Neuroactive steroids and rat CNS

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Umeå 2004
Neuroactive steroids and rat CNS
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ABSTRACT

Several studies suggest profound effects on mood and cognition by neuroactive steroids. Estrogen alone or in combination with antidepressant drugs affecting the serotonin system has been used to treat mood disorders. On the other hand, progesterone is related to negative effects on mood and memory. A major part of the progesterone effects on the brain can be mediated by its metabolite allopregnanolone, which is also de novo synthesized in the brain, and affects the GABAA receptors. It would be of great importance to find a substance that antagonize allopregnanolone adverse effects.

To investigate how long-term supplementation of estradiol and progesterone, resembling postmenopausal hormone replacement therapy, affects serotonin receptors in different brain areas important for mood and memory functions, we used ovariectomized female rats. After 2 weeks of supplementation with 17β-estradiol alone or in combination with progesterone, or placebo pellets, estradiol alone decreases but estradiol supplemented together with progesterone increases 5HT1A mRNA expression in the hippocampus. Estradiol decreases the 5HT2C receptor gene expression, while estradiol in combination with progesterone increases the 5HT2A mRNA expression in the ventral hippocampus. Thus, estradiol alone has opposite effects compared to the estradiol/progesterone combination. To detect if acute tolerance develops to allopregnanolone, an EEG method was used where male rats by continuous allopregnanolone infusion were kept on anesthesia level of the silent second (SS). After different time intervals (first SS, 30 min or 90 min of anesthesia) several GABAA receptor subunit mRNAs were measured for detecting if changed expression of any GABAA receptor subunits is involved in development of acute tolerance. There is development of acute tolerance to allopregnanolone and brain regions of importance are hippocampus, thalamus and hypothalamus. The GABAA receptor alpha4 subunit in thalamus and alpha2 subunit in the dorsal hippocampus are related to development of acute tolerance. For assessing allopregnanolone behavioral effects, we studied how this neurosteroid affects spatial learning in the Morris water maze task. Allopregnanolone inhibits spatial learning short after the injection and shows a specific behavioral pattern with swimming close to the pool wall. The steroid UC1011 can inhibit the increase in chloride ion uptake induced by allopregnanolone. UC1011 decreases allopregnanolone-induced impairment of spatial learning in the water maze, as well as the specific behavioral swim pattern.

In conclusion, the present work demonstrates that neuroactive steroids affect the 5HT and GABA systems in a brain region specific way. GABAA receptor subunit changes in hippocampus and thalamus are related to acute allopregnanolone tolerance. Allopregnanolone induces cognitive deficits, like spatial learning impairment and UC1011 can inhibit allopregnanolone-induced effects in vitro and in vivo.

Key words: Estradiol, progesterone, HRT, allopregnanolone, UC1011, serotonin receptor, GABAA receptor, mRNA, Morris water maze, silent second, tolerance.
Cover Illustration:
Rat brain hippocampus, Silent second in the electroencephalogram, Allopregnanolone chemical structure and a rat in a “water maze”

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Umeå University, 2004
To Ingvars, Madara, Beate and my parents

Brain: an apparatus with which we think we think

Ambrose Bierce
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ABSTRACT

Several studies suggest profound effects on mood and cognition by neuroactive steroids. Estrogen alone or in combination with antidepressant drugs affecting the serotonin system has been used to treat mood disorders. On the other hand, progesterone is related to negative effects on mood and memory. A major part of the progesterone effects on the brain can be mediated by its metabolite allopregnanolone, which is also de novo synthesized in the brain, and affects the GABAA receptors. It would be of great importance to find a substance that antagonize allopregnanolone adverse effects.

To investigate how long-term supplementation of estradiol and progesterone, resembling postmenopausal hormone replacement therapy, affects serotonin receptors in different brain areas important for mood and memory functions, we used ovariectomized female rats. After 2 weeks of supplementation with 17β-estradiol alone or in combination with progesterone, or placebo pellets, estradiol alone decreases but estradiol supplemented together with progesterone increases 5HT1A mRNA expression in the hippocampus. Estradiol decreases the 5HT2C receptor gene expression, while estradiol in combination with progesterone increases the 5HT2A mRNA expression in the ventral hippocampus. Thus, estradiol alone has opposite effects compared to the estradiol/progesterone combination. To detect if acute tolerance develops to allopregnanolone, an EEG method was used where male rats by continuous allopregnanolone infusion were kept on anesthesia level of the silent second (SS). After different time intervals (first SS, 30 min or 90 min of anesthesia) several GABAA receptor subunit mRNAs were measured for detecting if changed expression of any GABAA receptor subunits is involved in development of acute tolerance. There is development of acute tolerance to allopregnanolone and brain regions of importance are hippocampus, thalamus and hypothalamus. The GABAA receptor alpha4 subunit in thalamus and alpha2 subunit in the dorsal hippocampus are related to development of acute tolerance. For assessing allopregnanolone behavioral effects, we studied how this neurosteroid affects spatial learning in the Morris water maze task. Allopregnanolone inhibits spatial learning short after the injection and shows a specific behavioral pattern with swimming close to the pool wall. The steroid UC1011 can inhibit the increase in chloride ion uptake induced by allopregnanolone. UC1011 decreases allopregnanolone-induced impairment of spatial learning in the water maze, as well as the specific behavioral swim pattern.

In conclusion, the present work demonstrates that neuroactive steroids affect the 5HT and GABA systems in a brain region specific way. GABAA receptor subunit changes in hippocampus and thalamus are related to acute allopregnanolone tolerance. Allopregnanolone induces cognitive deficits, like spatial learning impairment and UC1011 can inhibit allopregnanolone-induced effects in vitro and in vivo.

Key words: Estradiol, progesterone, HRT, allopregnanolone, UC1011, serotonin receptor, GABAA receptor, mRNA, Morris water maze, silent second, tolerance.
LIST OF ORIGINAL PAPERS

The thesis is based on the following papers that will be referred to by their Roman numerals


II. Birzniece V, Johansson I-M, Wang MD, Bäckström T, Olsson T. Ovarian hormone effects on 5-hydroxytryptamine (2A) and 5-hydroxytryptamine (2C) receptor mRNA expression in the ventral hippocampus and frontal cortex of female rats. Neurosci Lett. 2002 Feb22; 319(3): 157-161.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3βHSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>5HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>5HTP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>AC</td>
<td>anterior commissure</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CA1v</td>
<td>ventral CA1 region</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CB</td>
<td>cerebellum</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CC</td>
<td>corpus callosum</td>
</tr>
<tr>
<td>CM</td>
<td>centromedial thalamic nucleus</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DHP</td>
<td>dihydroprogesterone</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRVL</td>
<td>ventrolateral part of dorsal raphe nucleus</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalography</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma aminobutyric acid</td>
</tr>
<tr>
<td>GABARAP</td>
<td>GABA receptor-associated protein</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>HIP, hipp</td>
<td>hippocampus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>HYP</td>
<td>hypothalamus</td>
</tr>
<tr>
<td>LDVL</td>
<td>ventrolateral part of laterodorsal thalamic nucleus</td>
</tr>
<tr>
<td>LSD</td>
<td>least significant difference</td>
</tr>
</tbody>
</table>
MAPK  mitogen-activated protein kinase
MDR  maintenance dose rate
mIPSC miniature inhibitory postsynaptic currents
MnR median raphe nucleus
MPD medial nucleus of amygdala, posteriodorsal part
MPV medial nucleus of amygdala, posterioventral part
mRNA message ribonucleic acid
NFκB nuclear factor-κB
NGFI nerve growth factor induced gene
NMDA n-methyl-d-aspartate
P450scc P450 side-chain cleavage enzyme
PCA principal component analysis
PET positron emission tomography
PKA protein kinase A
PKC protein kinase C
PLS partial least squares
PMDD premenstrual dysphoric disorder
PMS premenstrual syndrome
PR progesterone receptor
PREGS pregnenolone sulfate
RIA radioimmunoassay
Rma nucleus raphe magnus
RO nucleus raphe obscurus
RPa nucleus raphe pallidus
SEM standard error mean
SERT serotonin transporter
SRC steroid receptor coactivator
SRE serum response element
SS silent second
SSRI selective serotonin reuptake inhibitor
TH thalamus
THP tetrahydroprogesterone
TPH tryptophan hydroxylase
UC1011 3β-20β-dihydroxy-5α-pregnane
VM ventromedial thalamic nucleus
VPM ventral posteromedial thalamic nucleus
VPL ventral posterolateral thalamic nucleus
Ovarian steroids

Estradiol and progesterone (Fig 1) are the major female sex hormones. The precursor of all steroids is cholesterol, which can be obtained from the diet or synthesized de novo. The main steroid synthesis pathways are shown in Figure 2. In the adult women the principal sources of estradiol are the granulose cells of the developing follicle and the corpus luteum. The adrenal gland can produce androstenedione, which can be converted to estrone and estradiol, or to testosterone and then in fat, placenta, endometrium, liver, intestines, skin, muscle and brain to estradiol. Conversion of testosterone to estradiol is mediated by the aromatase cytochrome P450 enzyme. Progesterone is mainly synthesized in granulose cells of the corpus luteum, but also in the placenta, and the adrenals (Speroff, Glass and Kase, 1999). Following synthesis, most estradiol and progesterone are bound to plasma proteins (sex hormone-binding globulin, albumin, transcortin; Speroff et al., 1999). Bound hormone is relatively inactive, although the albumin-bound fraction may also be available for cellular action as this binding has low affinity.

Fig 1. Steroid structures.

Estrogens are required for the normal female phenotype, sexual maturation, female genital function, as well as for skeleton maintenance and are probably protective for the cardiovascular system (Speroff et al., 1999; Riggs et al., 2002;
Baker et al., 2003). Progesterone is a key hormone for conception and pregnancy maintenance. The ovarian steroids have profound effects on brain functions, including regulation of the reproductive neuroendocrine system, mood and cognition, as well as neuroprotective effects on neurons (Speroff et al., 1999; McEwen, 2001; Behl, 2002). Since steroid hormones are lipophilic and have a low molecular weight, estradiol and progesterone readily crosses the blood brain barrier and easily becomes available for their actions on the brain. The brain is also a significant site for progesterone metabolism.

Steroid hormone concentrations in plasma and the brain vary through the menstrual cycle. In women the menstrual cycle is divided into the follicular phase and the luteal phase, with ovulation as a cut-point, and is in average 28 days long (Fig 3). The duration of the estrous cycle in the rat is 4 or 5 days and is divided into proestrus (the time of late follicle growth, estrogen synthesis, and a
preovulatory progesterone peak), estrus (the time of ovulation and mating),
etestrus (the time of corpus luteum formation, if the mating has occurred), and
diestrus (start of new follicle growth). Changes in hormone concentrations during
the estrous cycle in rats and humans are shown in Figure 3.

![Hormone concentrations in peripheral blood of rats during the estrous cycle and human menstrual cycle](image)

**Fig 3.** Hormone concentrations in peripheral blood of rats during the estrous cycle and human menstrual cycle (adapted from Smith et al., 1975 and Speroff et al., 1999).

**Steroid hormone receptors**

Estrogen (ERα and ERβ) and progesterone (PRA and PRB) receptors belong
to a family of transcription factors, the nuclear receptor superfamily (Jensen and
DeSombre, 1972; Walter et al., 1985; Kuiper et al., 1996; Kuiper and Gustafsson,
1997). ERs consists of different domains: N-terminal domain, DNA-binding
domain, hinge region, large ligand binding domain, and C-terminal domain (Ruff
et al., 2000). Steroid hormones diffuse into the cell, bind to their individual
receptors and transformation and activation of the receptors occur. Activation is
dissociation of the receptor-heat shock protein complex (formed with unbound
receptor in order to stabilize, keep inactive and protect the receptor). The hormone-receptor complex dimerize, that is two activated receptors bind to each other. The dimer binds to specific DNA sites, hormone response elements, in the promoter region of target genes. This initiate transcription, subsequently leading to translation, synthesis of new proteins (Speroff et al., 1999). ERα and ERβ are able to form both homo- and heterodimers (Pettersson et al., 1997; Hart, 2002), the same as for PRA and PRB (DeMarzo et al., 1991). Two important classes of interacting proteins, co-activators and co-repressors, have been described. Co-activators (e.g., steroid receptor coactivator 1, CREB binding protein; Fig 4) bind to the receptor itself, and presumably serve as bridges to the general transcription factors. New co-activators have been described, like peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and PGC-1α related estrogen receptor coactivator (PERC), which seems to be important in modulation of ERα transcriptional activity (Tcherepanova et al., 2000; Kressler et al., 2002; Puigserver and Spiegelman, 2003). Co-repressors interact with the receptor in its hinge region and are typically released upon binding of ligand (Weigel, 1996). Many phosphorylation sites in the receptors have been identified and DNA binding and transcriptional activation are substantially modified by phosphorylation. Receptors usually are phosphorylated in the absence of ligand and exhibit increased phosphorylation upon ligand binding (Weigel, 1996). ER can also regulate transcription through binding to AP-1 response element (Paech et al., 1997). Interestingly, in HeLa cells transfected with either ERα or ERβ, estradiol promotes ERα-dependent transcription, whereas it has no effect on ERβ-dependent transcription from an AP1 site (Paech et al., 1997).

Estrogen receptors are distributed in many organs, like uterus, breast, ovaries, bone, lungs, kidney, and also throughout the brain (Kuiper et al., 1997; Shughrue et al., 1997, 1998). In the brain ER is localized predominantly in the limbic system, like amygdala, septum, and also in the hypothalamus, and are involved in emotional processing and cognition (Phillips and Sherwin, 1992; Sherwin and Tulandi, 1996; Shughrue et al., 1997; Osterlund et al., 1998, 2000a, c; Alves et al., 1998). In humans relatively high levels of ERβ are found in the hippocampus, cortex, and claustrum, whereas, in contrast to ERα receptor mRNA, low levels of ERβ transcript are present in hypothalamus and amygdala (Osterlund et al., 2000a). In primates and rodents both ERα and ERβ are found in many brain regions, whereas, in the hippocampus there is a high ERβ/ERα ratio (Shughrue et al., 1997; Register et al., 1998; Alves et al., 1998; Gundlah et al., 2000, 2001).
Progesterone receptors are also distributed in many tissues and brain areas (Perrot-Applanat et al., 1985; Bethea, 1993; Kato et al., 1994; Alves et al., 1998; Bethea and Widmann, 1998; Greco et al., 2001). Interestingly, coexpression of ERα, ERβ, and PR immunoreactivity is found in several brain areas and estradiol supplementation decreases ERs but induces progesterone receptors (Jung-Testas et al., 1992a; Alves et al., 1998; Petz and Nardulli, 2000; Greco et al., 2001).

Steroid hormone action in the cell could be direct genomic, indirect genomic or non-genomic (Lee and McEwen, 2001). As is shown in Figure 4, the direct genomic mechanism is via steroid hormone receptors that binds to the response element on the target gene, and indirect genomic – via steroid hormone receptor activation linked to second messenger systems. An example of a non-genomic effect can be estrogen stabilization of the mitochondrial membranes and reduction of the generation of free oxygen radicals, therefore having a neuroprotective effect (Mattson et al., 1997; Wang et al., 2001; Wise, 2002). The action of steroid hormones could also be through neurotransmitter systems (including the serotonin (5HT) and GABA systems; McEwen, 2001). Estradiol is excitatory and has modulatory effects via the glutamate system, increasing brain excitability and also synaptic spine density in the hippocampus. Estradiol induces NMDA receptor (NR1) expression in the CA1 region of the hippocampus and NMDA receptor antagonists block estrogen-induced synaptogenesis on dendritic spines (Woolley and McEwen, 1994; Gazzaley et al., 1996; Adams et al., 2001). Estrogen also decreases seizure threshold and direct application of estrogen to the brain causes epileptic focus and induces seizures (see in Morrell, 1999; Backstrom et al., 2003). On the other hand, progesterone exerts inhibitory action on the CNS, an effect thought to be mediated through its major metabolite, allopregnanolone (3alpha-hydroxy-5alpha-pregnan-20-one; Fig 9).
Fig 4. Estrogen and progesterone action in the cell. The direct genomic mechanism of estrogen involves estrogen-ER dimer complex association with estrogen response element (ERE) or with fos/jun heterodimers bound to activator protein 1 (AP1). Indirect genomic mechanism involves activation of ER linked to different second messenger systems (protein kinase A or C, mitogen-activated protein kinase (MAPK), extracellular signal regulated kinase (ERK), cAMP response element binding protein (CREB), nuclear factor-κB, etc). Similarly, progesterone has direct genomic (via progesterone receptors, coupled to co-activators – steroid receptor coactivator (SRC), CREB binding protein (CBP)), and indirect genomic (via G protein or through activation of GABAA receptor) mechanism of action.
Neurosteroids

Neurosteroids (a term introduced by Baulieu in 1981) are steroids synthesized in the central and peripheral nervous system, in myelinating glial cells, astrocytes and neurons (see in Baulieu and Robel, 1990; Compagnone and Mellon, 2000). The precursor cholesterol can be supplied by peripheral source, by biosynthesis, or can in many cells of the nervous system be derived from low density lipoproteins (Hu et al., 1989; Jung-Testas et al., 1992b; Jurevics and Morell, 1995). The cytochrome P450 side-chain cleavage enzyme (P450scc) is involved in the conversion of cholesterol to pregnenolone (Le Goascogne et al., 1987). Pregnenolone can be oxidized to progesterone by the 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD), Figure 2. 3βHSD mRNA expression has been determined in the adult rat brain by in situ hybridization, and type I and II are the major isoforms in the brain (Guennoun et al., 1995; Kohchi et al., 1998). Estradiol can also be classified as a neurosteroid. It can be synthesized de novo or converted from testosterone in the brain by aromatase P450, since the aromatase inhibitor formestane decreases the estradiol concentration in cortex and in the hippocampus of female rats (Amateau et al., 2004). In addition, enzymes needed for estradiol synthesis, P45017alpha and P450 aromatase, are localized in hippocampal neurons - in pyramidal cells of the CA1-CA3 regions, as well as in the granule cells of the dental gyrus (Hojo et al., 2004).

The enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase that are needed for the production of allopregnanolone from progesterone are in neurons and glial cells present in many areas of the brain, notably in cortex and the hippocampus (Compagnone and Mellon, 2000). Allopregnanolone is a neurosteroid that accumulates in the brain and increases in plasma during the luteal phase of the human ovarian cycle (Wang et al., 1996; Bixo et al., 1997; Genazzani et al., 1998). In plasma from fertile women the level of allopregnanolone is approximately 1 nM in the follicular phase and 4 nM in the luteal phase of the menstrual cycle, reaching the highest levels during the third trimester of pregnancy (more then 100 nM) (Purdy et al., 1990; Schmidt et al., 1994; Bicikova et al., 1995; Wang et al., 1996; Genazzani et al., 1998; Luisi et al., 2000). Allopregnanolone and pregnanolone have similar effects in the brain and in women pregnanolone plasma concentrations of 80-160 nM causes sedation, while concentrations of 530-700 nM is found during anesthesia (Carl et al., 1990; Sundstrom et al., 1999b). In women post-mortem brain levels of allopregnanolone are 30-130 nmol/kg (Bixo et al., 1997). In female rats
allopregnanolone plasma levels are around 9 nM but in male plasma the concentration is almost undetectable (Corpechot et al., 1993). In the brain there are variation in concentration between brain regions, i.e., in the cortex 13 nmol/kg in proestrus and 19 nmol/kg in estrus phase of the cycle has been detected, reaching approximately 38 nmol/kg of allopregnanolone in pregnancy and around 7.5 nmol/kg in male rat cortex (Purdy et al., 1991; Paul and Purdy, 1992). Plasma and brain concentrations of allopregnanolone also increase during stress (approximately up to 130 nM or 30 ng/g protein 30 min after foot shock stress in rodents; Barbaccia et al., 1997).

Allopregnanolone acts as a positive modulator of the GABAA receptor, similar to the action of benzodiazepines (Majewska et al., 1986; Gee et al., 1987). This enhancement of GABA mediated Cl− current results in inhibitory effects on neuronal functions. Systemic administration of progesterone or its metabolites, like allopregnanolone induces anticonvulsant, hypnotic and anxiolytic effects (Landgren et al., 1987; Norberg et al., 1987; Brot et al., 1997; Czlonkowska et al., 2001; Zhu et al., 2001). Interestingly, in rats activation of mitochondrial benzodiazepine receptors (which activation promotes the movement of cholesterol from the outer to the inner mitochondrial membrane, thereby increasing substrate availability to the cytochrome P450scc) in the hippocampus stimulates allopregnanolone synthesis and produces an anxiolytic effect, measured in the elevated plus maze (Bitran et al., 2000). Benzodiazepines have many side effects, from drowsiness, poor concentration, ataxia, motor incoordination, muscle weakness, to memory impairment (Longo and Johnson, 2000). Because of the similarities with benzodiazepines, allopregnanolone could also have similar adverse effects on the brain, including cognitive function decline and memory impairment (Sandstrom et al., 1999a; Holbrook et al., 2000). Interestingly, tolerance to several benzodiazepine, allopregnanolone mediated effects has been shown (Czlonkowska et al., 2001; Bateson, 2002; Palmer et al., 2002). But the mechanisms behind the tolerance are not clear, so it would be of great importance to obtain information concerning which brain regions or which GABAA receptor subunits that are involved in tolerance development.

The allopregnanolone 3β-hydroxy isomer isoallopregnanolone (3β-hydroxy-5α-pregn-20-one, Fig 9) is synthesized from 5α-dihydroprogesterone by 3β-hydroxysteroid dehydrogenase, which is present in the brain. It is thought, that 3β-hydroxyprogynane steroids are not active by itself at the GABAA receptor (Weir et al., 2004), but in vitro antagonism against potentiation of GABAA
receptor function by other neurosteroids have been shown. Thus, allopregnanolone and pregnanolone (3α-hydroxy-5β-pregnan-20-one) potentiates [3H]flunitrazepam binding at the GABAA receptor, whereas isoallopregnanolone and isopregnanolone (3β-hydroxy-5β-pregnan-20-one) do not produce a significant change in [3H]flunitrazepam binding themselves, but antagonize the potentiation of their 3α-hydroxy isomers (Prince and Simmonds, 1992, 1993). Moreover, 3β-hydroxyprogesterone steroids antagonize their 3α-hydroxy isomer induced enhancement of the GABA-mediated Cl⁻ currents (Maitra and Reynolds, 1998) and block inhibition of the population spike in the CA1 subregion of rat hippocampus (Wang et al., 2000). In addition, Lundgren et al. in 2003 showed that isoallopregnanolone selectively inhibits allopregnanolone induced Cl⁻ uptake, not affecting baseline Cl⁻ uptake in cortical homogenates from adult male rats (Lundgren et al., 2003). Nevertheless, all those studies show in vitro 3β-hydroxyprogesterone steroid antagonizing effects, in vivo data are missing.

There are many more neurosteroids present, and with different actions on neurotransmitter systems. For instance, pregnenolone sulfate (PREGS) act as an inhibitor of the GABAA receptor, and also potentiate the NMDA receptor (Wu et al., 1991). DHEAS (dehydroepiandrosterone sulfate) may act as a sigma receptor agonist, whereas progesterone behaves as an antagonist of this receptor (Monnet et al., 1995; Ueda et al., 2001). However, discussion in details on all neurosteroid action on the brain is beyond the scope of this thesis.

**Steroids and CNS**

**Ovarian hormone effects on mood and anxiety**

Several studies suggest gender differences in mood and memory, and major depression is more common among females (Burns et al., 2001). In a study by Nishizawa et al., women had a lower rate of brain serotonin (5HT) synthesis than men (analyzed by PET) and following acute tryptophan depletion (the substrate for serotonin synthesis) the reduction in serotonin synthesis was four times higher than it was in men (Nishizawa et al., 1997). In some studies estradiol alone or estradiol in combination with antidepressant drugs that affect the serotonergic system has been used as antidepressants (Schneider et al., 1997; Zweifel and O'Brien, 1997; Schmidt et al., 2000; Westlund Tam and Parry, 2003). Estrogen treatment also improves well-being and cognitive functions in postmenopausal
women (Rebar et al., 2000; Miller et al., 2002). However, with the combination of estrogen and progesterone the positive effect on mood and well-being disappears (Hays et al., 2003). In addition, in certain sensitive women use of hormone replacement therapy is well known to cause negative mood changes when the progesterone derivates are added (Hammarback et al., 1985; Magos et al., 1986; Bjorn et al., 2000). Ovarian hormones are not only involved in major depression but also in related disorders like premenstrual dysphoric disorder (PMDD), postnatal and postmenopausal depression. In PMDD the symptoms, like depressed mood, anxiety, lability, irritability, difficulty in concentrating, eating and sleeping disturbances, occur during the luteal phase (when progesterone and allopregnanolone are high), and only in ovulatory menstrual cycles when corpus luteum is present (Hammarback et al., 1991; Halbreich, 2003). Maximum severity of symptoms occurs during the last 5 days of the menstrual cycle and the first 2 days of next cycle, but variation in time can be seen (see in Backstrom et al., 2003; Freeman, 2003; Halbreich, 2003). Thus, progesterone might at least partly be responsible for these negative mood changes. Interestingly, treatment with mifepristone, a progesterone receptor antagonist, is not alleviating premenstrual symptoms (Chan et al., 1994). Therefore, non-genomic effects of progesterone or its metabolite allopregnanolone can be involved in the pathophysiology of premenstrual mood changes.

However, a higher estrogen dose in HRT increases negative mood when applied together with progestogen, but not when estrogen is used alone (Bjorn et al., 2003). In addition, in PMS patients negative mood symptoms during the luteal phase are more severe in cycles with high estradiol levels than in cycles with lower estradiol (Bjorn et al., 2003). So it seems, that estradiol alone has beneficial effect on mood but when used together with progesterone, negative effects appear.

Thus, ovarian steroids can play important roles in the modulation of anxiety, mood and cognition, but the mechanisms behind these effects are not clear. It is thought that the major estrogen effect on the brain is mediated via neurotransmitter actions. Since estrogen, progesterone and allopregnanolone are important for mood and the serotonergic system is highly involved in the pathogenesis of depression (the most frequently used drugs for treatment are SSRIs, selective serotonin reuptake inhibitors), my focus in this section mainly will be on the serotonergic system.
The serotonergic system

In serotonin biosynthesis L-tryptophan is converted to 5-hydroxytryptophan (5HTP) by tryptophan hydroxylase, found in most tissues, including the brain. 5HTP is almost immediately decarboxylated to serotonin and the enzyme responsible for this conversion is aromatic L-amino acid decarboxylase. The serotonin transporter (SERT) is responsible for reuptake of 5HT into cells. 5HT can be stored in the cytoplasm, transported to vesicles, or degraded by monoamine oxidase.

Fig 5. (A) Serotonergic system pathways of the human brain. (AC, anterior commissure; CB, cerebellum; CC, corpus callosum, HIP, hippocampus; HYP, hypothalamus; RD, dorsal raphe nucleus; RMa, nucleus raphe magnus; RMe, median raphe nucleus; RO, nucleus raphe obscurus; RPa, nucleus raphe pallidus; RPo, nucleus raphe pontis; TH, thalamus). (B) Serotonin receptor subtypes and their main effects.

Serotonergic neurons are localized in the raphe nuclei in mesencephalon and medulla oblongata. There are two main serotonergic pathways: ascending (from
median and dorsal raphe nuclei to frontal cortex, striatum, thalamus, amygdala, hypothalamus and hippocampus) and descending (from caudal raphe nucleus to the spinal cord), Fig 5A. The serotonin system is involved in a wide variety of complex physiological and behavioral functions such as mood, affect, learning, memory, sexual behavior, aggression, stress responses, sleeping, thermoregulation and eating. Abnormal serotonergic neurotransmission in various brain regions is thought to be one of the factors in development of depression and anxiety disorders. For over 30 years, the leading theory to explain the biological basis of depression has been the “monoamine hypothesis of depression”. This theory proposes that the biological basis of depression is a deficiency in one or more of the three key neurotransmitter systems, which are thought to mediate the therapeutic actions of virtually every known antidepressant agent. The important neurotransmitters are norepinephrine, dopamine and 5HT. The development and introduction of SSRIs, including fluoxetine, sertraline, paroxetine, fluvoxamine, and citalopram, represent an important advance in the pharmacotherapy of psychiatric disorders. SSRIs are not only used in depression, but also in a wide range of psychiatric disorders, e.g., panic disorder, obsessive compulsive disorder, eating disorders (Goodnick and Goldstein, 1998; Masand and Gupta, 1999; Vaswani et al., 2003). SSRIs are in women especially effective in pathologies like PMDD, postnatal depression, and perimenopausal depression (Goodnick et al., 2000).

SSRIs inhibit the reuptake of serotonin into the presynaptic nerve terminal, thus increasing the 5HT concentration in the synaptic cleft, prolonging its activity at postsynaptic receptor sites. After 2 to 3 weeks of treatment with SSRIs, desensitization of presynaptic 5HT1A receptors occurs. Since activation of those somatodendritic autoreceptors results in decreased firing activity along the serotonergic axon, decreased sensitivity of the receptor will result in enhancement of serotonin neurotransmission (see Chaput et al., 1986; Elena Castro et al., 2003; Hensler, 2003).

**Serotonin receptors**

There are at least 18 serotonin receptor subtypes present in the brain (Fig 5B), having different functions and different brain localizations (Barnes and Sharp, 1999). Most of the 5HT receptors are metabotropic (except the 5HT3 receptor), affecting G-protein-stimulated adenylyl cyclase or phospholipase activity,
depending on receptor subtype. Focus here will be on the 5HT1A, 2A and 2C receptors, since those are the major serotonin receptor subunits involved in regulation of mood and anxiety (Palvimaki et al., 1996; Blier et al., 1997; Toth, 2003; Van Oekelen et al., 2003).

5HT1A receptors are coupled via pertussis toxin-sensitive G proteins to the inhibition of adenylyl cyclase, or to the opening of potassium channels, so that activation of the 5HT1A receptor in the dorsal raphe opens potassium channels and inhibits cell firing (see Hensler, 2003). It has been shown, that activation of 5HT1A receptors on dorsal raphe neurons also directly inhibits voltage-dependent calcium currents (Penington and Kelly, 1990; Chen and Penington, 1996). 5HT2A and 5HT2C receptors are positively coupled through G-proteins to phospholipase C and phospholipase A2, and activation of these receptors leads to increased accumulation of inositol phosphates and intracellular Ca\(^{2+}\), causing cell excitation (Van Oekelen et al., 2003).

The 5HT1A receptor is in high density present in serotonergic cell body areas, in particular the dorsal and median raphe nuclei, as somatodendritic autoreceptors located on the serotonergic cell bodies and dendrites. In cortical and limbic areas (e.g. frontal cortex, entorhinal cortex, hippocampus, amygdala, septum) 5HT1A is present as postsynaptic receptors (Kia et al., 1996). 5HT1A receptors are also present in the hypothalamus, where they play an important role in the regulation of neuroendocrine function and responses to stress (see Van de Kar, 1991). The 5HT2A receptor is enriched in many brain areas including the frontal cortex, nucleus accumbens, striatum, ventral hippocampus, and amygdala (Cornea-Hebert et al., 1999). 5HT2C receptor expression is observed in the choroid plexus of all brain ventricles, pyriform cortex, amygdala, thalamic nuclei, hippocampus, and substantia nigra (Mengod et al., 1990).

Interestingly, 5HT1A receptor agonists and 5HT2A and/or 5HT2C receptor antagonists have antidepressant properties (Palvimaki et al., 1996; Blier et al., 1997). It has also been suggested that the 5HT7 receptor is involved in pathologies like anxiety, cognitive disturbances and migraine and some of 5HT2C receptor antagonists has also high affinity for 5HT7 receptors (Ruat et al., 1993; Garraway and Hochman, 2001; Thomas and Hagan, 2004). Administration of pindolol, a 5HT1A and beta-adrenoreceptor antagonist, accelerate the action of SSRIs by shortening the onset of the antidepressant effect (Artigas et al., 2001; Ballesteros and Callado, 2004). The 5HT1B receptor is a terminal autoreceptor, which stimulation inhibits the release of 5HT in different brain areas (Hjorth and Tao,
1991; Adell et al., 2001), and the increase of 5HT levels with systemic SSRIs is augmented by 5HT1B receptor antagonists (de Groot et al., 2003). 5HT2A and 5HT2C receptor subtypes are targets for antipsychotic drugs (Van Oekelen et al., 2003). These receptors have an atypical down-regulation after both agonist and antagonist treatment. Thus, chronic administration of antidepressants with 5HT2A or 5HT2C receptor antagonistic properties induced down-regulation of receptors in the brain (Van Oekelen et al., 2003). Regulation of the 5HT1A receptor is more complex, depending on applied substance, time of exposure and brain region.

**Ovarian steroid effect on the serotonin system**

In some experimental studies estradiol increases and progesterone has no effect on serotonin content in the brain, while the combination of both hormones decreases serotonin compared with estradiol alone (Di Paolo et al., 1986; Renner et al., 1987; Fabre-Nys et al., 1994). However, in the hypothalamus of guinea pigs, serotonin increased by estrogen in combination with progesterone, but not by estrogen alone (Lu et al., 1999). Brain serotonin levels are influenced by estrous cycle phase (Gundlah et al., 1998; Maswood et al., 1999). Tryptophan hydroxylase (TPH) protein and mRNA expression increases in the dorsal raphe nucleus after estradiol treatment in ovariectomized monkeys, but no effect on this enzyme mRNA is found in rats (Pecins-Thompson et al., 1996; Alves et al., 1998; Shively et al., 2003). Interestingly, in monkeys TPH remains elevated when natural progesterone is added to estrogen, but is reduced to levels of ovariectomized animals when medroxyprogesterone acetate is added (Bethea et al., 2000). Results of estradiol effects on the serotonin reuptake transporter (SERT) have been contradictory. In ovariectomized rats, acute estrogen treatment increases SERT protein and mRNA in the dorsal raphe nucleus, as well as the density of SERT binding sites in the amygdala, hypothalamus, thalamus, and septum, but decreases in the hippocampus (Mendelson et al., 1993; McQueen et al., 1997; Sumner et al., 1999). Estrogen supplementation for 2 days increases SERT binding in basolateral amygdala, ventromedial hypothalamus and hippocampus (Krajnak et al., 2003). Chronic estrogen treatment decreases SERT mRNA in the rat midbrain, whereas its down-regulation in hypothalamus by ovariectomy was reversed by estrogen, progesterone, or their combination (Attali et al., 1997; Zhou et al., 2002b). In primates, no change or a decrease of SERT
mRNA in the raphe nucleus, and an increase in hypothalamus has been detected after chronic estradiol alone and in combination with progesterone (Pecins-Thompson et al., 1998; Bethea et al., 2002b; Lu et al., 2003).

It is possible, that mediators of estradiol and/or progesterone effects on the serotonin system (and by that on mood) may be some of the 5HT receptor subtypes. The effect of estradiol on serotonin receptor expression depends on receptor subtype, brain area, strain and duration of treatment. Thus, in monkeys a decrease or no change of presynaptic and a decrease of hypothalamic 5HT1A receptor mRNA following chronic estradiol treatment was reported (Pecins-Thompson and Bethea, 1999; Lu and Bethea, 2002; Bethea et al., 2002b). Short-term administration of estradiol does not seem to affect 5HT1A gene expression or binding in the rat hippocampus (Clarke and Maayani, 1990; Sumner and Fink, 1993; Frankfurt et al., 1994; Österlund and Hurd, 1998). In a study by Österlund et al, two weeks of estradiol administration in rats decreased 5HT1A receptor binding in several hippocampal subregions, amygdala and cortex with unaltered 5HT1A receptor mRNA levels (Österlund et al., 2000b). However, recent study showed no change in 5HT1A receptor mRNA or binding in hippocampus, dorsal raphe, prefrontal and cingulate cortex by 2-week estrogen supplementation (Landry and Di Paolo, 2003). 5HT1 receptor densities also fluctuate during the estrous cycle, with lower receptor density in proestrus than in diestrus (Biegon et al., 1980).

Short-term estradiol treatment does not influence 5HT2A receptor mRNA levels in the dorsal hippocampus (Sumner and Fink, 1993), whereas treatment for 2 weeks with estradiol increase the 5HT2A receptor density in the rodent cerebral cortex (Biegon et al., 1983). In another study by Cyr et al., ovariectomy for 3 months decreased 5HT2A receptor mRNA and receptor binding in the frontal cortex, whereas estradiol supplementation for 2 weeks restored the receptor expression to control levels (Cyr et al., 1998). However, in the same study 2 weeks of estradiol supplementation in Sprague–Dawley rats did not influence 5HT2A receptor mRNA levels in the frontal cortex versus ovariectomized controls (Cyr et al., 1998). Furthermore, Sumner and Fink found increased levels of 5HT2A receptor density in frontal cortex after short-term (32 h) estrogen supplementation, but no changes were found in receptor mRNA levels in this brain region (Summer and Fink, 1995; Sumner and Fink, 1998). After short-term treatment an increase in 5HT2A receptor mRNA is detected in the dorsal raphe nucleus (Sumner and Fink, 1998), amygdala, hippocampus, nucleus accumbens,
and cortex (Osterlund et al., 1999). In a study by Gundlah et al. (1999) long-term estradiol was found to decrease the 5HT2C receptor mRNA signal in the hypothalamus (Gundlah et al., 1999). In rats estrogen supplementation for 3 weeks increases 5HT2C receptor mRNA in midbrain and hypothalamus (Zhou et al., 2002a).

Concerning the effects of combined estradiol and progesterone treatment on serotonin receptors, chronic treatment decreases 5HT1 receptor binding in the cerebral cortex in rats (Biegon et al., 1983), and 5HT1A receptor mRNA in the dorsal raphe nucleus and hypothalamus of monkeys (Pecins-Thompson and Bethea, 1999; Lu and Bethea, 2002). However, acute combined treatment has no effect on 5HT1A receptor binding in the hypothalamus and hippocampus (Frankfurt et al., 1994). Concerning 5HT2A receptors in humans, by the use of PET, an increase in cortex has been detected after long-term estrogen or combined estrogen and progesterone treatment (Moses et al., 2000; Kugaya et al., 2003; Moses-Kolko et al., 2003).

In summary, all these data indicates complexity of steroid hormone effects on the serotonin system. However, there are no clear data in the literature available on treatment resembling the most common HRT used in postmenopausal women with climacteric symptoms, namely the continuous combined treatment. Therefore a study on the effect of chronic estradiol alone versus estradiol in combination with progesterone on different serotonin receptor subtypes in different brain areas (which are involved in regulation of mood) is of interest. Thus, it is still of a great importance to collect more information in order to better explain through which receptors ovarian hormones affect mood and cognition.

**Steroids and serotonin - GABA system interaction**

Progesterone metabolites, like allopregnanolone, are also involved in the regulation of cognitive functions. Thus, mood changes during the menstrual cycle, postpartum, major depression, and epilepsy are pathologies associated directly or indirectly with allopregnanolone (Bicikova et al., 1998; McCoy et al., 2003; van Broekhoven and Verkes, 2003). The GABAergic system is involved in major depression, with decreased activity of enzymes needed for GABA synthesis and GABA levels in the brain (Brambilla et al., 2003). Especially occipital cortex is affected and after SSRI treatment there is an increased GABA levels in this brain region (Sanacora et al., 1999, 2002; Bhagwagar et al., 2004).
Thus, the GABA system is highly involved in pathologies like major depression. In line with this is also the decreased cerebrospinal fluid allopregnanolone concentration found in major depression, which increases in the brain (olfactory bulb, striatum, hippocampus and frontal cortex) after acute and chronic treatment with SSRIs (Uzunov et al., 1996; Uzunova et al., 1998). The mechanism by which SSRIs enhance allopregnanolone levels is thought to involve direct stimulation of 3α-hydroxysteroid dehydrogenase (Griffin and Mellon, 1999) and administration of indomethacin, an inhibitor of this enzyme, decreases the antidepressant like effect of allopregnanolone in the presence of SSRIs, measured as a decrease of immobility in the forced swim test (Khisti and Chopde, 2000).

Women with PMDD have decreased sensitivity towards GABAA receptor active substances, especially during the luteal phase (Sundstrom et al., 1998). The serotonin system is also involved, as serotonin reuptake inhibitors are effective in treatment of PMDD, the therapeutic effect is quickly achieved and even intermittent administration in the luteal phase is effective (Dimmock et al., 2000; Cohen et al., 2002; Halbreich, 2003). As a result of SSRI treatment of PMDD patients the decreased sensitivity to pregnanolone normalize, suggesting that the SSRI effect in PMDD is mediated via normalization of the developed tolerance to neurosteroids during the luteal phase (Sundstrom and Backstrom, 1998). A tolerance towards the SSRI effect in PMDD patients is noticed at continuous, but not at intermittent treatment (Wikander et al., 1998). In addition, SSRIs can be used in the treatment of epilepsy and of depression related to epilepsy (Prendiville and Gale, 1993; Favale et al., 1995).

It has been suggested that treatment with SSRIs increases inhibitory processes in brain limbic structures that are involved in regulation of emotional processes, due to hyperpolarization of neuronal membranes, probably with GABAB receptor involvement (Beck et al., 1997). SSRIs influences GABAA receptor function, thus low fluoxetine concentrations (1 nM) enhance GABA-stimulated Cl⁻ uptake in a rat cerebral cortical vesicular preparation. Whereas higher concentrations (0.1 - 1 mM) inhibit Cl⁻ uptake and concentrations above 10 μM also inhibit the binding of [3H]GABA and [3H]flunitrazepam to the GABAA receptor complex in brain cortical membranes (Tunnicliff et al., 1999). It is of interest that in vivo administration of low dose 5HT1A receptor agonist (reported to have anxiolytic effect) enhances GABA stimulated Cl⁻ uptake in cortico-hippocampal synaptoneurosomes (Soderpalm et al., 1997). In addition, coapplication of GABA and fluoxetine to cells expressing GABAA receptors increases the GABA
response in a concentration-dependent manner, while fluoxetine alone has no
effect (Robinson et al., 2003). There is also a direct interaction between the
GABA and the serotonin system in the hippocampus, where serotonin neurons
often end at inhibitory GABAergic interneurons (Gulyas et al., 1999). 5HT2A
and 3 receptors are present on GABA interneurons in cortex and hippocampus
and 5HT increases the firing rate of GABA interneurons in pyriform cortex, an
effect abolished by 5HT2A receptor antagonists (Gellman and Aghajanian, 1993,
1994; Morales et al., 1996; Willins et al., 1997; Jakab and Goldman-Rakic,
2000). In 5HT1A receptor knockout mice benzodiazepine insensitive anxiety and
changed GABAA receptor subunit composition in amygdala and hippocampus
has been shown (Parks et al., 1998; Sibille et al., 2000). Thus, there is a complex
interaction between the 5HT and the GABA systems, but nevertheless, both are
involved in regulation of cognitive functions.

The GABA system and neurosteroids

As discussed above, progesterone effects on mood and memory might be
cauised by progesterone itself or through CNS active progesterone metabolites,
like allopregnanolone (Majewska et al., 1986). Allopregnanolone does not act on
the progesterone receptor but has effects on the GABAA receptor. The interaction
of steroids with the GABAA receptor is dependent upon the structure of the
steroid. Thus, there are steroids with GABAA receptor agonistic
(allopregnanolone), antagonistic (pregnenolone sulfate, DHEAS) properties, and
3β-hydroxypregnane steroids. An antagonism against GABA was not noticed for
the 3β-hydroxypregnane steroids in rat cortical microsacs, but they act as
antagonists against potentiation of GABAA receptor function by 3α-
hydroxypregnane steroids (Lundgren et al., 2003).

GABAA receptors

The GABA (γ-aminobutyric acid) system is the major inhibitory system in the
mammalian CNS. In GABAergic neurons GABA is formed from glutamate in an
enzymatic reaction mediated by glutamic acid decarboxylase (GAD). The GABA
inhibitory action is mediated via the activation of specific receptors, e.g. the
GABAA receptor. The GABAA receptor belongs to the ligand gated ion channel
family, together with nicotinic acetylcholine, glycine and 5HT3 receptors. It consists of five subunits forming a chloride channel (Fig 6) and at least 18 different subunits have been described (6 α, 3 β, 3 γ, δ, ε, π, and 3 ρ; Mehta and Ticku, 1999). The distribution of different GABAA receptor subunits varies through the brain, and for many of the subunits the highest expression is in thalamus, hippocampus, cortex, or cerebellum (Wisden et al., 1992). Different combinations of subunits contribute to distinct pharmacological properties of the GABAA receptor and the expression of subunits is heterogeneous in the brain. The most common subunit compositions are α1β1γ2 or α1β2γ2. The activity of many GABAA receptor modulators depend upon the subunit composition of the receptor (see Korpi et al., 2002a).

The function of each subunit is not perfectly clear, but several studies point to especial importance of some subunits. For example, the sedative effect of benzodiazepines is mediated via α1 subunit containing GABAA receptors (Rudolph et al., 1999). The α2 subunit is considered to mediate benzodiazepine induced anxiolytic effects, since the knock-in point mutation His^{101} → Arg of the GABAA receptor α2 subunit in mice that makes receptors with this subunit insensitive to diazepam, and abolishes diazepam induced anxiolysis measured with the elevated plus maze test (Low et al., 2000). The GABAA receptor α2 subunit mRNA is mostly expressed in brain regions related to emotional stimulus processing, like the hippocampus and the amygdala (Wisden et al., 1992). Interestingly, microinjections of pregnanolone in the dorsal hippocampus give anxiolytic effect (Bitran et al., 1999). The GABAA receptor α4 subunit is also implicated in the regulation of anxiety (Gulinello et al., 2001). A concentration-dependent decrease of the α4 subunit is seen after 4 days application of allopregnanolone to developing neuronal cells (Grobin and Morrow, 2000), whereas in the hippocampus and cerebellum an increase in this subunit can be detected after withdrawal from chronic progesterone (or allopregnanolone) exposure and after short term treatment (Concas et al., 1999; Follesa et al., 2000; Gulinello et al., 2001). In addition, insensitivity to benzodiazepines after progesterone withdrawal has been shown and withdrawal induced increased susceptibility to seizure can be blocked using α4 subunit antisense (Smith et al., 1998). In the study by Li et al., the CA1 subregion of the hippocampus is the main brain region where down-regulation of benzodiazepine binding to the GABAA receptor α5 subunit was obtained after 4-week treatment with flurazepam (Li et al., 2000). In a recent study by Collinson et al., the GABAA
receptor α5 knockout mice had significantly better performance in a water maze model of spatial learning in comparison with wild-type mice (Collinson et al., 2002). In addition, a GABAA receptor α5 subunit gene point mutation decreases sensitivity to benzodiazepines and impairs hippocampal dependent memory related to fear conditioning (Crestani et al., 2002). The GABAA receptor β2 subunit has been shown to be involved in mediating the effect of anesthetic drugs, like etomidate, alphaxalone, pentobarbital and propofol (Belelli et al., 1997; Carlson et al., 2000). The γ2 subunit is involved in synaptic targeting and clustering, in anxiety regulation, and is changed during hormone manipulation and pregnancy (Essrich et al., 1998; Follesa et al., 1998; Concas et al., 1999; Crestani et al., 1999; Kittler et al., 2000b). The δ subunit is important for neurosteroid effects on the GABAA receptor (Stell et al., 2003) and receptor knockout studies revealed that absence of the δ subunit decreases the sensitivity to neuroactive steroids, like pregnanolone and alphaxalone, influencing the duration of anesthesia and anxiolytic effect of those steroids (Mihalek et al., 1999). Moreover, δ subunit knockout mice had fewer pups per litter than wild-type mice, showing that reduced sensitivity to neuroactive steroids can influence reproduction. Interestingly, in δ subunit knockout mice decreased α4 and increased γ2 subunits in areas normally expressing δ subunit (hippocampus, thalamus, striatum) has been shown (Korpi et al., 2002b; Peng et al., 2002). Studies of Xenopus laevis oocyte expression systems are of importance for determination of the actions of allopregnanolone on GABAA receptors with different subunit compositions. The α, β, or γ subunit isoforms are not greatly influencing the GABA-enhancing effect of allopregnanolone. However, in vitro there is a relative allopregnanolone insensitivity of the α4 and α5 subunits, but a 12-fold increase in potentiation of the GABA-evoked current when allopregnanolone is applied to GABAA receptors containing the α6 subunit (other alpha subunits show 6-7 fold increases). GABA A receptors containing the α2 subunit have been shown to react significantly less to allopregnanolone, compared with receptors including α1, α3, or α6 subunits (Belelli et al., 2002). There is a reduction in the maximal steroid effect by incorporation of the γ2 subunit, compared with receptors containing just α1β1 (see in Lambert et al., 2001), and replacement of the γ subunit with the δ subunit enhances steroid sensitivity of the receptor (Belelli et al., 2002).

These receptors can be modulated by a variety of substances and drugs, such as benzodiazepines, barbiturates, neurosteroids, anesthetics and ethanol.
The GABAA receptor is a critical component in the regulation of neuronal communication. The clustering of GABAA receptors at the postsynaptic terminals is essential for efficient neurotransmission. This process is facilitated by adaptor proteins that link the receptors to the underlying cytoskeleton. One such molecule is GABARAP, which interacts with the γ2 subunit of the GABAA receptor, the tubulin binding protein gephyrin, and the transferin receptor. Gephyrin stabilizes postsynaptic GABAA receptor clusters by preventing their internalization and is colocalized with the majority of GABAA receptor subtypes containing α1, α2, α3 or γ2 subunits (but not the α6 or δ subunit) in several brain regions (cerebellum, cortex, striatum, hippocampus, thalamus).
olfactory bulb and brainstem; Sassoe-Pognetto et al., 2000). Loss of GABAA receptor clusters in cultured cortical neurons in mice deficient in the γ2 subunit is paralleled with loss of the synaptic gephyrin and GABAergic function (Essrich et al., 1998). Moreover, in gephyrin knockout mice loss of GABAA receptor clusters in hippocampal neurons is observed and the GABAA receptor α2 and γ2 subunits appear in intracellular microclusters (Kneussel et al., 1999). Interestingly, microtubule polymerization disrupting agents, like colchicine, also decreases GABAA receptor clusters (Whatley et al., 1996). In that study colchicine inhibited ethanol-induced enhancement of muscimol-stimulated chloride uptake in mouse L(tk−) cells transfected with bovine α1β1γ2L or human α1β2γ2L subunits, whereas having no effect on flunitrazepam and pentobarbital enhancement of muscimol-stimulated chloride uptake, suggesting that microtubules play an important role in ethanol sensitivity (Whatley et al., 1996).

Phosphorylation is another mechanisms for controlling the functional properties of the GABAA receptor. It has been suggested that almost all GABAA receptor subunits (especially β1-3 and γ2) are substrates for phosphorylation (Macdonald, 1995), most commonly by protein kinase A (PKA) and/or protein kinase C (PKC) (McDonald et al., 1998; Brandon et al., 2003). It has been shown that inhibition of either PKA, or PKC significantly reduces the ability of allopregnanolone to prolong the miniature inhibitory postsynaptic currents (mIPSC) decay in the hippocampus CA1 neurons, however it is not the case in the cortex or oxytocin releasing neurons of the hypothalamus (Harney et al., 2003; Koksma et al., 2003). GABAA receptor phosphorylation causes enhancement or inhibition of GABA function depending on receptor subtype, brain area and location of the phosphorylation (Kapur and Macdonald, 1996; McDonald et al., 1998; Kumar et al., 2002; Brandon et al., 2002; Harney et al., 2003; Kittler and Moss, 2003). A recent study shows a role of hippocampal PKA in GABAA receptor dysfunction after 1-week treatment with benzodiazepines, when tolerance to anticonvulsant effect of flurazepam has developed (Lilly et al., 2003). Interestingly, the α4 subunit contains a consensus site for PKC and the β2 subunit is phosphorylated by PKC but not by PKA (Macdonald, 1995; McDonald et al., 1998). There are several isoforms of PKC, but only PKCγ is associated with the GABAA receptor subunits α1 and α4 in the cortex (Kumar et al., 2002). Moreover, it has been shown that PKC activity can modify receptor internalization (Connolly et al., 1999). Endocytosis is known to regulate the cell surface expression of neurotransmitter receptors, an important mechanism in
controlling the synaptic efficacy of neurotransmitters and a step in use-dependent tolerance development (Barnes Jr, 1996). GABAA receptor endocytosis is dependent on the clathrin adaptor complex AP2 adaptin, which is critical for recruitment of the receptor into clathrin-coated pits; then the receptor-adaptor complex interacts with clathrin, amphiphysin, and dynamin, which are key elements of the endocytotic machinery (Marsh and McMahon, 1999; Kittler et al., 2000a).

After receptor exposure to agonist down-regulation is expected. The receptor regulation could be at different levels: 1) desensitization (tachyphylaxis), 2) receptor internalization, 3) receptor subunit polypeptide degradation, 4) changed expression of receptor mRNA (Barnes Jr, 1996). Desensitization is a fading of receptor currents during continuous GABA application, associated with reduced frequency of channel opening that can be completed within a few seconds. Internalization is a slower process, completed within minutes to hours. Internalized receptors are targets for degradation or can be recycled. More prolonged exposure to agonist may cause change (usually reduction) in receptor mRNA. Thus, during chronic treatment with benzodiazepines tolerance gradually develops to the muscle relaxant, ataxic, locomotor and anticonvulsant effects of benzodiazepines (Bateson, 2002), often resulting in a down-regulation of benzodiazepine binding sites or GABAA receptor subunit mRNA expression in some brain regions (Kang and Miller, 1991; Zhao et al., 1994; Longone et al., 1996; Impagnatiello et al., 1996; Tietz et al., 1999; Li et al., 2000). In humans a tolerance towards the anxiolytic effect of benzodiazepines is a well known effect and limits the usage of these drugs in the treatment of anxiety (Lader and Petursson, 1981).

Tolerance is a decrease in sensitivity of the target organ to a drug within duration of exposure. Studies on tolerance have been focused on ethanol, as well as on others sedative and anesthetic drugs, such as barbiturates and benzodiazepines. For studying tolerance mechanisms, the GABAergic system has been in focus, since these substances are GABAA receptor modulators. The mechanism behind tolerance is not completely clear, but changes in the GABAergic system is noted and decreased GABAA receptor sensitivity to GABAA active substances are detected. This could be due to receptor desensitization, internalization, and/or decrease in synthesis of new receptor. Several neurotransmitter systems (GABA, serotonin, dopamine, acetylcholine, norepinephrine, NMDA) also seem to be involved. Acute tolerance to a single
hypnotic dose of ethanol develops more rapidly and persists many days longer in the alcohol-preferring than in the alcohol-nonpreferring rats. The alcohol-preferring rats also exhibit lowered serotonin levels in certain brain regions (Li et al., 1987). Furthermore, systemic ethanol administration dramatically elevates both plasma and cerebral cortical allopregnanolone levels in male and female rats. Ethanol induction of allopregnanolone is diminished in tolerant and dependent animals, showing the involvement of the GABA system in tolerance development (Morrow et al., 2001).

**Tolerance to neuroactive substances**

During the luteal phase of the menstrual cycle and during pregnancy the allopregnanolone concentration is increased, thus, in women there are situations when tolerance to prolonged progesterone and/or allopregnanolone exposure can occur. One sign of tolerance could be that during the first part of a pregnancy marked sleepiness is observed, which is substantially decreased later in the pregnancy, although progesterone and allopregnanolone levels are increasing. In postmenopausal women taking continuous combined estrogen/progestagen HRT, negative side effects arrive shortly after the introduction of the treatment but after 3-6 months the severity of symptoms decreases, indicating that an adaptation to the treatment has occurred (Ödmark et al., 2004). In women with PMS reduced benzodiazepine, ethanol and pregnanolone sensitivity occurs during the luteal but not the follicular phase of the menstrual cycle (Sundstrom et al., 1997, 1998). Interestingly, pretreatment with the SSRI citalopram during the luteal phase of one cycle normalize sensitivity to pregnanolone of PMS patients (Sundstrom and Backstrom, 1998). It was proposed, that PMS related anxiety symptoms might be related to progesterone and/or allopregnanolone withdrawal late in the luteal phase. In animal studies, increase in anxiety can be seen after withdrawal from 4-days exposure to progesterone, an effect mediated by the progesterone metabolite allopregnanolone (Gallo and Smith, 1993). 3 cycles of progesterone or allopregnanolone withdrawal in female rats have been shown to abolish the benzodiazepine enhancement of GABAA receptor currents in the hippocampus (Costa et al., 1995). After progesterone withdrawal increased susceptibility to seizure and insensitivity to benzodiazepines and allopregnanolone has been shown (Smith et al., 1998).
In rodents tolerance to the anticonvulsant and hypothermic effects of allopregnanolone develop after repeated administration (Czlonkowska et al., 2001; Palmer et al., 2002). Moreover, treatment with the neuroactive steroid minaxolone for 7 days results in development of tolerance to locomotor depression (Marshall et al., 1997). However, there is no tolerance development concerning sleep patterns after 5 day treatment with allopregnanolone (Damianisch et al., 2001), or epilepsy when pregnanolone (25 mg/kg) is given i.p. three times daily in 14 days, and no tolerance to the anticonvulsant effect of pregnanolone has developed (Kokate et al., 1998). Both pregnanolone and allopregnanolone enhance binding of flunitrazepam to the GABAA receptor, an effect abolished by chronic exposure of cultured cortical neurons to these steroids (Friedman et al., 1993). It has been shown that prolonged exposure to allopregnanolone can lead to down-regulation of the GABAA receptor α1-α5, β2, β3 and γ2L subunit mRNA expression in different brain areas, such as cortex, hippocampus, and cerebellum (Yu et al., 1996; Concas et al., 1999; Grobin and Morrow, 2000; Follesa et al., 2000). However, it is not clear which GABAA receptor subtypes that are involved in the development of tolerance.

**Neurosteroids and memory**

*Learning and memory*

Memory is a label for a diverse set of cognitive capacities by which humans and animals retain information and reconstruct past experiences, usually for present purposes. Memory could be described as retained knowledge and remembering is often related with emotions. The limbic system of the brain (hippocampus, amygdala, septum, entorhinal cortex, etc.) is highly involved in emotion perception and analysis. The hippocampus and amygdala are important brain regions for memory processes, showing that there is a powerful relationship between emotional situations and strong memories (Sutton, 2003).

Memory can be divided into several parts. Sensory memory is an experience that lasts for a very short time since it takes a second or two for the sensory neurons to recover from stimulation. The visual sensory memory is also called iconic memory, and last less than a second. The auditory version is called echoic memory, and lasts three or four seconds. Other senses have similar forms of sensory memory. Next in the time frame is working memory, which can be defined as the capacity to perform tasks that involve simultaneous storing and
manipulation of information. Long-term memory can be divided into several types. Non-declarative memory (implicit) is a memory of learnt skills and habits (using a knife and fork, dancing, etc.). A declarative memory (explicit) includes memory for facts (semantic memory) and memory for events (episodic memory). A declarative memory subtype is the spatial memory, memory for spatial information. Retrieval (remembering) comes in two forms: recognition and recall, which is more complex then recognition (Sutton, 2003).

As an in vitro model for long-term memory long-term potentiation (LTP) has been proposed. LTP is triggered by Ca$^{2+}$ influx following activation of NMDA receptors and gives a rapid and lasting increase in synaptic strength (see Lynch, 2004). However, there are controversial data available on the LTP requirement in memory formation, where LTP elimination in the CA1 subregion of the hippocampus is not influencing spatial memory (Meiri et al., 1998). For activation of the NMDA receptors and channel opening both presynaptic activity (glutamate release by axon terminal) and postsynaptic activity (depolarization that releases the Mg$^{2+}$ block) is required (Nowak et al., 1984; Riedel et al., 2003). The NMDA receptor subtype NR2A seems to be of special importance in LTP induction (Liu et al., 2004). Postsynaptic entry of calcium leads to activation of CaMKII (calcium/calmodulin-dependent protein kinase), which is required for consolidation of various forms of memory (Lynch, 2004). In CaMKII mutant mice hippocampal place cells (pyramidal cells which respond when the animal is in a particular location in the environment) are unstable, and spatial learning is impaired (Cho et al., 1998; Lisman et al., 2002). It is supposed that activation (by protein kinase A) of the transcription factor cAMP response element binding protein (CREB) is a key element in consolidation of memory. It has been suggested that an increase in cAMP concentration activates mitogen-activated protein kinase (MAPK/ERK). That activates several signaling cascades (e.g., cytoskeletal proteins MAP2, Tau, Arc; synaptosomal proteins, like synapsin; nuclear proteins CREB, c-fos, c-jun, NGFI-A, BDNF) that end with different neurotransmitter release, gene transcription and protein synthesis, leading to memory formation (see Lee et al., 2004; Lynch, 2004).

Thus, NMDA receptors are very important in memory formation and receptor antagonists and knockouts severely impair memory (Morris et al., 1986; Tsien et al., 1996; Nakazawa et al., 2002; Riedel et al., 2003). Other neurotransmitters, like acetylcholine, GABA, serotonin and dopamine are also involved in memory function. 5HT1A and 5HT2C receptor knockout/mutant mice also show impaired
spatial learning. Whereas 5HT1B knockout mice exhibit a facilitation in the acquisition of a hippocampal-dependent spatial reference memory task in the Morris water maze, but an impairment of delay-dependent working memory in a radial arm water maze (Heisler and Tecott, 1999; Sarnyai et al., 2000; Buhot et al., 2003a-b; Wolff et al., 2003). Interestingly, stimulation of the 5HT1B receptor inhibits the release of acetylcholine in the hippocampus, but stimulates release in the frontal cortex (Buhot et al., 2003a). Reduction in both cholinergic and serotonergic functions cause severe memory impairment in young as well as in aged rats (Richter-Levin and Segal, 1993). The dopamine striopallidal and strionigral systems are also involved in learning and memory, since striatum is a key brain region for the procedural part of spatial learning (Devan et al., 1996). The GABA system is the major inhibitory system in the brain and GABAA receptor active substances, like benzodiazepine, can inhibit learning and memory in humans and animals (Ghoneim et al., 1984; Cain, 1997; Holbrook et al., 2000). In addition, GABAA receptor activation with propofol can inhibit LTP induction (Wei et al., 2002).

Hippocampus and spatial learning

Anatomically, the hippocampus (Fig 7) can be divided into several subregions CA1 - CA4 (from Cornu Ammonis, because of its resemblance to a ram’s horn) and dental gyrus (DG) (Amaral and Witter, 1989; Brown and Zador, 1990). The dental gyrus molecular layer is a major recipient of the perforant path projections from the entorhinal cortex (layer II-III). The pyramidal cells in the CA3 subregion receive excitatory input from mossy fibers of the dental gyrus granule cells, the perforant path projections from the entorhinal cortex, and the recurrent collaterals of the CA3 pyramidal cells themselves. These CA3 pyramidal cells projects to CA1 via Schaffer collaterals and have output to the entorhinal cortex (layer IV-VI) (Brown and Zador, 1990). All hippocampal subregions are important in memory functions, such as contextual fear-conditioning (Lee and Kesner, 2004). Hippocampal CA1 pyramidal cells receive inhibitory input from local interneurons (Paulsen and Moser, 1998). The CA1 subregion is an essential site for successful storage of spatial memory (Tsien et al., 1996), thus output from the CA1 subregion of the hippocampus is of major importance.
In both humans and animals, the hippocampus is an important brain area for learning and memory functions (Farr et al., 2000; Astur et al., 2002). Both semantic and episodic memories involve the hippocampus, whereas procedural learning (like mirror-reading) is intact in amnesic patients (Cohen and Squire, 1980). The participation of hippocampus in encoding as well as retrieval and long-term consolidation of spatial memory has been demonstrated (Riedel et al., 1999). Hippocampus is essential for the formation of long-term declarative memories, but not for the retrieval of very remote spatial memories (Teng and Squire, 1999). If the damage is to the right half of the hippocampus, spatial memory is affected. Usually, damage limited to the left side causes little disruption of spatial memory, while verbal memory is affected. This left-right distinction is not true for everyone, though. Interestingly, positron emission tomography (PET) has been used to examine neural substrates of topographical memory retrieval in licensed London taxi drivers with many years of experience. Their recall of complex routes around the city resulted in activation of a network of brain regions, including the right hippocampus (Maguire et al., 1997). In addition, structural MRIs of the brains of taxi drivers show significantly larger posterior hippocampi (but smaller anterior hippocampi), compared with control subjects that do not drive taxi (Maguire et al., 2000). In this study the length of time spent as a taxi driver positively correlated with the volume of the right posterior hippocampus.

It seems that there are differences in hippocampal subregion involvement in learning and memory functions. Dendritic spines are the primary loci of excitatory synapses in central neurons and have been associated with neuronal
plasticity (Harris and Kater, 1994). Ovariectomy or gonadal steroid replacement is not affecting spine density of CA3 pyramidal cells or granule cells of the dentate gyrus but has effect just on the CA1 region (Gould et al., 1990). Excitatory NMDA receptors increases in the CA1 region of the dorsal hippocampus after 2-days estrogen supplementation (Weiland, 1992). Interestingly, adult mice lacking NMDA receptor-mediated synaptic currents and long-term potentiation in the CA1 synapses exhibit impaired storage and retrieval of spatial memory, but unimpaired non-spatial learning (Tsien et al., 1996). On the other hand, the CA3 region is important for associative memory recall (Nakazawa et al., 2002).

Spatial memory is a memory for spatial information. Spatial memory involves the ability to remember the spatial layouts of environments: knowing the locations of objects, yourself, and how to navigate from one place to another. In the rat hippocampus specific “place cells” have been described, thus pyramidal cells respond when the rat is at a particular location in the environment (O'Keefe and Dostrovsky, 1971; Redish, 2001). Primate hippocampal cells seems to show spatial view fields, where neurons respond selectively when a monkey view a particular portion of space (Rolls, 1999).

The Morris water maze is the favored test for the study of hippocampal-dependent spatial learning and memory in rats (Morris, 1984). It consists of a water pool with a hidden escape platform where the subject must learn the

![Fig 8. Morris water maze. (a) Rat performance at the start of testing and (b) after learning the task.](image)
location of the platform using distal cues (Fig 8). Performance in the Morris water maze depends on several mechanisms, from attention, learning and memory, to vision and motor coordination (D’Hooge and De Deyn, 2001). In navigation, the establishment of a spatial map is fast and involves the hippocampus, while to learn the routes and guidances are slow processes that involves caudate nucleus (see Redish, 2001).

Steroid hormone effect on learning

Estrogen has been shown to improve verbal memory and motor skills (Maki et al., 2001, 2002). After ovariectomy women treated for 2 months with estrogen scores better on recall of a story paragraph than placebo treated patients (Phillips and Sherwin, 1992). In women of fertile age a decrease of ovarial function with gonadotropin releasing hormone agonists also induce verbal memory deficit, what is reversed by “ad-back” of estrogen (Sherwin and Tulandi, 1996). The estrogen peak in the afternoon of the proestrus phase of the rat estrus cycle is associated with increased hippocampal long term potentiation (LTP) in the CA1 subregion (Warren et al., 1995). The number of dendritic spines and synapses in the hippocampal CA1 stratum radiatum decreases more than 30% between the proestrus (high estrogen) and estrus (low estrogen) phases of the rat estrous cycle (Woolley and McEwen, 1992). In addition, removal of circulating gonadal steroids by ovariectomy of adult female rats results in a decrease in dendritic spine density in the CA1 pyramidal cells of the hippocampus (Gould et al., 1990). In that study estradiol replacement prevented the observed decrease in dendritic spine density. Estrogen facilitates the spine-maturation process and may be associated with enhancement of hippocampal-dependent memory (Li et al., 2004). It has been suggested, that estradiol increased dendritic spine density is mediated via stimulation of the glutamate system with activation of NMDA receptors (Woolley and McEwen, 1994) or via reduction of GABA neurotransmission in hippocampal neurons (Murphy et al., 1998). Serotonergic system seems also of importance (Alves et al., 2002). In addition, estrogen has been shown to increase the activity of choline acetyltransferase (the enzyme for acetylcholine formation; Luine, 1985). However, it seems like the effect of estrogen alone on memory is different than in combination with progesterone. Importantly, hormone replacement therapy, where estrogen is combined with medroxyprogesterone acetate, increases the risk (almost doubles) for Alzheimer’s
disease in postmenopausal women with four years of treatment (Shumaker et al., 2003). It is worth to mention that estrogen used in this study by Shumaker et al., was conjugated equine estrogen which contains several equine estrogens not secreted by human ovaries and some metabolites has been shown to be cytotoxic (Liu et al., 2002; Turgeon et al., 2004). It is of interest that medroxyprogesterone acetate, used also in upper mentioned study, can induce anesthesia, probably via 3α-hydroxy-5α reduced metabolites that are active on the GABA receptor (Meyerson, 1967).

Cognitive disturbances are reported in pregnant women, including sleepiness, forgetfulness, reading difficulties, disorientation, poor concentration and coordination (for review see Brett and Baxendale, 2001). Complains of memory impairment in some women often begins in the second trimester (when very high progesterone and allopregnanolone levels are present in the brain), and appears to resolve soon after childbirth. Interestingly, decreased maternal brain size with slight ventricular increase have been shown late in pregnancy (Oatridge et al., 2002). In addition, women with PMDD often show difficulties in concentrating and impairment of working memory during the luteal phase of the menstrual cycle, when high circulating levels of progesterone and allopregnanolone are found (Man et al., 1999).

All hippocampal subregions are rich in GABA receptors and allopregnanolone can inhibit neural activity in the CA1 and the dental gyrus areas of the hippocampus (Wisden et al., 1992; Landgren et al., 1998). Treatment with another GABA receptor active substance, benzodiazepine, can inhibit learning and memory in humans and animals (Ghoneim et al., 1984; Cain, 1997; Holbrook et al., 2000). It is thus possible that allopregnanolone can mediate, supposedly via the GABA receptor, learning and memory disturbances in situations with high brain concentrations. Acute treatment with neurosteroids that have GABA agonistic effects (including allopregnanolone) impairs learning and memory (Mayo et al., 1993; Frye and Sturgis, 1995; Ladurelle et al., 2000; Vallee et al., 2001a; Matthews et al., 2002). On the other hand, steroids that act as GABA receptor antagonists enhance learning and memory (Mayo et al., 1993; Frye and Sturgis, 1995; Reddy and Kulkarni, 1998; Ladurelle et al., 2000; Vallee et al., 2001a). Thus, pregnenolone sulfate and DHEAS improve memory in aging mice and prevent pharmacologically induced amnesia (Vallee et al., 2001b). However, GABA receptor antagonism is potentially dangerous, and might induce seizures (Kokate et al., 1999). Because of that, selective antagonism towards GABA
receptor agonistic neurosteroids would be of great value. *In vitro* allopregnanolone-induced effects can be antagonized by 3β-hydroxypregnane steroids (Wang et al., 2000; Lundgren et al., 2003). Isoallopregnanolone (3β-hydroxy-5α-pregn-20-one; Fig 9) is an isomer of allopregnanolone with a hydroxygroup in the 3β position and does not have effects on CNS on its own (Lundgren et al., 2003; Weir et al., 2004). Nevertheless, all studies show *in vitro* 3β-hydroxypregnane steroid antagonizing effects, *in vivo* data are missing. It would be of great importance to discover or synthesize potential medications against allopregnanolone induced cognitive disturbances. Our research group has evaluated several neuroactive steroids as potent antagonists against allopregnanolone, verified by the Cl− uptake model in cortical homogenates and the model of Xenopus laevis oocyte expressing different GABAA receptor subunits. One of the most promising substances turned out to be UC1011 (3β-20β-dihydroxy-5α-pregnane), Fig 9.

Fig 9. Structures of neuroactive steroids.
AIMS

The overall goal with the present work was to contribute to the understanding of the neural mechanisms behind effects of ovarian- and neuro-steroids on the brain.

The specific aims of the different papers were:

- To investigate how long-term supplementation of estradiol and/or progesterone affects serotonin receptor 1A, 2A and 2C expression in different brain areas important for mood and memory functions (Paper I and II).

- To detect if acute tolerance develops to allopregnanolone and if changed expression of any GABAA receptor subunit is involved in development of acute tolerance (Paper III).

- To establish the effect of the neurosteroid allopregnanolone on spatial learning in the Morris water maze test (Paper IV).

- To study if allopregnanolone induced spatial learning impairment and GABA potentiation can be inhibited (Paper V).
MATERIAL AND METHODS

Experimental design

To investigate the effect of ovarian hormones on serotonin receptors adult female rats were used. In the normal rat estrus cycle the progesterone surge is very short - only one day. This has little similarity to the longer human menstrual cycle, pregnancy or postmenopausal HRT. Our hormone supplementation strategy includes a prolonged progesterone exposure and is thus more suitable for studies of the effects of long-term treatment with estradiol/progesterone than the normal estrus cycle. Thus, in Paper I and Paper II ovariectomized female rats received subcutaneous pellets containing different dosages of 17β-estradiol alone or in combination with progesterone, or placebo pellets (17β-estradiol 0.25 mg or 0.5 mg; or one 17β-estradiol pellet 0.5 mg plus one progesterone pellet 50 mg or 200 mg; or one estradiol placebo pellet (0.25 or 0.5 mg size); or one estradiol placebo (0.5 mg size) plus one progesterone placebo pellet (50 or 200 mg size)). Two weeks after pellet implantation animals were decapitated and trunk blood was collected for hormone assays. Brains were rapidly removed, frozen on dry ice and sectioned in a cryostat. 5HT1A receptor mRNA levels were analyzed by in situ hybridization in the dorsal hippocampus, dorsal raphe area, and entorhinal cortex. 5HT2A and 5HT2C receptor mRNA levels were analyzed in the ventral hippocampus and prefrontal cortex.

To study acute tolerance development to allopregnanolone (Paper III) adult male rats were infused intravenously with allopregnanolone (4 mg/kg/min). By continuous EEG recording the amount of allopregnanolone needed to reach the criterion “silent second” (SS – burst suppression of 1 second or more) was identified. The substance infusion was then stopped, and for longer anesthesia periods the infusion started again when there was no SS for a period of 1 min, and so forth. After different time intervals (first SS - control, 30 min or 90 min of anesthesia) the last infusion period to SS was followed by decapitation and samples from blood, different brain regions, muscle and fat tissue was analyzed for allopregnanolone content using celite chromatography followed by radioimmunoassay. Half the brain was used for GABAA receptor subunit mRNA detection in different brain regions. In situ hybridization for the GABAA receptor subunits α1, α2, α4, α5, α6, β2 and δ in different brain regions was performed.
In Paper IV to study the allopregnanolone effect on spatial learning adult male rats were injected with allopregnanolone (i.v., 2 mg/kg; 4 mg/kg/min), or vehicle, daily for 11 days. Eight or 20 minutes after the injection studies of place navigation were performed in the Morris water maze (for 9 days). Rats had a maximum of 120 s to search for the hidden platform, with four such trials per day. The allopregnanolone concentrations in plasma and in nine different brain areas where analyzed by radioimmunoassay.

In Paper V antagonism to allopregnanolone-induced effects was studied. Adult male rats were tested in the Morris water maze 8 min after daily i.v. injections of allopregnanolone 2 mg/kg, allopregnanolone:UC1011 (2:6, 2:8, 2:20 mg/kg), UC1011 20 mg/kg, or vehicle. Injection speed was 4 mg/kg/min of allopregnanolone. Studies of chloride ion uptake into cortical and hippocampal membrane preparations where also performed. Allopregnanolone concentrations in plasma and in 3 different brain areas where analyzed by radioimmunoassay.

Subjects

All experiments were performed on healthy adult female (Sprague-Dawley rats, 200–235 g; Paper I-II) or male rats (Sprague-Dawley rats, 348 ± 2.5 g in Paper III and Wistar rats, 240–320 g in Paper IV-V). The change of strains and sex was due to practical reasons. The estrous cycle in female rats would influence the outcome of results and would make larger variation in the groups. Detection of the phase of the cycle by vaginal smears is stressful to the animal and stress is known to influence neurosteroids and cognitive functions. Considering those circumstances, we used male rats in Papers III-V.

All experimental procedures were approved by the Regional Ethics Committee for Animal Experiment of Umeå University.

Drugs

Hormone or placebo pellets (Innovative Research of America, Sarasota, FL), designed to release hormones over a period of 21 days was used. Pellets contained either 17β-estradiol (0.25 mg or 0.5 mg), or progesterone (50 mg or 200 mg), or placebo for each hormone pellet (Paper I and II).
For the experiments in Papers III-V allopregnanolone (Umecrine AB, Umeå, Sweden, or from Dr. R.P. Purdy, Department of Psychiatry, College of Medicine, University of California, San Diego, CA, USA) was dissolved in 2-hydroxypropyl-β-cyclodextrin for intravenous injections. For Paper V UC1011 (3β-20β-dihydroxy-5α-pregnane) was purchased from Sigma Chemical Co. St. Louis, MO, USA. For in vivo experiments, allopregnanolone and UC1011 were dissolved by ultrasound in 10% 2-hydroxypropyl-β-cyclodextrin at a concentration of 2 mg /ml and these solutions were used to prepare mixtures of allopregnanolone and UC1011 with the concentration ratios, 1:3, 1:4 and 1:10. For in vitro studies (Paper V) picrotoxin was from FLUKA (Buchs, Switzerland), and 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) from Saveen Biotech (Malmö, Sweden), and the DC protein Assay was purchased from Bio-Rad (San Francisco, CA, USA). Other chemicals used were purchased from a local supplier. Steroids were dissolved in ethanol.

**Tissue preparation**

All animals were decapitated, trunk blood was collected for hormone assays and brains were rapidly removed. For Paper I and II, whole brain and for Paper III, half of the brain was frozen on dry ice, and stored at -80 °C. For the steroid analysis the other half of the brain was immediately dissected into different parts, with the dissection technique largely according to Glowinski and Iversen (Papers III - V; Glowinski and Iversen, 1966). White matter was carefully removed and after weighing, tissue was either frozen in dry ice and then stored at -80 °C until analysis or directly extracted with 99.5% ethanol for 7 days at 4 °C. For Paper III, abdominal fat tissue from the retroperitoneal area and part of the iliopsoas muscle was also dissected.

For in situ hybridization, brain sections (10 µm) at the level of the frontal cortex (bregma 3.7 to 3.2 mm), amygdala (bregma -2.3 to -2.8 mm), dorsal hippocampus (bregma -2.6 to -3.8 mm), ventral hippocampus (bregma -5.2 to -5.6 mm), dorsal raphe nucleus (bregma -7.3 to -8.0 mm), and cerebellum (bregma -10.0 to -10.5 mm; Paxinos and Watson, 1998) were cut on a cryostat, thaw-mounted on poly-L-lysine coated slides and stored at -80°C.

Cortical or hippocampal membranes (microsacs) for the chloride ion uptake experiment were prepared. Thus, cerebral cortex or hippocampus was rapidly removed after decapitation of animals. Cortices from two rats or hippocampi from
4 rats were homogenized with a glass and Teflon homogenizer in 10 mM Tris-HEPES buffer, pH 7.5, containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM d-glucose, and 1 mM CaCl₂. The homogenate was diluted with cold buffer to a final volume of 40 ml and filtered through two layers of 160 µm nylon mesh. The filtrate was washed twice by centrifugation, 1000Xg, 15 min, and the pellet resuspended in ice-cold buffer, to yield a homogenate concentration of 7-10 mg wet tissue/ml buffer.

**In situ hybridization**

Quantification of mRNA expression of different serotonin and GABAA receptor subunits was done using *in situ* hybridization. *In situ* hybridization is an imaging method to localize and detect specific mRNA sequences in morphologically preserved tissues sections or cell preparations by hybridizing the complimentary strand of a nucleotide probe to the sequence of interest.

For the 5HT1A receptor probe, a plasmid containing a 910bp fragment of the rat 5HT1A receptor cDNA was linearized with Hind III and transcribed with T7 RNA polymerase. For the 5HT2A receptor a 1kb fragment of the cDNA clone was used, the plasmid linearized with BamH1 and transcribed with T7 RNA polymerase. A 401 bp fragment of the cDNA clone for 5HT2C receptor was subcloned into plasmid, linearized with EcoRI and transcribed with T3 RNA polymerase (Holmes et al., 1995). *In vitro* transcription with [³⁵S]-UTP was utilized to synthesize labeled cRNA antisense riboprobes. Determination of the GABAA receptor α₁, α₂, α₄, α₅, α₆, β₂ and δ subunit mRNA expression was performed with antisense oligonucleotide probes (Wisden et al., 1992). Probes were 3´-end labeled using α-³⁵S-dATP by incubation with terminal deoxynucleotide transferase. As controls for unspecific binding sense probes were used.

Coronal cryostat brain sections were postfixed in cold 4% paraformaldehyde and washed in phosphate buffered saline followed by acetylation (to prevent non-specific binding of probe to positively charged amino groups) and dehydration. In the case of studying serotonin receptors prehybridization was used to decrease background binding of the probe. Denatured riboprobe, 2.0 x 10⁶ cpm, in 200 µl hybridization mixture was used per slide containing 3 consecutive sections from the same animal. Denatured oligoprobe for different GABAA receptor subunits, 1.0 - 4.0 x 10⁵ cpm in 100 µl hybridization mixture was used per slide containing
3 consecutive sections of half of the brain from the same animal. Slides were hybridized overnight and then washed and dehydrated before air drying.

After 2-10 days exposure to Hyperfilm β-max or Biomax MR-1 film (Amersham) slides were dipped in Kodak NTB2 photoemulsion, exposed at 4°C for 0.5-5.5 months and then developed followed by fixation. Before mounting with DPX sections were counter-stained with Pyronin Y.

The MCID M4 analyzing system (Imaging Research Inc. Ontario, Canada) was used for counting of silver grains in a fixed circular area with the approximate size of a pyramidal nucleus. Grains were counted over ten cell areas for each subregion on one section and three sections counted for each animal. Background, measured as the mean of grains in five "cell areas" randomly taken over white matter in each subregion and on each section, was subtracted. Specific expression was considered to exist when mean total counts were greater than twice the background. Counting was performed under blind conditions.

**Chloride ion uptake assay**

GABA produce its effects by enhancing the flux of chloride ions across nerve membranes and allopregnanolone can potentiate this effect (Majewska et al., 1986). Chloride ion influx can be measured by simultaneous addition of GABA and $^{36}$Cl$^{-}$ to membrane vesicles. By addition of the substance of interest, in our case steroid, GABA potentiation can be measured. Chloride ion influx is a fast process and therefore after a few seconds exposure the reaction is stopped with picrotoxin, a drug that blocks the GABA responses by closing the chloride channel. After filtration of microsacs (containing $^{36}$Cl$^{-}$) through glass fiber filters the filter-bound radioactivity of $^{36}$Cl$^{-}$ is measured by liquid scintillation spectroscopy. Thus, measured chloride ion amount reflects the chloride influx into microsacs when the GABAA receptor channel opens, and thereby reflects GABAA receptor function (Harris and Allan, 1985).

In *Paper V* rat brain cortical or hippocampal membrane preparations was diluted 1:2 with buffer and allowed to acquire room temperature for a minimum of 10 min. A Skatron (Lier, Norway) twelve-channel cell harvester and 96-well microplates were used to perform the chloride ion uptake assays. Steroids, dissolved in ethanol, were added to the wells, and ethanol allowed to evaporate. To each well $^{36}$Cl$^{-}$ (1.0 µCi/ml), various concentrations of steroids, buffer, and GABA, were added. The interaction was initiated by addition of 75 µl of
microsacs, to a final volume of 200 µl. At the same time as the test samples, controls were prepared containing isotope and buffer; isotope, buffer, and homogenate; or isotope, buffer, homogenate, and 10 µM GABA. The chloride ion uptake was terminated after 5 seconds by the addition of cold buffer containing 100 µM picrotoxin, followed by rapid filtration through glass fiber filters (Whatman GF/C, Millipore, Bedford, MA, USA) under vacuum. Filters were washed with cold buffer containing 100 µM picrotoxin and the filter-bound radioactivity was measured by liquid scintillation spectroscopy, Wallac 1409 DSA (Wallac, Turku, Finland). Each chloride ion influx test was conducted in quadruplicate determinations. Data are expressed as net chloride ion uptake (i.e., uptake in the presence of GABA and presence or absence of steroid, minus the basal uptake in the homogenate).

**EEG threshold method**

Acute tolerance is a decrease in sensitivity to a drug within the duration of a single continuous exposure (Kalant et al., 1971). For studies of acute tolerance to GABAA receptor active substances the EEG-threshold method has successfully been used (Bolander and Wahlstrom, 1988; Larsson and Wahlstrom, 1996; Korkmaz and Wahlstrom, 1997; Zhu et al., 2004). Thus, acute tolerance can be detected by comparing the dose of the administered substance needed to maintain anesthesia at the silent second level in the EEG. When combined with determination of substance concentrations in different brain regions, this method can be used to study areas in the brain that are important for tolerance development. The acute tolerance to allopregnanolone in rats was determined with this EEG threshold method. EEG was recorded continuously from subcutaneous stainless steel electrodes, while allopregnanolone was infused into the rat tail vein at a constant infusion rate (4 mg/kg/min). The infusion was stopped immediately when the first burst suppression period of 1 second or more (the “silent second”, SS) was noted in the EEG. Immediately after the first SS detection control animals were decapitated. To obtain longer anesthesia periods infusion started again when there was no SS noticed for a period of 1 min. Infusion continued until a new SS appeared in the EEG, and so forth. The time to reach the SS was recorded and the amount of steroid needed to induce the effect was calculated. When the predetermined time (30 or 90 min) of anesthesia was close, the injection-free time was slightly extended before the last infusion was
started and the first obtained SS was followed by decapitation of the animal. Cumulated doses were calculated with intervals of 5 min since the intervals between the allopregnanolone infusions varied between the animals. Maintenance dose rates (MDR, mg/kg/min) were calculated from cumulated doses where the difference in cumulated doses between two time points in the individual rats was divided by the used time interval. 20 min long time interval was used since several infusions had been performed during that time, giving an adequate certainty.

Fig 10. The change in EEG recording during continuous infusion of GABAA receptor active substances. I. the amplitude increases and the frequency slightly decreases; II.-III. the frequency gradually decreases and burst suppression periods appear; IV. The threshold criterion, silent second occurs; V. if the infusion is continued after the silent second, an isoelectric EEG is recorded (reprinted with permission from Mingde Wang, Ovarian steroids in the central nervous system: regulation of behaviour and neural function in rat and human, 1997).
Water maze test

The Morris water maze is the favored test for the study of hippocampal-dependent spatial learning and memory in rats (Morris, 1984), and the neurosteroid effect on spatial learning in Papers IV and V was evaluated using this task. Rats are placed into a large circular pool of water from which they can escape onto a hidden platform. Rats are natural swimmers and water controls for olfactory cues. Extramaze cues must be used and animals are supposed to locate themselves in space by looking on these cues, in order to find the hidden platform position for escaping from the water. In such learning tests animals do not need to be deprived of food to motivate learning, or use of electric shock to motivate escape. Occasionally the platform position is a fixed place in a pool (as in our case) but to test working memory the position of the platform can be changed. By taking away extramaze cues non-spatial learning can also be tested. The optimal trial number per day for our experiments was 4, each from different starting position. Since the allopregnanolone effect in the brain is short lasting, because of rapid metabolism/elimination, more trials in one day would influence the results. Therefore, to obtain spatial memory, several days are required.

In our studies the water maze was a black circular pool 180 cm in diameter and 60 cm in height, filled with water at 25 °C. A circular transparent platform, 10 cm in diameter, was for all trials placed in the middle of the northeast quadrant 1.5 cm below the water surface. The maze was placed in a semidark room with distal visual extra maze cues present (lamps, large geometric patterns on the walls, curtain). The experimenter and the equipment were located in an adjacent room.

Handling of animals was performed daily for 5 days until 2 days before the experiment. This included the same procedures as in the experiment, except for injections, i.e. warming the tail in hot water, holding the rat for injection. For the last 2 days swimming in the water maze was performed for 1 min, with no escape platform present in the pool. On each morning before the start of the experiment, rats were weighed, taken back to their home cage and left for 30 min in the experimental room for acclimatization.

Each session included 4 searches for the platform from different starting positions (randomly varying from day to day, but the same for all rats at each day). Rats were placed in the pool facing the sidewall and had a maximum of 120 s to search for the platform. Rats that did not find the platform were guided there by hand. All rats were allowed to sit on the platform for 30 s and were then taken up and dried with a towel for 30 s, until start of the next swim. The performance
of rats was monitored with an overhead video camera connected to an image analyzer (HVS Image, Hampton, UK) and analyzed by the water maze software HVS Water 2020. The primary measures were latency, in seconds, to find the platform, thigmotaxis (time spent close to the pool wall, shown as percentage of the total swimming time), and speed.

**Hormone assays**

Corticosterone and estradiol levels were measured in plasma using commercial radioimmunoassay (RIA) kits. Measurements of plasma progesterone were made by RIA using an antiserum against progesterone 11α-succinyl BSA-antigen.

For analysis of allopregnanolone plasma was extracted using diethyl ether, and tissue samples were extracted with 99.5% ethanol, for 7 days at 4 °C. The recovery of steroids by this procedure was previously shown to be 100% (Bixo et al., 1984). In Paper III and IV, allopregnanolone in tissue and plasma extracts was purified by celite chromatography, as described earlier (Backstrom et al., 1986; Corpechot et al., 1993). In Paper V, we used preparative High Performance Liquid Chromatography followed by Radioimmunoassay (HPLC-RIA) for quantification of allopregnanolone.

For measurements of allopregnanolone concentrations by radioimmunoassay allopregnanolone antiserum was raised against 3α-hydroxy-20-oxo-5α-pregn-11α-yl-carboxymethyl ether coupled to bovine serum albumin. Antiserum was kindly provided by Dr Robert Purdy (Department of Psychiatry, College of Medicine, University of California, San Diego, USA). Specificity and cross-reactivity of the antiserum has earlier been verified (Purdy et al., 1990).

**Statistical analysis**

For comparing body weight, plasma hormone levels and mRNA expression between groups one-way ANOVA was done, followed by the least significant difference post-hoc test (when the ANOVA test was significant). Differences in allopregnanolone concentrations in Paper IV were analyzed using one-way ANOVA followed by Dunnett’s T3 post-hoc test.

For the water maze performance (Paper IV and V) data were analyzed by repeated measures ANOVA, followed by the LSD post-hoc test, in order to test changes within animal groups over days as well as differences between different
treatment groups. For comparisons between groups on a single day, we used the Mann Whitney U test.

Repeated measures ANOVA was also used to evaluate the chloride ion uptake parameters. The independent factors were concentration of enhancing substance (GABA, allopregnanolone), and concentration of antagonizing substance (0–30 µM UC1011).

For Paper III the Student’s t-test for independent samples was used for analysis of differences in maintenance dose rate (MDR, mg/kg/min). Differences in allopregnanolone concentrations were analyzed using one-way ANOVA followed by LSD post-hoc test when p< 0.05. For mRNA analysis one-way ANOVA was also used. Two-way ANOVA (experimental groups vs. subregions of the brain) was used to detect an overall difference between groups in all subregions of one brain area. Linear parametric correlations (r) and regression coefficients (b) were used to evaluate simple statistical relations in the data set. Chi Square was used to test significances in frequency tables.

We used Principal Component Analysis (PCA) and Partial Least Squares (PLS) to evaluate overall models in the data set. Such multivariate statistical methods are used for analyzing large data sets where the number of variables is much larger than the number of objects. This is done by projecting down the objects in the multidimensional space onto a set of few principal components, which are derived so that they describe the direction in the multidimensional space with maximum variation (for review see Grainger, 2003).

PCA was applied to show the relationship between allopregnanolone exposure time and concentrations. The principal components are linear combinations of the original variables and uncorrelated to each other, and can be determined using the NIPALS algorithm (Wold et al., 1987).

PCA models were obtained using the following equation:

\[ X = t_1 p'_1 + t_2 p'_2 + t_3 p'_3 + \ldots + t_A p'_A + E = TP' + E \]

where \( A \) is the number of principal components and \( E \) is the residual matrix. R2X (fraction of the sum of squares of all the X’s explained by the current component) describes how much of the variance in the data that is described by the PCA model. The scores (t) obtained in this analysis show how the objects and experiments relate to each other. The loadings (p) reveal variables important for the patterns seen in the score plot.

PLS was used to correlate GABA\textsubscript{A} receptor subunit mRNA changes in the brain with MDR or allopregnanolone concentrations. PLS is a multivariate regression
method that relates a data matrix $X$ to a $y$-response that can be either single ($y$) or multiple ($Y$). PLS has proved to be a powerful tool for finding relationships between descriptor matrices and responses. Especially when there are more variables than observations and the variables are collinear to each other and noisy. The PLS theory and methods discussed here concern single $y$-responses. As in PCA, principal components are constructed to reduce the dimensions of $X$. In order to obtain the principal components, PLS maximizes the covariance between the response variable $y$ and a linear combination of the original variables $t = Xw$, where $t$ is the score vector, $X$ is the data matrix and $w$ is the weight vector. 

$W$ describes the correlation between the data matrix and the response and can be analyzed in the same way as $p$. For a more in-depth description of PLS, see (Wold et al., 2001; Grainger, 2003) and references therein.

$$X = t_1 p_1' + t_2 p_2' + t_3 p_3' + \ldots t_A p_A' + E = TP' + E$$

$$Y = t_1 c_1' + t_2 c_2' + t_3 c_3' + \ldots + t_A c_A' + F = TC' + F$$

$A$ = the number of PLS components, $t$ = score vector for $X$, $p$ = loading vector for $X$, $c$ = loading vector for $Y$, $E$ = residual matrix for $X$, $F$= residual matrix for $Y$. The Q2Y value (the fraction of the total variation of the $Y$’s that can be predicted by a component, as estimated by cross-validation) reflects predictive power of the models while the R2Y value (fraction of the sum of squares of all the $Y$’s explained by the current component) shows how much of the variance in the response that is described by the models. PCA and PLS calculations were performed using the Evince software (UmBio Umeå, Sweden).

Statistical calculations were performed using the SPSS program, unless stated differently.
SUMMARY OF RESULTS

Effects of ovarian steroids on serotonin receptors

Effects of two-week estrogen and progesterone treatment on 5HT1A, 2A and 2C receptor expression in different brain areas, such as cortex, raphe nuclei, hippocampus (*Paper I and II*)

Weight difference

Two weeks after ovariectomy the mean body weight of animals that received placebo pellets was significantly higher than the weight of animals receiving hormone pellets. The lowest weight gain was seen in the high dose estradiol treated rats. This is consistent with findings in the literature (Varma et al., 1999; Matsuda et al., 2002).

**Fig 11.** Histograms representing 5HT1A mRNA expression in different subregions of the dorsal hippocampus in animals treated with estradiol alone (A) or in combination with progesterone (B). Analyses by grain counting over individual neurons. * - P<0.05 versus the placebo group; Ec - placebo for 17β-estradiol; E+Pc - placebo for 17β-estradiol and progesterone; Ei - 17β-estradiol 0.25 mg; Eh - 17β-estradiol mg; Pl - progesterone 50 mg; Ph - progesterone 200 mg.

**5HT1A**

Estradiol treatment alone reduced 5HT1A gene expression in the dentate gyrus and the CA2 region of the dorsal hippocampus (17 % and 19 % decrease, respectively; *Fig 11A*). Estradiol combined with progesterone supplementation increased 5HT1A gene expression versus placebo in the CA1 and CA2
subregions of the dorsal hippocampus (16 % and 30 % increase, respectively; Fig 11B). Concomitantly, 5HT1A mRNA expression was decreased by 13 % in the ventrolateral part of the dorsal raphe nuclei when estradiol was combined with high dose of progesterone. No changes were found in the median raphe nucleus and entorhinal cortex from any hormone supplementation.

5HT2C

5HT2C receptor gene expression was in the ventral hippocampus decreased in the CA2, ventral CA1 and the subiculum subregions by high-dose estradiol treatment (8-20 % decreases; Fig 12A).

5HT2A

Estradiol treatment in combination with low-dose progesterone increased 5HT2A receptor mRNA by 43 % in the CA2 region of the ventral hippocampus, while estradiol combined with high-dose progesterone increased the expression of this gene by 84 % in ventral CA1 (Fig 12B). 5HT2A mRNA expression in the frontal cortex was not influenced by the hormone manipulation.

![Fig 12. Histograms representing (A) 5HT2C mRNA and (B) 5HT2A mRNA expression in different ventral hippocampus regions. Analyses by grain counting over individual neurons. *-indicate significant (P<0.05) difference versus placebo group. E_c - placebo for 17β-estradiol; E+P_c - placebo for 17β-estradiol and progesterone; E_i - 17β-estradiol 0.25 mg; E_h -17β-estradiol 0.5 mg; P_i - progesterone 50 mg; P_h - progesterone 200 mg.](image)
Acute tolerance to allopregnanolone
(Paper III)

Initial sensitivity to allopregnanolone

The initial sensitivity to allopregnanolone (threshold dose) for the first silent second induction did not differ between experimental groups. However, there was individual variation between rats in the allopregnanolone dose needed to induce the first SS in the EEG (mean ± SD of initial dose for the induction of first SS was 10.46±1.43 mg/kg; range 7.27-14.13 mg/kg). The dose needed to induce first SS showed positive correlation with the GABA\(_A\) receptor \(\alpha_1\) subunit mRNA expression in the thalamus (VPM and VM), \(\delta\) subunit mRNA expression in VPM of thalamus and \(\alpha_2\) subunit mRNA expression in the hypothalamus (p<0.05).

Acute tolerance

The dose rate of allopregnanolone needed to maintain anesthesia (MDR) was significantly (p<0.001) higher in the time period 65-85 min (0.98±0.04 mg/kg/min) compared with the period 10-30 min (0.67±0.03 mg/kg/min; Fig 13A). Since we keep animal on the same anesthesia level (SS) but the dose of allopregnanolone required to reach the SS is higher in the later time-points than in the beginning of anesthesia, this means that acute tolerance to allopregnanolone has developed during the anesthesia period.

Fig 13. (A) Maintenance dose rates in different time intervals in the 90 min group. * p < 0.001 versus 10-30 min period. (B) Concentrations of allopregnanolone in serum, hippocampus, striatum, MTH (midbrain, thalamus, hypothalamus), brain stem (pons, medulla oblongata), cerebellum and fat tissue. * p < 0.05 versus control (first SS) group, # p < 0.05 versus 30 min group.
The allopregnanolone concentrations differ between groups in the serum, hippocampus and midbrain/thalamus/hypothalamus (MTH) regions (Fig 13B). After 90 min of anesthesia there was a significant increase in allopregnanolone concentration in the hippocampus (58 %) and MTH (67 %) compared with the first SS group. Since we inject allopregnanolone in higher dose in the later time-points to maintain anesthesia, but an increased concentration in the brain is obtained just in those two regions at that time, this points out plausible primary sites for the allopregnanolone action in the development of tolerance.

**GABAA receptor subunit mRNA changes**

mRNA for the GABAA receptor subunits α1, α2, α4, α5, α6, β2 and δ were analyzed in various subregions of the hippocampus, thalamus, amygdala, raphe nucleus, cortex, and in hypothalamus and cerebellum. There was an increase in the GABAA β2 subunit mRNA expression in the CA3 and DG subregions of the dorsal hippocampus in the 90 min group, compared to 30 min of allopregnanolone infusion. A decrease in the GABAA α4 subunit mRNA expression was detected in the ventral posteromedial thalamic nucleus (VPM) in animals with 90 min of anesthesia, compared to animals killed at the first silent second (p<0.05; Fig 14A). Using two-way ANOVA (experimental groups vs. all subregions of the hippocampus or thalamus) overall differences between groups were found for the GABAA receptor α5 and β2 mRNA in the hippocampus, and for α4 subunit mRNA in the thalamus, showing the same pattern of changes between groups for subunit mRNA expression in all subregions of the dorsal hippocampus or thalamus.

The individual increases in the dose of allopregnanolone (mg/kg/min) needed to retain the silent second from the period 10-30 min of anesthesia to the period 65-85 min negatively correlated with the mRNA expression of the GABAA receptor α4 subunit in the VPM of thalamus (Fig 14B). There was a positive correlation between MDR in the period 65-85 min and the GABAA receptor α2 mRNA in the hippocampus (CA1, CA4, DG). Moreover, the MDR difference between the time periods 65-85 min and 10-30 min also correlates with α2 mRNA in the CA1 region, showing possible involvement of the GABAA receptor α2 subunit in the development of acute tolerance.

Overall correlation matrices for the GABA_A receptor δ subunit mRNA expression in the hippocampus, thalamus, hypothalamus and cortices, showed a
high number of significant positive correlations just in the 90 min group of animals between δ mRNA in one region vs. δ mRNA in another brain region (40 correlations out of 45 possible). Meaning that there is a synchronization of expression pattern for the GABAA receptor δ subunit in tolerant rats.

Fig 14. GABAA receptor α4 subunit mRNA expression in the thalamus. Analyses by grain counting over individual neurons. #p < 0.05 versus control (first SS) group (A). Linear regression plot for the GABAA receptor α4 mRNA expression in the VPM of thalamus and the increase in allopregnanolone dose per min needed to maintain the anesthesia (B).

**Allopregnanolone effects on spatial learning**

*(Paper IV)*

The time to find the hidden platform was longer 8 minutes after the allopregnanolone injection (2mg/kg; p<0.003), while normal learning was seen after 20 minutes (Fig 15A). Swim speed did not differ between groups. A higher number of rats were swimming close to the pool wall (thigmotaxis) in the 8 minutes allopregnanolone group compared to the other groups (Fig 15B).

The allopregnanolone concentrations in the brain tissue were at 8 minutes 1.5 to 2.5 times higher then at 20 minutes after the allopregnanolone injections (table1). After vehicle injections the brain concentrations of allopregnanolone were at physiological levels. Plasma concentrations of allopregnanolone followed the same pattern as in the brain, with the exception of an increase 8 minutes after vehicle injections (table1). Corticosterone levels were in our study not
significantly different between the different injections or between the two time points after the injections.

**Fig 15.** (A) Latency to find the hidden platform in the Morris water maze and (B) the time each group of rats spent close to the pool wall (within 10% of the pool diameter) shown as percentage of the total swimming time, mean ± SEM. The four groups of animals started swimming 8 minutes (filled squares) or 20 minutes (filled circles) after the allopregnanolone injection, and 8 minutes (unfilled squares) or 20 minutes (unfilled circles) after the vehicle injection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Allo 8</th>
<th>Allo 20</th>
<th>Mean decrease a)</th>
<th>Vehicle 8</th>
<th>Vehicle 20</th>
<th>Allo 8 vs Allo 20 b)</th>
<th>Vehicle 8 vs Vehicle 20 b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>(%)</td>
<td>Mean</td>
<td>SEM</td>
<td>p&lt;</td>
<td>p&lt;</td>
</tr>
<tr>
<td>Plasma</td>
<td>1538 ± 121</td>
<td>809 ± 29</td>
<td>47</td>
<td>10.7 ± 0.93</td>
<td>4.4 ± 0.76</td>
<td>0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2063 ± 58</td>
<td>820 ± 33</td>
<td>60</td>
<td>4.3 ± 0.18</td>
<td>7.8 ± 1.00</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>1394 ± 98</td>
<td>571 ± 21</td>
<td>59</td>
<td>1.3 ± 0.40</td>
<td>2.0 ± 0.18</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>2661 ± 99</td>
<td>1254 ± 89</td>
<td>53</td>
<td>3.7 ± 0.67</td>
<td>1.9 ± 0.33</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>1606 ± 85</td>
<td>818 ± 64</td>
<td>49</td>
<td>ND</td>
<td>ND</td>
<td>0.001</td>
<td>-</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1624 ± 127</td>
<td>937 ± 122</td>
<td>42</td>
<td>2.3 ± 0.32</td>
<td>1.3 ± 0.11</td>
<td>0.014</td>
<td>NS</td>
</tr>
<tr>
<td>Striatum</td>
<td>2933 ± 120</td>
<td>1790 ± 86</td>
<td>39</td>
<td>2.7 ± 0.52</td>
<td>1.3 ± 0.14</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>995 ± 92</td>
<td>613 ± 25</td>
<td>38</td>
<td>ND</td>
<td>2.6 ± 0.58</td>
<td>0.026</td>
<td>-</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2570 ± 104</td>
<td>1680 ± 144</td>
<td>35</td>
<td>2.3 ± 0.25</td>
<td>1.6 ± 0.22</td>
<td>0.004</td>
<td>NS</td>
</tr>
<tr>
<td>Midbrain</td>
<td>1579 ± 148</td>
<td>1076 ± 53</td>
<td>32</td>
<td>4.1 ± 0.55</td>
<td>2.9 ± 0.42</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

a) Decrease in concentration from 8 to 20 minutes; b) Dunnett T3 Post hoc test; ND = Not detected; NS = Not significant

**Table 1.** Allopregnanolone concentrations (mean ± SEM) in plasma (nmol/l) and CNS tissue (nmol/kg) eight minutes (Allo 8) and 20 minutes (Allo 20) after i.v. injection of allopregnanolone or after i.v. injection of vehicle (Vehicle 8 and Vehicle 20).
Allopregnanolone induced GABA potentiation and learning impairment can be inhibited by UC1011

(Paper V)

UC1011 effects on allopregnanolone increased GABA mediated chloride ion uptake

For studies of GABA mediated chloride ion uptake membrane preparations from cerebral cortex and hippocampus of adult male rats were used. In the Figure 16, the percentage increase in chloride ion uptake in the presence of 10 μM GABA and increasing dosages of allopregnanolone ± UC1011 are shown. 100% corresponds to the chloride ion uptake without any allopregnanolone present. The chloride ion uptake was significantly increased by the allopregnanolone concentrations 150 nM - 3 μM (p<0.001). The increase in chloride ion uptake induced by allopregnanolone was significantly inhibited by the UC1011 dosages 10 and 30 μM (p<0.029, Fig 16). UC1011 in the concentrations 3 - 30 μM did not show any significant inhibitory effect on the chloride ion uptake induced by 10 μM GABA.

![Graph showing chloride ion uptake](image)

**Fig 16.** Interaction between allopregnanolone and UC1011 in the presence of 10 μM GABA. Percentage change in chloride ion uptake with increasing dosages of allopregnanolone ± 3, 10 and 30 μM UC1011. Chloride ion uptake with only 10 μM GABA present was set as 100%.

UC1011 effects on allopregnanolone decreased learning in the Morris water maze test

Allopregnanolone (2 mg/kg) injected together with either 6 or 8 mg/kg of UC1011, eight min before the start of swimming, doesn’t improve learning
compared to allopregnanolone alone. However, with increased dosage of UC1011 (20 mg/kg) it was possible to decrease the negative effect that allopregnanolone induced on learning in the Morris water maze.

As shown in Fig 17A, the latency to find the platform was in the allopregnanolone-injected group still above 80 s. after six days with practice. Whereas, rats injected with a mixture of allopregnanolone and UC1011 (2, 20 mg/kg, respectively) had significantly lower latency from day 3, p<0.05, compared with the allopregnanolone-injected group. The group that only received UC1011 learned to find the platform as quick as the control group (Fig 17A).

There was no significant difference in mean swim speed between the four groups. Thigmotaxis in the allopregnanolone-injected group was higher than in the UC1011 and the vehicle injected groups (p<0.001 day 1-6). In the group injected with the mixture of allopregnanolone and UC1011 (2:20 mg/kg, respectively) thigmotaxis was significantly decreased (p<0.05) day 3-6, compared to the allopregnanolone-injected group (Fig 17B).

![Graphs](https://example.com/graphs.png)

**Fig 17.** Allopregnanolone (2 mg/kg), allopregnanolone and UC1011 (2:20 mg/kg, respectively), UC1011 (20 mg/kg), or vehicle was injected (i.v) 8 min before start of each swim session. Data shown are mean ± SEM of all four swim trials each day. A) Latency time to find the hidden platform, B) thigmotaxis - the fraction of the total path that the rats swam close to the pool wall.

**Allopregnanolone concentration**

In the group injected with allopregnanolone and UC1011 (2:20 mg/kg) the allopregnanolone levels were lower in the brain regions (hippocampus, cortex, striatum), compared with levels in the group only injected with allopregnanolone. The differences were statistically significant in striatum and cortex.
GENERAL DISCUSSION

The present thesis concerns the effects of sex hormones and their metabolites effects in the brain on a molecular and behavioral level. I have chosen to addresses two main issues: The first one is effects of estrogen, progesterone and the neurosteroid allopregnanolone on serotonin and GABAA receptor expression in different brain regions. These studies could contribute to the understanding of the mechanisms behind effects of steroids on the brain. The second issue concerns how neurosteroids affect learning and if the allopregnanolone effect on spatial learning can be inhibited.

Long-term supplementation of estradiol and/or progesterone effect on serotonin receptor 1A, 2A and 2C expression in different brain areas important for mood and memory functions

The major finding in Paper I is the opposite effect on 5HT1A receptor mRNA expression induced by combined estradiol and progesterone treatment compared with estradiol alone. Thus, we found a decrease in 5HT1A receptor gene expression after chronic estradiol supplementation in the dentate gyrus and the CA2 region of the dorsal hippocampus, but an increase in the CA1 and CA2 subregions after progesterone treatment in combination with high-dose estradiol. Chronic estradiol treatment had no significant effect on 5HT1A receptor mRNA expression in the dorsal raphe nucleus, whereas decreased autoreceptor mRNA expression in the ventrolateral subregion of the dorsal raphe nuclei was obtained after combined hormone treatment. These results points out that the ovarian hormones singly or in combination differently regulate serotonin autoreceptors and postsynaptic receptors in the brain.

Paper II is the first study in the literature to explore 5HT2A and 5HT2C receptor mRNA expression changes in the ventral hippocampus after chronic gonadal hormone manipulation. Of major interest is the difference in the regulation of 5HT2A and 5HT2C receptor mRNA expression by estradiol alone versus combined estradiol and progesterone supplementation. Estradiol alone thus decreases 5HT2C receptor gene expression whereas combined hormone
supplementation increases 5HT2A receptor expression in specific subregions of the ventral hippocampus.

These findings are interesting in light of earlier data showing that the effect on mood by estradiol alone is completely different from the effect together with progestagens (Hammarback et al., 1985; Magos et al., 1986; Bjorn et al., 2000). In postmenopausal women the HRT estrogen-only phase is associated with positive mood, but a higher dose of estradiol given together with progestagens induces more negative mood symptoms compared to a lower dosage (Bjorn et al., 2000, 2003).

For more detailed comparison with other studies on ovarian hormone effects on serotonin receptor expression available in literature, see the individual papers (Paper I and II).

There is no completely clear explanation of the obtained results, but some points follow.
1. The estradiol induced decrease in postsynaptic serotonin receptor 1A mRNA expression may be due to a negative feedback regulatory mechanism induced by increased neurotransmitter levels in the synaptic cleft. This statement is made due to the knowledge that estrogen can increase serotonin levels and decrease SERT in specific brain areas. However, the somatodendritic autoreceptor gene expression in the median raphe nucleus did in Paper I not change. This area is thought to be highly connected particularly to the dorsal hippocampus (Vertes et al., 1999). Thus, it might be that autoreceptor sensitivity in median raphe is affected without alterations in receptor mRNA levels. Importantly, it has been shown that even after prolonged treatment with SSRIs residual raphe 5HT1A autoreceptor capacity remains functional (Hjorth and Auerbach, 1999).
2. In the dorsal raphe the decrease in 5HT1A autoreceptor expression by progesterone in combination with estradiol may be expected to increase serotonin neural firing, thereby increasing serotonin neurotransmission, mainly in the frontal cortex. However, the combination of estradiol with progesterone decreases serotonin levels in the brain (at least in the mediobasal hypothalamus) compared with estradiol alone (Fabre-Nys et al., 1994). Thus, the actual consequences of such a change in the mRNA is hard to know, but of importance is that autoreceptors and postsynaptic receptors are regulated in opposite directions.
3. Both estrogen receptors (ERα and ERβ) are present in the raphe nucleus, whereas, in the hippocampus there is a relatively high ERβ/ERα ratio (Shughrue
et al., 1997; Register et al., 1998; Alves et al., 1998; Gundlah et al., 2000, 2001). Thus, ERβ activation may be of importance for estradiol effects in the hippocampus. As the presence of ERα and ERβ varies between different brain regions (Shughrue et al., 1997), this might contribute to brain region-specific changes in ER-dependent transcription.

4. Recent studies indicate the presence of both progesterone receptor isoforms in the hippocampus (Guerra-Araiza et al., 2001). Notably, the progesterone receptor A isoform was selectively up-regulated in this brain region after estradiol supplementation to ovariectomized rats (Camacho-Arroyo et al., 1998; Guerra-Araiza et al., 2003). It is worth noting that progesterone receptor immunoreactivity was almost absent in the hippocampus in a study by Weiland et al. (Weiland et al., 1997). This suggests that progesterone effects on the serotonin system in the hippocampus might be mediated through PRA, or via the raphe nuclei, or indirectly by affecting the GABAA system.

5. There are not any known binding sites for the ER or PR within the 5HT1A receptor promoter region, while induction of gene expression have been shown by the transcription factors NFκB and Sp1 (Parks and Shenk, 1996; Wissink et al., 2000). NFκB may interact with steroid hormone receptors and mutual repression has been shown with both ER and PR (Kalkhoven et al., 1996; Cerillo et al., 1998). However, this mechanism seems to be cell-type specific (Cerillo et al., 1998), and if this interaction is functional within the hippocampus is not known. Although, the yet unpublished observation that estrogen alone or together with progesterone reduced number of NFκB positive cells in the dorsal raphe of monkeys was discussed in a review article by Bethea et al. (see in Bethea et al., 2002a). It is also of interest that estrogen deficiency results in enhanced levels of free Sp1 in bone stromal cells (Srivastava et al., 1998). If this effect is similar within the brain estradiol supplementation might decrease the amount of active Sp1, resulting in decreased expression of the 5HT1A receptor gene. Thus, steroid hormone manipulation could directly influence 5HT1A receptor gene expression in the presence of tissue-specific regulatory factors.

6. We are not aware of data indicating estrogen or progesterone response elements in neither the 5HT2A nor the 5HT2C receptor promoter. However, several transcription factors can interact with the 5HT2A receptor promoter, including Sp1 and the CRE binding proteins (Zhu et al., 1995). The progesterone receptor has been shown to up-regulate gene transcription through interaction
with DNA bound Sp1 (Owen et al., 1998). Thus, it is possible that progesterone can affect 5HT2A receptor expression via Sp1.

7. The estrogen receptor can probably influence serotonin receptor gene expression through binding to an AP1 enhancer element (Paech et al., 1997). In HeLa cells transfected with either ER\textalpha or ER\textbeta, estradiol thus promoted ER\textalpha-dependent transcription, whereas the same hormone had no effect on ER\textbeta-dependent transcription from an AP1 site (Paech et al., 1997).

In summary, activation of target protein transcription by steroid hormones are brain region, ligand and promoter dependent. Opposite 5HT receptor change in the hippocampus from estrogen alone or in combination with progesterone could contribute to a better explanation for opposite effects on mood exerted by those hormones.

Mechanisms behind development of acute tolerance to allopregnanolone

In Paper III development of acute tolerance to continuous i.v. injected allopregnanolone was established. The hippocampus and MTH (midbrain, thalamus, hypothalamus) regions showed increased levels of allopregnanolone in the animal group with 90 min of anesthesia. This is also verified using PCA where the hippocampus and MTH regions are the most important regions concerning the interaction between allopregnanolone concentrations and the anesthesia time 90 min. A rapid elimination of free allopregnanolone from the brain was indicated by the massive redistribution of the steroid to the fat. Since tolerance to the anesthetic effect of allopregnanolone has developed as indicated by the requirement of a higher amount of allopregnanolone needed to reach the criterion SS, the increase in allopregnanolone concentration in just some brain regions specify these particular areas as sites for tolerance development.

Using in situ hybridization, we found that several of the GABAA receptor subunits might be involved in the development of acute tolerance. Reduced GABAA receptor \(\alpha4\) subunit mRNA levels in the VPM might be the primary component in the system that caused the increase in dose of allopregnanolone needed to maintain the anesthesia (Fig 14). A reduced number of \(\alpha4\) subunits could mean decreased number of functional GABAA receptors or a change in efficacy of the receptor by a changed subunit composition. With regard to number of GABAA receptors we did not measure the amount of receptors present in different areas, but each GABAA receptor includes two \(\alpha\) subunits and we did
not find any compensatory up-regulation in thalamus of any of the other α subunits analyzed. Therefore there is no evidence for a change in subunit composition where the α4 subunits would be replaced with other α subunits. The GABA system is inhibitory and a decreased number of functional GABAA receptors will cause less inhibitory activity. Since we keep rats at the anesthesia level of the silent second, we have to increase the dose of neurosteroid to maintain anesthesia in situation with lower GABA system inhibitory activity. Importantly, in tolerant animals there is a negative correlation between the individual increases in the dose of allopregnanolone needed to retain the silent second from the period 10-30 min of anesthesia, to the period 65-85 min, and the mRNA expression of the GABAA receptor α4 subunit in the VPM of thalamus. This indicates that the stronger the tolerance has developed in the individual animals, the lower the GABAA receptor α4 subunit it is in the VPM of thalamus. Thalamic VPM integrates sensor input to the primary somatosensory cortex (Jones, 1998) and could be involved in anesthesia. Our detected decrease in GABAA receptor α4 subunit mRNA in the VPM of tolerant animals together with increased allopregnanolone levels in thalamus points to pharmacodynamic processes in this brain region. In tolerance specific neuronal adaptation occurs and more drug is required to overcome this new neuronal adaptation to produce an equivalent pharmacologic effect, in our case SS. From studies on epilepsy, an emerging view is that α4 subunit containing GABAA receptors are highly plastic and are rapidly changed in response to changes in neuronal activity (see in Sur et al., 1999). Previous studies have shown that the GABAA receptor α4 subunit is induced by allopregnanolone (10 mg/kg i.p. once a day) in parallel with increased anxiety, but after treatment for four days the α4 subunit expression and the anxiety decreases. A withdrawal from allopregnanolone again resulted in increased α4 subunit in the hippocampus in parallel with increased anxiety (Gulinello et al., 2001).

Another subunit found to be important for the mechanisms of tolerance development was the GABAA receptor α2 subunit in the hippocampus. Both MDR in the period 65-85 min, and the MDR difference between the time periods 65-85 min and 10-30 min, positively correlate with the GABAA receptor α2 mRNA amount in animals with 90 min of anesthesia. Moreover, using the PLS statistical method, a rather good model was obtained for the GABAA receptor α2 mRNA in the hippocampus, using MDR in the period 65-85 min as a response, supporting evidence of α2 subunit involvement in tolerance development to
allopregnanolone. The GABAA receptor $\alpha_2$ subunit mRNA is mostly expressed in brain regions related to emotional stimulus processing, like the hippocampus and the amygdala (Wisden et al., 1992) and is considered to mediate anxiolytic effect (Low et al., 2000). Thus, an increase in the hippocampal $\alpha_2$ subunit mRNA in rats with high tolerance to allopregnanolone could be related to the anxiolytic effect of allopregnanolone.

The GABAA receptor $\beta_2$ subunit has been shown to be involved in mediating the effect of anesthetic drugs (Belelli et al., 1997; Carlson et al., 2000) and in primary cultures of cerebellar granule cells, GABA specifically stimulates an increase in $\alpha_1$ and $\beta_2$ subunit mRNA (Kim et al., 1993). Importantly, in the 90 min anesthesia group we had difference in $\beta_2$ subunit expression compare to animals with 30 min of anesthesia, but not to the first SS group, meaning V-shape change with time for subunit expression. We did not obtain any correlation between $\beta_2$ subunit mRNA changes and MDR, it might be possible that the obtained $\beta_2$ subunit mRNA difference between experimental groups is due to the anesthetic effect of allopregnanolone or other receptor regulatory events, and not to the tolerance state.

For the GABAA receptor $\delta$ subunit we found a synchronization of the expression pattern in rats with tolerance and therefore also possible involvement in development of acute tolerance. Receptor knockout studies revealed that absence of the $\delta$ subunit decreases the sensitivity to neuroactive steroids, like pregnanolone and alphaxalone, influencing the duration of anesthesia and anxiolytic effect of those steroids (Mihalek et al., 1999). This corresponds well with the high allopregnanolone response found in $\delta$ containing GABAA receptors in vitro (Belelli et al., 2002).

It is thought, that $\alpha_5$ subunit containing GABAA receptors are extrasynaptic in the hippocampus, mediating tonic inhibition in the hippocampal CA1 region, and as an extrasynaptic receptor desensitizes more slowly that synaptic GABAA receptors in hippocampal neurons (Caraiscos et al., 2004). Thus, our tendency of a decrease in $\alpha_5$ subunit (especially in the CA1 region of the hippocampus) could probably become significant with longer anesthesia times.

In summary, changes in the GABA$_A$ receptor $\alpha_4$ and $\alpha_2$ subunit mRNA expression in the thalamus and the hippocampus seem important in development of acute allopregnanolone tolerance. Our findings of the GABA$_A$ receptor $\alpha_4$, $\alpha_2$, and also possibly $\delta$, $\alpha_5$ and $\beta_2$ subunit involvement in acute tolerance development to allopregnanolone is of great importance. However, our detected
GABAA receptor mRNA changes due to allopregnanolone supplementation are restricted to just some receptor subunits and specific brain areas. It is thus possible that in this experimental paradigm rather receptor function is changed, than the actual number of the mRNA coding for the subunits. Thus, it could be change in GABAA receptor number and/or sensitivity, internalization or receptor trafficking that causes the acute tolerance.

Allopregnanolone effects on spatial learning

The novel finding in Paper IV is the major decrease in spatial learning after i.v. administration of allopregnanolone. This effect is not caused by impairment of motor function, as swim speed is unaffected. The increased swimming close to the pool wall is found in most of the rats injected with allopregnanolone 8 minutes before the start of swimming trials.

We found learning impairment 8 min following the injection but not 20 min after the injection of allopregnanolone. There was also a clear-cut difference in brain and plasma concentrations of allopregnanolone at 8 min compared to 20 min after the allopregnanolone injection. This indicates that the allopregnanolone effects is short lived and suggests that the concentration needed to cause impairment of the learning ability was present at 8 min, while concentrations at 20 min after injection were below the threshold of learning impairment. Notably, hippocampus was the area with the largest difference in allopregnanolone concentration between the two time points. The brain allopregnanolone concentrations obtained in this study are around 10 - 20 times lower than at anesthesia (Zhu et al., 2001; Paper III). Whether these concentrations are possible to obtain under physiological conditions like chronic stress is not know.

Allopregnanolone is a positive GABA system modulator, enhancing the GABAA receptor effect on chloride flux, resulting in inhibition of neural activity (Majewska et al., 1986; Gee et al., 1987), which is similar to the effect of benzodiazepines and barbiturates. Memory impairment after benzodiazepine administration is well known (Ghoneim et al., 1984; Holbrook et al., 2000). The hippocampus is a key brain area for learning and memory, especially spatial learning (Riedel et al., 1999), and from earlier studies we know that allopregnanolone can inhibit neural activity in the hippocampus (Landgren et al., 1998). Information flow and processing in hippocampal neuronal network is regulated also by GABA interneurons that control large number of principal cells.
Thus, GABAA receptor activation can inhibit septo-hippocampal, raphe-hippocampal pathways and an interaction with the serotonin system seems to be important in regulation of learning and memory processes (Freund et al., 1990). It has been shown that around 90% of the serotonin 3 receptor expressing cells in the hippocampus are GABAergic (Morales et al., 1996). Suppression of GABA-mediated inhibition by 5HT3 receptor antagonists results in cortical acetylcholine increase and the cholinergic system is involved in learning and memory (see Sarter and Bruno, 1994; Diez-Ariza et al., 1998). In addition, GABAA receptor activation can inhibit LTP induction and NMDA receptors, which are involved in regulation of hippocampal dependent spatial memory (Morris et al., 1986; Staley and Mody, 1992; Tsien et al., 1996; Nakazawa et al., 2002; Wei et al., 2002; Riedel et al., 2003). The GABAA receptor α5 subunit is almost exclusively expressed in the hippocampus and seems to be involved in spatial learning regulation, since α5 knockout mice had significantly better performance in a water maze model of spatial learning in comparison with wild-type mice (Collinson et al., 2002). Thus, allopregnanolone could impair memory processes by activating GABAA receptors.

The increased swimming close to the pool wall found in most of the rats 8 min after the allopregnanolone injection could be due to induction of specific behavioral abnormality resulting in loss of strategy how to search for the platform. This type of sensorimotor disturbance has been described after administration of the benzodiazepine diazepam as well as after treatment with a NMDA antagonist (Cain et al., 1996; Cain, 1997). Rats with caudate-putamen lesions are also characterized by a tendency to swim near the wall, thigmotaxis, a behavior thought to be an impairment of learning the procedural parts of the place navigation (Devan et al., 1996). It is of interest that the allopregnanolone concentration in our study is highest in the striatum of the brain areas studied and GABAA receptors are also present in this brain area (Wisden et al., 1992). However, there was no significant correlation between thigmotaxis and allopregnanolone concentrations within striatum of the allopregnanolone-injected animals.

A strong driving force to leave the wall is the learning that there is a possibility to escape from the water and that this is not achieved by swimming close to the wall. It is first thereafter a true spatial learning of the platform position can take place (Morris, 1984). Thus, the allopregnanolone treated rats are stuck in an early phase of the task, and the endpoint is a slower acquisition of the task. Preliminary
data indicate that also learning of a non-spatial version of the water-maze task is negatively affected by the same dose of allopregnanolone (unpublished observation), again pointing in the direction of a more general learning disruption caused by allopregnanolone.

In summary, allopregnanolone impairs the procedural strategy of learning and inhibits spatial learning shortly after the injection. The explanation for the learning disability after allopregnanolone treatment might be a combination of changed swimming behavior and difficulties in spatial navigation.

**Allopregnanolone induced spatial learning impairment and GABA potentiation can be inhibited**

In Paper V a functional antagonism of an in vivo allopregnanolone-induced condition is shown for the first time. The drug used as an antagonist for allopregnanolone was UC1011 (3β-20β-dihydroxy-5α-pregnane), a steroid that as far as we know not occur naturally. In preliminary studies we found UC1011 to be one of the most effective in vitro allopregnanolone antagonists. The UC1011 antagonistic effect against allopregnanolone was thoroughly evaluated with studies of chloride ion uptake into membrane preparations from the cerebral cortex and the hippocampus. UC1011 significantly decrease the allopregnanolone-induced increase in chloride ion uptake seen in the presence of GABA in both brain regions analyzed. Thus, it is probable that the allopregnanolone-induced decreased learning is caused by increased GABAA activation, and that the UC1011 reduction of the allopregnanolone effect depends on decreased GABAA receptor activation.

To get a positive in vivo effect of UC1011 we needed a high concentration of the drug, 20 mg/kg, which is ten times the concentration of allopregnanolone infused. On the last day, the latency for this group was close to the time that control rats needed to find the platform. Animals that only received UC1011 (20 mg/kg) learned the task just as good as control rats, which were injected with vehicle.

The group of rats that learnt the task did not find the platform quicker because of better swim performance, since there was no significant difference in swim speed between the groups. Instead, there was a clear difference in the pattern of swimming between groups, i.e. differences in thigmotaxis (percentage of total path length spent close to the pool wall). While the allopregnanolone treated rats
largely swam around the wall, the rats with the combined treatment (allopregnanolone and UC1011), as well as the groups treated with only UC1011, or with vehicle, swam less in the periphery. UC1011 then either decreases the negative swimming pattern or restore the motivation to escape.

In the present study, we found decreased levels of allopregnanolone within cortex and striatum, but not hippocampus, after injection of allopregnanolone together with UC1011 (20 mg/kg). This decrease in brain tissue concentrations of allopregnanolone corresponded with a small nonsignificant increase in plasma allopregnanolone. In the striatum, the allopregnanolone concentration was comparable to the concentration found 20 min after an allopregnanolone injection from *Paper IV*. Thus, the decrease in allopregnanolone concentration within striatum might be of importance for the UC1011 positive effect on learning. It seems that a change in strategy how to search for the platform is essential for the learning disability caused by allopregnanolone injections and that UC1011 could exert antagonism against allopregnanolone induced adverse effects especially in this brain region. We are at the moment not able to analyze the concentration of UC1011, meaning that we do not know how much of the drug that is actually present within the brain. The finding of decreased CNS concentration of allopregnanolone after treatment with allopregnanolone together with UC1011, suggests existence of pharmacokinetic interactions. The interaction may occur at several levels: the uptake of allopregnanolone to the brain from the blood might be impaired; there can be a reduction in the number of high affinity binding sites for allopregnanolone; the blood flow can be changed and influence the distribution of allopregnanolone.

The *in vitro* antagonism of UC1011 on chloride ion uptake indicates that the antagonism of the allopregnanolone effect is at the GABAA receptor level. This is supported by results with other 3β-pregnan-steroids tested on recombinant GABAA receptors expressed in *Xenopus* oocytes (Wang et al., 2002), as well as the isoallopregnanolone inhibition of allopregnanolone induced effects on the population spike in hippocampal slices (Wang et al., 2000). Importantly, isoallopregnanolone selectively inhibits allopregnanolone induced Cl⁻ uptake, not affecting baseline Cl⁻ uptake in cortical homogenates from adult male rats (Lundgren et al., 2003).

Our group has also shown that women with premenstrual dysphoric disorder have changed sensitivity towards different GABAA receptor modulators during the luteal phase (Sundstrom et al., 1997, 1998). Allopregnanolone also increases
during stress (Purdy et al., 1991), and stress is related to impaired cognitive function (de Quervain et al., 1998). Recently, a large clinical trial showed an increased frequency of Alzheimer’s dementia among patients taking postmenopausal estrogen/progestagen replacement therapy (Shumaker et al., 2003). The progestagen used in the study, medroxypregesterone-acetate, can in high dosages induce anesthesia (Meyerson, 1967), probably via metabolism into GABA receptor active metabolites. Thus, there is a need for a drug that can inhibit negative effects caused by allopregnanolone, or other GABA receptor active metabolites.

Importantly, the tested concentrations of UC1011 had no significant effect on the amount of chloride flux, when allopregnanolone was absent. There was also a lack of change in learning seen when UC1011 was given alone to the animals before the Morris water maze test, pointing out the fact, that UC1011 is not active by itself in the brain. This is a good quality for a potential medication against allopregnanolone-induced conditions, as an antagonism to GABA is potentially dangerous, and might induce seizures (Kokate et al., 1999).

In summary, UC1011 antagonizes the allopregnanolone effects on chloride ion uptake and spatial learning impairment. Thus, such steroids, like UC1011 (having no effect by itself on the GABAA receptor but antagonizing allopregnanolone induced effects), would be of great importance in finding effective medication against progesterone/allopregnanolone induced cognitive disturbances.
CONCLUSIONS

1. The higher the estradiol supplementation dose to ovariectomized rats, the lower the weight gains.

2. Ovarian hormone manipulation influence 5HT1A, 2A and 2C receptor mRNA expression in a site-, and hormone-specific manner in different brain areas important for mood and memory functions.

3. In the hippocampus long term estradiol alone has opposite effect on 5HT1A mRNA expression compared with estradiol supplemented together with progesterone.

4. Long-term estradiol decreases the 5HT2C receptor gene expression, while estradiol in combination with progesterone increases the 5HT2A mRNA expression in the ventral hippocampus in a region specific manner.

5. It is possible to induce acute tolerance to allopregnanolone and the GABAA receptor alpha2 subunit in the dorsal hippocampus and alpha4 subunit in thalamus might be involved in development of tolerance. Alpha5, beta2 and delta subunits could also be of importance.

6. Allopregnanolone inhibits spatial learning in the Morris water maze shortly after the injection. Allopregnanolone injected rats show a specific behavioral pattern with swimming close to the pool wall.

7. The increase in chloride ion uptake induced by allopregnanolone can be inhibited by UC1011.

8. UC1011 significantly decreases allopregnanolone-induced impairment of spatial learning in the water maze, as well as the specific behavioral swim pattern.
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