the most common [26, 27]. In addition, the genetic set-up of the mother, in combination with environmental factors like stress, life experiences and previous reproductive/hormonal history, may also influence the individual risk of developing depression during pregnancy [28].

Depression is associated with altered levels of monoamine neurotransmitters, especially noradrenalin and serotonin, in the brain. These altered levels can be caused by a genetic predisposition, tissue damage, or, as in the case of pregnancy, by altered hormone levels [29-31]. Clinical studies on neurotransmitter adaptations in the central nervous system during pregnancy are rare, primarily due to a precautionary approach to the effects of imaging techniques on the fetus. However, positron emission tomography studies have revealed significantly increased monoamine oxidase A (MAO-A) activity (i.e. increased breakdown of serotonin, norepinephrine, and dopamine), throughout all analyzed brain regions in the early postpartum period of healthy women [32]. Likewise, studies on serum indicators of serotonergic activity in healthy women a few days after delivery strengthen the idea that the early postpartum is a state of serotonin deficiency [33-35]. Indirect evidence of central nervous changes also include markedly decreased maternal serum levels of brain-derived neurotrophic factor (BDNF), both before and after delivery, and at both time-points BDNF was correlated with decreased serotonin levels [35].

Both increased and decreased levels of cortisol have been associated with depression [36, 37]. Stressful life events (e.g. parental loss, major childhood stress or abuse, deteriorating physical health) can increase cortisol levels, which lead to an increased vulnerability to depression [38]. At the end of the pregnancy, the cortisol levels have increased three fold compared to those of non-pregnant women. At the same time, the hypothalamus-pituitary-adrenal (HPA) axis reactivity to stressful stimuli is dampened [39]. In addition, by positive feedback from cortisol, the placenta also secretes large amounts of corticotropin releasing hormone (CRH), resulting in 100 - 1,000-fold increased CRH levels in maternal serum during pregnancy [40]. After birth, the cortisol and CRH levels drop dramatically, but the responsiveness of the HPA axis will not return to normal until approximately 6 - 8 weeks postpartum [41, 42]. These perturbations in the HPA axis across pregnancy and
postpartum have repeatedly been held responsible for the mood symptoms that may develop, but findings in pregnant (and postpartum) women are thus far mixed [43]. For instance, previous research from our department has indicated that women with antenatal depression have similar cortisol awakening response [44] and similar CRH levels (Hannerfors et al., submitted manuscript) in comparison with healthy pregnant women.

The effect of antenatal depression on pregnancy outcomes, fetal development and offspring

A number of negative effects of antenatal depression have been described in the literature. However, when reading through the list of possible outcomes, it should be emphasized that the association between exposure (antenatal depression) and outcome may not always be as straightforward as individual reports may claim. It is quite possible that risk factors such as smoking, obesity, drug or alcohol use, or domestic violence and abuse, which are common in women with mental health problems, explain some of the effects that have been attributed to the antenatal depression, as these comorbidities are also risk factors for adverse obstetric and child outcomes. Thus, to separate the effects of mental health problems in pregnancy from these confounders, large sample sizes are needed. Furthermore, there may be additional factors that researchers thus far have not paid attention to. For instance, Conroy and colleagues [45] showed that children with behavioral disorders are predominantly found when the mother is suffering from comorbid depression and personality disorder, whereas these problems were not found in children of women with only depression. Also, poor outcomes in children occur predominantly in families living with socioeconomic difficulties, and high educational level in the depressed mother is, in fact, protective [46, 47]. Taken together, these findings suggest that not only the depression per se, but also other factors influence the outcome of a child.

Nevertheless, antenatal depression has been associated with an increased risk of preeclampsia, preterm birth, decreased fetal head growth, impaired fetoplacental function, and increased risk of neonatal complications [48-50]. Maternal depression has also been associated with low birth weight; however, this effect was predominantly found in low-income countries and in countries with marked health inequalities [51]. Several behavioral problems and developmental delays have been described in children exposed to antenatal depression (in utero). Developmental delays were found in 18-month-old children [49, 50]. Behavioral problems were found in both young and old children, where children of 3 and 4 years of age had increased behavioral, emotional and attention problems. Children aged 6 to 9 years had increased levels of anxiety, and in adolescence, children exposed to antenatal depression showed emotional disorders like violent behavior [49, 50, 52]. Howev-
er, this association was only significant in girls, suggesting that there might be a gender difference in how antenatal depression affects the offspring.

The obstetric and fetal outcomes in relation to anxiety disorder are sparsely studied, and, thus far, findings are mixed. A recent systematic review reported on six studies where premature birth or infant weight had been assessed in relation to anxiety disorders [8]. While no meta-analytic approach was possible, the review concluded that half of studies found no influence on these outcomes, whereas remaining studies did show some association with premature birth and/or birth weight [8]. Even more preliminary, two small studies, possibly on the same material, suggested lower BDNF [53] and higher circulating tumor necrosis factor-alpha (TNFα) [54] levels in the cord blood of newborns to mothers with GAD. Yet another study reported on higher stress-induced cortisol levels (but similar behavioral response) in 7-month old infants of prenatally anxious mothers [55]. Thus, given the commonly encountered comorbidity between depressive and anxiety disorders, the long-term effects on offspring to depressed mothers may equally well be a result of peripartum anxiety.

Selective serotonin reuptake inhibitors (SSRIs)

In the 1980s selective serotonin reuptake inhibitors (SSRIs) (Table 1) were introduced and soon became the primary antidepressants used during pregnancy due to their good efficacy and relatively few side-effects [56]. With time, SSRIs were found to be associated with rare but significant side effects, discussed below. As of today, 1 - 3% of European women use antidepressants during pregnancy [48, 57] and in the U.S. as many as 4 - 13% of pregnant women are using antidepressants [58, 59]. In addition, Alwan and colleagues [60] have shown that these numbers are increasing. In 1998, 2.5% of pregnant women reported using antidepressants at some time during pregnancy. In 2005, this number had increased to 8.1% and the numbers are still increasing. However, studies have shown that 75% of SSRI-treated women in the U.S. discontinue antidepressant treatment either before pregnancy, at discovery of pregnancy, or during the first trimester [59].

Serotonin (5-HT) is one of the neurotransmitters that regulate mood and emotions, depending on how much of the protein is released into the synaptic cleft (Fig. 1). To regulate the levels of serotonin in the extracellular cleft, the released serotonin is reuptaken to the presynaptic neuron by serotonin transporters [61]. SSRIs inhibit the reuptake of serotonin, thereby increasing the levels of serotonin in the synaptic cleft between two neurons (Fig. 1). SSRIs cross the placenta and have been found in cord blood and amniotic fluid [62-65], which means that the fetus is exposed to SSRIs during its development, either via the blood or by swallowing and inhaling the amniotic fluid.
Table 1. SSRI substances and medical products available for prescription in Sweden according to FASS (*Farmaceutiska Specialiteter i Sverige*). Number of prescriptions of each substance to Swedish women in 2014 according to The National Board of Health and Welfare in Sweden (shown as % of total SSRI prescriptions in Sweden)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Medical product</th>
<th>Prescriptions to women in Sweden (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citalopram</td>
<td>Cipramil, Citalopram</td>
<td>48.32</td>
</tr>
<tr>
<td>Sertraline</td>
<td>Oraline, Sertraline, Sertrone, Zoloft</td>
<td>28.88</td>
</tr>
<tr>
<td>Escitalopram</td>
<td>Cipralex, Premalex</td>
<td>11.31</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Fluoxetine, Fontex</td>
<td>7.35</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>Paroxetine, Paroxiflex, Seroxat</td>
<td>4.09</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>Fevarin</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Figure 1. The function of SSRI on serotonin reuptake in the extracellular synaptic cleft (Olivier *et al.* (2013) Front Cell Neurosci vol. 7:73, p. 1-15).
Serotonin and its role during embryo development

**Embryo development**

The development of a child is a nine month long process that begins with the fertilization of an egg and ends at the delivery of the child. During this development, a number of processes and events have to occur in a structured and chronological order. The development of an embryo, from the first cell division to the formation of a blastocyst, has to follow certain critical steps. Even if these initial events proceed according to plan, nine months remain during which the embryo/fetus continues to develop, and is affected by the surrounding environment *in utero*. The embryo development can be divided into four periods: pre-embryo period (from fertilization until blastocyst formation); implantation period (day 6 until 7); post-implantation period (day 9 until week 4) when the organs and organic systems are developed; and fetal period (from week 4 until birth) when the organic systems grow and mature [66, 67].

After fertilization of the oocyte, the single cell divides into two cells and continues to divide until it reaches compaction and forms the morula (Fig. 2). In the morula, a cavitation is formed and the embryo starts to expand into a blastocyst with an outer layer of trophoblastic cells, the trophectoderm, and an inner cell mass, the embryoblast (Fig. 2). The blastocyst is surrounded by the *zona pellucida*, but at day 6 the blastocyst breaks out from the *zona*, an event called hatching (Fig. 2). By this time, the blastocyst has travelled through the fallopian tubes and has reached the uterine cavity. The trophoblasts invade the outer cell layers of the endometrium to implant. Later on, the trophectoderm develops into the placenta and the inner cell mass forms the fetus and the amniotic cavity [68-70].

**Scoring of embryo development**

Endogenous embryonic gene expression first occurs between the four- to eight-cell stages [71]. Based on studies within the *in vitro* fertilization (IVF) field, the timing of the different embryo developmental stages has been shown to be of importance. If the timing between embryo developmental stages is too short, the quality of the embryo is negatively affected [72-76]. Neither is it beneficial for a proper embryo development, if the timing is too long [73, 77]. For IVF, it is important to carefully select which embryo to transfer back to the woman after embryo culturing. Therefore, the timing of developmental stages has been carefully studied and a scoring system has been developed [76, 78, 79].

To monitor development of an embryo, embryologists have started to use a closed system consisting of an incubator with a time-lapse camera, e.g. EmbryoScope® (Unisense FertiliTech, Denmark). Timing between cell divisions, compaction, cavitation and formation of blastocyst is analyzed, together with recording whether the embryo contains fragments reflecting cell
deaths or improper cell division. All these events are scored and a total final score is set for each embryo. The inner cell mass and the trophectoderm are scored from A (the best) to D (the worst), and the developmental stage is scored from 1 to 6, where 6 refers to a hatched blastocyst [70, 76, 79]. The best total score for an embryo is 6AA. Gardner and colleagues [80] found that embryos with a score of 3AA had an implantation rate of 70%.

Figure 2. Pictures of embryo development monitored in an EmbryoScope®. A) embryo at 4 cell stage, B) embryo at 8 cell stage, C) morula, D) beginning of cavitation (cav), E) blastocyst with trophectoderm (t) and inner cell mass (i), and F) hatching.

Implantation window
One major event with high impact on a successful pregnancy rate is the implantation of the embryo, which occurs in the luteal phase in the menstrual cycle in humans. This well-orchestrated cross-talk between the blastocyst and the uterine endometrium is referred to as the window of implantation [81]. The window of implantation consists of four major events, i.e. the blastocyst activation, uterine receptivity, blastocyst attachment and uterine decidualization, the latter meaning that the endometrial stroma undergoes cellular transformation to facilitate embryonic growth and invasion [81]. A
prerequisite for attachment to the uterine endometrium is hatching of the blastocyst, in which the blastocyst bursts out of the zona pellucida. The presence of an embryo in the uterus stimulates the endometrium to become more receptive for embryo attachment [82]. During the window of implantation, estrogen and progesterone induce cells in the endometrium to proliferate and differentiate and to secrete proteins that affect trophoblast development. Also, Altmäe and colleagues [83] confirmed in their study that many genes and signaling pathways are involved in human endometrium receptivity and the embryo-endometrium interaction. Some of the genes and pathways included are JAK-STAT signaling pathways, inflammatory pathways, leukemia inhibitory factor (LIF), leptin (LEP), fibroblast growth factor 7 (FGF7), and heparin-binding EGF-like growth factor 7 (HB-EGF) [82]. Once the endometrium has become receptive to the embryo, the embryo attaches to pinopodes in the endometrium [84] and the decidualization of the endometrium is initiated. Also, serotonin may have a role in the implantation process. Mitchell and colleagues [85] showed that rats subcutaneously injected with serotonin had an impaired implantation due to suppression of decidualization, indicating that serotonin has an impact on embryo implantation. After decidualization, the trophoblasts can invade the endometrium, the embryo can become embedded, and the placenta is eventually formed [69].

**Embryo susceptibility to teratogens**

During the post-implantation period in the embryo development, when the majority of the organs are developed, the embryo is most susceptible to teratogens [86]. Teratogens are substances, drugs or infectious agents, which cause congenital malformations in the fetus. The timing of drug administration during pregnancy is crucial: use of drugs early in pregnancy can have severe effects on the offspring. Administration later in pregnancy reduces the risk of congenital malformations, but there is a risk of toxicological or pharmacological effects on the fetus. Sometimes, the combination of two drugs can increase the teratogenic effects and there may also be a correlation between drug dose and severity of malformations [86].

There is evidence that the effects of teratogens on individual humans vary considerably [87] and there have been attempts to elucidate the mechanisms behind the individual differences. Genetic predisposition for increased susceptibility has been suggested [88] as well as genetic polymorphisms of placental transporter proteins [89]. Unfortunately, the available data are often conflicting and methodological tools need further development.

**Serotonin and its role for embryo development**

Serotonin is found in follicular and fallopian tube fluid (at least in female rats) [90, 91]. Serotonin transporters and tryptophan hydroxylase 2, the enzyme responsible for serotonin synthesis, are expressed as early as in two-cell mice embryos [92, 93]. In addition, expression of some serotonin recep-
tors has been demonstrated in mouse preimplantation embryos, such as 5-HT$_{1D}$ and 5-HT$_{7}$, but thus far, none of the 5-HT$_{3}$ receptors have been identified [94]. The exact role of serotonin at this stage is not known; it has merely been speculated that the serotonin transporter expressed in embryos is important for transport of serotonin from surrounding fluid. During embryogenesis, high and low levels of serotonin can have both positive and negative effects on embryo development, depending on the stage of development. Mouse embryos exposed to serotonin, from 4-cell stage until blastocyst, showed significantly reduced number of cells and there was an increased incidence of dead cells [95]. However, Khozhai and colleagues [96] showed that, by reducing the levels of maternal serotonin by administration of a tryptophan hydroxylase inhibitor to pregnant mice, the development of mouse preimplantation embryos was impaired. Altered expression of different G-protein coupled serotonin receptors during the preimplantation period could explain these differences in response to serotonin, since the different serotonin receptors could activate different signaling pathways at different developmental stages [94]. Kim and colleagues [97] treated mouse 2-cell stage embryos with fluoxetine, a selective serotonin reuptake inhibitor, and found that embryos exposed for a short period to low-dose fluoxetine had an increased percentage reaching blastocyst stage compared with controls. This was probably mediated through activation of Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII)-dependent signaling pathways. Also, long-term exposure decreased mouse early embryo development through inhibition of the potassium channels TREK-1 and TREK-2. This indicates that both the exposure time and the developmental stage of the embryo at exposure, has an influence on mouse embryo development. However, studies in human embryos are still lacking.

Serotonin and its role during placental development

The placenta is an organ that develops during pregnancy. It protects and provides the fetus with nutrients, proteins and hormones to secure normal fetal development. The placenta serves as an endocrine organ throughout fetal development and synthesizes and secretes a variety of steroid and protein hormones, including estrogen, progesterone, human chorionic gonadotropin, and placental lactogen. In addition, it also produces and secretes a number of substances important for the development of the fetal brain [98].

During placentation, the trophoblasts of the blastocyst invade the epithelial cells of the uterus [68]. This step is of great importance since dysregulation and improper implantation can lead to miscarriage [99]. From the myometrium, spiral arteries stretch towards the intervilluos space of the placenta [68]. Early in pregnancy, the spiral arteries are composed of smooth muscle cells. Later on in pregnancy, cytotrophoblasts from the fetal part of the placenta invade the spiral arteries, making them more dilated and less sensi-
tive to changes in blood pressure. Improper invasions of trophoblasts are involved in e.g. preeclampsia, where the spiral arteries have kept some of their ability to respond to changes in blood pressure [100].

Serotonin is important for placentation [101] and acts as a vasoconstrictor in the placenta [102]. Oufkir and colleagues [101] demonstrated that activation of the serotonin receptor 5-HT$_{2A}$ in trophoblast cells activates intracellular signals that are known to regulate survival, differentiation, migration and invasion, suggesting a role for serotonin in placentation. This fits well with the suggestions that serotonin plays a role in preeclampsia [103] and in the pathophysiology of gestational diabetes [104].

During brain development, serotonin acts as a neurotrophic factor regulating e.g. cell division, differentiation and synaptogenesis [105]. The placenta has been shown to serve as a serotonin source and serotonin produced by the placenta is accumulated in the fetal forebrain where it is involved in fetal brain development [106].

The effects of SSRIs on pregnancy and fetal development

As for antenatal depression, the reported risks associated with SSRI use during pregnancy need to be interpreted with caution. Most epidemiological studies rely on registers, but these registers rarely include information on health behaviors in pregnancy, and thus, important confounders may be lacking in the analyses. Furthermore, pharmaco-epidemiological studies need to consider confounding by indication (i.e. the underlying effects of the psychiatric disorder) when examining adverse effects of SSRIs. This is typically accomplished either by defining a comparison group who have discontinued SSRI use prior to pregnancy or by using women with untreated psychiatric disorders for comparison. In addition, in most epidemiological research SSRI exposure relies on filed prescriptions, but women may not always use their medication as prescribed, ultimately resulting in misclassification bias.

These problems were elegantly demonstrated by a recent Danish study on the risk of miscarriage in SSRI-treated women. With previous reports suggesting an increased risk for miscarriage in SSRI users [107, 108], the Danish study came to quite different conclusions. Being able to adjust for underlying psychiatric diagnoses, the study found that women who discontinued SSRI treatment had higher risk of miscarriage than those who continued treatment and non-users. While the SSRI users also had an increased risk of miscarriage, this finding no longer remained when psychiatric diagnoses were adjusted for. In fact, women on SSRI who had no psychiatric diagnosis (suggesting that they were treated in primary health care rather than in psychiatric care) had lower risk of miscarriage in comparison with untreated women who had a psychiatric diagnosis [109]. Furthermore, the risk for second trimester miscarriages were the same regardless of whether the depressed women were using SSRI or not [109]. Similarly, no risk for still-
birth, neonatal mortality or postneonatal mortality has been noted in SSRI users [110].

SSRI treatment during pregnancy has been associated with preterm birth [59, 111-113], decreased birth weight (or small for gestational age), reduced fetal head growth, and impaired fetoplacental function [48, 114-118]. However, there are also several studies that show no effect of SSRI on preterm birth and birth weight [113, 119, 120], suggesting that it is the depression per se and/or other associated behaviors or conditions, such as smoking, obesity or drug abuse, that causes these outcomes. Of specific relevance for this thesis, an association with preeclampsia has also been described [116, 121-123], which appears relevant in relation to the pathophysiology of the disorder, i.e. increased risk if treatment is continued into the second trimester in comparison with those who discontinued during the first trimester. However, it should be emphasized that risk estimates for development of preeclampsia are higher for serotonin and noradrenaline reuptake inhibitors (SNRI) and tricyclic antidepressants than for SSRI monotherapy [122].

The effects of SSRIs on the offspring

Infants exposed to SSRIs during pregnancy run the risk of poor neonatal adaption, such as jitteriness, convulsions, respiratory distress, hypoglycaemia and feeding difficulties, also referred to as the neonatal adaptation syndrome (reviewed in [49, 124]). There are several studies showing that infants antenatally exposed to SSRIs more often need neonatal intensive care and have a higher rate of neonatal seizures [115, 117, 125].

Other, less frequent, effects of antenatal SSRI exposure are physiological malformations. There have been several reports on the association between SSRI exposure and congenital malformations like omphalocele, anencephaly and craniostenosis [126-129]. Also, cardiac malformations have been found in infants exposed to SSRIs in utero [120, 130]. However, risks of congenital anomalies in association with the use of SSRIs (or other drugs) might be underestimated if elective terminations of pregnancies are not taken into account. Kieler and colleagues [131] recently addressed this concern, but found no association between the use of SSRIs and elective termination of pregnancy for fetal anomalies. As expected, induced abortion due to maternal ill health or socio-economic disadvantage was more common in women using antidepressants.

Another rare condition found in infants prenatally exposed to SSRI, is persistent pulmonary hypertension (PPHN) [57, 114, 127, 132]. In PPHN, the pulmonary vasculature does not relax after birth, which leads to hypoxemia. There is significant evidence of an increased risk of PPHN due to SSRI exposure, but the actual risk difference between exposure and non-exposure is very small (an increase of 2 per 1000) [14].

22
Later in life, children exposed to SSRIs during pregnancy are at greater risk of developing behavioral disorders; like autism spectrum disorders [133, 134]. Externalizing behavior has been found in 4-year-old children after antenatal exposure to SSRI [135], but Oberlander and colleagues later also reported on internalizing behaviors in 3- and 4-year-old children following SSRI exposure [136]. Again, there are also studies suggesting no association between prenatal SSRI exposure and internalizing behaviors [137].

Treatment of mental health problems during pregnancy

International guidelines for treatment of antenatal health problems are available. The American College of Obstetricians and Gynecologists (ACOG) published their recommendations in 2008 [138], and The National Institute for Health and Care Excellence (NICE) recently published their updated recommendations on clinical management and service guidance for women with antenatal and postnatal mental health problems [14]. Both guidelines stress the importance of preconception counselling, and the importance of integrated care plans for women who present in antenatal care with mental health problems. In addition, both guidelines emphasize that the potential risk of SSRI use in pregnancy must be considered in the context of the risks associated with untreated mental health problems and the risk of relapse of depression if treatment is discontinued in pregnancy. Treatment with SSRIs or other antidepressants during pregnancy should be individualized. However, it is interesting that the ACOG guidelines strongly recommend that paroxetine use in pregnant women and women planning pregnancy should be avoided.

The NICE guidelines, being issued by psychiatrists, are more detailed in their treatment recommendations and emphasize the role of high-intensity psychological interventions. For women with mild to moderate depression, psychological intervention is the first line of treatment. Pharmacological treatment is only recommended for women with moderate or severe depression if the woman has expressed a preference for medical treatment, or declines psychological treatment, or has not responded to psychological treatment. However, the NICE guidelines suggest that in women with a history of severe depression who present even with mild symptoms of depression in pregnancy, pharmacological treatment should be considered. As to choice of antidepressant treatment, NICE is less specific than ACOG but stresses that the drug with lowest risk profile and at the lowest effective dose should be used (taking into account that dosages may need to be adjusted during pregnancy). In line with ACOG, single drug treatment is advocated. NICE guidelines also mention the risk of neonatal adaptation syndrome in babies exposed to paroxetine and venlafaxine.
The role of NGF in neuronal and non-neuronal development

In the 1950s, Levi-Montalcini discovered a protein later called the nerve growth factor (NGF), reflecting its ability to stimulate growth and differentiation of neuronal cells [139].

The most obvious role of NGF is in neurogenesis and neuritogenesis (reviewed in [140]), but NGF is also found in a number of other tissues, suggesting a role in other non-neuronal processes like angiogenesis [141] and immune response [142].

Ovarian production of NGF is involved in folliculogenesis, where it seems to be important for a proper ovulation to occur [143-145]. But, an excessive production of NGF has been shown to have an opposite effect, where mice with an overproduction of NGF are sub-fertile [146].

During pregnancy, NGF is expressed in the placenta and plays a crucial role in placentation and pregnancy maintenance [147]. NGF expression was also found in the maternal decidua and the decidual vessels, indicating a role for NGF in implantation and formation of vessels in the decidua [147]. Also, there are studies showing that NGF is involved in stress-induced abortion and preterm birth [148, 149].

Signaling pathways downstream of the NGF receptor

NGF binds to two different receptors, tropomyosin receptor kinase A (TrkA) and p75 [150-152]. Upon binding to TrkA, the receptor becomes autophosphorylated on several tyrosine residues, initiating a number of signaling cascades. In this thesis, focus is on the rat sarcoma (Ras)-rapidly accelerated fibrosarcoma (Raf)-mitogen-activated protein kinase (MAPK) and Ras-related C3 botulinum toxin substrate (Rac)-Ras homolog gene family member A (RhoA) pathways (Fig. 3).

When TrkA is phosphorylated on tyrosine residue 490, Src homology 2 containing (SHC) protein is bound to the receptor and forms a complex with son of sevenless (SOS) and growth factor receptor-bound protein 2 (Grb2) (Fig. 3) [153]. This initiates a signaling cascade that involves Ras, Raf, mitogen/extracellular signal-regulated kinase 1/2 (MEK1/2) and extra-cellular signal-regulated kinase 1/2 (ERK1/2), which leads to differentiation and survival of a cell [154-156].

Another pathway down-stream of TrkA is the Rac-RhoA signaling pathway [157, 158], where RhoA further activates Rho-associated, coiled-coil-containing protein kinase 1/2 (ROCK1/2) [159, 160].

ROCK1/2 can also be activated via Ras-Raf-MAPK [161], which is then mediated via ribosomal S6 kinase (RSK) [162, 163].

NGF also cross-talks with other signaling pathways. Both vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are up-regulated by
Figure 3. Schematic figure on NGF signaling pathways with focus on Ras-Raf-MAPK and Rac-Rho pathways.
over-expression of NGF in brown adipose tissue in mice and this up-
regulation induces angiogenesis [164].

Takei and Laskey [165] found that NGF induces expression of tumor ne-
crosis factor-α (TNFα) in the undifferentiated SH-SY5Y neuroblastoma cell
line. The increased TNFα expression suppresses the NGF-induced ERK
activation, thereby blocking NGF-induced differentiation of the cells. Also,
mouse macrophages treated with NGF induces TNFα expression and this
increased production was mediated via the MAPK-ERK pathway [166].

In human monocytes, the NGF-receptor TrkA, the epidermal growth fac-
tor receptor (EGFR) and the formyl peptide receptor (FPR) cross-talks via
e.g. ERK when modulating pro-inflammatory mediators [167].

Raf-1 can also be activated by the phorbolester 12-O-tetradecanoyl-
phorbol-13-acetate (TPA) in a protein kinase C (PKC)-dependent manner
[168, 169] and NGF-induced neurite outgrowth has been shown to involve
PKCδ [170]. PKC can also increase the activation of Ras, which causes an
activation of Raf-1[171].

One of PKC’s substrates is myristoylated alanine-rich C-kinase substrate
(MARCKS) [172, 173], which is a protein important for normal cerebral
development in mice [174]. Since this protein is endogenously expressed in
cells, measurement of phosphorylation of MARCKS in vivo is a commonly
used method to measure the activation of PKC.

Neuroblastoma cell line SH-SY5Y as a model system

Neuroblastoma is a tumor primarily found in children. It consists of imma-
ture neuronal cells that have stopped differentiating and started to proliferate
instead [175]. The neuroblastoma cells can be arrested at different stages
during development, which is the reason why there are different stages of
neuroblastoma found in children and some which spontaneously regress
without treatment [176]. Several neuroblastoma cell lines have been estab-
lished over the years and they make perfect models for studying neuronal
development, since they can be stimulated to differentiate by different treat-
ments [175].

In study IV, the neuroblastoma cell line SH-SY5Y was used. These cells
can be induced to differentiate by treatment with the phorbolester TPA
[177]. Even though SH-SY5Y cells are of a neuronal phenotype, they do not
differentiate when treated with NGF, possibly due to the low expression of
the NGF receptor TrkA [178]. When transfecting SH-SY5Y cells with hu-
man exogenous TrkA, the cells respond to NGF treatment by formation of
neurites [179].
Aims

The main aim of this thesis was to investigate whether SSRI treatment during pregnancy has an effect on human placenta and early embryo development. The specific aims in each study were:

I. To investigate the impact of antenatal depression and antidepressant treatment during pregnancy on gene expression in the fetal placenta.

II. To investigate the independent influence of SSRI treatment and antenatal depression on placental proteins in the NGF signaling pathway.

III. To evaluate whether fluoxetine exposure influences the timing of different embryo developmental stages, and furthermore, to analyze what protein networks are affected by fluoxetine in the early development of an embryo.

IV. To investigate whether treatment of SH-SY5Y/TrkA cells with TPA and NGF leads to activation of Raf-1 and, if so, to investigate the involvement of the upstream signaling molecules Ras and PKC.
Materials and methods

Material

Women and placentas (study I and II)

Placental tissue was collected from women participating in an ongoing longitudinal study on antenatal and postpartum depression called the BASIC (the Biology, Affect, Stress, Imaging and Cognition in Pregnancy and the Puerperium) project. All women attending the routine ultrasound at gestational week 16-18 at Uppsala University Hospital are asked to participate in the BASIC study, which results in a population-based cohort. Oral and written information was given and an informed written consent was obtained. The women filled out web-based questionnaires at gestational age 17 and 32 weeks, including questions on physical and socio-demographic characteristics, medical, psychiatric, gynecologic and obstetric history variables, lifestyle, medication parameters, and the Swedish version of the Edinburgh Postnatal Depression Scale (EPDS). Information concerning maternal depression, SSRI use, delivery and neonatal outcomes were retrieved from the medical records.

For the specific aim of these sub-studies, the inclusion criteria were women of Western European descent, normal pregnancies, normal deliveries and healthy offspring (no diagnoses and no admittance to neonatal care). Exclusion criteria were smoking or alcohol use during pregnancy, any daily use of prescribed drugs during pregnancy, any other chronic conditions or disease, gestational age <35 weeks, and maternal age <18 or >42 years. Depressed women had EPDS scores corresponding to major depression, in combination with medical records confirming depression and ongoing treatment for their depression in terms of psychotherapy. Women on SSRI treatment used their treatment during the pregnancy in clinically relevant doses, i.e. low-dose use was excluded.

In study I, five women with ongoing depression, five women on SSRI treatment and ten women with normal pregnancies were included for the microarray analysis. The control group was divided into two groups and was matched with depressed or SSRI-treated women, respectively, by age, body mass index (BMI) and gestational length on an individual level.
For the validation of the microarray analysis in study I, a larger number of women was included in each group: 24 depressed women, 29 SSRI-treated women and 31 healthy pregnant women (Table 2). All the women from the microarray analysis were included in the study groups. The depressed women had an EPDS score of >12 in gestational week 17 and 32 (except for one woman with EPDS >14 at one time point).

In study II, there were 12 women in the depressed, SSRI-treated and healthy control groups, respectively (Table 3). The depressed women had EPDS scores of 13 or higher in gestational weeks 17 and 32, together with hospital records confirming major or minor depressive disorder and ongoing treatment in terms of psychotherapy. The groups were matched on maternal age.

Placental biopsies were collected at delivery from two different locations on the placenta and all the way through the placenta, containing both the maternal and fetal side. The biopsies were rinsed in cold sterile phosphate-buffered saline (PBS), frozen on dry ice within 60 minutes after delivery and stored at -70°C until further use.

Embryo collection (study III)
Couples that had undergone IVF treatment at the Center for Reproduction in Uppsala, Sweden, were asked to donate surplus embryos that would have been destroyed after 5 years of cryopreservation, according to Swedish law. A written informed consent was obtained from the couples donating the embryos.

Before freezing, the embryos had been inseminated with sperms and, after evaluation of fertilization, zygotes with 2 pro-nuclei were selected for further culturing in a humidified incubator.

Cell culture and treatments (study IV)
SH-SY5Y cells transfected with full-length human TrkA (SH-SY5Y/TrkA) were cultured under ordinary conditions. For experiments on serum-deprived cells, the cells were cultured in serum-free SHTE medium.

SH-SY5Y/TrkA cells were treated with 100 ng/mL NGF (Promega Corp., US) and 16 nM TPA (Sigma-Aldrich Corp., US) with or without serum. Treatment with 4 µM GF109203X (PKC inhibitor, Boehringer Mannheim GmbH, Germany) was performed 30 minutes before the addition of NGF or TPA.
Table 2. Demographic data on the depressed, SSRI-treated and healthy women included in the qRT-PCR validation of the microarray results. Data are presented as mean ± SD or median (min – max).

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n=31)</th>
<th>Depressed women (n=24)</th>
<th>SSRI-treated women (n=29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.4 ± 3.9</td>
<td>31.1 ± 4.3</td>
<td>31.2 ± 4.1</td>
</tr>
<tr>
<td>Parity (n, median, range)</td>
<td>0 (0-3)</td>
<td>1 (0-2)</td>
<td>1 (0-3)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.0 ± 4.9</td>
<td>24.0 ± 6.2</td>
<td>27.2 ± 4.9</td>
</tr>
<tr>
<td>Systolic blood pressure in first trimester, mmHg</td>
<td>119 ± 13</td>
<td>111 ± 12</td>
<td>118 ± 12</td>
</tr>
<tr>
<td>Diastolic blood pressure in first trimester, mmHg</td>
<td>73 ± 9</td>
<td>69 ± 7</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>Systolic blood pressure at last visit, mmHg</td>
<td>125 ± 11</td>
<td>119 ± 11</td>
<td>125 ± 10</td>
</tr>
<tr>
<td>Diastolic blood pressure at first visit, mmHg</td>
<td>79 ± 8</td>
<td>76 ± 6</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>Lowest hemoglobin level during pregnancy, g/dl</td>
<td>11.7 ± 0.8</td>
<td>11.4 ± 0.8</td>
<td>11.0 ± 0.9 c</td>
</tr>
<tr>
<td>Birth weight (gram)</td>
<td>3577 ± 351</td>
<td>3546 ± 499</td>
<td>3589 ± 400</td>
</tr>
<tr>
<td>Gender offspring (% boy)</td>
<td>65</td>
<td>54</td>
<td>45</td>
</tr>
<tr>
<td>Gestational length</td>
<td>281 ± 7</td>
<td>275 ± 11</td>
<td>273 ± 8 c</td>
</tr>
<tr>
<td>EPDS score week 17</td>
<td>2.9 ± 1.8</td>
<td>15.0 ± 4.2 a</td>
<td>7.7 ± 4.9 b</td>
</tr>
<tr>
<td>EPDS score week 32</td>
<td>2.9 ± 1.8</td>
<td>15.9 ± 3.6 a</td>
<td>8.7 ± 5.3 b d</td>
</tr>
</tbody>
</table>

a significantly higher than all other groups, P<0.001, ANOVA post hoc Bonferroni
b significantly higher than healthy control group, P<0.001, ANOVA post hoc Bonferroni.
c significantly lower than healthy control group, P<0.01, ANOVA post hoc Bonferroni.
d significantly lower than depressed group, P<0.001, ANOVA post hoc Bonferroni.
<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n = 12)</th>
<th>Depressed women (n = 12)</th>
<th>SSRI-treated women (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, year</strong></td>
<td>28.5 (25.0 – 33.0)</td>
<td>31.5 (26.0 – 36.0)</td>
<td>29.0 (25.0 – 35.0)</td>
</tr>
<tr>
<td><strong>Parous women, n (%)</strong></td>
<td>3 (25.0%)</td>
<td>10 (83.3%)*</td>
<td>8 (66.7%)*</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>24.2 (20.1 – 31.7)</td>
<td>24.3 (18.2 – 45.4)</td>
<td>27.0 (22.7 – 35.8)*</td>
</tr>
<tr>
<td><strong>MAP first trimester</strong></td>
<td>97.5 (82.5 - 112.0)</td>
<td>85.2 (77.5 – 104.0)*</td>
<td>93.0 (85.0 – 102.0)</td>
</tr>
<tr>
<td><strong>MAP partus</strong></td>
<td>103.8 (87.5 – 120.0)</td>
<td>99.5 (89.5 – 110.0)</td>
<td>101.2 (94.0 – 113.0)</td>
</tr>
<tr>
<td><strong>Smokers, n (%)</strong></td>
<td>0</td>
<td>1 (8.3%)</td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td><strong>IVF treatment</strong></td>
<td>0</td>
<td>0</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><strong>Gestational length, days</strong></td>
<td>282 (272 – 289)</td>
<td>277 (263 – 293)</td>
<td>272 (263 – 284)**</td>
</tr>
<tr>
<td><strong>Sex of child, n of girls/boys (%)</strong></td>
<td>5 / 7 (41.7% / 58.3%)</td>
<td>5 / 7 (41.7% / 58.3%)</td>
<td>8 / 4 (66.7% / 33.3%)</td>
</tr>
<tr>
<td><strong>Birth weight, grams</strong></td>
<td>3520 (3110 – 4080)</td>
<td>3730 (2890 – 4540)</td>
<td>3510 (3170 – 4230)</td>
</tr>
<tr>
<td><strong>EPDS gestational week 17</strong></td>
<td>3.0 (0.0 – 5.0)</td>
<td>17.0 (4.0 – 24.0)***</td>
<td>10.5 (0.0 – 20.0)**,a</td>
</tr>
<tr>
<td><strong>EPDS gestational week 32</strong></td>
<td>3.5 (1.0 – 6.0)</td>
<td>16.0 (11.0 – 23.0)***</td>
<td>11.5 (3.0 – 24.0)***, a</td>
</tr>
</tbody>
</table>

* p < 0.05, significantly different in comparison with controls, Pearson Chi-Square test
** p < 0.01, significantly different in comparison with controls, Mann-Whitney U test
*** p < 0.001, significantly different in comparison with controls, Mann-Whitney U test

a p < 0.05, significantly different compared with depressed, Mann-Whitney U test
Methods

Study I

RNA isolations from placenta
A 3 mm³ cube was taken from the fetal side of the placenta biopsies.

For isolation of total ribonucleic acid (RNA) for the microarray analysis, miRNeasy mini kit (Qiagen, Germany) was used. The tissue was lysed with QIAzol (Qiagen, Germany) using a rotor-stator homogenizer and chloroform was added for phase-separation.

Total RNA isolation for the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis was done similarly, except that RNeasy mini kit (Qiagen, Germany) was used and tissue homogenization was performed by using stainless steel beads in a TissueLyser (Qiagen, Germany).

After phase-separation, the protocol in the miRNeasy mini kit and RNeasy mini kit handbooks was followed. The RNA quality was determined using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, US).

Microarray analysis
250 ng of total RNA was used to generate amplified and biotinylated sense-strand complementary DNA (cDNA) from the entire expressed genome using Ambion WT Expression kit and Affymetrix GeneChip® WT Terminal Labeling and Hybridization User Manual (Affymetrix Inc., US). GeneChip® ST Arrays were hybridized and, after the incubation, the arrays were washed, stained and scanned using the GeneChip® Scanner 3000 7G.

cDNA synthesis and qRT-PCR analysis
From 250 ng of total RNA, 20 µL of cDNA was synthesized using random primer and SuperScriptIII reverse transcriptase (Invitrogen, US) according to the manufacturer’s protocol.

qRT-PCR was performed using the StepOne Plus qPCR machine (Applied Biosystems, Life Technologies, US). For normalization, GAPDH and β-actin were selected as reference genes. TaqMan Gene Expression Assay primers, probes and master mix (all Applied Biosystems, Life Technologies, US) were used and the qRT-PCR was run according to the manufacturer’s protocol.

Study II

Protein isolation
Pieces were taken from the fetal side of the placenta biopsies and total protein lysates were prepared using RIPA lysis buffer (Sigma-Aldrich Corp.,
US) containing Protease inhibitor cocktail (Sigma-Aldrich Corp., US), PMSF and ortovanadate. The biopsies were homogenized and, after centrifugation, the supernatants containing the total protein lysates were collected. The proteins were separated by NuPage Novex 4-12% Bis-Tris gels (Invitrogen, Life Technologies, US).

**Western blot (WB)**

After separation, the proteins were transferred to Immobilon-FL PVDF membrane (Merck Millipore, US). The proteins were detected by protein specific primary antibodies (Table 4). Protein levels were normalized against β-actin. Secondary antibodies were labelled with IRDye 680RD and IRDye 800CW, which enables detection of two proteins at the same time using Odyssey Image Scanner (LI-COR Biosciences Inc., US).

**Immunohistochemical staining (IHC)**

Paraffin-embedded placental biopsies were sectioned into 5 µm slices and mounted on slides. After deparaffinization and rehydration, antigenic retrieval was performed by heating the slides in citric acid. The sections were fixated using an ice-cold acetone/methanol mix and incubated against endogenous peroxidase activity. Non-specific binding was blocked by incubating the sections in PBS containing horse or goat serum. Proteins of interest were detected using primary antibodies (Table 4). After incubation with biotinylated secondary antibodies, the sections were incubated with avidinD conjugated horseradish peroxidase, followed by staining/developing using liquid 3,3’-diaminobenzidine tetrahydrochloride (DAB) + substrate chromogen system (Dako, Denmark), and counterstaining with Mayer hematoxylin (Histolab Products, Sweden). The sections were dehydrated and mounted under cover glass.

Scoring of the staining intensity was done visually in a light microscope (Axio Observer.Z1) (Carl Zeiss AG Corp., Germany) using a scale from 1 to 4, where 1 corresponds to the lowest intensity and 4 the highest. A double-blinded validation of the scoring was performed: a second person scored the slides in the same way as the first scorer, without knowing the initial results.
Table 4. Primary antibodies used in Western blot and immunohistochemistry on placenta material

<table>
<thead>
<tr>
<th>Target</th>
<th>Catalogue no</th>
<th>Commercial supplier</th>
<th>Used for WB and/or IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>sc-548</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>TrkA</td>
<td>06-574</td>
<td>Upstate, Merck Millipore, US</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>pTrkA (pTyr490)</td>
<td>ab1445</td>
<td>Abcam plc, England</td>
<td>WB</td>
</tr>
<tr>
<td>pTrkA (pTyr496)</td>
<td>sc-7987-R</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>WB</td>
</tr>
<tr>
<td>Raf-1</td>
<td>sc-133</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>pRaf-1 (pTyr340/341)</td>
<td>sc-16806</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>IHC</td>
</tr>
<tr>
<td>RhoA</td>
<td>sc-418</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>ROCK1</td>
<td>sc-5560</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>ROCK2</td>
<td>sc-1851</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>WB and IHC</td>
</tr>
<tr>
<td>pROCK2 (pSer1366)</td>
<td>PA5-34895</td>
<td>Thermo Scientific Inc., US</td>
<td>IHC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>sc-47778</td>
<td>SantaCruz Biotechnology Inc., US</td>
<td>WB</td>
</tr>
</tbody>
</table>

Study III

Embryo culturing and developmental scoring

Cryopreserved two-day embryos were thawed and transferred to equilibrated culture medium used for human cleavage stage embryos (CCM) (Vitrolife, Sweden), which were the culturing conditions for control embryos. For fluoxetine (FLX) treatment of the embryos, FLX (Sigma-Aldrich Corp., US) was added to the culture medium to reach a final concentration of 0.5 µM. The FLX dose was chosen based on levels found in cord blood and amniotic fluid of FLX-treated pregnant women.

The embryos were cultured in an EmbryoScope® (Unisense FertiTech, Denmark) with a time-lapse camera taking images every 15 minutes. The embryo development and quality was evaluated and scored retrospectively using standard morphological criteria developed by several research groups [70, 76, 78, 79].
**Bottom-up shotgun proteomics of embryos**

Embryos were snap frozen after culturing and dissolved in 2 M thiourea/6 M urea in ammonium bicarbonate for lysis and protein extraction. Proteins were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide (IAA) before enzymatic digestion, first by Lys-C (Wako Chemicals GmbH, Germany) and then by trypsin (Promega, US). The peptides were solid phase extraction purified by ZipTips® (Merck Millipore, US), dried in a SpeedVac® system, and analyzed by high resolution tandem mass spectrometry.

The analysis were performed using a QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) after on-line separation of the digested peptides by reversed phase liquid chromatography. A 150 min. linear gradient from 4% to 100% acetonitrile at 250 nL/min was used to elute the peptides.

**Proseek Multiplex Immunoassay analysis on embryo culture medium**

For the detection of proteins secreted into the culture medium, 20 µL of the culture medium was snap frozen after embryo culturing. The protein analysis was performed using the Proseek Multiplex Immunoassay analysis with the Inflammation 1 panel. The analysis method is based on the Proximity Extension Assay technology and was run at Olink Bioscience facilities in Uppsala, Sweden.

**Immunohistochemical staining**

After culturing, the embryos were fixed in 2.5% paraformaldehyde (PFA) in PBS and stored in PBS/polyvinylpyrrolidone (PVP) until later. The embryos were permeabilized in 0.25% Triton X-100 in PBS/PVP, blocked in blocking solution containing bovine serum albumin and Tween20 and incubated with primary antibodies against urokinase-type plasminogen activator (uPA) and NGF (both from SantaCruz Biotechnology Inc., US). The embryos were incubated with secondary antibodies labeled with AlexaFluor488 or AlexaFluor594 (Life Technologies, Thermo Scientific Inc., US), which makes it possible to detect two proteins at the same time. After incubation, the embryos were mounted on a slide in 5 µL VectaShield (Vector Laboratories Inc., US), surrounded by Vaseline, covered by a cover slip and sealed with nail varnish.

The stainings were evaluated using a fluorescence microscope (Axio Observer.Z1, Carl Zeiss Ag Corp., Germany).
Study IV

**Mobility shift assay of Raf-1**

Total protein lysates from SH-SY5Y/TrkA cells treated with 100 ng/mL NGF or 16 nM TPA were separated by 7% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Raf-1 was detected by Western blot using primary antibody specific for Raf-1 (C-12, Santa Cruz Biotechnology Inc., US). The protein was visualized using enhanced chemiluminescence (ECL) (Amersham, UK).

**Immunoprecipitation of Raf-1 and in vitro kinase assay**

Total protein lysate from NGF- or TPA-treated SH-SY5Y/TrkA cells was incubated with Raf-1 antibody (C-12, SantaCruz Biotechnology Inc., US) and, after incubation, the antibodies were bound to protein A Sepharose (Pharmacia, Sweden). The immunocomplexes were washed and incubated in a kinase buffer containing glutathione S-transferase (GST)-MEK1 substrate (Upstate Biotechnology Inc., US, and Dr Stephen Pelech, Kinetek Pharmaceuticals, US) and 5 µCi [γ-32P] ATP (Amersham, UK). The samples were separated by 7% SDS-PAGE and phosphorylated GST-MEK1 was visualized by autoradiography and quantified using phosphorimage analysis (Fuji Bas 2000).

**Ras activity measurement**

SH-SY5Y/TrkA cells were treated with NGF or TPA and total protein lysates were prepared. The Ras-binding domain (RBD) of Raf-1 (kindly provided by Dr Julian Downward, Imperial Cancer Research Fund Lab. London UK), expressed as a GST fusion protein, was used to bind active Ras (Ras-GTP). The precipitates were separated by 12.5% SDS-PAGE and Ras was detected using Western blot and anti-Ras antibody (Ro2120, BD Transduction Laboratories™, BD, US). Total amount of Ras was analyzed in total lysates from the same samples and detected using the same Ras antibody.

**Phosphorylation of the PKC substrate MARCKS**

Serum-starved SH-SY5Y/TrkA cells were incubated with [32Pi] in phosphate-free Eagle’s medium containing bovine serum albumin for 4 h before treatment with NGF or TPA. The incubation was terminated by washing the cell cultures with ice-cold TRIS-buffered saline (TBS). Protein lysates were boiled in Triton-X100 and separated by 8% SDS-PAGE. The phosphorylation of MARCKS was measured and quantified by phosphorimage analysis.

**Subcellular fractionation of cells and Western blot for PKC isoforms**

SH-SY5Y/TrkA cells treated with NGF or TPA were incubated in digitonin buffer and homogenized using a Dounce homogenizer with a glass pestle.
One part of the homogenate was incubated with Triton-X100, followed by centrifugation. The supernatant was collected and denoted total lysate (T).

The rest of the homogenate was separated into a cytosol (S) and particulate fraction (P) by ultracentrifugation. Proteins in the cytosolic fraction were precipitated with trichloroacetic acid (TCA) and the pellet was dissolved in sodium hydroxide (NaOH)/hydrochloric acid (HCl). The particulate fraction was incubated with digitonin/Triton-X100 buffer and centrifuged to remove the insoluble cytoskeletal fraction.

Equal amounts of proteins from each fraction were separated by 8% SDS-PAGE and the PKC isoforms were detected using Western blot analysis. Primary antibodies used were anti-PKC-α (Gibco BRL, Life Technologies, US), anti-PKC-δ and anti-PKC-ε (SantaCruz Biotechnology Inc., US), and the immunoreactivity was detected using ECL (Amersham, UK).

[^32Pi]-orthophosphate labelling of cells, in situ trypsin digestion and 2D phosphopeptide mapping of Raf-1

Serum-starved SH-SY5Y/TrkA cells were pre-incubated with 3 mCi[^32Pi]-orthophosphate (PBS.43) in phosphate-free Eagle’s medium, before treating the cells with 100 ng/mL NGF or 16 nM TPA. After treatment, total protein lysates were prepared. RNase A was added to the lysates and Raf-1 was immunoprecipitated using Raf-1 antibody (C-12, SantaCruz Biotechnology Inc., US).

The immunoprecipitates were separated on SDS-PAGE, transferred to Hybond C-extra membranes, trypsin digested and prepared for two-dimensional phosphopeptide mapping [180]. The samples were separated using a cellulose thin-layer chromatography plate, first in one dimension in electrophoresis buffer and then in a second dimension by ascending chromatography in isobutyric acid buffer. The radiolabelled peptides were visualized using phosphorimage analysis.

Statistics and bioinformatics

All statistical analyses were performed by the Statistical Package for the Social Sciences 20.0 (SPSS Inc., US) for Windows software package.

All significance tests were two-tailed and p-value < 0.05 was considered statistically significant. For normally distributed data, ANOVA was used (study I) and for non-normally distributed data, Mann-Whitney U test was used (study II and III). Categorical data were compared using Pearson Chi-Square test (study II).

Differentially expressed genes between the groups (study I) was defined by using an empirical Bayes moderated t-test with robust regression (Smyth, 2004). To visualize differentially expressed genes, the Genesis software, version 1.7.1 (http://genome.tugraz.at/) was used, also to produce hierar-
chical clustering. The expression data was analyzed using Ingenuity pathway analysis (IPA), which calculates the top genes that are the most deregulated and identifies pathways and networks in which the focus genes are involved.

Results from the mass spectrometry analysis of embryos (study III) were analyzed using the Mascot™ search algorithm (Matrix Science, UK) embedded in Proteome Discoverer software (ThermoFisher Scientific, Germany) and searched against human proteins in the UniProtKB/Swiss-Prot database. At least two matching peptides of 95% confidence per protein counted as protein identification.

A Venn diagram (study III) was made using software freely available on the internet (Oliveros, J.C. (2007). http://bioinfogp.cnb.csic.es/tools/venny/), to detect and illustrate which proteins are expressed in SSRI-treated women, healthy controls or in both groups. IPA analysis was performed to discover which pathways and networks are associated with the proteins of interest.

**Ethical approvals (study I to III)**

The BASIC study, including these sub-studies, and the embryo study were approved by the Regional Ethics Review Board in Uppsala, Sweden (approval numbers 2009/171 and 2014/298, respectively).
Summary of Results

Study I

The gene expression in human placenta from SSRI-treated women, untreated depressed women and healthy controls were studied.

A microarray analysis was performed on biopsies collected from the fetal side of the placenta from depressed women, SSRI-treated women and healthy controls. Demographic data of the women participating in the study is presented in Table 2. The analysis revealed 109 genes that were differentially expressed between SSRI-treated women and controls, 82 genes were down-regulated and 27 were up-regulated. In depressed women, 108 genes had an altered expression compared with healthy controls, 100 genes were down-regulated and eight genes were up-regulated. IPA analysis revealed that 17 genes in the depressed group and 20 genes in the SSRI-treated group had the most significant alterations compared with healthy controls (Table 5 and 6). Of all genes that had an altered expression in depressed women and SSRI-treated women compared with controls, 20 genes were overlapping (Table 7).

The IPA analysis also detected significant canonical pathways of interest when comparing depressed women with healthy controls, as well as SSRI-treated women with healthy controls (Table 8 and 9).

For further validation of the microarray results, we selected seven genes that were more prominently altered in their expression between groups, or found in the pathway or canonical pathway analysis in placenta from depressed and SSRI-treated women. We validated the expression by performing a qRT-PCR analysis on a larger number of women in each study group (Fig. 4). In placenta from SSRI-treated women, ROCK1 and ROCK2 were significantly down-regulated when compared with healthy women (Fig. 4B). Also, GCC2, KTN1 and DNM1L were significantly down-regulated in women treated with SSRI compared with controls. For C12orf39, there was only a tendency for down-regulation in SSRI-treated women compared with healthy women. The gene expression of NEXN did not differ between SSRI-treated and healthy women.

In depressed women, there was a tendency for down-regulation of ROCK2 compared with controls (Fig. 4A). There was also a significant down-regulation of placental gene expression of C12orf39 in depressed
Table 5. Significantly up- and down-regulated top molecules in the control vs depressed group

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene title</th>
<th>log2 fold change*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTRNA1-2</td>
<td>vault RNA 1-2</td>
<td>2.28</td>
<td>0.026</td>
</tr>
<tr>
<td>PGF</td>
<td>placenta growth factor</td>
<td>0.70</td>
<td>0.013</td>
</tr>
<tr>
<td>RNH1</td>
<td>ribonuclease/angiogenin inhibitor 1</td>
<td>0.57</td>
<td>0.036</td>
</tr>
<tr>
<td>ITPK1</td>
<td>inositol 1,3,4-triphosphate 5/6 kinase</td>
<td>0.55</td>
<td>0.037</td>
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<tr>
<td>Mir-503</td>
<td>microRNA 503</td>
<td>0.52</td>
<td>0.047</td>
</tr>
<tr>
<td>RAD23A</td>
<td>RAD23 homolog A (S. cerevisiae)</td>
<td>0.52</td>
<td>0.013</td>
</tr>
<tr>
<td>APOC1</td>
<td>apolipoprotein C-I</td>
<td>0.50</td>
<td>0.037</td>
</tr>
<tr>
<td>USP15</td>
<td>ubiquitin specific peptidase 15</td>
<td>-0.87</td>
<td>0.026</td>
</tr>
<tr>
<td>PPP1R12A</td>
<td>protein phosphatase 1. regulatory (inhibitor) subunit 12A</td>
<td>-0.88</td>
<td>0.021</td>
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<tr>
<td>FNBP1L</td>
<td>formin binding protein 1-like</td>
<td>-0.89</td>
<td>0.046</td>
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<tr>
<td>SMC6</td>
<td>structural maintenance of chromosomes 6</td>
<td>-0.92</td>
<td>0.026</td>
</tr>
<tr>
<td>SMC4</td>
<td>structural maintenance of chromosomes 4</td>
<td>-0.92</td>
<td>0.024</td>
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<tr>
<td>ROCK2</td>
<td>Rho-associated. coiled-coil containing protein kinase 2</td>
<td>-0.92</td>
<td>0.021</td>
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<tr>
<td>AKAP9</td>
<td>A-kinase (PRKA) anchor protein (yotiao) 9</td>
<td>-0.94</td>
<td>0.028</td>
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<tr>
<td>COPS2</td>
<td>COP9 constitutive photomorphogenic homolog subunit 2 (Arabidopsis)</td>
<td>-0.98</td>
<td>0.008</td>
</tr>
<tr>
<td>GCC2</td>
<td>GRIP and coiled-coil domain containing 2</td>
<td>-1.02</td>
<td>0.019</td>
</tr>
<tr>
<td>ROCK1</td>
<td>Rho-associated. coiled-coil containing protein kinase 1</td>
<td>-1.07</td>
<td>0.028</td>
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</table>

* “-” = reduction in expression levels
Table 6. Significantly up- and down-regulated top molecules in the control vs SSRI group

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene title</th>
<th>log2 fold change*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12orf39</td>
<td>chromosome 12 open reading frame 39</td>
<td>1.28</td>
<td>0.009</td>
</tr>
<tr>
<td>RNU4-1</td>
<td>RNA, U4 small nuclear 1</td>
<td>0.91</td>
<td>0.026</td>
</tr>
<tr>
<td>KRT81</td>
<td>keratin 81</td>
<td>0.78</td>
<td>0.007</td>
</tr>
<tr>
<td>RNU4-2</td>
<td>RNA, U4 small nuclear 2</td>
<td>0.71</td>
<td>0.043</td>
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<tr>
<td>SERINC2</td>
<td>serine incorporator 2</td>
<td>0.64</td>
<td>0.038</td>
</tr>
<tr>
<td>APLN</td>
<td>apelin</td>
<td>0.63</td>
<td>0.014</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>angiopoietin-like 4</td>
<td>0.62</td>
<td>0.047</td>
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<td>TUBA1C</td>
<td>tubulin, alpha 1c</td>
<td>0.56</td>
<td>0.027</td>
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<td>S100A3</td>
<td>S100 calcium binding protein A3</td>
<td>0.54</td>
<td>0.042</td>
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<tr>
<td>TECR</td>
<td>trans-2,3-enoyl-CoA reductase</td>
<td>0.54</td>
<td>0.048</td>
</tr>
<tr>
<td>ANKRD12</td>
<td>ankyrin repeat domain 12</td>
<td>-0.78</td>
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<td>ZNF721</td>
<td>zinc finger protein 721</td>
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<td>ROCK1</td>
<td>Rho-associated, coiled-coil containing protein kinase 1</td>
<td>-0.80</td>
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<td>AKA9P9</td>
<td>A-kinase (PRKA) anchor protein (yotiao) 9</td>
<td>-0.84</td>
<td>0.017</td>
</tr>
<tr>
<td>SYCP2</td>
<td>synaptonemal complex protein 2</td>
<td>-0.87</td>
<td>0.042</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosome antigen 1</td>
<td>-0.88</td>
<td>0.015</td>
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<tr>
<td>ANKRD36B</td>
<td>ankyrin repeat domain 36B</td>
<td>-0.88</td>
<td>0.041</td>
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<tr>
<td>COPS2</td>
<td>COP9 constitutive photomorphogenic homolog subunit 2 (Arabidopsis)</td>
<td>-0.88</td>
<td>0.007</td>
</tr>
<tr>
<td>GCC2</td>
<td>GRIP and coiled-coil domain containing 2</td>
<td>-0.92</td>
<td>0.032</td>
</tr>
<tr>
<td>NEXN</td>
<td>nexilin (F actin binding protein)</td>
<td>-1.00</td>
<td>0.044</td>
</tr>
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</table>

* "-" = reduction in expression levels
### Table 7. Genes with altered expression in both depressed and SSRI-treated women compared with controls

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Probe Set ID</th>
<th>Log2 Fold change SSRI</th>
<th>Log2 Fold change Depressed</th>
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<tbody>
<tr>
<td>jumonji domain containing 1C</td>
<td>JMJD1C</td>
<td>7933877</td>
<td>-0.612753894</td>
<td>-0.86686842</td>
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<tr>
<td>dynamin 1-like</td>
<td>DNM1L</td>
<td>7954752</td>
<td>-0.713859601</td>
<td>-0.716232722</td>
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<tr>
<td>ERGIC and golgi 2</td>
<td>ERGIC2</td>
<td>7962013</td>
<td>-0.65241833</td>
<td>-0.820896474</td>
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<tr>
<td>kinectin 1 (kinesin receptor)</td>
<td>KTN1</td>
<td>7974483</td>
<td>-0.712691264</td>
<td>-0.866627304</td>
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<tr>
<td>AT rich interactive domain 4A (RBP1-like)</td>
<td>ARID4A</td>
<td>7974621</td>
<td>-0.602522967</td>
<td>-0.661691075</td>
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<tr>
<td>serologically defined colon cancer antigen 1</td>
<td>SDCCAG1</td>
<td>7978866</td>
<td>-0.717656644</td>
<td>-0.776418378</td>
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<tr>
<td>COP9 constitutive photomorphogenic homolog subunit 2 (Arabidopsis)</td>
<td>COPS2</td>
<td>7988605</td>
<td>-0.883531246</td>
<td>-0.975928968</td>
</tr>
<tr>
<td>Rho-associated, coiled-coil containing protein kinase 1</td>
<td>ROCK1</td>
<td>8022441</td>
<td>-0.804644966</td>
<td>-1.074633032</td>
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<tr>
<td>zinc finger protein 146</td>
<td>ZNF146</td>
<td>8028186</td>
<td>-0.679184287</td>
<td>-0.584609444</td>
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<td>zinc finger protein 100</td>
<td>ZNF100</td>
<td>8035808</td>
<td>-0.534310441</td>
<td>-0.741413733</td>
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<td>GRIP and coiled-coil domain containing 2</td>
<td>GCC2</td>
<td>8044236</td>
<td>-0.919095326</td>
<td>-1.024026926</td>
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<tr>
<td>Rho-associated, coiled-coil containing protein kinase 2</td>
<td>ROCK2</td>
<td>8050302</td>
<td>-0.73784498</td>
<td>-0.924567683</td>
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<td>PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)</td>
<td>PRPF40A</td>
<td>8055913</td>
<td>-0.641694744</td>
<td>-0.652162751</td>
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<td>natural killer-tumor recognition sequence</td>
<td>NKTR</td>
<td>8079079</td>
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<td>8098287</td>
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<td>-0.658215787</td>
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<tr>
<td>polymerase (DNA directed) kappa</td>
<td>POLK</td>
<td>8106303</td>
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<td>-0.814486001</td>
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<tr>
<td>RAD50 homolog (S. cerevisiae)</td>
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<td>8107942</td>
<td>-0.732319601</td>
<td>-0.82709801</td>
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<tr>
<td>LysM, putative peptidoglycan-binding, domain containing 3</td>
<td>LYSMD3</td>
<td>8113064</td>
<td>-0.675591155</td>
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<td>A-kinase (PRKA) anchor protein (yotiao) 9</td>
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<td>PHD finger protein 20-like 1</td>
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<td>8148358</td>
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<td>-0.551986952</td>
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Table 8. Canonical pathway analysis of the depressed group

<table>
<thead>
<tr>
<th>Canonical pathway</th>
<th>Genes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin Nucleation by ARP-WASP Complex</td>
<td>PPP1R12A, ROCK1, ROCK2</td>
<td>0.003</td>
</tr>
<tr>
<td>RhoA Signaling</td>
<td>KTN1, PPP1R12A, ROCK1, ROCK2</td>
<td>0.029</td>
</tr>
<tr>
<td>VEGF Signaling</td>
<td>PGF, ROCK1, ROCK2</td>
<td>0.011</td>
</tr>
<tr>
<td>Protein Kinase A Signaling</td>
<td>AKAP9, ANAPC4, PDE3B, PPP1R12A, ROCK1, ROCK2</td>
<td>0.011</td>
</tr>
<tr>
<td>1D-myo-inositol Hexakisphosphate Biosynthesis V (from Ins(1,3,4)P3)</td>
<td>ITPK1</td>
<td>0.015</td>
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</table>

Table 9. Canonical pathway analysis of the SSRI-treated group

<table>
<thead>
<tr>
<th>Canonical pathway</th>
<th>Genes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ephrin A Signaling</td>
<td>EFNA5, ROCK1, ROCK2</td>
<td>0.001</td>
</tr>
<tr>
<td>RhoA Signaling</td>
<td>CDC42EP1, KTN1, ROCK1, ROCK2</td>
<td>0.002</td>
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<tr>
<td>PEDF Signaling</td>
<td>FAS, ROCK1, ROCK2</td>
<td>0.004</td>
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<tr>
<td>Breast Cancer Regulation by Stathmin 1</td>
<td>ARHGEF5, ROCK1, ROCK2, TUBA1C</td>
<td>0.012</td>
</tr>
<tr>
<td>Signaling by Rho Family GTPases</td>
<td>ARHGEF5, CDC42EP1, ROCK1, ROCK2</td>
<td>0.021</td>
</tr>
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</table>
Figure 4. Gene expression detected by qRT-PCR in placenta comparing depressed (A) or SSRI-treated women (B) with healthy women. A) *P = 0.05, #P = 0.08, B) *P ≤ 0.05, **P < 0.01, #P = 0.07.
women compared with healthy controls. No other genes were differentially expressed in placentas between depressed and healthy women.

Our results indicate that SSRI treatment and depression per se alters the expression of many genes in placenta, although not in the same way.

Study II
To further investigate whether the altered gene expression of ROCK1 and ROCK2 found in study I could also be detected in placenta on a protein level, SSRI-treated, untreated depressed and healthy pregnant women were compared. The demographic data is shown in Table 3. ROCK1 and ROCK2 are involved in RhoA signaling, which is also one of the signaling pathways downstream of the NGF receptor TrkA. We therefore studied the NGF signaling pathways and also investigated which pathway from the receptor could lead to altered ROCK levels (Fig. 3).

By performing Western blot analysis on total protein lysates from placenta, we did not find any significant changes in protein levels of the proteins investigated.

Immunohistochemical (IHC) staining of placental sections showed that the staining pattern was almost the same for all the proteins investigated, with the strongest staining in the trophoblasts, weaker in the endothelial cells, and the weakest staining in the stromal cells (Fig. 5). Only ROCK1 had a different staining pattern, with the strongest staining in the endothelial cells, weaker in the trophoblasts, and the weakest in the stromal cells. As an indication of active signaling, the phosphorylated forms of Raf-1 and ROCK2 were accumulated in the nucleus of the cells, whereas the staining for total Raf-1 and ROCK2 was found both in the nucleus and the cytoplasm (Fig. 5).

When performing IHC staining on placental sections, NGF levels were increased in trophoblasts and endothelial cells in SSRI-treated women compared with depressed women and healthy controls (Fig. 6A and B). The NGF staining intensity in endothelial cells was decreased in depressed women compared with controls (Fig. 6B).

Phosphorylated Raf-1 was increased in stromal cells in both SSRI-treated and depressed women compared with controls (Fig. 6C). No other differences in levels of phosphorylated Raf-1 were found between groups in trophoblasts or endothelial cells.

There was a tendency towards increased ROCK2 levels in placental trophoblasts and endothelial cells in SSRI-treated women compared with healthy controls (Fig. 6A and B). In stromal cells, ROCK2 levels were significantly higher in SSRI-treated women than in controls (Fig. 6C). However, ROCK2 levels in endothelial cells were significantly lower in women treated with SSRI compared with depressed women (Fig. 6B).
depressed women had increased levels of ROCK2 in all cell types compared with controls (Fig. 6A-C).

The staining intensity of phosphorylated ROCK2 was significantly increased in all three cell types when comparing SSRI-treated women with depressed women and healthy controls (Fig. 6A-C). Also, the placental staining intensity of phosphorylated ROCK2 was significantly higher in endothelial and stromal cells of depressed women than in healthy controls (Fig. 6B and C).

No significant differences in staining intensities of TrkA, Raf-1, RhoA and ROCK1 were detected between SSRI-treated, depressed and healthy women in trophoblasts, endothelial or stromal cells.
**Figure 6.** Protein levels in different cell types of placenta detected by IHC. A) Trophoblasts, B) Endothelial cells and C) Stromal cells. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, #1 = 0.065, #2 = 0.056, Mann-Whitney U test.

**Study III**

Embryos treated with 0.5 µM FLX needed a significantly shorter time to reach formation of a morula/compaction compared with controls (Table 10). Also, the time to reach other developmental stages tended to be shorter in FLX-treated embryos compared with controls. FLX treatment did not significantly alter the number of embryos developing into blastocysts or the quality of the embryos.
Table 10. Timing of developmental stages in human embryos treated with or without 0.5 µM FLX

<table>
<thead>
<tr>
<th>Time difference (hours)</th>
<th>Control n = 10</th>
<th>0.5 µM FLX n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thawing - first cell division, median (min – max)</td>
<td>8.60 (2.20 – 38.90)</td>
<td>13.55 (1.00 – 19.30)</td>
</tr>
<tr>
<td>Thawing - fourth cell division, median (min – max)</td>
<td>29.20 (2.90 – 58.20)</td>
<td>20.15 (5.20 – 37.40)</td>
</tr>
<tr>
<td>Thawing - compaction, median (min – max)</td>
<td>56.75 (29.10 – 72.00)</td>
<td>44.05 (34.90 – 51.60)*</td>
</tr>
<tr>
<td>Thawing - cavitation, median (min – max)</td>
<td>57.55 (40.90 – 84.90)</td>
<td>47.50 (41.90 – 71.40)</td>
</tr>
<tr>
<td>Thawing - start of expansion, median (min – max)</td>
<td>71.90 (56.90 – 90.20)</td>
<td>67.90 (53.40 – 93.20)</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with control, Mann-Whitney U test

By performing a bottom-up shotgun proteomics analysis on whole embryo, we investigated whether FLX induces unique protein expression patterns in the embryo. We discovered that 36 proteins were uniquely expressed in the FLX-treated embryos and 48 proteins were detected in both groups (Fig. 7). In order to determine the biological relevance of the 36 proteins expressed in FLX-treated embryos, an IPA analysis was performed. Three networks of relevance were detected in embryos treated with FLX (Table 11).

![Figure 7. Venn diagram illustrating proteins detected in FLX-treated embryos and controls by mass spectrometry.](image)

48
Table 11. Networks identified by IPA, focusing on the proteins detected by mass spectrometry in FLX-treated embryos compared with controls

<table>
<thead>
<tr>
<th>IPA networks top 3</th>
<th>Proteins</th>
<th>IPA score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Death and Survival, Cellular Growth and Proliferation, Hematological Disease</td>
<td>ENO1, HBB, HNRNPA2B1, HNRNPC, NCL, NPM1, PEBP1, PFN1, PPIA, RPL8, RPS16, YBX1</td>
<td>23</td>
</tr>
<tr>
<td>Cancer, Organismal Injury and Abnormalities, Reproductive System Disease</td>
<td>ALDOA, CLTC, P4HB, PARK7, PGK1, PRDX1, RPLP2, STMN1, TPI1, TUBB4A</td>
<td>18</td>
</tr>
<tr>
<td>Inflammatory Response, Cell Death and Survival, Digestive System Development and Function</td>
<td>ATP1A1, C1QBP, HP, HPX, KRT8, NSF, PPIA, SOD1</td>
<td>14</td>
</tr>
</tbody>
</table>

By using Proseek Multiplex Inflammation Immunoassay from Olink, protein secretion from embryos with or without FLX treatment was investigated. Culture medium in which the embryos had been cultured was analyzed. Several proteins, after normalization, were detected above the limit of detection. Levels of urokinase-type plasminogen activator (uPA) tended to be higher in FLX-treated embryos compared with controls (Table 12). For other proteins, there were either no significant differences between the groups or the number of embryos where the protein could be detected was too limited for statistical analyses.

Table 12. Top 10 proteins detected by Multiplex Immunoassay analysis and above levels of detection in medium from 0.5 µM FLX-treated and control embryos

<table>
<thead>
<tr>
<th>Protein</th>
<th>UniProtKB</th>
<th>Control n = 10</th>
<th>0.5 µM FLX n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n median (min – max)</td>
<td>n median (min – max)</td>
<td></td>
</tr>
<tr>
<td>uPA</td>
<td>P00749</td>
<td>9 1.30 (0.40 – 3.20)</td>
<td>10 2.15 (0.90 – 3.40)</td>
</tr>
<tr>
<td>IL-6</td>
<td>P05231</td>
<td>8 1.35 (0.80 – 3.20)</td>
<td>10 2.05 (0.50 – 4.90)</td>
</tr>
<tr>
<td>STAMPB</td>
<td>O95630</td>
<td>7 0.50 (0.40 – 1.10)</td>
<td>6 0.55 (0.40 – 1.00)</td>
</tr>
<tr>
<td>ADA</td>
<td>P00813</td>
<td>7 1.70 (1.10 – 2.30)</td>
<td>5 1.50 (1.10 – 1.80)</td>
</tr>
<tr>
<td>CST5</td>
<td>P28325</td>
<td>6 0.10 (0.10 – 0.20)</td>
<td>1 0.10 (N/A)</td>
</tr>
<tr>
<td>Beta-NGF</td>
<td>P01138</td>
<td>5 0.60 (0.40 – 1.50)</td>
<td>2 0.25 (0.20 – 0.30)</td>
</tr>
<tr>
<td>FGF-23</td>
<td>Q9GZV9</td>
<td>5 0.40 (0.30 – 0.40)</td>
<td>1 0.30 (N/A)</td>
</tr>
<tr>
<td>IL-8</td>
<td>P10145</td>
<td>4 1.15 (0.10 – 5.20)</td>
<td>5 1.30 (0.20 – 3.20)</td>
</tr>
<tr>
<td>IL-10</td>
<td>P22301</td>
<td>4 0.40 (0.30 – 0.40)</td>
<td>1 0.10 (N/A)</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>P15692</td>
<td>3 0.60 (0.10 – 1.80)</td>
<td>8 0.85 (0.20 – 1.70)</td>
</tr>
</tbody>
</table>
Immunohistochemical staining of the embryos was performed to visualize the true presence of some of the proteins of interest. uPA and NGF were chosen because of their high ranking in the Multiplex Immunoassay analysis. Also, NGF protein levels in placenta had been shown to be altered in SSRI-treated women compared to healthy controls (study II). uPA and NGF staining were found in the cytosol and cellular membrane of the trophectoderm (Fig. 8). NGF was furthermore found in the inner cell mass, whereas uPA had only a weak staining in the inner cell mass of the embryos (Fig. 8).

Figure 8. IHC staining of human embryos. A-D are embryos cultured in control medium, E-F are embryos treated with 0.5µM FLX. DAPI staining (blue) shows cell nuclei. Staining for NGF is shown in green and uPA in red. D and H are overlay pictures.

Study IV

Signal transduction down-stream of the NGF receptor TrkA in human neuroblastoma cell line SH-SY5Y transfected with TrkA was studied.

In SH-SY5Y/TrkA cells treated with TPA or NGF for 5 minutes, 30 minutes, 1 hour and 2 hours, both TPA and NGF caused a rapid activation of Raf-1. Raf-1 had an almost threefold increase in activation by NGF (Fig. 9). TPA also induced an activation of Raf-1, although this was weaker.

Raf-1 became phosphorylated to a higher extent after TPA treatment than after NGF treatment, shown by a mobility shift assay (Fig. 10A). The NGF-induced phosphorylation and activation of Raf-1 was not inhibited by the PKC-inhibitor GF109203X (Fig. 10B and 11).
Figure 9. Activation of Raf-1 in SH-SY5Y/TrkA cells after treatment with 100 ng/mL NGF (black bars) or 16 nM TPA (grey bars). The cells were treated for 5 minutes, 30 minutes, one hour and two hours. The values are mean ± SEM.

Figure 10. Phosphorylation-dependent decelerated mobility of Raf-1 in SY-SY5Y/TrkA cells. Phosphorylated proteins have a higher molecular weight, resulting in a band detected higher up in the Western blot. A) Raf-1 phosphorylation after treatment with 100 ng/mL NGF or 16 nM TPA. B) The effect of PKC inhibitor GF109203X on NGF-induced phosphorylation of Raf-1.
NGF treatment also induced an activation of Ras, whereas TPA had no effect on Ras activity (Fig. 12). Also, NGF did not notably activate different PKC isoforms, whereas TPA induced a strong activation of PKC, detected as phosphorylation of MARCKS (Fig. 13).

In conclusion, the results indicate that Raf-1 can be activated and phosphorylated by both NGF and TPA in SH-SY5Y/TrkA cells, but via different pathways. NGF-induced activation of Raf-1 is mediated via Ras, whereas the TPA-induced activation is mediated via PKC. Our results also show that, even though wild-type SH-SY5Y cells with a very low amount of endogenous TrkA do not respond to NGF treatment, the signaling pathways downstream of the TrkA receptor are still intact.
Figure 12. Ras activity in SH-SY5Y/TrkA cells treated with 16 nM TPA or 100 ng/mL NGF. Immunoprecipitation (Ip) of active Ras was performed using the Ras binding domain (RBD) as bait and Ras was detected by immunoblotting (Ib) for Ras.

Figure 13. PKC activation in SH-SY5Y/TrkA cells after treatment with 16 nM TPA or 100 ng/mL NGF. PKC activation was measured by phosphorylation of MARCKS, where the lane to the very right is a positive control. The average level of MARCKS phosphorylation (measured as number of pixels) is shown by numerical values beneath each condition.
Discussion

Methodological considerations

Choice of control group

In studies on SSRI, the effect of the underlying condition that is being treated must be isolated. In epidemiological studies this is typically done by including control groups that either have discontinued SSRI use prior to pregnancy, or who have untreated mental health conditions. Either choice is associated with its own pitfalls. In this thesis, we have chosen women with untreated depression (in the sense that women were not medically treated - several women we’re receiving counseling or therapy) as controls. It could be argued that women who had discontinued SSRI would have made a stronger control group, but information on pre-pregnancy drug treatment was not available in the BASIC study questionnaires, and information on already discontinued treatment is rarely disclosed to the mid-wife at first antenatal booking. Furthermore, timing of discontinuation would be critical for the experiments of this thesis. While women may state at a first antenatal booking that they have discontinued treatment, they may not have done so until they discovered the pregnancy, ultimately exposing the early placentation process to SSRI. In addition, 75% of women who are on SSRI treatment when they start planning a pregnancy or realize they are pregnant will discontinue [59]. At present, there is no information on why some women decide to quit their medication while others continue. It can be assumed that women who chose to continue SSRI during pregnancy are the ones in greatest need of treatment. Possibly, they had a severe episode when treatment was initiated, possibly they had been on treatment for a longer time, possibly prior attempts to terminate treatment had failed, and possibly a co-morbid anxiety disorder made it impossible to stop treatment during pregnancy. Whatever reason they may have had for continuing treatment, we are left with the possibility that their depression is more severe than the depression of untreated depressed women.

However, SSRIs are not only used in depression but also in anxiety disorders. We chose untreated depression as control, but it may be argued that untreated anxiety could have been a relevant alternative. However, our choice was merely based on the fact that depression is far more studied than
anxiety in pregnancy. Furthermore, anxiety disorders are relatively heterogeneous, with some diagnoses stressing situational anxiety (specific phobias, panic disorder) whereas other disorders may be characterized by more generalized anxiety symptoms.

In choosing untreated depression, it should also be acknowledged that depression is not a simple and straightforward phenotype. The diagnosis of depression is based on the presence of a group of symptoms, but the underlying causes of the depressive episode may vary; life stress, trauma, infection, severe medical conditions, drug or alcohol abuse, just to mention a few, all of which may influence the placental function differently. In addition, the depressive episode may also differ in its duration and severity, which in turn, may affect the associated disturbances in the HPA axis [24]. All in all, the complexity of depression is illustrated in study I, where among the genes chosen for biological validation of the microarray, only 2 were validated with qRT-PCR for depressed women, whereas 6 (out of 7) genes were validated among women who had been treated with antidepressants. As more placental gene alterations were validated in a larger subset of SSRI-treated women compared to those with antenatal depression, it was concluded that for these subset of genes, the effects of SSRI intake during pregnancy appear more robust. Presumably, a more homogeneous group of depressed women could have been assembled if we had added some type of biological criteria to the diagnosis, for instance depression with elevated baseline cortisol levels.

It may also be argued that the severity of depression actually differed between SSRI cases and women with untreated depression. Because the placental studies were performed within the BASIC study framework we had access to self-rated depression scores for all participating women in gestational week 17 and 32. Indeed, from these scores it was apparent that women on SSRI treatment displayed lower depression scores than the untreated controls. Had we chosen a control group with similar depression scores to the SSRI users, they would not have been depressed. Alternatively, finding SSRI users with high depression scores would impact greatly on the number of cases available.

In study II and in the validation experiment of study I, untreated depression was merely defined by the EPDS scores of participating women. Preferably, a group with diagnosed major depressive disorder should have been included in all experiments, but most women with depressive disorders go undetected throughout pregnancy [2]. In addition, it should also be pointed out that, while EPDS may fail to detect depression in almost half of cases, its specificity for depressive disorders is very high at the cut-offs we were using [14].

Depression is also associated with a number of factors that may influence placental function. Smoking, obesity, young age, drug or alcohol use, and domestic violence all contribute to the risk of adverse perinatal outcomes, and smoking and obesity are specifically associated with altered placental
function [181-183]. We have tried to control for these associated factors by matching controls by age (study I and II) and BMI (study I), or adjusting for these factors in the statistical analyses. Severe medical conditions, daily medication and obstetric complications were exclusion criteria for both papers. Smoking was not an exclusion criteria but was rare in our study population. Similarly, none of the subjects acknowledged alcohol use during pregnancy.

**Sample size**

Ideally, sample size is determined by *a priori* power analyses. Adequate sample sizes are important not only to avoid type two errors (failure to detect true findings) but also to protect against false significant findings. However, to be completely honest, the sample sizes used in the present thesis were to a large extent based on available tissue. The BASIC study started to collect placental tissue in 2010, and by the time the initial microarray analysis was planned, approximately 200 placentas were available. With time, the placenta biobank has grown to include more than 50 women who have used SSRIs during the entire pregnancy, but all cases cannot be used due to the presence of obstetric complications. Presumably due to positive attrition, the proportion of SSRI users is greater than in the general population (approximately 5.5% in comparison with the expected 2.5%) [57]. In addition, the sample size for study II also depended on availability of formalin-embedded placental tissue. However, while the sample sizes for the microarray analysis were extremely small, i.e. 5 depressed and 5 SSRI-treated women, each compared with 5 healthy controls (in total 10 controls), the microarray analysis revealed many genes to be differentially expressed between the groups. Also, these results were validated by qRT-PCR in a larger population, where significantly altered gene expressions could be confirmed, at least in the SSRI cases.

Obviously, the above limitation has precluded any differentiation between different SSRIs, and for the validation of the microarray, a few cases using other types of antidepressants were also included. This may have influenced the outcome of the gene expression in the SSRI treatment group.

Similarly, the number of embryos used in study III is very low, but for obvious reasons, the number of human embryos that can be used for research is not unlimited. The relatively low number of embryos in this study could have an impact on the statistics. However, several significant alterations in embryos treated with FLX were discovered. Also, several changes were noted in e.g. protein expression in embryo culture medium that tended to differ between treated and untreated embryos.
Human embryos

The use of human embryos for study III distinguishes us from all previous attempts to elucidate the effects of SSRIs on embryo development. However, with no previous studies to rely on, a number of methodological issues were encountered. The aim was to investigate the effects of pharmacologically/therapeutically relevant levels of FLX on human early embryo development. The dose used in the study corresponded to physiological umbilical cord and amniotic fluid levels of FLX-exposed fetuses in women with commonly used doses of FLX [64, 65, 184]. A small dose-response pilot study was also conducted before we started the trial; with higher concentrations of FLX (1 µM) added to the culture medium, the number of dead embryos increased. These dual effects of SSRI have been noted earlier with mice embryo, where FLX induced an enhanced embryo development into blastocysts up to a certain dose, followed by an inhibition of blastocyst formation at higher doses [97].

The treated embryos in culture correspond to embryos in early pre-implantatory phase. In a spontaneous pregnancy this means that the placenta has not yet been developed and there is no amniotic fluid or umbilical cord through which FLX could reach and affect the embryo. However, during the pre-implantation period, the embryo might be exposed to FLX via the surrounding environment (follicular fluid in ovary, fallopian tubes, endometrium and myometrium in uterus).

The analysis method used for the proteomics has been used in several previous studies within reproductive medicine [185-187], but the complexity of the method should be taken into consideration when interpreting and analyzing the results.

Another possible limitation of the study is that the development of an embryo in culture is different from maturation and development in an intrauterine environment. However, this also holds true for embryos that are transferred back to the utero during in vitro fertilization (IVF), meaning that these embryos are in an environment that allows them to develop into blastocysts and further on into a fetus.

Experimental considerations

To prepare and isolate RNA is already known to be quite difficult and to prepare RNA from placenta (study I) needed an optimized protocol. RNA easily degrades and degraded RNA is useless for down-stream applications. However, the speed of degradation can be reduced by immediate freezing of the tissue after collection. In our study, placenta was collected at birth and frozen almost at bedside. However, our experience was that RNA from placenta seems to degrade quickly and that it is not always easy to handle sample collection in the delivery room.
A full-length RNA is of great importance for both microarray and qRT-PCR analysis. For microarray analysis, a poor quality RNA reduces the chances of identifying genes of importance. For qRT-PCR, the cDNA used needs to be of full-length, to avoid any false negative findings or misinterpreted results. Since cDNA synthesis uses RNA as a template, a good quality RNA is crucial.

Other limitations for qRT-PCR analysis are the primers and probes, which need to be selected carefully so that they have as good hybridization capacity as possible for the gene of interest. The primer and probes used in study I are validated for each gene detected, but of course these validations are made by the manufacturer and could be biased.

Performance of immunohistochemistry (IHC) (study II and III) is highly dependent on the primary antibody and its specificity for the targeted protein. A poor specificity can give both false positive and negative results. Also, a highly purified secondary antibody is important to avoid false binding to other compounds and materials, creating a high background or false positive findings. The dependency on the specificity of the primary and secondary antibodies also holds true for Western blot (WB) analysis (study II and IV).

For WB, the enhanced chemiluminescence (ECL) method using films to identify proteins (study IV) has, since the paper was published, been further developed. If the study had been performed today, the detection of chemiluminescence would probably have been made by sensitive screens and/or scanners. Alternatively, fluorescently labeled antibodies detected by imagers could have been used, making it possible to analyze Western blots more quantitatively (as was done in study II).

When scoring IHC stained placental sections (study II), this was done by visual inspection in a light microscope. This could be a limitation to the study, since such scoring can be subjective. To avoid biased results, a double-blinded scoring was performed in the same way by a second person who had no access to the initial results.

The effects of SSRI and antenatal depression on placenta

Many studies have been conducted in order to investigate the effects of antenatal depression and SSRI treatment during pregnancy, but the underlying biological mechanisms for these are largely unknown. The placenta is an important organ in the development of the fetus and this thesis includes two studies which: 1) investigated the gene expression in the fetal side of the placenta in order to find gene expression patterns in mothers with antenatal depression and in mothers using SSRI treatment during pregnancy (study I);
and 2) investigated, based on the results in study I, the NGF signaling pathway in placenta from mothers with antenatal depression and using SSRI treatment during pregnancy (study II).

ROCK1 and ROCK2

ROCK1 and ROCK2 are part of the Rho-associated coiled-coil kinase family [188, 189] and are downstream effectors of RhoA-GTP. Rho-ROCK signaling pathways are involved in the regulation of actin cytoskeleton, cell migration and proliferation [190]. In mice, ROCK1 is highly expressed in the lung, liver, spleen, kidney and testis, whereas ROCK2 is most abundant in the brain and heart [189, 191, 192].

No altered protein levels of ROCK1 was seen between the groups by IHC, but elevated levels of phosphorylated ROCK2 were detected in all placental cell types in SSRI-treated women compared with depressed and healthy controls. These findings might seem contradictory to the results in study I, where ROCK1 and ROCK2 gene expressions were down-regulated in SSRI-treated women. Also, phosphorylated ROCK2 levels were slightly elevated in all placental cell types in depressed women compared with controls (although lower than in SSRI-treated women), whereas a tendency towards lower ROCK2 gene expression was noted in depressed women compared with controls. This discrepancy between protein levels and gene expression might be explained by the fact that in gene expression analysis, pooled placental cells were used. However, gene expression and protein expression do not necessarily mirror each other and the results found could also be explained by different post-translational modification in SSRI users and depressed women [193, 194].

Also, IHC revealed alterations in protein levels, but this was not confirmed by WB analysis. This is most likely explained by the fact that in WB analysis all cell types in the placenta are pooled, meaning that a decrease in levels in one cell type might mask an increase in another cell type and vice versa, or a small effect in only one cell type might not be seen when all cell types are mixed together.

It is interesting though, that SSRI treatment alters both gene expression and protein levels of ROCK2 in placenta. The role of the Rho/ROCK family in cardiovascular diseases has been extensively studied [195]. Cardiac malformations [130], including pulmonary hypertension [57, 114, 127, 132], have also been reported in the SSRI-exposed offspring. ROCK2 has been found in vascular smooth muscle cells and has been shown to play a role in hypoxia-induced pulmonary hypertension in mice [196], a rare but important complication from SSRI use during pregnancy [57].

In addition, normal ROCK1 and ROCK2 activity is required for normal inner cell mass morphogenesis, which is of importance for successful fetal development [197]. The findings in study I indicate that antidepressant use
during pregnancy may influence pregnancy complications and fetal development, and that ROCK1 and ROCK2 may be involved with these processes.

Due to lowered ROCK2 gene expression found in the fetal placenta of SSRI-treated women compared with depressed women, it is tempting to speculate that a normal expression of ROCK2 in placenta is important for normal functioning of the cardiovascular system in the fetus. Ongoing studies in the lab on SSRI-treated heterozygous serotonin transporter knock-out rats exposed to maternal deprivation (leading to a depressive-like phenotype) may shed light on the SSRI-induced effects on offspring cardiovascular system.

Trophoblasts, placenta and pregnancy complications

Trophoblasts are important in exchanges between the fetus and the mother and possess endocrine activity releasing hormones that are important in the maintenance of pregnancy [198]. In addition, trophoblasts are involved with the secretion of placental growth hormone and are important in the development of the placenta [199].

NGF is found to be expressed in human placenta [200, 201], where it is involved in important functions such as placentation [202] and pregnancy maintenance [142]. In study II, elevated protein levels of NGF in placental trophoblasts and endothelial cells in SSRI-treated women were found. Previous animal and human research has shown that NGF signaling is involved in miscarriage and preterm birth [148, 149, 203], so our study could indicate that NGF might have a role in SSRI-induced pregnancy outcomes. Inadequate trophoblast invasion and endothelial dysfunction are important features in the development of preeclampsia.

Also, as shown by IHC, higher ROCK1 levels were detected in endothelial cells, whereas ROCK2 was predominantly found in trophoblasts. A role for ROCK1 has been suggested in hypertension [204-207] and increased ROCK2 expression has been described in pre-eclamptic human placentas [208]. This indicates that ROCK1 and ROCK2, even though both in the NGF signaling pathway, might play different roles in the development and function of the placenta.

Since SSRI-treated women had increased protein levels of ROCK2 in trophoblasts, it may be speculated that NGF signaling also plays a role in preeclampsia. Besides the role of the Rho kinase pathway in cardiovascular diseases, a role has also been proposed for the modulation of the placental vasculature. It has been shown that, although the gene expression of ROCK1 and ROCK2 was not different, a higher RhoA mRNA expression was found in placentae from women who suffered from preeclampsia compared with placentae from those that were normotensive [209]. Interestingly, the use of antidepressants has been associated with an increased risk for preeclampsia.
and this risk also depends on duration of SSRI use during pregnancy; i.e. women using SSRIs during the entire pregnancy have an increased risk in comparison to those who discontinue before gestational week 20, and in comparison with non-users [111, 113, 116].

In study I, down-regulated *KTN* gene expression was found in SSRI-treated women compared with healthy controls. *KTN* (the full-length kinec-tin) is mainly expressed in the brain, liver, ovarian, and hematopoietic cells and is found in the endoplasmic reticulum, where it is responsible for the transport of vesicles along microtubules [210, 211]. Of interest is that kinec-tin can interact with RhoA [212], and, as mentioned above, the RhoA signaling pathway is affected in the depressed and antidepressant-treated women. Normal RhoA signaling is important to prevent pregnancy complications, where elevated levels of RhoA have been found in placenta from women suffering from preeclampsia [209]. However, more research is needed to investigate the consequences for the fetus due to altered gene expression of *KTN1* in the placenta.

The validation of the microarray analysis in study I revealed that the gene expression of *C12orf39* was down-regulated in both depressed and SSRI-treated women. Also, a down-regulated expression of *DNM1L* was found in women treated with antidepressants. Interestingly, *C12orf29* and *DNM1L* are found in trophoblasts. *C12orf39* is located in the villous trophoblasts and is mainly expressed in the placenta and brain, suggesting a function for *C12orf39* in these active secretory tissues [213]. These findings suggest that *C12orf39* is involved in the regulation of placenta development through its role in the biological functions of the trophoblasts and that antenatal depression during pregnancy may influence this development. The fact that in antidepressant-treated women, in study I, the effect is no longer significant, which may indicate that antidepressants may restore the effects of the depression-induced down-regulation of *C12orf39*.

*DNM1L* is a GTPase regulating the mitochondrial fission and ablation of the *DNM1L* gene induced defects in trophoblast giant cells and cardiomyocytes in mice [214]. Also, brain-specific *DNM1L* ablation caused developmental defects in the cerebellum [214]. Although these results were found in knockout mice it is tempting to speculate that the down-regulation of the *DNM1L* found in the antidepressant-treated women in study I, might have an effect on embryo and brain development as well. However, again, this effect remains to be established.

Study I shows that both depressions *per se* and SSRI treatment alter the expression of several genes in the placenta. These results are confirmed in study II, where both depression and SSRI treatment have an effect on the protein levels in placental trophoblasts. Taken together, this indicates that depression and SSRI treatment have an effect on trophoblast development and placental function, which could ultimately affect pregnancy outcome and fetal development.
Developmental programming

Adequate implantation/placentation is a prerequisite for a pregnancy to occur, and a well-functioning placenta is of major importance for the intrauterine development and growth of a child [215, 216]. Barker [217] hypothesized that diseases that might manifest later in life can be traced back to early development, and a mechanistic role of the placenta in fetal programming has been discussed during recent years [215]. This phenomenon is usually referred to as the Barker hypothesis and the relevance of this has also been confirmed by others [218-221].

The two major hypotheses regarding intrauterine developmental programming are exposure to either malnutrition or stress (i.e. increased cortisol levels). Indeed, in many mammalian species, maternal under-nutrition or exposure to stress or glucocorticoid excess leads to reduced birth weight and may cause persisting hypertension, hyperglycemia, affective dysfunction, and neuroendocrine abnormalities in the offspring [222, 223]. Both placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) and the glucocorticoid receptor (NR3C1) have been implicated in the prenatal glucocorticoid programming. While glucocorticoids are important for fetal growth and organ maturation, the enzyme 11β-HSD2, which converts cortisol to inactive cortisol, is strategically positioned in the syncytiotrophoblast to protect the fetus from the pregnancy-induced glucocorticoid excess in the maternal circulation [224]. Indeed, placental 11β-HSD2 inactivates the majority of maternal glucocorticoids passing to the fetus in humans, and inhibition of fetoplacental 11β-HSD-2 leads to long-standing perturbations in the HPA axis and anxiety-like behavior in the offspring [225, 226]. Maternal depression during the third trimester of pregnancy has been associated with increased methylation of NR3C1, and greater methylation of placental NR3C1 or 11β-HSD-2, in association with depression and anxiety, respectively, were related to hypotonia and lethargy in infants [227, 228]. Parenthetically, no differences in NR3C1 or 11β-HSD-2 placental gene expression have been noted in SSRI users or women with untreated depression (Edvinsson et al., preliminary results).

Based on the results of this thesis, it could be speculated that SSRI exposure may also have fetal programming effects. It has relatively recently been shown that the placenta may synthesize serotonin and that placental serotonin is accumulated in the fetal forebrain during an important growth period [106, 229]. While it may be argued that the epidemiological evidence suggesting behavioral disturbances in infants exposed to SSRI would strengthen such an assumption, the clinical evidence at present is extremely preliminary [133-137]. Until further long-term follow-ups of exposed children (with the use of relevant controls) are available, this will merely remain a speculation.
Additive or separate effects?

In study I, antenatal depression and antidepressant exposure during pregnancy both had influences on the gene expression of the placenta. In the microarray, 108 genes were differentially expressed in women with antenatal depression, while 109 genes were differentially expressed in antidepressant-treated women. However, only 20 genes were overlapping between depressed women and women on antidepressant treatment. Because we were not able to match SSRI users and untreated depressed women (due to availability of tissue at that stage), we refrained from direct comparison of these two groups. With more tissue now available, a repeated microarray experiment could be considered, using only women with untreated and treated depression. Nevertheless, while the microarray data relied on few individuals, the gene expression pattern suggested mostly separate effects of the two exposures.

In addition, this pattern of mixed additive and separate effects was also repeated in study II. Additive effects by depression and SSRI use were found on certain aspects of NGF signaling (ROCK2 and phosphorylated ROCK2). Other parts of the signaling pathway, however, showed opposite effects of depression per se and SSRI use, as in the case of NGF levels in endothelial cells. Other findings from our lab are also emerging, suggesting that depression and SSRI may have separate effects; for instance, SSRI treatment is associated with increased placental corticotropin releasing hormone (CRH), whereas depression on the contrary seems to result in decreased CRH levels, at least in comparison with the SSRI users (Hannerfors et al., submitted manuscript).

An alternative interpretation could, of course, be that while we attempt to control for depression in our experiments, we may not have fully accomplished that.

Although these studies are not able to elucidate the role of these alterations for all of these maternal and fetal complications, these findings nevertheless points towards important distinctions between how the exposure to depression per se and SSRI treatment of depression affects placental function. However, it remains to be established how these differences influence the development of the child, and whether these differences are found in the fetus as well.

Fluoxetine affects embryo development

In study I and II, SSRI treatment in pregnant women altered gene expression and protein levels in the placenta. This could affect the function of the placenta, and consequently also the developing fetus, but the fetus can also be exposed to SSRI more directly. Based on this, and considering that serotonin is involved in embryogenesis [230] and that the serotonin transporter is ex-
pressed in early embryos [92], the effects of fluoxetine (FLX) on early human embryo development were studied (study III).

FLX affected the timing to different embryo developmental stages, with a significant shorter time between thawing and compaction compared with controls. The same tendency was noted for times to other embryo developmental stages. The timing between different developmental stages of the embryo has been shown to be crucial for embryo quality and implantation rate after assisted reproduction [72-75]. The results in study III indicate that FLX may alter the time between embryonic gene activation, which is initiated at the 4- to 8-cell stage of the embryo [71], and the formation of morula. The impact of this alteration on the future development of the fetus remains to be determined. However, effects of in vitro culturing conditions have been shown to have an effect on fetal development in cattle and mice [231, 232]. Also, embryo culture medium used in IVF is shown to have an effect on the perinatal outcome [233, 234]. It has been suggested that these outcomes are due to epigenetic changes in the placenta and/or fetus [235-237].

In the mass spectrometry analysis of embryos, several important pathways, including cellular growth, proliferation and survival, seem to be affected by FLX. When analyzing proteins secreted into the culture medium from the embryo, significantly increased levels of uPA were found in medium from FLX-treated embryos. uPA is involved in cell proliferation, differentiation and migration [238] and uPA in complex with its receptor is involved in cell signaling during neuronal migration and neuritogenesis [239].

In study III, uPA was detected by immunohistochemistry in the trophectoderm of the embryo. Also, NGF was detected in the trophectoderm, which is in line with the findings in study II, where the levels of NGF were increased in trophoblasts in placenta from SSRI-treated women compared with healthy controls. Again, the embryonic trophectoderm will develop into the placenta and alterations in protein levels in the placenta could lead to changes in the placental function, which could ultimately affect the development of the fetus.

In conclusion, this study shows that FLX affects embryo development as well as the embryo protein expression. However, the impact on the developing fetus remains to be investigated.

NGF signaling pathways

In study IV, the NGF signaling pathway in the neuroblastoma cell line SH-SY5Y was investigated. Since the wild-type cell line does not respond to NGF, due to low expression levels of the receptor TrkA, SH-SY5Y was transfected with exogenous TrkA. In contrast to TPA treatment of SH-SY5Y/TrkA, the NGF-induced activation of Raf-1 in SH-SY5Y/TrkA cells was not mediated via PKC. SH-SY5Y/TrkA cells do respond to NGF treat-
ment by neurite outgrowth and this response was mediated via the Ras-Raf-1 pathway. This is in line with the fact that SH-SY5Y cells treated with NGF in combination with the growth inhibiting agent aphidicholin, induced an up-regulation of the receptor TrkA after 3 days of treatment [240]. After 3 to 4 days of treatment, the cells begun to differentiate in response to NGF.

It was concluded that, since SH-SY5Y/TrkA cells respond to NGF treatment by neuronal differentiation, the Ras-Raf-MAPK pathway was intact in these cells. However, one could speculate about how accurate it is to use tumor cell lines to investigate certain aspects of signaling cascades. The neuroblastoma cell line SH-SY5Y is developmentally arrested and defects causing this arrest may interfere with pathways investigated. Even though it was elucidated that Raf-1 activation was mediated by Ras, other signaling proteins might also be involved. However, the aim of study I was to investigate whether Ras and/or PKC were involved in NGF-mediated Raf-1 activation and to conclude that the developmental arrest of these neuroblastoma cells are not due to a defective signaling cascade down-stream of the TrkA receptor.

The focus of study II was the effects of antenatal depression and SSRI use during pregnancy on proteins in the NGF signaling pathway. In study I, altered ROCK1 and ROCK2 gene expression in depressed and SSRI-treated women had been detected. Study IV had revealed that NGF-induced differentiation of neuroblastoma cells was mediated via activated Raf-1 and the most well-known down-stream mediators for Raf-1 signaling are the MAP kinases. But Raf-1 can also activate ROCK1 and ROCK2 via ribosomal s6 kinase (RSK) [241]. Therefore, study II also investigated whether the effect of antenatal depression and SSRI on ROCK1 or ROCK2 were mediated via RhoA or Raf-1. Immunohistochemical staining of placental sections showed increased levels of ROCK2 and phosphorylated ROCK2 in SSRI-treated women compared with healthy controls. SSRI use or depression per se did not alter the RhoA levels; however, increased levels of phosphorylated Raf-1 were found in stromal cells in SSRI-treated women compared with controls, indicating that the altered levels of ROCK2 and phosphorylated ROCK2 are mediated via Raf-1.

NGF can also cross-talk with other signaling pathways, i.e. vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNFα) and epidermal growth factor (EGF) [164, 165, 167]. This means that alterations in the NGF signaling pathway induced by SSRI treatment or depression, could also affect other signaling pathways that may contribute to the associated pregnancy outcomes and fetal development. And vice versa, signaling pathways not investigated in this thesis could be differentially regulated by SSRI-treatment or depression per se, and add to the altered gene expressions or protein levels found in this thesis.
Conclusions and remarks

Even though significant alterations in placental gene expression and protein levels due to SSRI treatment and depression were found in this thesis, it is still difficult to conclude what clinical outcomes these alterations might lead to. But, the fact that differences in gene expression could also be seen at protein level further strengthens the hypothesis that depression and SSRI treatment during pregnancy have an effect. Also, some of the findings in this thesis could be important for the understanding of the clinical effects that have been associated with SSRI use.

Also, FLX clearly has an effect on embryo development and protein expression, but it is hard to be certain whether these effects can be directly correlated to pregnancy outcomes of increased risk of miscarriage, and malformation and behavioral defect in infants. Based on findings from study II, it seems appropriate for the NICE guidelines to recommend the lowest possible dose of SSRI to women who consider continuing treatment during pregnancy.

In addition, this thesis has also pointed out that the choice of appropriate control groups for epidemiological research should be reconsidered, or at least be interpreted with caution. We chose untreated depression, but instead of the additive effects we had expected, we ended up having more findings that separated the two groups. Additional experiments with women who have discontinued SSRI use prior to their pregnancy might shed light on what would be the best controls for epidemiological research.

However, taken together, the results in this thesis show that both depression per se and SSRI has an effect on the placenta and that FLX affects embryo development. These findings contribute to the overall understanding about the effects of SSRI and could be an important part of the knowledge base used when deciding about whether or not to treat a depressed woman with SSRI during pregnancy.
Summary of conclusions

- SSRI treatment of pregnant women alters gene expression in the placenta compared with depressed and healthy women. Also, proteins in the NGF signaling pathway have altered levels in placenta from SSRI-treated women. In placenta from depressed women, gene expression and NGF signaling protein levels are also altered, but in a different way compared with SSRI-treated and healthy women. Altered placental gene and protein expression could lead to an altered placental function, which in turn could affect the development of the fetus.

- Fluoxetine treatment of human embryos alters the timing to different developmental stages. Also, fluoxetine induces the expression of several proteins in the embryo, as well as proteins secreted by the embryo, compared with control embryos. This indicates that the embryonal exposure to fluoxetine in utero could lead to changes in embryo development.

- In SH-SY5Y cells transfected with TrkA, both TPA and NGF activates Raf-1, but to a different extent and via different pathways. TPA activates Raf-1 via PKC and NGF via Ras. Although SH-SY5Y cells express very low amounts of endogenous TrkA, the signaling pathway down-stream of the receptor seems to be intact.
Sammanfattning på svenska (Summary in Swedish)

Under år 2014 hämtade 10,4% av alla kvinnor i barnafödande ålder i Sverige ut antidepressiv medicin minst en gång. Även om inte alla är gravida då de börjar sin behandling, hamnar några av dem förr eller senare i ett läge då de blir gravida och behöver ta ställning till om de ska fortsätta med sina antidepressiva läkemedel eller ej. Depressionssymtom är vanliga under graviditet och omkring 4 - 7% uppfyller kriterier för diagnosen pågående medelsvår depression.

En depression äventyrar inte bara mammans hälsa och välmående, utan kan också påverka fostrets utveckling och ge beteendestörningar hos både yngre och äldre barn. Havandeskapsförgiftning och förtidsbörd är några av de graviditetskomplikationer som beskrivits hos deprimerade gravida kvinnor. Barnen kan också födas tillväxthämmande och försenas i sin utveckling.


En välfungerande placenta är inte bara viktig för näringsförsörjningen till barnet, den fungerar också som en barriär för icke önskvärda substanser, så som mediciner och giftiga ämnen. I delstudie ett och två i denna avhandling studerades uttrycket av gener och proteiner i vävnadsprover från placenta från SSRI-behandlade och friska gravida kvinnor, men även hos obehandlade deprimerade gravida kvinnor. Att ha med deprimerade kvinnor i studien var av största vikt, då det annars skulle vara svårt att avgöra om eventuella effekter berodde på SSRI-behandlingen eller depressionen i sig. Det visade sig att både SSRI-behandling och depression påverkade uttrycket av flera olika gener i placenta jämfört med den friska kontrollgruppen, en del på ett unikt sätt för de olika grupperna av kvinnor, medan en del gener påverkades

68
på samma sätt i båda grupperna. Två gener som nedreglerades (uttrycktes mindre av) i placenta hos både SSRI-behandlade och deprimerade kvinnor var *ROCK1* och *ROCK2*. Dessa gener är involverade i flera olika intracellulära signaleringsvägar, bl.a. i neurotrofiners signalering. Neurotrofiner är nervtillväxtfaktorer som framför allt styr tillväxten av nervceller och deras utskott, s.k. neuriter. Delstudie två visade att nivåerna av vissa proteiner av betydelse i olika signaleringsvägar nedströms om nervtillväxtfaktorn NGF ökade hos SSRI-behandlade och deprimerade kvinnor jämfört med nivåerna hos de friska kvinnorna. Mönstret skiljde sig dock något mellan de båda grupperna. Förändringar i placentans gen- och proteinuttryck kan påverka hur den fungerar, vilket i sin tur kan påverka hur embryot och fostret utvecklas.

SSRI anses kunna passera placentan och halter av SSRI har uppmätts i både navelsträngsblod och fostervatten. Detta betyder att fostret exponeras för SSRI genom blodet, men också genom att svälja fostervatten. I delstudie tre undersöktes hur direktxponering av SSRI påverkar embryoets utveckling och proteinuttryck. Humana embryon behandlades med SSRI-preparatet fluoxetin (FLX) och deras utveckling följdes i ett mikroskåp. Det visade sig att FLX-behandlade embryon genomgår en lite snabbare utveckling jämfört med obehandlade embryon, vilket mättes genom skillnader i tidsintervall mellan olika embryologiska utvecklingsstadijer. FLX-behandlingen påverkade också wilka proteiner som producerades av embryot, detta gällde såväl intracellulär produktion som utsöndring. Två speciellt intressanta proteiner var urokinasliknande plasminogenaktivator (uPA) och NGF. uPA är involverad i bl.a. bildandet av nervceller, men också i tillväxten av neuriter. Både uPA och NGF återfanns i den del av embryot som senare utvecklas vidare till placentan.

I delstudie fyra undersöktes vilka proteiner som är involverade i nervtillväxtfaktorn NGFs signalering. En cell-linje från neuroblastom (SH-SY5Y/TrkA) användes som modellsystem. Neuroblastomceller är omogna nervceller, som kan fås att mogna ut om de behandlas med t.ex. NGF. Det visade sig att NGF-signaleringen i dessa celler framför allt går via proteiner- na Ras och Raf-1. Raf-1 signalerar huvudsakligen via MAPK-vägen, men det har även visats att Raf-1 kan aktivera ROCK1/2, vilket delstudie två i avhandlingen också indikerar.

Sammanfattningsvis visar denna avhandling att SSRI och depression påverkar både gen- och proteinuttryck i placenta om än på lite olika sätt, samt att embryots utveckling och proteinuttryck påverkas av FLX. Även om det är svårt att påvisa vilka kliniska effekter dessa förändringar ger, bör de beaktas när beslut fattas gällande en gravid kvinnas fortsatta behandling eller ej under en pågående graviditet.
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References


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