Stress and the female brain. The effects of estradiol on the neurobiological reactions to chronic stress.

Gerrits, Marjolein

General discussion

7

General discussion

The aim of this thesis was to elucidate the role of estradiol on neurobiological mechanisms related to stress. Many studies determining the long-term effects of estradiol make use of estradiol releasing pellets, inducing invariable plasma concentrations. Here, we introduced a cyclic administration paradigm to investigate the effects of estradiol under experimentally controlled physiological conditions. Ovariectomized rats underwent a cyclic estradiol administration paradigm inducing alternating high and low plasma levels of estradiol to maintain adequate ER function. The stress reactions of ovariectomized and estradiol-treated rats were compared. Where acute stress provokes a healthy reaction of the nervous system, chronic stress can induce several neurobiological aberrations possibly responsible for abnormalities seen in patients suffering from affective disorders.

Stress and estradiol in the PVN

The PVN serves as a main relay station of emotional stimuli. Signals from the limbic neurocircuits involved in the processing of emotions converge in the vicinity of the PVN and indirectly modulate PVN activity through GABA-ergic interneurons (reviewed by Herman et al., 2002b). Glutamate-GABA connections can inhibit PVN activation; excitation of the PVN is possible through GABA-GABA connections that disinhibit the local activation. The PVN can be subdivided in a parvocellular and a magnocellular part each executing different functions, although all parts directly or indirectly have an influence on the HPA-axis (Figure 1; reviewed by Herman et al., 2002a). The dorsomedial parvocellular zone (mpd) contains CRH positive neurons projecting to the median eminence, which induce ACTH release from the pituitary (Swanson et al., 1987; Whitnall, 1993). Expression of glucocorticoid receptors in the PVNmpd reveals a role in the negative feedback of the HPA-axis at the level of the PVN (Liposits et al., 1987).

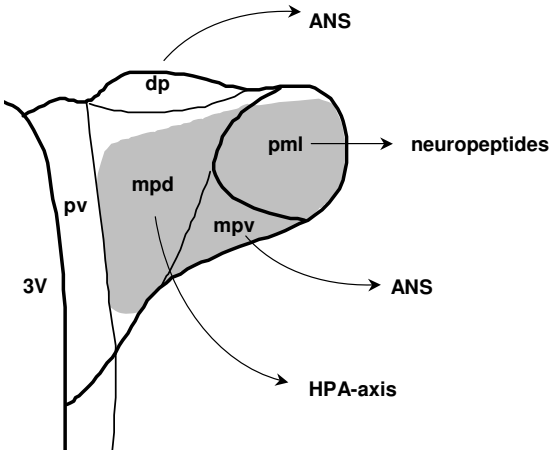

Figure 1: Subdivisions of the PVN. ERβ is distributed in the shaded area. 3V, third ventricle; pv, periventricular zone; mpd, dorsomedial parvocellular zone; mpv, ventral medial parvocellular zone; dp, dorsal parvocellular zone; pml, lateral posterior magnocellular zone; ANS, autonomic nervous system; HPA-axis, hypothalamic pituitary adrenal-axis.

Neurons of the lateral zone of the posterior magnocellular division (pml) of the PVN directly release neuropeptides as vasopressin and oxytocin into the systemic circulation (Swanson et al., 1983). Systemic vasopressin and oxytocin plays in particular a role in controlling body fluid and electrolyte balance, blood pressure, lactation and parturition; however, modulation of the ACTH release during stress has been reported as well (Dohanics et al., 1991). Projections to brainstem and spinal cord originate in the dorsal (dp) and ventral medial (mpv) parvocellular part of the PVN and play a role in the autonomic responses to stress, for example by evoking catecholamine secretion (Swanson et al., 1983). Increased autonomic activation can either directly or indirectly influence the HPA-axis (Jasper et al., 1997).
Acute stress increased the neural activity in the PVN of ovariectomized rats severely as determined with the number of cells expressing c-Fos protein. After 3 weeks of stress the neuronal activity in the PVN was still elevated. In contrast, total habituation of the c-Fos response in the PVN has been reported after recurrent exposure to a predictable stressor like restraint (Chen et al., 1995; Stamp et al., 1999). This indicates that in our model, where stress is applied with several unpredictable parameters, activation of the PVN is provoked even after repeated exposure. Specifically, the stress in the current paradigm induces increased PVN output even after 3 weeks.
Altogether, increased neuronal activity in the PVN can cause stress-induced changes in neuroendocrine or autonomic systems, which may be adaptive on the short-term but can provide a basis for structural damage on the long-term.

Cyclic estradiol administration reduced the number of c-Fos positive cells in the PVN induced by acute and chronic stress, but the attenuation was significant only when the rats were sacrificed within 24 hours after the last injection (so when plasma estradiol levels were high). In contrast to the estradiol-mediated reduction of c-Fos protein expression in the PVN, this finding was not replicated for the c-Fos mRNA expression. The c-Fos mRNA expression in ovariectomized and estradiol-treated rats is similar after chronic stress. Therefore, it seems that estradiol directly or indirectly mediates posttranscriptional mechanisms, causing attenuated protein expression despite mRNA elevation. Modulation of AP-1 sites, and therefore neuronal activation, is induced by dimers of c-Fos and Jun proteins, so c-Fos protein appears to be a more reliable measure for activity changes because of the dissociation between mRNA and protein.
In a subsequent experiment, ovariectomized rats were allowed to recover for 3 weeks after chronic stress (Figure 2). Re-exposure to the shock box after this washout period increased the neuronal activation pattern in the PVN dramatically. Enhanced sensitization of the PVN to a stressor after a washout period was reported before (Bruijnzeel et al., 1999). Long-term sensitization to stress may have consequences for the PVN output.
Similarly as found before, cyclic estradiol administration attenuated this enhanced response. However, in this experiment there were 5 weeks of estradiol depletion (corresponding to 8 estrus cycles) before the treatment started. Moreover, the estradiol treatment started after the chronic stress period instead of simultaneous to the stress.

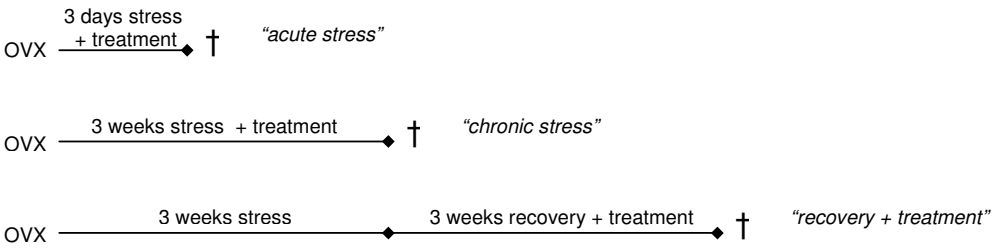

Figure 2: Simplified scheme of the experimental design of subsequent studies.

Despite these two altered factors in hormone treatment estradiol could still reduce the stress-induced activation of the PVN, indicating the robustness of this response. Not only the reaction to acute and chronic stress was attenuated, but also the reaction to a stressor after a recovery phase was reduced.
On the other hand, cyclic estradiol could not lower the number of c-Fos expressing cells in the PVN when the stress was continued during the treatment. Possibly other still unknown factors overrule the estradiol effects when stress is applied for such a long time.

ERβ is the main ER in the rat PVN and is expressed in the mpd, mpv and pml of the caudal portion (Figure 1) (Simonian et al., 1997; Laflamme et al., 1998; Price, Jr. et al., 2000; Shughrue et al., 2001; Hrabovszky et al., 2004). The high expression of ERβ in the PVN may provide the initial conditions for a mechanism in the attenuation of c-Fos by estradiol during stress. Indeed, chronic stress elevated the number of ERβ expressing cells in the PVN.
ERβ is colocalized with several neuropeptides in the PVN. ERβ abundantly colocalizes with oxytocin (Simonian et al., 1997; Laflamme et al., 1998; Alves et al., 1998; Hrabovszky et al., 1998; Isgor et al., 2003; Hrabovszky et al., 2004). Estradiol can induce oxytocin synthesis by activating the ERE on the oxytocin gene (Richard et al., 1990; Mohr et al., 1991; Adan et al., 1993; Loven et al., 2001). Oxytocin produced in the magnocellular neurons of the PVN is transported to the posterior pituitary and released into the systemic circulation, however parvocellular oxytonergic neurons project to brain areas like the amygdala and hippocampus and nuclei of the autonomic nervous system and give rise to local dendritic release (Swanson et al., 1980; Sawchenko et al., 1982).
The reduction of stress-induced c-Fos positive cells in the PVN by estradiol may entail a mechanism involving oxytocin (Figure 3). Stress increased ERβ immunoreactivity, in combination with estradiol this can result in elevated oxytocin release in the PVN. As reported before, oxytocin in the PVN can reduce stress-induced c-Fos activation (Windle et al., 2004). Moreover, central oxytocin administration can reduce stress-induced corticosterone secretion and anxiety behavior (Windle et al., 1997). The attenuated stress-induced c-Fos response induced by estradiol is dependent on the plasma estradiol level, high repression of the c-Fos activation pattern is displayed when plasma estradiol levels are high and low

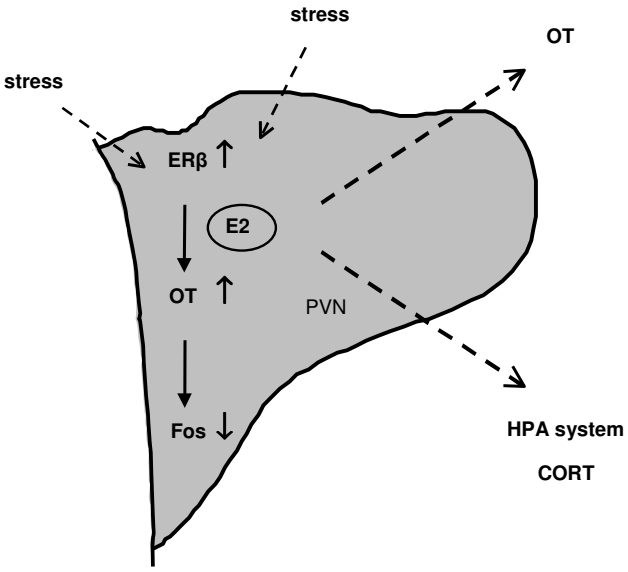

Figure 3: Schematic representation of the attenuating effects of estradiol on the stress-induced activation of the PVN. Chronic stress increased the number of cells in the PVN expressing ERβ, which can induce oxytocin gene transcription. Oxytocin release in the PVN can reduce the stress-induced activation (Windle et al., 2004). The estradiol-induced attenuating effect on the stress-induced Fos response is dependent on the plasma estradiol level, showing high repression of the Fos activation when plasma estradiol levels are high and low repression when plasma estradiol levels are low. Reduced activity of the PVN consequently can lead to decreased output to the autonomic and endocrine system.

repression when plasma estradiol levels are low, which fits to the proposed model. Oxytocin may influence processes involved in the translation of c-Fos mRNA into protein.

A limited number of CRH-containing neurons in the PVNmpd express ERβ (Laflamme et al., 1998; Hahn et al., 2003; Isgor et al., 2003; Hrabovszky et al., 2004). Direct interaction of estradiol through ERβ on CRH in the PVN is still unclear, although the CRH gene contains an ERE (Vamvakopoulos et al., 1993; Itoi et al., 2004). Cyclic estradiol administration for 5 weeks lowered the basal CRH mRNA expression in the PVN, which may dampen the absolute levels of HPA-axis activation. However, the reactivity of CRH mRNA as a response to chronic stress was similar in both ovariectomized and estradiol-treated rats.

Additionally, ERβ is expressed in the vasopressin-containing neurons in the parvocellular PVN (Alves et al., 1998; Hrabovszky et al., 1998; Hrabovszky et al., 2004). Moreover, the promoter of the vasopressin gene contains an ERE (Shapiro et al., 2000). However, central vasopressin infusion had no effect on either HPA-axis responses or neuronal activation in response to stress (Windle et al., 2004).

Besides these well-known neuropeptides also colocalization of ERβ and the precursor of orphanin FQ (OFQ) prepro-OFQ and its opioid receptor-like (ORL1) receptor has been described in the PVN (Isgor et al., 2003). OFQ is an endogenous anxiolytic and local infusion attenuates behavioral responses to stress (Jenck et al., 1997; Griebel et al., 1999). OFQ and ORL1 are widely distributed throughout the brain (Neal, Jr. et al., 1999; Nomura et al., 2002). This opioid peptide and its receptor resemble a relatively new discovered modulatory system of stress processing.

No persistent disturbances of plasma corticosterone levels were found after chronic stress when the stress was applied in the dark phase of the circadian cycle, which was in contrast to studies in cyclic female rats that were stressed in the light phase describing enhanced corticosterone responses to chronic stress (Kuipers, 2004). It has been reported before that the impact of stress is maximal when applied in the early light phase of the cycle, when the basal plasma corticosterone concentration is lowest (Allen-Rowlands et al., 1980; Retana-Marquez et al., 2003).
The role of estradiol in HPA-axis responses has been thoroughly described in literature. However, most studies used invariable and high levels of estradiol instead of cyclic estradiol administration, and reported enhanced corticosterone release and longer prolongation of the response compared to ovariectomized rats, in contrast to our results (Burgess et al., 1992; Lunga et al., 2004; Lund et al., 2005).
ERα and ERβ have opposite effects on corticosterone release; administration of selective agonists revealed that the increase in circulating corticosterone levels is due to ERα activation, while ERβ activation inhibits stress-induced corticosterone secretion (Lund et al., 2005). Invariable high levels of estradiol, as managed with a pellet, cause down-regulation of ERβ in the PVN (Suzuki et al., 2004) and thereby may induce enhanced corticosterone release following stress. After cyclic administration of estradiol, as used in the current experiments, no down-regulation of ERβ in the PVN has been demonstrated, which may explain the lack of effect on corticosterone release by estradiol.
Adrenal hypertrophy after chronic stress was seen in ovariectomized rats but not in estradiol-treated rats. Likewise, a group of unsynchronized female rats showed a non-significant increase in adrenal weight after chronic stress. Previous studies showed that individual housing is stressful in female rats, causing higher adrenal weights compared to socially housed female rats (Westenbroek et al., 2005). The female rats in the current experiments were individually housed, which makes it possible that the adrenal glands are maximally enlarged by isolation already and that the stress paradigm does not further increases the adrenal weight of estradiol-treated rats.

Altogether, cyclic estradiol administration attenuates stress-induced PVN activation, lowers basal CRH mRNA expression, but does not affect corticosterone response in the dark phase of the circadian rhythm although it prevents significant adrenal hypertrophy. To draw unambiguous conclusions about the effects of cyclic estradiol administration on stress-involved HPA-axis functions more data are needed. The ACTH response to a stressor is missing; moreover, a temporal analysis of the corticosterone release following a stressor seems necessary, as well as measurements during both phases of the circadian cycle.
The attenuating effect on the PVN by estradiol is very straightforward and established in several designs. The high percentage of colocalization of ERβ and oxytocin or prepro-OFQ in the PVN, and the minimal colocalization of ERβ and CRH may suggest that ERβ is not directly involved in the regulation of the HPA-axis via traditional neuroendocrine-mediated pathways. If the estradiol-induced attenuation of the PVN activation is not affecting neuroendocrine responses, possibly other PVN outputs, as the regulation of the autonomic

stress responses, may be modulated.
Indeed, estrogen replacement attenuated the elevation of mean arterial pressure to restraint stress; an effect centrally mediated by the autonomic neurotransmitter nitric oxide (NO) (Cherney et al., 2003). NO in the PVN exerts inhibitory effects on autonomic output to the periphery. Moreover, estradiol induced changes in endothelial nitric oxide synthase expression (eNOS) in the PVN are ERβ dependent (Gingerich et al., 2005). Besides central suppression of the autonomic stress response by elevating local eNOS in the PVN through estradiol administration, also over-expression and activation of eNOS in the periphery has been demonstrated in association with reduced cardiovascular responses to stress in estradiol-treated ovariectomized rats (Morimoto et al., 2004).
This raises the intriguing question what comes first, the central or the peripheral effect. Normally, higher brain areas perceive stress during increased cardiovascular responses to stress; the brain detects increased blood pressure or heart rate. Vise versa, detection of stress-induced changes by the brain provokes autonomic activity. When the cardiovascular responses are attenuated by estradiol less perception of stress by the brain will be induced and subsequently less stress-induced brain activity will result in lowered autonomic activity.

Stress and estradiol in the mPFC

The IL and PL regions of the mPFC have been associated with emotional and cognitive processes (reviewed by (Vertes, 2004). Each subdivision has specific projections in the basal forebrain, hypothalamus, amygdala and brainstem corresponding to individual functions. The main projections of the IL include areas involved in the control of visceral and autonomic activity, while the PL projects to areas regulating limbic and cognitive functions.
Several animal studies reveal the extreme vulnerability of the mPFC to stress. Reduced length and branch numbers of the apical dendrites, loss of dendritic spines, and disturbed expression of pERK2 and pCREB have been reported in the mPFC after chronic stress (Trentani et al., 2002; Kuipers et al., 2003; Radley et al., 2004; Radley et al., 2006).

Like in the PVN, acute stress in ovariectomized rats increased the neuronal activation of the IL and PL areas of the mPFC. However, chronic stress abolished c-Fos protein activation in the mPFC, despite significantly increased c-Fos mRNA expression. Therefore, the attenuated c-Fos protein response in the mPFC does not simply indicate habituation to the stressor, but may suggest that chronic stress affects the translation of the mRNA into functional protein through yet unidentified mechanisms, leading to a failing response to long-term stress with increased c-Fos protein expression. Stress may mediate posttranscriptional mechanisms. Another possibility lies in the differential temporal expression of mRNA and protein. c-Fos mRNA is induced within minutes after a stimulus, while protein reaches maximum levels after 2 hours (Kovacs, 1998). To overcome this discrepancy, for mRNA determination the rats were sacrificed immediately after stress exposure while for protein

determination the rats were placed back in the home cages and sacrificed 1.5 hour later. Possibly the familiar environment of the home cage suppresses translation of c-Fos mRNA into protein after recurrent exposure (Westenbroek, 2004).
As a consequence there is less AP-1 activation in the mPFC after chronic stress by c-Fos. However ΔFosB, another Fos protein, accumulates after chronic stress because of its long half-life and is also associated with activation of the transcription of AP-1 sites (Chen et al., 1997; McClung et al., 2003). The transient increase of AP-1-mediated transcription by c-Fos in the mPFC shifts to long-term cellular alterations mediated by ΔFosB after chronic stress.

Cyclic estradiol treatment could preserve the neuronal activity of the IL cortex after chronic stress. However, the sensitization of the mPFC after re-exposure could not be attenuated by estradiol when administered in the recovery phase. Moreover, when the stress was continued for 6 weeks and estradiol treatment started after the initiation of the first stress-induced aberrations, cyclic estradiol administration had no effect. Also ΔFosB accumulation, a marker for sustained AP-1 mediated transcriptional changes, was not affected by cyclic estradiol treatment.
Therefore, the putative protective effect of estradiol as shown after 3 weeks stress in preserving the capacity of the IL cortex to respond to a stressor is abolished when stress already has induced aberrations. The positive actions of estradiol in the mPFC have a more preserving than a restoring character. Moreover, the long-term depletion might negatively influence the beneficial effects of estradiol.

Beside markers of transient or sustained AP-1 activation, also pERK1/2 was determined. Phosphorylated ERK1/2 is implicated in neuronal plasticity and survival (Bonni et al., 1999; Sweatt, 2001). A transient increase of pERK1/2 is associated with enhanced activation of CREB and increased transcription of pro-survival genes (Wu et al., 2001); however, persistent activation can lead to inhibition of CREB-mediated gene expression (Wang et al., 2003) and is associated with pathological mechanisms (Colucci-D'Amato et al., 2003). Neuronal pERK1/2 expression in the mPFC was increased after chronic stress. However, cyclic estradiol administration prevented the stress-induced pERK1/2 accumulation, which may increase the neuronal viability and enhance mPFC functioning under stressful conditions.
The phosphorylation of ERK1/2 by estradiol is thought to be mediated by a putative membrane bound receptor, mER-X (Kuroki et al., 2000; Toran-Allerand, 2004). This receptor is not detected by the currently available antibodies and remains still undetermined. Nuclear ER expression (ERα or ERβ) is sparsely detected in the mPFC (Shughrue et al., 1997). Possibly the lack of transcriptional receptors in the mPFC explains the moderate effects of estradiol in this brain area detected so far. Additionally, estradiol may mediate its effects through mER-X in the mPFC.

As demonstrated in the current and previous studies, chronic stress can induce several aberrations in the mPFC. In addition, patients suffering from affective disorders display dysfunction of the mPFC, established by decreased number and size of neurons and glia and disturbed cerebral blood flow and metabolism (Drevets et al., 1997; Ongur et al., 1998; Galynker et al., 1998; Rajkowska, 2000; Drevets, 2000; Lanius et al., 2001). Moreover, the PFC of suicide subjects shows a reduction in BDNF and TrkB expression, indicating reduced neuronal plasticity (Dwivedi et al., 2003). Dysfunction of the mPFC is one of the pathological aspects in affective disorders that may disarrange adequate processing of emotional stimuli. A dysfunctional mPFC in intact cyclic female rat has several consequences on stress processing. Female rats with an mPFC lesion show a reduction in the onset of appearance of stress-induced behavioral changes. Where unlesioned female rats show increased total distance moved, increased distance moved in the inner zone of the open field and decreased time spent in the area of a familiar object after 3 weeks of stress, rats with an mPFC lesion display these changes already after 2 weeks of stress. Moreover, the regulation of the catecholamine response to chronic stress was blunted in lesioned rats; plasma noradrenaline and adrenaline concentrations were elevated at least up to 2 hours after the latest stressor. The stress-induced activation of the PVN and DMH, brain areas controlled by the mPFC, was more enhanced in rats with an mPFC lesion.
Altogether, a very small lesion of the PL cortex severely exacerbates chronic stress processing. These data were achieved in non-synchronized cyclic rats. It is possible that if this experiment was reproduced in ovariectomized and cyclic estradiol-treated rats, the negative effects of an mPFC lesion would have been more pronounced in the ovariectomized rats than in the estradiol-treated rats. Nevertheless, a vicious circle can be proposed: stress affects mPFC functioning and thereby subsequent stress processing is affected. Estradiol may increase the neuronal viability of the mPFC by preventing pERK1/2 accumulation and therefore postpone the deleterious effects of chronic stress (Figure 4).

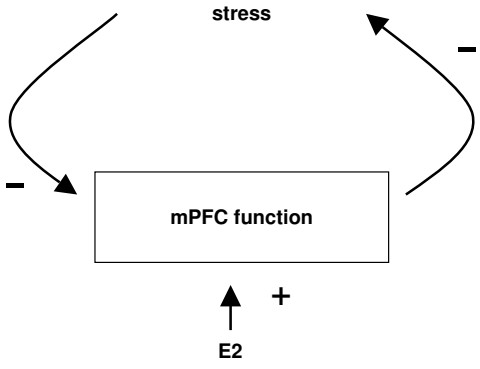

Figure 4: Stress negatively affects mPFC functioning and thereby subsequent stress- processing can be disturbed. Cyclic estradiol treatment may enhance mPFC function and prevent (postpone) the deleterious effects of stress.

Remarks concerning the experimental setup

The studies described in the current thesis were all performed in the dark phase of the rats. That was on purpose. The thesis included a behavioral study and we are convinced to see more of the rat's natural behavior, as far as possible in a laboratory set-up, when this is done in the active period of the circadian cycle. However, many other studies ignored the nocturnal character of rodents and performed behavioral studies in the light phase, although under dimmed-light conditions.

The stress in our paradigm is also applied in the dark phase. However, it is known from literature that a stressor in the light phase is more effective. Baseline levels of corticosterone and c-Fos expression during rest are very low; therefore, the relative increase is much higher. Moreover, stress during the light period also includes sleep disturbance, especially in a paradigm where the stress is applied for a couple of hours each day. We tried to imitate the clinical situation as much as possible in our experimental set-up. In human, stressful events are mostly received during the day. However, the perception of stress in human can also be at night. Traumatic events can be re-experienced, and patients may be awake during the night concerned about future prospects. However, these kinds of mechanisms are, if they exist, very difficult to measure in rat.

Another drawback of these studies is the fact that the mRNA and protein data are not obtained from the same animals. Several studies revealed that pre- or postnatal stress can affect the stress reactivity in later life (McCormick et al., 1995; Meaney et al., 1996; Matsumoto et al., 2005). Breeding conditions, a factor difficult to control when rats are ordered from a commercial company, can have been slightly different between the batches of rats, affecting the outcomes of the stress experiments. Immunohistochemistry is a useful qualitative tool for investigating the distribution of protein expression throughout the brain. However, once the material is fixated and sliced there is not much space for techniques revealing mRNA expression. One option would be to split the brain after removal and use one hemisphere for immunohistochemistry and one hemisphere for, for example, real time PCR. This would reduce the number of animals, would save a lot of time and will increase the amount of information within each experiment. However, this combination of techniques is not optimized yet. Another possibility would be to combine real time PCR with western blotting on punches of specific brain areas giving a more quantitative measure of protein and mRNA expression, however, punching has a much lower spatial resolution compared to microscopy unless you use very advanced laser micro dissection techniques.

Estrogen receptor subtypes

Apart from the brain, ERs are located all over the body. ERs are detected in the reproductive organs such as the mammary glands, the ovaries, the vagina, and the uterus but also the bone, the cardiovascular system, the kidneys, the immune system, and the liver are potent targets for estrogens (Diel, 2002). ERα and ERβ can have agonistic or antagonistic

functions depending on the tissue. Considering the wide distribution of ERs and the differential pattern of actions of estradiol, administration of estradiol can induce a number of unwanted effects, such as endometrical and breast cancer. The use of specific estrogen receptor modulators (SERMs) that act locally may reduce the side effects.
The biological activity of a SERM is determined by three factors; first the conformation of the SERM-receptor complex, second the ER-coupled-signaling mechanism and third the tissue co-regulator composition (McDonnell, 1999; Sandberg, 2002). Co-activators and co-repressors mediate the contact between the receptor and the transcriptional apparatus. In detail, co-activators act as bridging molecules for interactions with the transcription machinery. Moreover, they help unravel target regulatory regions and increase accessibility to these areas, and they mediate cross talk within the receptor molecule (Diel, 2002).
ERα plays a critical role in reproductive functions (Ogawa et al., 1998a; Hewitt et al., 2003). Female ERα knockout mice are infertile; they are anovulatory, have a disruption in the neuroendocrine regulation of LH secretion, and are insensitive to the uterotrophic actions of estradiol (Hewitt et al., 2003). Moreover, female ERα knockouts were deficient in sexual behavior (Ogawa et al., 1998a). Conversely, ERβ does not seem to be critical for reproductive function; although ERβ knockout females have reduced ovulatory function they remain fertile (Hewitt et al., 2003). Interestingly, female ERβ knockout mice display increased anxiety compared to wild type and ERα knockout mice, while ERα knockouts do not display abnormal anxiety (Krezel et al., 2001; Imwalle et al., 2005). Additionally, a study in ERβ knockout mice reveals the ERβ dependency of estradiol-mediated anxiolytic behavior (Rocha et al., 2005). These data strongly suggest a role of ERβ in mediating the effects of estradiol in the regulation of stress-related pathways. Indeed, female ERβ knockout mice exhibit reduced threshold for the induction of synaptic plasticity in the basolateral amygdala (Krezel et al., 2001), decreased concentrations of 5-HT in the bed nucleus of the stria terminalis, preoptic area and hippocampus and decreased dopamine in the caudate putamen (Imwalle et al., 2005). There is little or no expression of ERβ in the reproductive tissues such as the uterus (Couse et al., 1999), which may indicate that specific modulators of ERβ may emulate effects of estrogens on mood without unwanted side effects.
Phytoestrogens have a higher binding affinity to ERβ than to ERα, although the binding is still weaker than 17β-estradiol (Kuiper et al., 1998). This selectivity may implicate clinical usefulness of phytoestrogens (Ruggiero et al., 2002). A phytoestrogen-rich diet in rats, present via soy, produced marked anxiolytic effects in the elevated plus maze (Lund et al., 2001). Moreover, an extract of *Cimicifugae racemosae rhizoma*, containing phytoestrogens, has been shown to decrease mood-related menopausal symptoms without evidence of peripheral estrogen-like effects (Liske et al., 2002).
Recently two highly specific ER agonists are developed. Propyl pyrazole triol (PPT) is a selective agonist for the ERα subtype of ER, displaying a 410-fold binding selectivity over ERβ, while diarylpropionitrile (DPN) is a potent ERβ agonist with a 70-fold selectivity over ERα. Both PPT and DPN are able to pass the blood-brain-barrier after systemic administration (Harris et al., 2002; Lund et al., 2005). The anxiolytic effect found after estradiol treatment in ovariectomized rats (Rachman et al., 1998; Marcondes et al., 2001;

Bowman et al., 2002) was reproduced by acute administration of DPN or coumestrol (coumestrol is also a ERβ agonist, but less potent than DPN) in the elevated plus maze, open field and several other anxiety-related behavioral paradigms and could be blocked by co-administration of the non-selective ER antagonist tamoxifen (Lund et al., 2005; Walf et al., 2005). Moreover, ERβ or coumestrol administration induced decreased immobility and increased struggling and swimming in the forced swim test, which can be interpreted as an antidepressive effect (Walf et al., 2004). PPT administration on the other hand, was not able to induce anxiolytic or antidepressive behavior (Walf et al., 2004; Lund et al., 2005; Walf et al., 2005).
Since DPN and PPT are rather new compounds, practically no other experiments besides *in vitro* tests and behavioral studies considering the acute effects have been performed until now. Neurobiological examination after long-term DPN or PPT administration in combination with stress will be very helpful in understanding and unravel the roles of the separate ERs in stress processing in future studies.
Although the modulation of ERβ by specific agonists seems promising in reducing anxiolytic or stress-induced behavior without many of the estradiol-induced side effects one should be careful with the extrapolation of experimental data to human. After all, the most remarkable effect of estradiol in the currently used animal model for affective disorders has been found in the PVN and is thought to be ERβ-mediated; however, the human PVN expresses more ERα than ERβ. On the other hand, it is possible that the effect achieved is not solely a PVN-mediated effect but is the sum of actions of other limbic nuclei.
Besides the intriguing role of specific ERβ agonists in the treatment of mood disorders, also co-regulators may contain interesting sites of action for therapeutic intervention in signal transduction cascades linked to the effects of estrogen.

Clinical relevance

Estrogen replacement therapy

As described in the introduction, affective disorders occur more often in women than in men during the reproductive part of the life cycle. Moreover, the perimenopause and the postnatal period are both associated with an increased prevalence of mood problems. During the perimenopausal period the estradiol plasma levels increase and display rigorous, irregular and sudden changes at the same time, and after giving birth the estradiol levels decline severely. Several studies suggest that ERT improves the depressive symptoms (Carranza-Lira et al., 1999; Soares et al., 2001; Halbreich et al., 2001; Friebely et al., 2001). ERT in women suffering from estradiol-related mood symptoms may stabilize the tremendous changes in plasma levels and may therefore exert a beneficial effect. However, the exact mechanism behind ERT is not understood yet.
The current study investigated the effects of physiological levels of estradiol administered in a cyclic manner compared to estradiol-deficient rats on stress-related neurobiological outcomes. Cyclic estradiol administration was able to attenuate the excessive PVN activa-

tion, preserves responsiveness of the prefrontal cortex and reduces stress-induced elevation of prefrontocortical pERK1/2 expression after stress. Mitigation of stress-induced activity in the PVN is suggested to be ERβ-mediated. However, the distribution pattern of ERs in this brain area is specie dependent; the human PVN expresses substantial levels of ERα but practically no ERβ (Osterlund et al., 2000), which makes the translation to the human situation complex as pointed out in the previous section. Moreover, ERT has some serious adverse effect on tumor growth and cardiovascular parameters, which makes the administration of estrogens discussable.
Women on ERT receive estrogens daily resulting in invariable estradiol concentrations. As explained in the Introduction invariable plasma levels are likely to induce ER down-regulation and consequently reduce the modulation of EREs limiting the possibility for estradiol to exert its actions. A potential improvement of the currently used ERT may be the development of an administration paradigm with intermitting and lower doses.

Polymorphisms and splice variants of ER in estradiol responsiveness

A selective group of women seems to be extremely sensitive to estrogens. It has been reported that women with a history of postpartum depression (PPD) have a differential sensitivity to changing levels of gonadal steroids (Bloch et al., 2000). Moreover, women with premenstrual syndrome (PMS) display destabilization of mood to normal changes in hormone levels (Schmidt et al., 1998). Expression of a differential estrogen receptor can induce deviant reactions downstream and may underlie this increased sensitivity to changing estradiol.
PvuII and XbaI polymorphisms of ERα are characterized by restriction fragment length polymorphism (RFLP) sites in intron 1. Smaller amygdalar volumes have been reported in women with the presence of this restriction site (Den Heijer et al., 2004). One may speculate that the change in amygdale size indicates an association of polymorphisms in the ERα gene and anxiety (Comings et al., 1999; Prichard et al., 2002). However, another study reports that ERα polymorphisms are not associated with the emotional symptoms of postmenopausal depression (Malacara et al., 2004).
Another basis of variability in receptor function and dysfunction are ER splice variants causing differential protein despite an intact gene. Several splice variants of ERβ have been described (reviewed by Nilsson et al., 2001). Some have an extended N-terminus; ERβ-530 has an extension of 45 amino acids, while ERβ-485 is 64 amino acid residues longer than the original ERβ clone. In addition to extensions of the N-terminus, an isoform named ERβ-503 with an in-frame insertion of 18 amino acids in the ligand-binding domain has been reported. The affinity of ERβ-503 for estradiol is reduced and the estradiol-dependent transcriptional activation of a reporter gene is 100- to 1000-fold lower.
ERβcx is identical to ERβ-530 in exons 1-7, but exon 8 is completely different. The last 61 C-terminal amino acids (exon 8) have been replaced by 26 unique amino acid residues. Due to the exchange of the last exon, ERβcx lacks amino acid residues important for ligand binding and those that constitute the core of the AF2 domain. Therefore, ERβcx cannot

bind estradiol and has no capacity to activate transcription of an estradiol-sensitive reporter gene. Surprisingly, in view of its intact DNA-binding domain, ERβcx does not bind to a ERE. Furthermore, ERβcx shows preferential heterodimerization with ERα rather than with ERβ, and thereby inhibits ERα DNA binding (Ogawa et al., 1998b).
Another study described five ERβ isoforms (ERβ1-5). As with ERβcx, neither of the novel C-terminus splice variants (ERβ2-5), can be expected to bind E2 or activate transcription from an ERE-driven reporter, as they all lack amino acids important for ligand binding as well as the core of AF2. In contrast to what was reported for ERβcx, ERβ2 and 3 do bind to EREs (Moore et al., 1998).
Since the interaction between ERα and ERβ is delicately balanced it seems plausible that ER polymorphisms or receptor splice variants play a role in altered tissue reaction to estradiol. Future studies are necessary to investigate this interesting issue further.

Oral contraceptives

Some women using oral contraceptives experience depressive symptoms. This is even one of the main reasons to discontinue the use of oral contraceptives (Sanders et al., 2001). A plausible explanation lies in the fact that the standard oral contraceptive regimen does not mimic the normal menstrual cycle as they expose women to estrogen en progestin on a daily basis for 3 weeks alternated with a hormone-free week. The normal cycle is disturbed and as claimed in this thesis, this may interfere with neurotransmitter systems in the brain, which are securely tuned to physiological levels and changes. Moreover, it is possible that especially women sensitive for estrogens due to a genetic predisposition as proposed above report negative effects on mood after use of oral contraceptives. Indeed, twin studies indicate that the liability to depressive symptoms related to use of oral contraceptives is influenced by genetic factors (Kendler et al., 1988).

Drug development and testing

About 50 years ago it was discovered by serendipity that depressive symptoms could be treated with monoamine oxidase inhibitors (MAOIs) or tricycic antidepressants (TCAs). MAOIs inhibit the enzyme monoamine oxidase, thus prevent the breakdown of serotonin, dopamine and noradrenaline, and thereby increase the availability of monoamines in the synaptic cleft. TCAs exert their antidepressant effect by inhibition of serotonin and noradrenaline reuptake transporters. Besides the TCAs and MAOIs several newly developed selective reuptake inhibitors support today's treatment, however the underlying mechanism remains more or less the same (me-too compounds).
Compared to other severe or life threatening diseases, like infections, heart diseases or cancer for which major improvement in drug development has taken place in the last decades and curative treatments have been developed, the development of new drugs against

affective disorders does not show much progress. The effects of antidepressants are limited, there is a delay in response of about 6 weeks and the drugs have only palliative actions but do not induce complete and permanent remission.
In other areas of medicine, for example heart disease or cancer, treatment development begins by defining the pathological mechanism. Investigators try to identify a potential target through cell biology or epidemiological studies (Insel et al., 2006). The approach in psychiatry is principally based on developing drugs modeled on an existing, insufficient drug without knowledge of the underlying psychopathology (Insel et al., 2006).
Additionally, testing newly identified drugs in animal models is performed in a debatable manner. As described above stress models are widely recognized as valid animal models for affective disorders (Willner, 1997) and drugs can be tested in their efficacy to change these stress reactions. Nevertheless, most studies make use of acute stress responses instead of a recurrent stress paradigm. As we demonstrated in Chapter 4, the reactions to acute stress differ from the reactions to chronic stress. It is described before that recurrent stress may result in a vicious circle leading to severely disturbed neurobiological reaction patterns, which may underlie affective disorders. Therefore, a valid animal model for testing antidepressant drug should embrace a chronic stress paradigm instead of an acute stressor.
In our view the most important mistake scientists have made in the experimental setup for testing antidepressants is that in almost all studies the animals were treated first and subjected to the stress protocol afterwards. In such a design it is impossible to determine the efficacy of antidepressants in restoring the stress-induced changes. The only way to test a drug for the usefulness in the treatment of a certain disease properly is to induce pathology first, like it is done in testing treatments against heart failure, renal failure, cancer and so on. In Chapter 6 of the current thesis we described this new approach in drug testing. We subjected the rats 3 weeks to stress, which induces neurobiological changes in the reactions to subsequent stress. After that period, we started to treat the animals for 21 days and tested the reaction to re-exposure to the stressful environment after this treatment phase. Shocking was the lack of effect of mirtazapine treatment in this study design and it would be of great interest to test the efficacy of other well acknowledged antidepressants, for example citalopram or fluoxetine, in a similar setup.
Major improvement in the research and development of drugs treating affective disorders can be made by identifying molecular or cellular targets important in the psychopathology and by testing potential compounds to reverse pathology in appropriate animal models.

Summary of the main findings

Chronic stress	**Cyclic estradiol administration**
Increases number of c-Fos positive nuclei PVN	Attenuates excessive stress-induced PVN activation
Enhances number of ERβ positive nuclei PVN	Reduction of c-Fos activation through ERβ-mediated mechanisms
Raises number of pERK1/2 positive nuclei in the mPFC	Reduces stress-induced elevation of prefrontocortical pERK1/2 expression
A dysfunctional mPFC in female rats has negative effects on future stress processing	Preservation of IL cortex responsiveness
Re-exposure after recovery induces a strong response in the PVN, DG, mPFC, MeA and CeA	Prevents sensitization in the PVN and CeA